Efecto anti-inflamatorio e inmunomodulador de una intervención con dieta mediterránea sobre los marcadores de inflamación de la pared vascular y de la placa inestable. Estudio PREDIMED

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“Cerrar un proceso no es cerrar la mirada que nos lleva a su fin. Y es que proceso es des-entender, de-construir, crearse. Proceso es aprender a ver, proceso es entender que el proceso jamás se cierra del todo”.

Helena Guerrero

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ÍNDICE

ABREVIACIONES

AGS	Ácidos grasos saturados
ALA	Ácido α-linolénico
AGMI	Ácidos grasos monoinsaturados
AGPI	Ácidos grasos poliinsaturados
AOVE	Aceite de oliva virgen extra
ApoE	Apolipoproteína E
ASP	Proteína estimulante de acilación
bFGF	Factor de crecimiento básico de fibroblastos
CAM	Moléculas de adhesión
CCL	Quimiocina (C-C motivo) ligando 5
CCR2	Receptor de CCL2
CD	Cluster de diferenciación
CG	Carga glicémica
C-HDL	Lipoproteínas de alta densidad
C-LDL	Lipoproteína de baja densidad
CML	Células del músculo liso
CMLV	Células de músculo liso vascular
COX-2	Ciclooxigenasa-2
PCR	Proteína C reactiva

CSF	Colony-stimulating factor
DietMed	Dieta mediterránea
DM2	Diabetes mellitus tipo 2
ECV	Enfermedad cardiovascular
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-Derived Hyperpolarizing Factor
ELAM-1	Molécula 1 de adhesión de los leucocitos al endotelio
GIP	Polipéptido inhibidor gástrico
GlyCAM-1	Glicosilación dependiente de molécula de adhesión celular 1
GLP-1	El péptido-1 similar al glucagón
GMP-140	Platelet alpha granule membrane protein
Hcy	Homocisteina
IAM	Infarto agudo de miocardio
ICAM-1	Molécula de adhesión intercelular-1
IG	Índice glicémico
Igs	Inmunoglobulinas
IGF-1	Insulina tipo 1
IL	Interleucina
IFN-γ	Interferón gamma

IP-10	Proteína- 10 inducible
I-TAC	Quimioatrayente α de las células IFN-γ inducible
LECAM-1	Lectina de la molécula de adhesión de neutrófilos 1
LFA-1	Antígeno asociado a función leucocitaria-1
Lp-PLA2	Lipoprotein-associated phospholipase A2
LDLox	LDL-oxidadas
MAdCAM	Mucosal vascular addressin cell adhesion molecule 1
MCP-1/CCL2	Proteína quimiotáctica de monocitos
MIF	Factor inhibidor de la migración de macrófagos
Mig	Monocina inducible por IFN-γ
MMP	Metaloproteasa de la matriz extracelular
MPO:	Mieloperoxidasa
MT1-MMP	Tipo de membrana 1 metaloproteasa
NF-κβ	Factor de transcripción nuclear Kappa β
OMS	Organización Mundial de la Salud
ON	Óxido nítrico
PAI-1	Inhibidor del activador del plasminógeno-1
PAPP-A	Proteína A del plasma sanguíneo asociada al embarazo
PDGF	Factor de crecimiento derivado de las plaquetas

PDM	Patrón de dieta mediterránea
PECAM-1	Molécula de adhesión de célula endotelial y plaquetas
PGE2	Prostaglandina 2
PGI2	Prostaciclina 2
PSGL-1	Glicoproteina P-selectina ligando-1
SAA	Proteína amiloide sérica A
TG	Triglicéridos
TGF-β	Factor de crecimiento transformante beta
TLR	Toll-like receptors
TNFR	Receptores del TNF
RCV	Riesgo de cardiovascular
SCA	Síndrome coronario agudo
sCD40L	Ligando de CD40 soluble
TA	Tejido adiposo
TAV	Tejido adiposo visceral
TIMP	Inhibidores tisulares de metaloproteinasas
TNF-α	Factor de necrosis tumoral α
VLA-4	Antígeno de activación tardía-4
VCAM-1	Molécula de adhesión celular vascula

INTRODUCCIÓN

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El término arterosclerosis hace referencia a un conjunto de trastornos de las arterias caracterizados por el engrosamiento y endurecimiento de la pared arterial. Y es el resultado de dos fenómenos íntimamente relacionados: la ateromatosis, que es la acumulación lipídica focal intra y extracelular, con formación de células espumosas y reacción inflamatoria, y la esclerosis, endurecimiento cicatrizal de la pared arterial (Arce-Torres, 2008). Ross y cols, integraron ambas teorías al considerar la aterosclerosis como un proceso de respuesta inflamatorio a una agresión endotelial (Ross, 1999). Es un proceso de evolución lenta, que se inicia en edades muy tempranas (nacimiento y niñez) y es a partir de los 50 años, cuando progresa de manera más rápida, severa e intensa, dando lugar a la Enfermedad Cardiovascular (ECV) que afecta a todos los órganos pero especialmente corazón (cardiopatía isquémica), cerebro (ictus) y extremidades inferiores (vasculopatía periférica), etc. (Arce-Torres, 2008).

A pesar del descenso del casi 50% en la incidencia de ECV en las últimas tres décadas, continua siendo la causa más frecuente de muerte en el mundo. Los países occidentales, incluidos los Estados Unidos y Europa (norte y este), continúan teniendo una tasa absoluta de morbilidad y mortalidad cardiovascular inaceptablemente alta, del 35% y adquiere características de epidemia encubierta en países en vías de desarrollo (Reddy, 2004; World Health Organization, 2003 a y b). De acuerdo con las previsiones de la Organización Mundial de la Salud, la ECV continuarán siendo la principal causa de muerte en el año 2030 (Mathers, 2006). Sin embargo, algunas áreas del mundo, como los países mediterráneos o Japón, muestran una incidencia menor de ECV que países del norte y este de Europa o EEUU (Tunstall-Pedoe, 1999; Rosamond, 2007). Descartada la causa genética, estas diferencias podrían explicarse por unos hábitos de vida más saludables como la dieta y la actividad física.

En este sentido, actualmente, existe suficiente evidencia científica que demuestra el papel protector de la Dieta Mediterránea (DietMed) en la prevención de la ECV a través de diversos mecanismos como un mejor control de los factores clásicos de riesgo cardiovascular (FRC) (DM, HTA, sobrepeso, etc.) (Vincent-Baudry, 2005; Estruch, 2006). Más concretamente se ha descrito que la DietMed también ejerce un papel anti-inflamatorio, inmunomodulador y antioxidante que retrasaría la progresión de la ECV (Estruch, 2006; Esposito, 2004; Mena, 2008). Quedan sin embargo incógnitas que resolver como la duración del efecto anti-inflamatorio e inmunomodulador de la DietMed y en qué etapas de la formación de la placa actúa. Tampoco se conocen otros aspectos como el papel de las adipocitocinas o de alimentos específicos de la DietMed en este efecto inmunomodulador. Esta tesis doctoral pretende resolver estas incógnitas.

1. FISIOPATOLOGÍA DE LA ATEROSCLEROSIS

La aterosclerosis se caracteriza por el engrosamiento y endurecimiento de la pared vascular a causa de una compleja interacción entre ésta y las células circulantes. Intervienen procesos de inflamación, crecimiento, proliferación y migración de depósitos de lípidos y síntesis de matriz extracelular, y que progresivamente dan lugar a las placas de ateroma que reducen la luz arterial pudiendo llegar a disminuir el flujo sanguíneo en el territorio dependiente (Ross y Fuster, 1996). La formación de un trombo sobre la placa de ateroma provocará la oclusión total de la arteria y por tanto, isquemia en el territorio dependiente dando lugar a la aparición de eventos clínicos. Este proceso inflamatorio crónico de baja intensidad en la pared arterial se traduce a nivel histológico en 4 fases: iniciación de la lesión o formación de la estría grasa, formación de la placa fibrosa, lesiones avanzadas y ruptura de la placa y trombosis (Ross, 1999; Libby, 2002; Glass i Witzum, 2001; Berliner, 2002; Hansson, 2005). A continuación, describimos los fenómenos que se suceden a nivel celular/molecular en el desarrollo de la placa de ateroma.

1.1. DISFUNCIÓN ENDOTELIAL

La *disfunción endotelial* es la primera manifestación de la aterosclerosis. El endotelio vascular está constituido por una monocapa de células que recubre el interior de las arterias y que sintetiza sustancias vasodilatadoras (ON, PGI, PGE y EDHF) y vasoconstrictoras (endotelina y EDCF) (Widlansky, 2003). Las principales funciones del endotelio incluyen evitar la adhesión plaquetaria y leucocitos (monocitos/linfocitos T) y evitar la formación de trombos, regulación del sistema fibrinolítico, control de la actividad de las CMLs y modulación del tránsito de macromoléculas (lipoproteínas, etc) (Stenvinkel, 2001). El endotelio disfuncionante debido a agresiones de diverso tipo pierde total o parcialmente estas funciones y facilita las primeras etapas de la

aterosclerosis. Los FRC (hipertensión arterial, tabaquismo, diabetes tipo 2, hipercolesterolemia, etc) son entre otros, capaces de inducir disfunción endotelial (Ross, 1999; Widlansky, 2003).

1.2. MOLÉCULAS DE ADHESIÓN

Son proteínas que se expresan en diferentes tipos celulares y permiten la interacción entre ellos. Una de las primeras consecuencias de la disfunción endotelial es que aumenta la adhesividad del endotelio para los linfocitos y monocitos al aumentar la expresión de proteínas de superficie de la membrana celular que actúan como moléculas de adhesión (CAM) para receptores específicos de leucocitos circulantes (Herman 2001; Badimón, 2006). Las CAM se agrupan fundamentalmente en tres familias (**tabla 1**):

Tabla 1. Moléculas de adhesión implicadas en las interacciones leucocito-endotelio.

Clasificación-CD	Nombre alternativo	Ligando	Expresión	Etapa donde interviene
Selectinas				
CD62E	E-sectina, ELAM-1	Syalil Lewis x, Syalil Lewis a, PSGL-1	Endotelio	Rodamiento
CD62L	L-selectina, LECAM-1	GlyCAM-1, MAdCAM-1, CD34	Todos los leucocitos	Rodamiento
CD62P	P-selectina, GMP-140	PSGL-1	Endotelio y plaquetas	Rodamiento
Superfamilia IgG				
CD54	ICAM-1	LFA-1, MAC-1	Endotelio, monocitos	Activación/Adhesión
CD102	ICAM-2	LFA-1	Endotelio	Activación/Adhesión
CD106	VCAM-1, PECAM	VLA-4	Endotelio	Activación/Adhesión
Integrinas				
CD11a/CD18	LFA-1	ICAM-1, ICAM-2	Todos los leucocitos	Activación/Adhesión
CD11b/CD18	MAC-1	ICAM-1	Granulocitos, monocitos	Activación/Adhesión
CD49d/CD29	VLA-4	VCAM-1, SC-1, Fibronectina	Linfocitos, monocitos	Activación/Adhesión

Las interacciones leucocito-endotelio se dividen en 4 fases (**figura 1)**:

A. Fase rodamiento: Es la primera interacción entre leucocitos y las células endoteliales, y está mediada fundamentalmente por los receptores de selectinas (E- y P-selectinas). En esta fase los leucocitos disminuyen sensiblemente su velocidad sobre el endotelio vascular facilitando su interacción con los mediadores de la inflamación liberados por el endotelio (TNF-α, lL-1, etc).

B. Aumento de la afinidad de las integrinas mediada por quimiocinas: Los leucocitos circulantes expresan integrinas que son activadas como respuesta a la señalización de los receptores de las quimiocinas (CCR2). De esta forma se produce una unión más fuerte de los leucocitos a la superficie endotelial.

C. Fase adhesión firme: Una vez activados los leucocitos y las células endoteliales tiene lugar la adhesión firme entre ellos. Aquí, la expresión de los ligandos de las

integrinas, principalmente VCAM-1 (el ligando de la integrina VLA-4) e ICAM-1 (el ligando de las integrinas MAC-1 y LFA-1) también se verá incrementada.

D. Fase migración: Finalmente, después de la adherencia transitoria (*rodamiento*) y más tarde de la firme (*adhesión*), los leucocitos migran al espacio subendotelial.

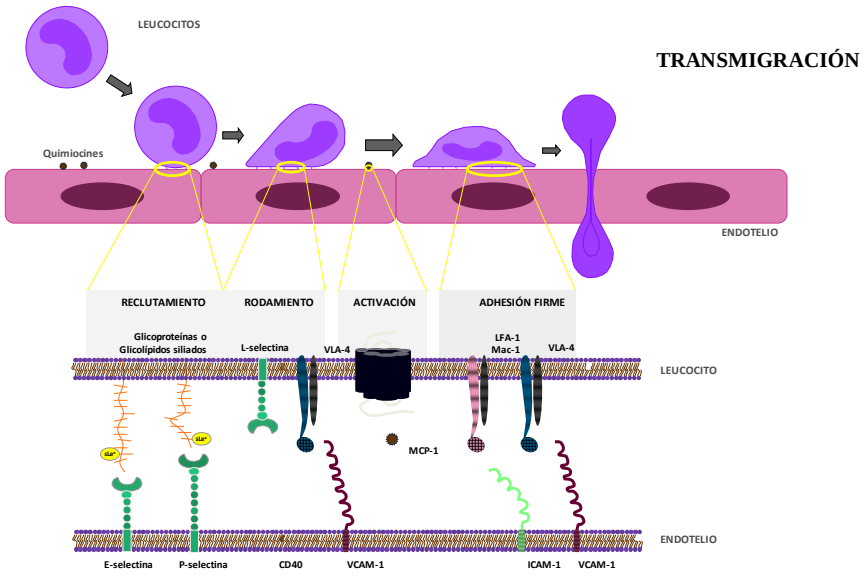

Figura 1. Etapas de la interacción leucocito-endotelio. (Bhargava, 2012)

1.3. CITOCINAS Y QUIMIOCINAS

Las quimiocinas constituyen un grupo numeroso de citoquinas proinflamatorias. El papel más importante que desempeñan es el de actuar como quimioatrayentes para guiar la migración celular (monocitos y linfocitos T fundamentalmente). Su liberación es a menudo estimulada por citoquinas pro-inflamatorias tales como IL-1. La **tabla 2 (de I a III)** muestra un resumen de las principales citocinas, quimiocinas y otras moléculas implicadas en el desarrollo de la aterosclerosis (Hansson, 2011; Wan, 2013; Vilahur, 2013).

Tabla 2. Citocinas implicadas en el desarrollo de la aterosclerosis (I)

MOLÉCULA	CLASIFICACIÓN	EXPRESIÓN	FUNCIONES
Interleucinas			
IL-1β	Superfamilia de les interleucinas-1	Monocitos, macrófagos	Induce la activación de monocitos y la expresión de MA en las CE, induce la secreción de otras citoquinas, quimioquinas y factores de crecimiento y estimula la proliferación de las CML.
IL-4	Citoquina anti-inflamatoria	Células T (TH2)	Induce la proliferación de células T, coestimula la producción de células B
IL-5	Interleucina proinflamatoria	Linfocitos T (TH2)	Coestimula la proliferación de las células B
IL-6	Interleucina que actúa tanto como una citocina proinflamatoria y anti-inflamatoria	Macrófagos, células TH2, monocitos, células endoteliales, células vasculares del músculo liso.	↑ reactantes de fase aguda
IL-8	Citocina de la familia de las quimiocinas. Proinflamatoria	Macrófagos, monocitos, fibroblastos	Activación, quimiotaxis
IL-10	Citocina antiinflamatoria. Se clasifica como Citocina de clase II	Monocitos, macrófagos, células TH2	↓ producción IL-1, TNF-α, y IL-2 Niveles bajos de IL-10 son marcadores de la inestabilidad de placa
IL-12	Citocina proinflamatoria	Monocitos, macrófagos	Esencial para la diferenciación, proliferación y mantenimiento de T helper 1 (Th1)
IL-13	Citocina no glicosilada. Del tipo II. Anti-inflamatoria	Linfocitos T principalmente	Activa las células NK para secretar IFN-γ
IL-17	Citocina proinflamatoria. Familia de IL-17	Linfocitos T, células musculares lisas	Función similar al IFN-γ. Induce el factor de proliferación de las células B.
IL-18	Citocina de la Superfamilia IL1. Proinflamatoria	Macrófagos	Induce la producción de IFN- γ. Quimiotaxia de CD4

Tabla 2. Citocinas implicadas en el desarrollo de la aterosclerosis (II)

MOLÉCULA	CLASIFICACIÓN	EXPRESIÓN	FUNCIONES
Receptores de interleucinas			
IL1R1	Receptor de la familia interleucina 1 (IL1), concretamente IL1α,β e IL1RN	Membrana celular	Media la activación de NF-κβ, y otras vías de MAPK.
Interferones			
IFN-γ	Citoquina. Pertenece a la familia de Interferones del tipo II	Linfocitos T. Tejido adiposo blanco.	Activación de los macrófagos y NK. Coestimula la producción de la célula T, induce la secreción de IL-6, IL-8 y G-CSF.
Factores de necrosis tumorales (TNF)			
TNF-α	Superafamilia TNF	Monocitos, linfocitos, neutròfils, macrófagos	Activa el NF-κβ, y estimula la expresión de E-Selectina, ICAM-1, VCAM-1, así como la adhesión y transmigración monocitaria y de neutrófilos al endotelio. Estimula la síntesis de algunos reactantes de fase aguda
Colony-stimulating factor (CSF)			
GM-CSF	Glicoproteína (citocina que funciona como un factor de crecimiento de glóbulos blancos)	Células endoteliales, macrófagos	Estimula la proliferación de supervivencia es decir, la diferenciación, y la función de precursores de neutrófilos y neutrófilos maduros
G-CSF	Glicoproteína (citoquina que funciona como un factor de crecimiento de glóbulos blancos)	Macrófagos, linfocitos T, células madre, células NK, endoteliales, fibroblatos	Estimula la producción de monocitos

Tabla 2. Citocinas implicadas en el desarrollo de la aterosclerosis (III)

MOLÉCULA	CLASIFICACIÓN	EXPRESIÓN	FUNCIONES
Factores transformadores del crecimiento (TGF)			
TGF-β	Citocina de superfamilia de factores de crecimiento transformante beta	Células T Células B, linfocitos T, monocitos, células endoteliales	Inhibe la proliferación de IL-1 e IL-2. Inhibe la secreción y la actividad de IFN-γ y TNF-α y varias interleucinas y citocinas. Disminuye los niveles de expresión de receptores de citoquinas como el receptor de IL-2.
Quimiocinas			
MCP1 o CCL2	Familia de les citocinas C-C	Macrófagos, monocitos	Reclutamiento de células monocitarias a las zonas ateroscleróticas. Receptor CCR2.
IP10 o CXCL10	Familia de les citocinas C-X-C	Monocitos, células endoteliales	Quimiotaxis de los monocitos / macrófagos, células T y promoción de la adhesión de células T a las células endoteliales, actividad antitumoral.
MIP1b o CCL4	Familia de les citocinas C-C	Macrófagos, linfocitos	Es un quimioatrayente para las monocitos y una variedad de otras células inmunes.
Rantes o CCL5	Familia de les citocinas C-C	Células T, macrófagos	Quimiotáctica para las células T, y desempeña un papel activo en el reclutamiento de leucocitos en los sitios inflamatorios
ENA78 o CXCL5	Familia de les citocinas C-X-C	Eosinófilos	Atrae y activa neutrófilos (quimiotáctica)
ITAC o CXCL11	Familia de les citocinas C-X-C	Leucocitos	CXCL11 es quimiotáctico para las células T activadas.
Metaloproteasas			
MMP-9	Familia de las Metaloproteasa	Neutrófilos, macrófagos	Involucrada en la degradación de la matriz extracelular en procesos fisiológicos normales

1.4. FORMACIÓN DE LA PLACA DE ATEROMA. CONCEPTO PLACA ESTABLE/INESTABLE

Tal y como ya hemos citado anteriormente, son cuatro las etapas que principalmente intervienen en el desarrollo de la placa de ateroma:

Iniciación de la lesión o formación de la estría grasa

El proceso aterosclerótico comienza con la acumulación del colesterol de las lipoproteínas de baja densidad (C-LDL) en la matriz subendotelial siendo oxidadas por la presencia de radicales libres y dando lugar a las LDL-oxidadas (LDLox) (Libby y Aikawa, 2000; Libby, 2002). Estas lipoproteínas oxidadas estimulan la producción de moléculas proinflamatorias y la expresión de las moléculas de adhesión celular (VCAM-1, PCAM-1, ICAM-1, E- y P- Selectina), MCP-1 y M-CSF en el endotelio. Todo ello provoca la atracción de monocitos y linfocitos a la pared arterial. Estos macrófagos presentan en su superficie unos receptores conocidos como *"scavengers"* (CD36 entre otros) que reconocen y captan las LDLox y otras partículas (Lutgens, 2006). El macrófago cargado de LDLox se transforma en *Célula espumosa o foam cell*. Las células espumosas también contienen receptores conocidos como *"toll-like"* los cuales contrariamente a los *Scavengers*, pueden iniciar una cascada de señalización que lleva a la activación celular produciendo citocinas inflamatorias, PCR, proteasas y radicales citotóxicos de oxígeno y nitrógeno (Hansson, 2005).

Formación de la placa fibrosa

La placa fibrosa se inicia con la migración de las CMLs desde la capa media hacia la matriz subendotelial (íntima). Allí estas células proliferan y captan las lipoproteínas modificadas, contribuyendo así, junto con las células espumosas al engrosamiento del núcleo lipídico. Estas células a su vez secretan proteínas de la matriz extracelular, conduciendo a la formación de la placa fibrosa (Glass y Witztmun, 2001).

Lesiones avanzadas: placa de ateroma estable e inestable

La respuesta proliferativa de las CMLs (lesión fibroproliferativa) y de las células inflamatorias conduce a que la estría grasa evolucione a una placa aterosclerótica más compleja llevando la lesión a estadíos más avanzados y que pueden implicar calcificación, muerte celular, ruptura de las placas y trombosis. En las placas ateromatosas más avanzadas aparece la *"lesión necrótica"* caracterizada por la formación de un *núcleo necrótico*. El crecimiento de la placa reduce la luz de la arteria hasta poder llegar a ser una obstrucción crítica.

Las placas de ateroma pueden ser estables, con poca predisposición a la ruptura o pueden ser inestables, propensas a la ruptura, facilitando la formación de un trombo oclusivo y a acontecimientos isquémicos agudos. Las placas estables se caracterizan por una cubierta fibrosa densa, un núcleo lipídico pequeño y una inflamación de bajo grado. Por el contrario, las placas inestables o «vulnerables» tienen una cubierta fibrosa fina, un contenido lipídico elevado y unos niveles de actividad inflamatoria local elevados que pueden producir la degradación de la capa fibrosa, y facilitar así, la ruptura de la placa y posteriormente, trombosis en esa zona. En la **tabla 3** se resumen las características de ambos tipos de placa.

Tabla 3. Características de la placa de ateroma estable e inestable.

Placa inestable	Placa estable
Ateromatosa	Fibrosa
Cubierta fibrosa fina	Gruesa capa fibrosa
Gran núcleo lipídico	Núcleo pequeño de lípidos
Poco de colágeno	Mucho en colágeno
Calcificada	No calcificación
Ulceración	No ulceración
Hemorragia intraplaca	No hemorragia intraplaca
Muchas células inflamatorias	Pocas células inflamatorias
Proteólisis y remodelación	Escasa remodelación y proteólisis

1.5. MECANISMOS DE RUPTURA DE LA PLACA DE ATEROMA

Si el proceso inflamatorio se mantiene porque los factores de riesgo que la provocan persisten, el núcleo necrótico puede seguir creciendo produciéndose de esta manera una degradación de la matriz extracelular por las MMPs secretadas por los leucocitos activados. Además, las citocinas proinflamatorias limitan la síntesis de colágeno, y así disminuyen el grosor de la capa de fibrosa y facilitando que la placa sea más susceptible a la ruptura (Hansson, 2005). Con la ruptura de la cobertura fibrosa, los factores de la coagulación del torrente sanguíneo pueden acceder al centro lipídico trombogénico y desencadenar la formación de un trombo local que puede causar isquemia como resultado la aparición de un accidente cerebrovascular, IAM, angor, ictus, etc. En este punto, pueden pasar dos cosas: que el trombo se mantenga (isquemia aguda) o bien, que el trombo no se propague bien y sea lisiado. En este último caso podría pasar desapercibido clínicamente o bien, producir isquemia transitoria (Malpartida, 2007; Shah, 2009; Dégano, 2013).

El equilibrio entre la síntesis y la degradación del tejido conectivo es el que va a determinar si se produce o no la ruptura. La ruptura de la placa ocurre preferentemente en los puntos en los que la cubierta fibrosa es más delgada o está parcialmente erosionada (Falk, 1995). En estos puntos, se produce una gran cantidad de moléculas inflamatorias y enzimas proteolíticos debido a la activación de las células inmunitarias. Así, los macrófagos estimulados por la IL-1β o por el TNF-α, son capaces de reducir la población de CMLs lo que conduce a una reducción de la síntesis de colágeno y a la producción de metaloproteasas. Estas MMPs degradan el tejido conectivo y lo convierten en fragmentos de bajo peso molecular. Las metaloproteasas, tales como MMP-1, MMP-3, MMP-8 y MMP-9, se encuentran altamente sobreexpresadas en las placas rotas y vulnerables en comparación con las placas estables, siendo particularmente activas en los puntos más vulnerables de la

misma (Hellings, 2007). La **Figura 2**, muestra algunos de los marcadores séricos que pueden ser utilizados como marcadores predictores de estabilidad (IL-10 o TGF-β) e inestabilidad de placa (PCR, IL-6, IL-10, IL-18, , MMP-9 o TIMP-1) (Hermus, 2010).

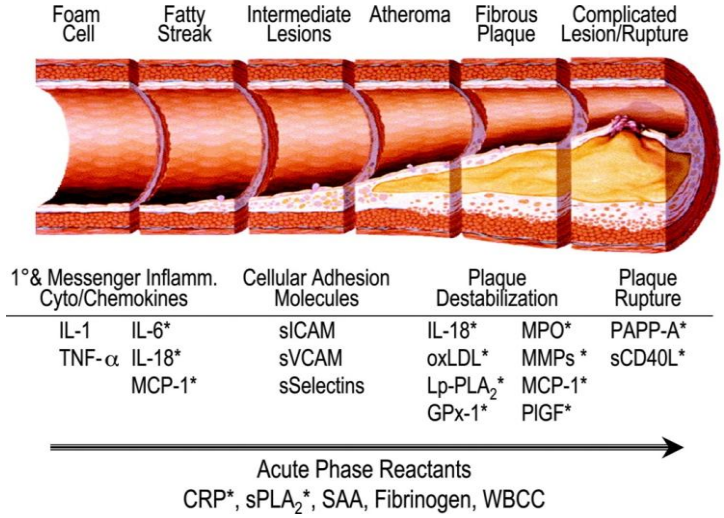

Figura 2. Los marcadores de la inflamación y la inestabilidad de la placa: de células espumosas a la ruptura de placa (Koenig, 2007).

1.6. PAPEL DEL TEJIDO ADIPOSO Y LAS ADIPOQUINAS EN LA PATOGENIA DE LA ATEROSCLEROSIS

El tejido adiposo es un órgano endocrino y paracrino, que produce un gran número de citocinas como TNF-α, IL-6 o PCR y de mediadores bioactivos, designados como adipoquinas (**figura 3**). Estas moléculas influencian no solo la homeostasis del peso corporal, sino están vinculados con inflamación, coagulación y fibrinólisis (Do Nascimento, 2009; Anfossi, 2010).

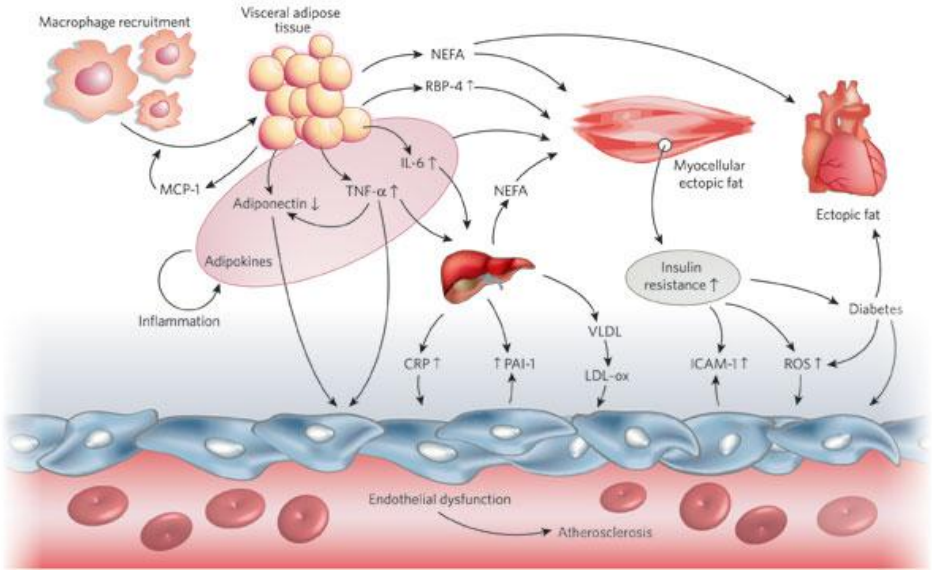

Figura 3. Acción de las adipoquinas secretadas por el tejido adiposo sobre la aterosclerosis

Las adipoquinas o adipocitoquinas (IL-6 o TNF-α, leptina y adiponectina, resistina, visfatina entre otras) son proteínas producidas por el tejido adiposo que pueden ser liberadas a la circulación y llegar a otros tejidos donde pueden ejercer diferentes funciones (**Tabla 4**) (Do Nascimento, 2009).

Adiponectina

La adiponectina es una hormona polipeptídica de 247 aminoácidos con una amplia actividad biológica y originada de manera exclusiva en el tejido adiposo por el adipocito maduro. Ésta ejerce un efecto anti-aterogénico y anti-inflamatorios predominantemente (**tabla 4**) (Ouchi, 2001; Herder y Hauner, 2006). Existen diversos estudios tanto en animales como en humanos, que ratifican la fuerte correlación negativa entre niveles circulantes de adiponectina y resistencia a la insulina y obesidad (Arita, 1999; Akihiko, 2011), diabetes (Hotta, 2000; Akihiko, 2011), síndrome

metabólico (Trujillo, 2005; Akihiko, 2011), ECV (Kumada, 2003; Akihiko, 2011) y HTA (Ouchi, 2003; Akihiko, 2011).

Visfatina

La visfatina fue inicialmente denominada nicotinamidaciltransferasa (Nampt), la cual presenta dos formas, una intracelular (iNampt), y otra extracelular (eNampt) denominada también como factor de incremento de colonia de células pre β (PBEF) (Revollo, 2007). Ésta es liberada por macrófagos infiltrados en el tejido adiposo visceral, en respuesta a un proceso inflamatorio. Tiene acción endócrina, parácrina y autócrina, siendo su rol autócrino importante en la regulación de la sensibilidad a la insulina en el hígado (Saddi-Rosa, 2010). Diversos estudios clínicos señalan a la visfatina como un marcador de disfunción endotelial e inflamación (Malyszko, 2010; Malyszko, 2009). En sujetos con síndrome metabólico también se han observado mayores concentraciones séricas de visfatina (Zhong, 2008; Omer, 2009), siendo aún más elevados en aquellos pacientes que además presentaron placas de ateroma en la arteria carótida (Omer, 2009). Además, la alta inmunoreactividad de visfatina en CMLV de aorta y coronarias, además de la presencia de esta citoquina en células espumosas de lesiones arterioescleróticas ponen de manifiesto su correlación positiva con procesos inflamatorios y con la desestabilización de las placas de ateroma (Spiroglou, 2009; Dahl, 2007). Sin emabrgo, diversos estudios han mostrado el papel de la visfatina como mediador en la síntesis de ON endotelial (activaría la proteinquinasa Akt, la cual interviene en la activación postranscripcional de la eNOS) (Lovren, 2009).

Leptina

La leptina es una hormona específica del adipocito que posee una función importante en la regulación del peso corporal, a nivel hipotalámico, debido a la supresión del apetito y el aumento del gasto de energía. Además, también tiene carácter

proinflamatorio (Nava-Santana, 2013). Existen personas con resistencia a la leptina en los que paradójicamente coexisten concentraciones elevadas de leptina y obesidad, lo que conlleva una mayor morbilidad por ECV (Sharma, 2010). El *West of Scotland Coronary Prevention Study* mostró que las concentraciones elevadas de leptina eran un FRC independiente de la asociación de la obesidad a diabetes, hipertensión e hipercolesterolemia (Bodary, 2002). Sin embargo, al igual que la visfatina, esta proteína también favorece la biodisponibilidad de ON en los vasos sanguíneos (Wang, 2010).

PAI-1

El Inhibidor del factor activador del plasminógeno 1 (PAI-1) pertenece a la superfamilia de las serpinas (inhibidoras de serina proteasas). Es una proteína reguladora de la cascada de la coagulación con propiedades antifibrinolíticas. Es secretado por el hígado y por el adipocito en respuesta a la insulina, VLDL y ácidos grasos libres entre otros factores. Las concentraciones plasmática de PAI-1 son elevadas en la obesidad (Berg y Scherer, 2005; Darvall et al, 2007), lo que favorece fenómenos protrombóticos y es considerado predictor del desarrollo de IAM y de DM (Correia y Hayes, 2007). Puede de inhibir la leptina segregada por el tejido adiposo (Correia y Hayes, 2007) y predispone la formación de placas de ateroma, incrementando la concentración lipídica dentro de los miocitos (Linjnen, 2005). En estados inflamatorios, el TNF-α o IL-6 estimulan su producción e interviene directamente en la formación y ruptura de la placa de ateroma, y es bien conocida su estrecha relación con el síndrome metabólico, la resistencia a la insulina (Cesari, 2010) y a HTA (Dimitrijevic-Sreckovic, 2007).

Resistina

Es segregada por los adipocitos y por las células estromales del tejido adiposo, principalmente macrófagos (Gualillo, 2007). La función más importante de esta

proteína es la regulación de insulina, glucosa y por lo tanto, se asocia a la aparición de diabetes tipo 2. La resistina favorece la inflamación subclínica y la activación endotelial (Harsch, 2004), y su efecto puede ser inhibido por la adiponectina (Zulet, 2007).

En un estudios con ratas a las que le habían inducido síndrome metabólico, encontraron un aumento de la expresión de resistina y una disminución de la expresión de adiponectina en el tejido adiposo visceral, y un incremento del estrés oxidativo y de inflamación vascular (VCAM-1), así como un aumento de la expresión de NF-κβ con respecto a ratas controles, que fue revertida al administrarles vino tinto de manera moderada (Vazquez Prieto, 2011). Todo ello sugiere el papel de esta molécula en la aparición del síndrome metabólico.

Otras adipoquinas

La **grelina** es una hormona peptídica producida principalmente en el estómago que aumenta durante el ayuno y disminuye después de la ingesta de alimentos, por tanto, estimula el apetito y promueve la formación de tejido adiposo mediante el neuropéptido Y (Gaigai, 2010). Se postula como una citocina cardioprotectora reduciendo la presión arterial, protegiendo la célula endotelial mediante la inhibición de procesos inflamatorios, migración endotelial estimulada por angiotensina II, promoviendo la contractibilidad cardíaca y la recuperación tras un evento cardiovascular, además de poseer efectos antiapoptóticas (Gaigai, 2010; Cjia-Chi, 2010; Beiras, 2011).

La **adipsina** es una proteasa de serinas idéntica al factor D y se expresa principalmente en adipocitos y monocitos en humanos. Favorece la litogénesis. En humanos, la obesidad y la alimentación incrementan su expresión, mientras que ayuno, caquexia y lipodistrofia la disminuyen (Napolitano, 1994). Por otro lado la

adipsina contribuye con la síntesis de ASP, una proteína implicada en el metabolismo lipídico que estimula la síntesis y almacenamiento de triacilglicéridos (Yasruel, 1991).

Tabla 4. Efectos de las adipoquinas en la formación de la placa de ateroma

Adipoquinas	Funciones	Efecto pro-aterogénico	Efecto anti-aterogénico
Adiponectina	↑ IL-10 , IL-1RA, TIMP-1		+
	↓ TNF-α y IL-6, IL-8, IFN-γ , NF-κβ, PCR		+
	↓ paso de macrófago a célula espumosa		+
	↓ Señalización, proliferación, agregación, reclutamiento		+
	↓ Moléculas de adhesión (VCAM-1, ICAM-1, E-Selectina)		+
	↑ Producción ON		+
Visfatina	↑ NF-κβ	+	
	↑ Moléculas de adhesión (VCAM-1, ICAM-1)	+	
	↑ IL-6, PCR o TNF-α	+	
	↑ Señalización, proliferación, agregación, reclutamiento		
	↑ Producción ON endotelial	+	+
Leptina	↑ Activación de células T, natural killer y macrófagos	+	
	↑ IL-6, TNF-α	+	
	↑ MCP-1	+	
	↑ TGF- ß 1	+	
	↑ Producción ON		
	↑ Oxidación	+	
	Regula proliferación, y migración de CE		+
PAI-1	Regula proliferación y migración de CE		+
	↑ TNF-α, PCR		
	↑ Diferenciación preadipocitaria y cúmulo lípidico en adipocitos	+	
	↑ Adhesión y migración celular	+	
	↑ Formación de placas de ateroma	+	
	↑ Estrés oxidativo	+	
Resistina	↑ NF-κβ	+	
	↑ IL-6, TNF-α, PCR	+	
	↑ MCP-1	+	
	↑ Moléculas de adhesión (VCAM-1, ICAM-1)	+	
	↑ Proliferación y migración de CE y CML	+	
	↑ Formación de células espumosas	+	

2. FACTORES DE RIESGO CARDIOVASCULAR CLASICOS

Los estudios epidemiológicos de los últimos 50 años, iniciados con el estudio *Framingham* (Dawber y Kannel, 1966), identificaron hipercolesterolemia, diabetes Mellitus (DM), hipertensión arterial (HTA), obesidad y tabaquismo como los factores de riesgo “mayores” o “causales” de la ECV y son conocidos como FRC.

2.1. HIPERCOLESTEROLEMIA

La relación entre las concentraciones elevadas de colesterol en plasma y el RCV está bien establecida, desde el estudio Framingham (Kannel et al., 1979). Es conocido, el papel protector de la fracción HDL colesterol frente a la ECV, de forma que por cada mg/dl de aumento de esta lipoproteína, se reduce el riesgo un 2% en hombres y un 3% en mujeres (Gordon y Probstfield, 1989). Mientras que, el aumento de LDL-colesterol favorece la formación de la placa de ateroma (Diaz et al., 1997) y aumenta la densidad de la sangre favoreciendo la aparición de trombos. Existe una relación lineal entre el colesterol LDL y la cardiopatía isquémica y a partir de los estudios de intervención se pudo estimar que por cada descenso de 30 mg/dl de esta lipoproteína se consigue una reducción del 30% del riesgo de padecer un IAM/Angor (Grundy, 2004).

Diversos estudios en prevención secundaria han demostrado que las dietas hipocalóricas, bajas en grasas saturadas y ricas en vegetales, verduras y frutas y ácidos grasos mono y poli-insaturados reducen el riesgo de ECV a través de una reducción del ratio LDL /HDL-colesterol (Singh, 1992; Lohse, 2010).

2.2. DIABETES MELLITUS

Estudios epidemiológicos, como el estudio Framingham, han mostrado que la incidencia ECV se multiplica por 2 y 3 respectivamente en hombres y mujeres diabéticas respecto a los que no lo son (Kannel & McGee, 1979). Además, el estudio MRFIT (*Multiple Risk Factor Intervention Trial*) reveló que la diabetes era el FRC de mayor importancia en comparación a HTA, hipercolesterolemia o tabaquismo (Stamler, 1993). Además, los diabéticos presentan una evolución más rápida de la ECV debido a la combinación de hiperglucemia sostenida, perfil lipídico aterógenico y aumento de la inflamación (Mazzone, 2008). La hiperglicemia aumenta la expresión de genes proinflamatorios en células endoteliales, monocitos y células musculares lisas, promoviendo la adhesión leucocitaria al endotelio mediante la sobreregulación de la expresión de las proteínas de adhesión de la superficie celular (E-selectin, VCAM-1, and ICAM-1) (Morigi, 1999).

2.3. HIPERTENSIÓN ARTERIAL

La HTA es el factor más frecuente de riesgo en el desarrollo de arteriosclerosis (Mata López, 2001). Un incremento de 20 mm Hg en la PAS y 10 mm Hg en la PAD duplica el riesgo de mortalidad por ECV (Perk, 2009). En un metaanálisis sobre los resultados de nueve estudios epidemiológicos internacionales (Mahon Stephen, 1990), con un seguimiento a 10 años, se observó que la correlación entre la PAD y la enfermedad coronaria y cerebrovascular era lineal, encontrando que una reducción de 7,5 mmHg en la PAD puede conllevar una reducción del riesgo coronario de casi un 30% (Stamler, 1993). Fisiopatológicamente, la HTA provoca la activación del endotelio (Brown y Hu, 2001), lo que conduce al engrosamiento de la pared arterial y por tanto, al desarrollo de la placa de ateroma por la activación de moléculas de adhesión (ICAM y VCAM) e infiltración de células sanguíneas a la pared vascular (BNF, 1992). Además, la HTA es responsable de la reducción en la producción del ON en el

endotelio, facilitando así la adhesión leucocitaria (Komatsu y cols., 1997; Griendling, 1997).

2.4. SOBREPESO Y OBESIDAD

Obesidad y arteriosclerosis son dos procesos multifactoriales entre los que existen numerosos puentes de unión que explican, en parte, la mayor morbimortalidad cardiovascular del obeso. La obesidad desencadena una cascada de alteraciones (resistencia a la insulina, intolerancia a la glucosa, cambios en el perfil lipídico y actividad procoagulante y antifibrinolitica, HTA o altas concentraciones de leptina e insulina, deposición de grasa subepicárdica), que explicarían la asociación entre obesidad y ECV (Lee, 2008; Despres, 2008). También induce inflamación, con aumento de citocinas proinflamatorias y del estrés oxidativo y escasa producción de adiponectina, (Visser, 1999; Haslam, 2005). El estudio INTERHEART confirmó la importancia de la adiposidad abdominal como factor de riesgo de IAM, al tratarse de un factor predictivo de RCV más fiable que la cantidad total de grasa corporal (Yusuf, 2005). Como consecuencia, las personas obesas tienen el doble de riesgo de sufrir insuficiencia cardiaca que los sujetos con un IMC normal (IMC=18,5- 24,9) (Krum, 2009).

2.5. TABAQUISMO

El consumo de tabaco se asocia a un aumento de la progresión de la aterosclerosis y de los fenómenos trombóticos pero es potencialmente reversible. Así, en pacientes con ECV, el riesgo de padecer un nuevo episodio coronario disminuye a los 2-3 años de la cesación tabáquica al mismo nivel de los que nunca habían fumado, mientras que en prevención primaria el riesgo de un primer episodio coronario no se reduce

hasta el nivel de los no fumadores hasta pasados los 10 años de cesación del hábito tabáquico (Wood, 1998).

El tabaquismo se asocia con concentraciones elevadas de colesterol total y bajas de HDL-colesterol, así como con una hipertrigliceridemia (HTG) postpandrial (Kabagambe, 2009), mayor agregación plaquetaria y actividad vasomotora (Glantz, 1995). La acción proaterogénica de los productos derivados del humo del tabaco se ejerce fundamentalmente sobre el tejido elástico de la pared arterial disminuyendo la cantidad de elastina y aumentando la actividad de las elastasas (Francès, 1991).

3. FACTORES DE RIESGO VASCULAR EMERGENTES

La existencia de ECV en sujetos sin FRC clásicos, ha sugerido la existencia de otros factores "nuevos" que pueden contribuir al desarrollo de la ECV. Se han estudiado los siguientes:

3.1. HIPERTRIGLICERIDEMIA

La HTG se debe a un aumento de la síntesis hepática de las lipoproteínas de muy baja densidad (VLDL), asociándose a niveles bajos de HDL-colesterol, y a la formación de partículas LDL, que son muy aterogénicas lo que justifica que la HTG sea un FRCV independiente (Ros y Laguna, 2006).

En los últimos años se ha puesto de manifiesto que la hipertrigliceridemia (HTG) también se asocia ECV. Así, estudios observacionales han mostrado la relación existente entre concentraciones elevadas de triglicéridos (TG) y ECV. Así, en el *estudio de Copenhague* (Jeppesen, 1998) con una muestra de 2906 varones entre los 53 y 74 años sin evidencia de ECV, y seguidos durante 8 años, se observó una

relación positiva entre niveles elevados de TG y riesgo de ECV. En el estudio PROCAM (*Prospective Cardiovascular Munster Study*) se observó una correlación positiva y significativa entre el número de eventos cardiacos (IAM fatal, no fatal y muerte súbita) y la concentración de TG en sangre superior a 200 mg/dl (Assmann, 1992). También, un metanálisis de 17 estudios prospectivos (Hokanson, 1996), con una muestra de 46413 varones y 10864 mujeres, mostró un incremento del RCV, de un 30% y un 75% respectivamente, cuando se asociaban concentraciones altas de TG. Ensayos clínicos como el *Helsinki Heart Study* también observó la relación HTG - niveles bajos de HDL como FRCV, particularmente en la Cardiopatía Isquémica (Manninen, 1992). Por otra parte, numerosos estudios han confirmado que la ingesta de omega-3 con pescado azul se asocia a menor incidencia de cardiopatía isquémica, aceite de pescado o cápsulas de aceite concentrado de pescado, tiene la capacidad de reducir los TG. Una revisión de 65 estudios clínicos aleatorizados y controlados con placebo en humanos, concluyó que la ingesta de un promedio de 3-4 g de omega-3 de origen marino reducía las cifras de TG un promedio de 25-35%, lo cual se asociaba con aumentos del cLDL de un 5 a 10% y del cHDL de un 1-3% y por tanto, a un menor riesgo de IAM (Ros y Laguna, 2006).

3.2. HIPERHOMOCISTEINEMIA

Numerosos estudios epidemiológicos y meta-análisis (Rasouli, 2005) demuestran que la homocisteína plasmática total (tHcy) constituye un factor de riesgo ECV y predice la mortalidad en pacientes con enfermedad coronaria arterial, así como la aparición de nuevos eventos cardiovasculares, independientemente de los factores de riesgo tradicionales. Además, la presencia de hiperhomocisteinemia (tHcy>12 µmol/L) predice de forma independiente la progresión de la placa coronaria evaluada mediante la tomografía computarizada por haz de electrones (Rasouli, 2005). Según los datos

de un metaanálisis, por cada 4 µmol/l de aumento en la concentración de homocisteína se produce un aumento del riesgo relativo de enfermedad coronaria de un 32% (Boushey, 1995). También se ha visto que concentraciones elevadas de homocisteína plasmática (> 14 µmol/l) un parecen ser un factor de riesgo más fuerte (1,9 veces) para la mortalidad en pacientes diabéticos tipo 2 que en sujetos no diabéticos (Hoogeven, 2000).

Los mecanismos a través de los cuales la homocisteína podría causar daño vascular son: a) aumento del estrés oxidativo, que contribuye a la disfunción endotelial y fibrosis miocárdica (Nappo, 1999; Chambers, 1999); b) reduciendo la disponibilidad de ON y por efecto directo promitogénico en las CML (Ungvari, 2003); c) Aumentando la adhesividad plaquetaria, la oxidación del LDL col y su depósito en la pared arterial, y activando la cascada de la coagulación (Bensusan, 2011).

3.3. LIPOPROTEÍNA A

La lipoproteína (a) o Lp(a) es una molécula que tiene una estructura similar a la del LDL -colesterol incluyendo su núcleo rico en ésteres de colesterol y una molécula de apolipoproteína B-100 (ApoB 100). La Lp(a) contiene además una glicoproteína, la Apo(a), que se relaciona con la ApoB a través de una unión disulfuro. El exceso de Lp(a) está asociado con un elevado RCV (Bensusan, 2011). Un metaanálisis de 27 estudios prospectivos con un seguimiento medio de 10 años mostró que niveles superiores de Lp (a) a 30 mg/dl incrementaron aproximadamente 60% veces el riesgo de ECV (Danesh, 2000). En un estudio de seguimiento realizado recientemente en el que se incluyeron casi 10.000 individuos pertenecientes a población general, el aumento de las concentraciones de Lp(a) se asoció a mayor mortalidad cardiovascular para ambos sexos después de ajustar los resultados por otros factores de riesgo sin existir un valor umbral para este efecto (Kamstrup, 2008; Merino-Ibarra, 2007).

Sin embargo, no está claro aún si el aumento del riesgo de ECV asociado a la Lp(a) es independiente de los factores de riesgo tradicionales, especialmente de las concentraciones del colesterol LDL. Tampoco si esta lipoproteína podría ser un buen marcador pronóstico de ECV ya que la mayoría de los pacientes con niveles elevados de Lp(a) son diabéticos o tienen niveles elevados de LDL- colesterol. Tampoco hay estudios aleatorizados que hayan demostrado que el descenso de las concentraciones de Lp(a) disminuya el RCV (Bensusan, 2011).

La Lp(a) podría promover ECV a través de 2 mecanismos: 1) Favoreciendo la trombogénesis al tener una estructura similar al plasminógeno; 2) Facilitando la aterogénesis a través de su función con LDL- colesterol (Bensusan, 2011).

Por otro lado, la Lp(a) está asociada con elevados niveles del inhibidor PAI-1 y disminuye la actividad del activador tisular del plasminógeno (t-PA). Estos efectos promueven la trombosis e inhiben la fibrinólisis. Parece ser, que la Lp(a), al igual que la molécula de LDL-colesterol, podría ser captada por los macrófagos para terminar formando parte de la placa aterosclerótica (Bensusan, 2011).

3.4. APOLIPROTEÍNA A1 y APO B

Las apolipoproteínas (Apo) son componentes proteicos estructurales de las lipoproteínas. La Apo A1 es el principal componente proteínico de las HDL, que eliminan el colesterol de las células y, por tanto, tienen un efecto de protección frente a la ateroesclerosis (Bhatnagar, 1997). La Apo B se le llama también Apo B100. La Apo B es el principal componente proteínico de las LDL-colesterol, que transportan el colesterol a las células y, consecuentemente, contribuyen a la formación de las placas ateroscleróticas en las arterias (Rifai, 1999).

Concentraciones elevadas de Apo B se asocian a ECV, debido a la estrecha relación entre la Apo B y el grado de la aterosclerosis. Los estudios epidemiológicos demuestran una estrecha correlación entre las concentraciones bajas de HDL o Apo A1 y la prevalencia de las enfermedades coronarias. Se ha planteado que la razón apolipoproteína B / apolipoproteína A1 (Apo B/Apo A1) como un buen predictor de ECV (Wilson, 1999) como así lo demuestra el estudio INTERHEART (Moqueen, 2008). En otro estudio, los niveles de Apo A1 y Apo B fueron significativamente superiores a los mostrados por los lípidos convencionales y otros FRC (tabaquismo, dislipidemia, hipertensión, obesidad, diabetes y PCR) para la predicción de muerte por ECV (Sierra-Johnson, 2008). El *estudio AMORIS* concluyó que la razón ApoB/ApoA1 debería ser incluida en la evaluación del RCV (Walldius, 2001).

3.5. MARCADORES INFLAMATORIOS

La evidencia que demuestra que la aterosclerosis es una reacción inflamatoria de baja intensidad de la pared vascular ha conducido al estudio de diferentes moléculas inflamatorias como predictoras de ECV (**tabla 5**):

Tabla 5. Marcadores inflamatorios séricos emergentes predictores de ECV.

PCR	CD40L
SAA	sP-Selectina
Citocinas	MIF
IL-6	Lp-PLA2
IL-18	Glutation peroxidasa
TNF-α	PAPP-A
Moléculas de Adhesión	Matriz metaloproteasas
sICAM, sVCAM, E-selectina	MPO
MCP-1	

3.5.1. hs-PCR

La PCR es reactante de fase aguda y actualmente reconocida como un importante factor predictor de ECV en el humano (Deveraj, 2003; Albert, 2000). Se produce casi exclusivamente en el hígado como parte de la respuesta aguda estimulada por la IL-6, y en menor grado, por el TNF-α, y la IL-1α. La PCR contribuye en la formación de la placa de ateroma a través de varios mecanismos. PCR induce disfunción endotelial atenuando la producción de ON (Verma, 2002), participa en el reclutamiento de monocitos al interior de la placa (Torzewski, 2000), facilita la captación de LDL por los macrófagos (Zwaka, 2001) al activar el complemento (Torzewski, 1998; Bhakdis,1999) e inducir la producción de citocinas proinflamatorias y moléculas de adhesión como IL-6, ICAM-1, VCAM-1 y MCP-1 (Verma, 2002; Pasceri; 2001). Estudios prospectivos en humanos demuestran que la hs-PCR puede predecir de forma independiente la aparición de ECV (Koenig, 1999; Danesh, 2004). En un estudio con 543 hombres, sanos y seguidos durante 8 años, se observó una asociación entre niveles basales de hs-PCR más altos en pacientes que desarrollaron IAM o ACV (Ridker, 1997). Otros estudios como en el *Women's Health Study*, que incluyó 28.263 mujeres postmenopáusicas, el estudio ARIC (*Atherosclerosis Risk In Communities*) (Ballantyne, 2004), the *Nurses' Health Study*, and *Health Professionals Follow-up Studies* (Pai, 2008), el estudio MONICA (*Monitoring of Trends an Determinants in Cardiovascular Disease*) (Koenig, 1999), *the Reykjavik Health Study* (Danesh, 2004) y el *Cardiovascular Health* Study (Cushman, 2005) se obtuvieron resultados similares que los obtenidos por Ridker y cols. Los niveles plasmáticos de PCR también son capaces de discriminar entre enfermedad de la arteria coronaria estable e inestable (Koenig, 2006; Hermus, 2010).

3.5.2. CD40L

El ligando CD40 (CD40L) es una glicoproteína que se une a su receptor CD40 en la superficie de las células T, B, CML, CE, etc. (Armitage, 1992). Tras esta unión el receptor CD40 se activa y se internaliza dentro de la célula (Anand, 2003) uniéndose a miembros del receptor de TNF-α y dando lugar a toda una serie una expresión de genes proinflamatorios y proaterogénicos (TNF-α, IFN-γ, IL-12, IL-10, MMP, etc.) (Chen, 2006). El ligando CD40 soluble y su receptor CD40 están sobreexpresados en las lesiones ateroscleróticas, lo que conduce a un incremento de los mediadores del desarrollo de la placa de ateroma, y ambos contribuyen de manera importante al proceso inflamatorio que conduce a la aterosclerosis y a la trombosis.

A nivel clínico se ha visto que concentraciones elevadas de CD40L se observan en pacientes con IAM, angina inestable y tras intervenciones coronarias percutáneas, así como en mujeres aparentemente sanas que desarrollaron IAM o muerte cardiovascular, los niveles de sCD40L se han visto incrementados (Tanne, 2006). Estos mismos resultados fueron observados en el estudio CAPTURE en pacientes con síndrome coronario agudo (Koenig, 2007). Por otro lado, en personas aparentemente sanas, concentraciones elevadas de sCD40L se correlaciona un mayor riesgo de presentar lesiones ateroscleróticas y accidentes cardiovasculares (Schonbeck, 2001).

3.5.3. TNF-α

El TNF-α participa en el desarrollo de la aterosclerosis ya que está involucrado en la síntesis de proteínas de fase aguda, como la PCR, y de otras citocinas, como la IL-1, IL-6 o IL-8, y receptores de quimiocinas en las CE, así como en la inducción de la expresión de moléculas de adhesión (ICAM- 1 y VCAM-1), todas ellas consideradas como factores de riesgo en la ECV. En las placas de la arteria coronaria, el TNF-α provoca daño e inestabilidad (**tabla 2**) (Luo, 2010).

El TNF-α señaliza a través de dos receptores triméricos de membrana: los receptores I y II de TNF-α (TNFR-I y TNFR-II). Los valores elevados de TNF-α y de sus receptores solubles (TNF-RI y TNF-RII) en plasma se han asociado con cardiopatía isquémica siendo predictores independientes de mortalidad en la insuficiencia cardíaca. Más concretamente, un estudio sugirió que la forma soluble del TNFR- I era el principal predictor, tanto a corto como a largo plazo, de mortalidad y eventos cardiovasculares en pacientes que presentaban IAM (Fragoso-Lona, 2009).

3.5.4. MCP-1

Esta quimiocina es la principal encargada del reclutamiento de monocitos a los tejidos en que hay respuesta inflamatoria activa, como es la lesión aterosclerótica. Sin embargo, hay resultados contradictorios, por un lado en el estudio Orbofiban (de Lemos et al, 2003), concentraciones elevadas de MCP-1 se asociaron con riesgo de muerte o IAM después de 10 meses de intervención, mientras que, en el estudio MONICA / KORA Augsburgo (Herder et al, 2006), MCP-1 no pudo ser considerado predictor independiente de riesgo de muerte o IAM.

3.5.5. Moléculas de adhesión solubles

Como se ha comentado, las moléculas de adhesión son cruciales en el reclutamiento celular hacia el interior de la pared vascular y algunas de estas se han evaluado como posibles marcadores de ECV.

Diversos han estudios han mostrado que los sujetos sanos con concentraciones elevadas de ICAM-1 y VCAM-1 tienen más riesgo de IAM (Ridker, 1998; Ridker, 2000). El estudio ARIC (*Atherosclerosis in Risk Communities*) mostró que sICAM-1 era capaz de predecir los eventos coronarios y el desarrollo de aterosclerosis carotídea (Hwang, 1997). Otro estudio mostró que en los sujetos con ECV los niveles de ICAM-1 y

VCAM-1 eran mayores en los pacientes que experimentaron eventos cardiovasculares (Blankenberg, 2001). En pacientes con síndrome coronario agudo, se ha visto que sVCAM-1 era un predictor de futuros eventos cardiovasculares, pero no sICAM-1 (Mulvihill, 2001). El estudio PRIME evidenció que la sICAM-1 era predictor tanto de síndrome coronario agudo como de angina estable (Empana, 2008). También se ha reportado que las formas solubles de E-selectina están asociadas con la mortalidad en pacientes con enfermedad arterial coronaria (Fragoso-Lona, 2009) y que la P-selectina soluble se ha asociado como un factor de riesgo independiente de futuros eventos cardiovasculares en individuos aparentemente sanos y con enfermedad arterial coronaria (Ridker, 1998; Hajilooi, 2004).

3.5.6. Marcadores de estabilidad / inestabilidad de placa

IL-6

IL-6 es una citocina proinflamatoria (**tabla 5**) que también tiene propiedades proaterogénicas y que puede contribuir a la inestabilidad de la placa de ateroma. Ésta es producida por diferentes tipos de células en la placa aterosclerótica en los que amplifica la cascada inflamatoria y también es una citocina pro-coagulante (Koenig, 2006; Hermus, 2010). El aumento de expresión de IL-6 se muestra especialmente en las regiones de placa inestable. IL-6 es capaz de estimular los macrófagos para secretar MCP-1 y participa en la proliferación de CMLs y potencia la expresión sICAM-1 en las CEs (Koenig, 2007).

Varios estudios prospectivos han demostrado que las concentraciones basales de IL-6 elevadas es un potente predictor de futuros eventos CV (coronario o cerebral) en sujetos asintomáticos (Ridker y Rifai, 2000; Pradhan, 2002; Volpato, 2001). Estos mismos resultados se han obtenido en individuos de alto riesgo vascular (Ridker, 2000).

Asimismo, valores elevados de IL-6 circulante es un predictor de mortalidad en individuos con enfermedad arterial coronaria inestable (Pai, 2004). Diversos estudios han mostrado una asociación entre valores elevados de la IL-6 e IAM, lo que confirma nuevamente que la IL-6 es un marcador independiente de otras proteínas como la PCR para futuros eventos de IAM (Fragoso-Lona, 2009). En los pacientes con angina inestable, como en el estudio FRISC II, valores elevados de IL-6, 48 horas después de la admisión, se asociaron con un aumento de la morbilidad y mortalidad hospitalaria (Biasucci, 1999; Lindmark, 2001).

IL-10

La IL-10 es considerada la interleucina anti-inflamatoria (**tabla 5**) por excelencia, disminuye la función de los macrófagos e inhibe la producción de citocinas proinflamatorias (IL1-α, IL 1-β, IL-6, IL-8, TNF-α, o el INF-γ) (de Waal, 1991) y antiaterogénicas como IL-18 y de la MMP-9 (Kleemann, 2008; Fragoso-Lona, 2009). IL-10 también, inhibe la producción de ON e intermediarios de oxígeno en macrófagos, así como la adherencia de macrófagos (Moore, 1993). La IL-10 parece ser protectora desde las etapas iniciales de la aterosclerosis a las etapas más avanzadas. Estudios in vitro han puesto de manifiesto este efecto antiaterogénico y ateroprotector, ya que IL-10 es capaz de inhibir la adhesión de monocitos a células endoteliales (Tedgui, 2006; Kleemann, 2008; Fragoso-Lona, 2009). Mientras que la deficiencia de IL-10 en ratones C57/BL6 alimentados con una dieta con una dieta aterogénica promueve una precoz formación de lesión aterosclerótica caracterizada por una gran infiltración de células inflamatorias, mayoritariamente células T activadas, y por una elevada producción de citocinas proinflamatorias (Mallat y Besnard, 1999; Kleemann, 2008). Todos los efectos de IL-10 parecen que son mediados a través de la inhibición del factor nuclear NF-κβ.

Desde el punto de vista clínico, el estudio CAPTURE demostró que niveles elevados de IL-10 se asociaron con un mejor pronóstico en pacientes con síndrome coronario agudo y niveles elevados de PCR (Heeschen, 2003). Por el contrario, un nivel reducido de IL-10 es un buen marcador de placa inestable y por tanto de riesgo de síndrome agudo coronario, y además, resulta un buen marcador incluso después de que el evento haya finalizado.

IL-18

IL-18 promueve la respuesta inmune Th-1 y también aumenta la producción de MMPs de la matriz favoreciendo la inestabilidad de la placa (**tabla 5**). Se cree que el mecanismo por el cual IL-18 ejerce sus principales efectos proaterogénicos es de manera directa o bien induciendo la producción de IFN-γ (Trøseid, 2010), a través de su receptor el cual es capaz de activar a NF-κβ (De Martin, 2000). Como marcador de riesgo resulta controvertido, ya que estudios relativos a las asociaciones entre IL-18 y la aterosclerosis han mostrado que la expresión de IL-18 se encuentra incrementada, especialmente en las lesiones ateroscleróticas inestables (Mallat, 2001). Además, los valores séricos de IL-18 también han demostrado ser un factor pronóstico de futuros eventos coronarios (Blankenberg, 2003), y se correlacionan positivamente con el grosor de la íntima media carotídea (Korshunov, 2006). Sin embargo, los resultados de un gran estudio prospectivo, llevado a cabo en 1.229 pacientes con enfermedad coronaria angiográfica demostraron que los niveles aumentados de IL-18 al inicio del estudio se asociaron independientemente con la futura muerte CV durante una media de 3,9 años de seguimiento, pero a los 5,9 años, las concentraciones IL-18 ya no fueron predictivos de los resultados, cuestionando así su valor como un marcador de riesgo especialmente a muy largo plazo (Koenig, 2007).

En el estudio PRIME, las elevadas concentraciones de IL-18 en pacientes sanos al inicio del estudio se asociaron con un mayor riesgo de posteriores eventos

cardiovasculares. Sin embargo, el estudio de casos y controles MONICA / KORA Augsburgo no mostró asociación entre la concentración de IL-18 y eventos cardiovasculares en hombres o mujeres (Koenig, 2007). En resumen, IL-18 ofrece resultados controvertidos para ser utilizado como marcador pronóstico de riesgo cardiovascular.

Metaloproteasas y TIMP-1

Las MMPs son endopeptidasas dependientes de zinc que se expresan ampliamente en monocitos / macrófagos, células endoteliales, CMLs y fibroblastos. Las MMPs se clasifican en diferentes grupos según la organización del dominio y su especificidad por el sustrato. Las MMPs son capaces de degradar los componentes de la matriz extracelular lo que promueve el adelgazamiento de la capa fibrosa y la posterior desestabilización de las lesiones ateroscleróticas (**tabla 5**). Las *gelatinasas* (MMP-2 y -9) son capaces de degradar el colágeno tipo IV, V, y VII y elastina, mientras que las *estromelisinas* (MMP-3, -10, y -11) son activas contra los proteoglicanos y la elastina. Las *colagenasas* (MMP-1, -8, y -13) degradan los colágenos fibrilares tales como el colágeno de tipo I, II, III, y V. Las *matrilisinas* (MMP7), *metaloelastasas* (MMP12), metaloproteasas unidas a la membrana (MMP-14 a MMP-17). Las gelatinasas, estromelisinas y colagenasas son de particular interés con respecto a las placas ateroscleróticas (Koenig, 2007; Skjøt-Arkil, 2010). La presencia o actividad de las enzimas proteolíticas dentro de una placa no está necesariamente asociada con la inestabilidad, pero un desequilibrio entre las MMPs degradantes de la matriz y sus inhibidores tisulares TIMPs pueden conducir a la degradación de la matriz y desestabilización de la placa (Hermus, 2010; Hansson y Vasan, 2011). El desequilibrio MMP-TIMP que induce un descontrol en el balance de la matriz está relacionado con otras patologías como artritis, cáncer, enfermedad cardiovascular, nefritis, etc.) (Woessner, 1998).

En placas ateroscleróticas humanas inestables y en las regiones vulnerables de las placas, se ha observado una mayor expresión y concentración de MMP-9. Es sabido que altas concentraciones séricas de MMP-9 se asocian con aterosclerosis y es predictor de ECV y se asocia a mayor riesgo de accidente cerebrovascular y muerte cardiovascular (Skjøt-Arkil, 2010). Kai et al. fueron los primeros en estudiar los niveles plasmáticos de MMP-2 y MMP-9 en 33 pacientes con síndrome coronario agudo (SCA) y 17 pacientes con angina estable. Tanto, en los pacientes con SCA y como con angina estable, los niveles plasmáticos de MMP-2 fueron altos, mientras que los niveles de MMP-9 solo fueron elevados en pacientes con SCA, pero no en los pacientes con angina estable. Estos niveles elevados de MMP-9 fueron mantenidos 3 días, y solo volvieron a la normalidad transcurridos 7 días después del evento coronario agudo (Hermus, 2010).

Estos hallazgos indican que la MMP-2 juega un papel importante en aterosclerosis, pero es la MMP-9 quien parece tener una mayor implicación en la ruptura de la placa y por lo tanto, resulta un marcador más interesante pero se necesitan más estudios para probar o refutar su utilidad clínica para la evaluación de riesgos (Hermus, 2010).

Al igual que en caso de las MMPs, los niveles circulantes elevados de TIMP-1 presagian un peor pronóstico para la ECV (Hansson, 2011).

TGF-β1

Diversos autores han demostrado que varios componentes de las vías de señalización de la superfamilia de TGF-β (**tabla 5**) tienen importancia como marcadores pronóstico de la ECV. En los últimos años, se ha propuesto que el papel protector en la aterogénesis de la TGF- β1 se da mediante la inhibición de la migración y la proliferación de CML y macrófagos y ejerciendo un efecto protector en la función endotelial (Aihara, 2011). Se ha observado que el TGF- β1 disminuye la adhesividad

de las células endoteliales a los leucocitos y linfocitos, inhibiendo la expresión de VCAM-1, que es regulada por el TGF- β1 (Fragoso-Lona, 2009). Además, parece ser que el TGF-β1 reduce la producción de colagenasa y acelera la expresión de inhibidores tisulares de las MMPs, lo que conlleva a la inhibición total de la degradación de la matriz extracelular. Diversos estudios han postulado que las vías de señalización de TGF-β1 activado conducían a la estabilización de la placa coronaria en pacientes ateroscleróticos. Parece ser que esta estabilización vendría dada por un aumento de los niveles plasmáticos de TGF-β1 (Porreca, 2002; Cipollone, 2004; Fragoso-Lona, 2009). A pesar de que TGF-β1 parece tener un efecto positivo en la estabilizaciσn de la placa, hay estudios que han demostrado el efecto contrario, un efecto negativo sobre dicha estabilizaciσn (Aihara, 2011). TGF- β1 podría tener un efecto proinflamatorio, participando en la excesiva acumulación de matriz extracelular en las paredes de los vasos dañados, lo que es muy desfavorable para éstos (Dabek, 2006; Fragoso-Lona, 2009; Aihara, 2011). En resumen, TGF-β1 resulta un marcador de riesgo controvertido necesitándose más estudios para aclarar su papel en la ECV.

Tabla 5 Citocinas proaterógenicas y citocinas antiaterogénicas

	Función proaterógenicas	Función antiaterogénicas
Familia TNFR	TNF-α	
Familia IL-1 **Citocinas clase I**	IL-1 IL-18 IL-2 IL-4 IL-6 IL-12	IL-1ra IL-18BP IL-6
Citocinas clase II	IFN-γ	IL-10
Factores Hematopoyéticos	M-CSF	
Receptores Quimiocinas	MCP-1 IL-8 RANTES	
Familia TGFβ	TGF-1	TGF-1

4. DIETA MEDITERRÁNEA EN LA PREVENCIÓN DE LA ECV. BASES FISIOPATOLÓGICAS

El papel protector de la "Dieta Mediterránea" (DietMed) en la ECV se conoce desde la década de los 60 cuando se publicó el *Seven Countries Study* (Keys, 1961; Keys, 1980) que demostró una mayor adherencia a la dieta tiene mayores efectos protectores sobre la mortalidad global y la mortalidad de origen cardiovascular. A partir de aquí, diversos estudios epidemiológicos (Trichopoulou, 1997; Keys, 1986; Trichopoulou, 2005) han demostrado que una mayor adhesión a la DietMed no sólo se asocia con una menor mortalidad e incidencia de ECV, sino también a una reducción del riesgo de desarrollar síndrome metabólico, diabetes tipo 2, algunas enfermedades neurodegenerativas y cáncer. Más recientemente, el estudio PREDIMED (Estruch,

2013) ha demostrado que una intervención con DietMed suplementada con AOVE o frutos secos reduce la incidencia de eventos cardiovasculares en un 30% respecto a una dieta baja en grasas en sujetos sanos pero con elevado riesgo de padecer ECV.

Se considera que no existe una DietMed única, pero sí un patrón de dieta mediterránea (PDM), que se caracteriza por el alto consumo de frutas, verduras, cereales (preferentemente en forma de grano entero), legumbres y frutos secos, así como consumo moderado de pescado y mariscos, carnes blancas, huevos y productos lácteos (queso y yogur). Por el contrario, incluye pequeñas cantidades de carnes rojas y carnes procesadas, así como alimentos ricos en azúcares. El aceite de oliva es la principal fuente de grasa de la dieta. El consumo moderado de vino se recomienda preferentemente con las comidas, así como una adecuada ingesta de agua (Trichopoulou, 1997; Castro-Quezada, 2014). La DietMed se caracteriza por una ingesta relativamente alta en grasa (40% -50% del total de calorías diarias), de las cuales los ácidos grasos saturados (AGS) representan ≤ 10% y los ácidos grasos monoinsaturados (AGMIs), principalmente de aceite de oliva, es superior al 20% de las calorías totales. El PDM se caracteriza por una alta ingesta de ácidos grasos omega-3 cuyas principales fuentes de obtención son el pescado azul y los vegetales, así como un relación baja de omega-6: omega-3 de 2:1-1:1 comparada con la relación 14:1 de Europa (Trichopoulou, 1997; Kris-Etherton, 2001). La DietMed se caracteriza por un alto consumo de fibra dietética (Estruch, 2009) y una baja carga glucémica e índice glucémico (Vasto, 2012), poseer efectos anti-inflamatorios (Estruch, 2006), y compuestos antioxidantes propios de la dieta (Visioli, 2004; Pitsavos, 2005).

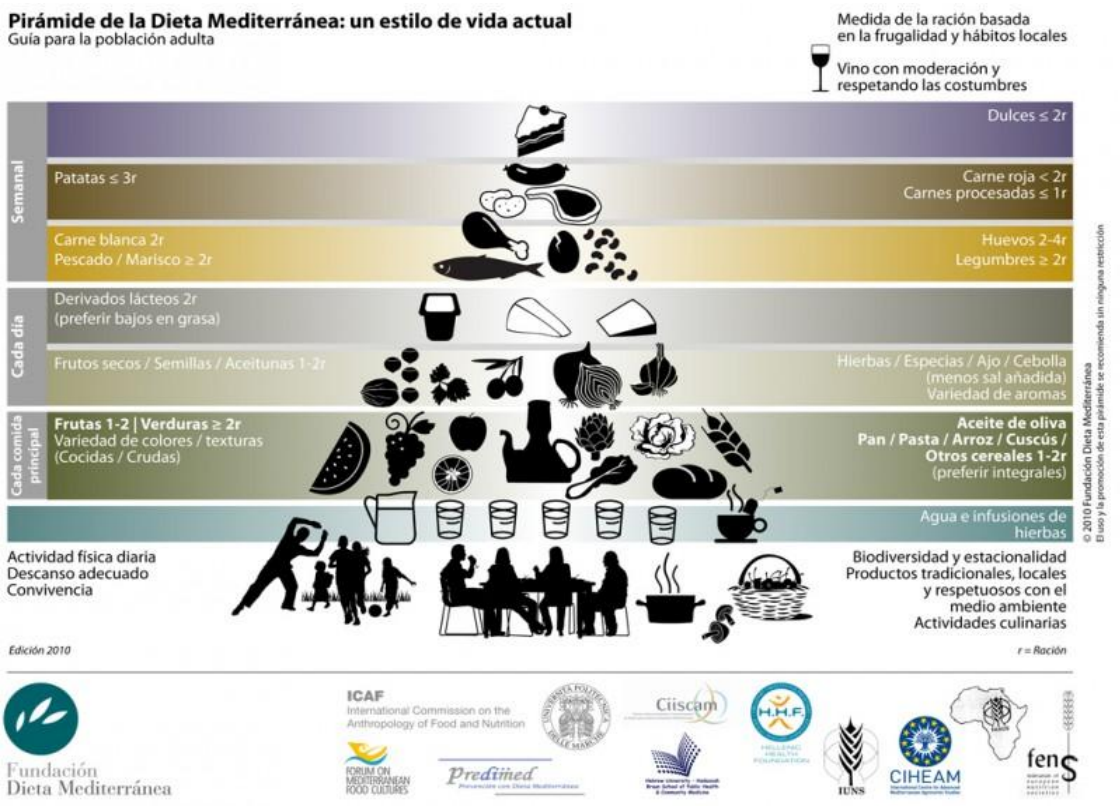

Figura 4. Pirámide nutricional. (Fundación dieta mediterránea)

4.1. ALIMENTOS DESTACADOS DE LA DIETA MEDITERRÁNEA

4.1.1. Aceite de oliva virgen extra

El aceite de oliva ha sido uno de los pilares básicos de la alimentación en la cuenca mediterránea. El aceite de oliva se caracteriza por ser un aceite rico en ácidos grasos monoinsaturados (AGMI) y con una concentración moderada en ácidos grasos saturados (AGS) y poliinsaturados (AGPI). El aceite de oliva es rico fundamentalmente en ácido oleico (AGMI), pero también contiene fitoesteroles y compuestos fenólicos como el tirosol y el hidroxitirosol (Galli, 2001). También tiene un alto contenido en α-tocoferol (52-87%), así como γ-tocoferol (15-20%) y β-tocoferol (7-23%). La vitamina E

o α-tocoferol, clave en mantener la defensa antioxidante de las células (Alarcón de Lastra, 2001).

4.1.2. Frutos secos

Las nueces, almendras, avellanas y otros frutos secos, como los piñones, son ingredientes comunes de la DietMed tradicional. Tradicionalmente los frutos secos han sido considerados alimentos de alta densidad energética debida principalmente a su elevado contenido graso.

Los frutos secos se caracterizan por el bajo contenido en AGS, y AGMI (fundamentalmente ácido oleico), que constituyen alrededor de la mitad de la grasa total en la mayoría de los frutos secos, con la excepción de las nueces. Éstas últimas son particularmente ricas en AGPI (ácido α-linolénico y ácido linoleico, fundamentalmente). El ácido α-linolénico (ω-3) tiene un notable efecto antiaterogénico. Los frutos secos también son ricos en arginina, ácido fólico, vitamina E y polifenoles antioxidantes, fitoesteroles y otros compuestos fitoquímicos como fitosteroles, carotenoides, compuestos fenólicos (como el resveratrol o los flavonoides, entre otros) y la melatonina, todos ellos con efectos beneficiosos para la salud (Ros, 2010).

4.1.3. Vino

El vino es una bebida de composición compleja que se obtiene del zumo de la uva exprimido y fermentado, que contiene más de 250 sustancias diferentes lo que le proporciona una variabilidad notable. Entre los componentes del vino se encuentran alcoholes, compuestos carbonílicos, ésteres, derivados nitrogenados, compuestos fenólicos y otros productos orgánicos e inorgánicos. El vino es un producto de uso común en la dieta mediterránea. En las últimos 30 años, se han publicado múltiples estudios que indican que tanto el consumo moderado de alcohol como de bebidas

fermentadas (vino fundamentalmente) podría tener efectos beneficiosos (protectores) sobre la salud como reducir de manera significativa la mortalidad global, la ECV, la incidencia de diabetes tipo 2, cánceres, enfermedades neurodegenerativas, y osteoporosis (Sacanella, 2011; Lamuela, 2006). Parece que el efecto cardioprotector del vino, no depende de un único compuesto sino del conjunto de sus componentes (Lamuela, 2006). Algunos de los elementos más importantes son los compuestos polifenólicos que se producen de forma natural y que determinan la pigmentación o color del vino, y a la vez contribuyen a sus aromas y sabores característicos (Lamuela, 2006).

Los polifenoles son los antioxidantes más abundantes en la dieta. Dentro de los polifenoles cabe destacar el *Resveratrol*. Es un componente que se encuentra fundamentalmente en la piel y en la semilla de la uva negra y pasa a los vinos durante la fermentación (Lamuela, 2006).

4.2. DIETA MEDITERRÁNEA EN LA PREVENCIÓN DE LA ECV Y OTRAS ENFERMEDADES CRÓNICAS. EVIDENCIAS CIENTÍFICAS

Varios estudios clínicos han demostrado que las dietas de estilo mediterráneo reducen la incidencia de factores de RCV que favorecen la ECV, tales como el síndrome metabólico (Kastorini, 2011), diabetes (Salas-Salvadó, 2011), hipertensión arterial (Doménech, 2014) o la dislipemia (Vincent-Baudry, 2005). Este patrón dietético también se asocia a menor riesgo de enfermedades degenerativas como cáncer (Gotsis, 2014) o alzheimer y demencia (Valls-Pedret, 2012).

4.2.1. Dieta Mediterránea y Enfermedad Cardiovascular

Actualmente está establecido que el aceite de oliva virgen extra tiene un papel preventivo y beneficioso en el tratamiento de las ECV. Distintos estudios, desde el estudio observacional *Seven Countries Study* (Keys, 1980), hasta estudios como el EUROLIVE y otros (Pérez-Jiménez, 2002; Covas, 2006; Machowetz, 2007), han puesto de manifiesto que el consumo de AOVE y de una dieta rica en AGMI conduce a una reducción del RCV. En la mayoría de los estudios llevados a cabo en sujetos sanos, la sustitución de grasa saturada por AGMI condujo a una disminución de los niveles de colesterol total y LDL-colesterol (Kris-Etherton, 1999), y que fue asociado a un aumento de los niveles de HDL-colesterol (Thomsen, 1999). Por otra parte, otros estudios han encontrado que el AOVE reduce la presión arterial sistólica en individuos hipertensos (Perona, 2004). Este efecto inicialmente fue atribuido a los AGMI pero un elevado número de autores han puesto de manifiesto que este efecto podría deberse a la presencia de sustancias antioxidantes (Vitamina E principalmente) en el AOVE (Visioli y Galli, 1998). También diversos estudios han puesto de manifiesto el efecto protector del AOVE frente a la oxidación de las LDL (Fitó, 2002; Marrugat, 2004; Visioli, 2005). El AOVE también parece tener un efecto sobre los procesos de inflamación que se desencadenan en las primeras etapas del proceso aterosclerótico. Así, un estudio a doble ciego en hombres adultos mostró que el consumo durante 2 meses de AOVE llevó a un descenso de ICAM-1 plasmático (Yaqoob, 1998). También estudios *in vitro* han mostrado un descenso de la expresión de VCAM-1 y NF-κβ (Massaro, 1999) así como una reducción de adhesión monocitaria a CE (Carluccio, 2003).

Los estudios realizados sobre el efecto de los frutos secos han mostrado que la frecuencia de consumo de los mismos se asocia inversamente con RCV, así los individuos que ingerían frutos secos 5 o más veces por semana presentaban una reducción del 50% en el riesgo de ECV en comparación a los que no consumían (Fraser, 1992). Más recientemente, los resultados de otros tres estudios de cohortes,

el Iowa Women's Health Study, el Nurses' Health Study, y el Physician's Health Study, han confirmado que la ingesta frecuente de frutos secos se asocia a un menor riesgo de ECV (Ellsworth, 2001; Hu, 1998; Albert, 2002). Un metaanálisis analizó los cuatro estudios citados, observó una reducción media del riesgo de muerte por enfermedad coronaria del 37% (RR: 0,63; IC 95%: 0,51-0,83) (Kelly y Sabaté, 2006). También, los estudios prospectivos realizados con amplias cohortes han evidenciado que el consumo frecuente de frutos secos conlleva una reducción riesgo de mortalidad por ECV o muerte súbita del 36-54% (Kelly y Sabaté, 2006). También, estudios clínicos de intervención dietética a corto y medio plazo en voluntarios sanos indican que la colesterolemia puede verse reducida por el consumo moderado y diario de frutos secos (Kris-Etherton, 2001).

Asimismo, se ha observado que el consumo moderado de alcohol (20 g y 10 g diarios en hombres y mujeres, respectivamente) reduce cerca de un 24% la mortalidad global (Estruch, 2014), reduce el riesgo de accidentes vasculares cerebrales en un 20%, IAM un 37% y vasculopatía periférica un 20-25%, e incluso se ha asociado a una menor incidencia de diabetes tipo 2, y una reducción de casi un 50% en el riesgo de desarrollar una insuficiencia cardiaca. Entre ellos podemos destacar el *Copenhagen City Heart Study,* el *Nurse's Health Study,* el *First large-scale study on mainland China* o el *Health Professionals Follow-up Study* son algunos de los estudios que ponen de manifiesto los efectos cardioprotectores del alcohol*,* mostrando una asociación negativa entre el riesgo relativo de IAM y el consumo moderado de bebidas alcohólicas (Sacanella, 2011; Lamuela, 2006; Chiva-Blanch, 2013).

4.2.2. Dieta Mediterránea y Diabetes

Recientemente, los estudios de intervención han confirmado el posible efecto beneficioso de la DietMed contra la diabetes. Esposito y cols. realizaron un ensayo

controlado aleatorizado en 215 pacientes con un nuevo diagnóstico de diabetes. Los pacientes fueron asignados al azar a una DietMed o una dieta baja en grasas. Este estudio mostró que la DietMed puede modificar favorablemente el estado glucémico, controlar los factores de RCV, y retrasar la necesidad de una terapia hipoglucemiante (Espósito, 2004). De una manera similar, otro estudio de intervención llevada a cabo por Estruch y cols. fue capaz de dar resultados comparables a los obtenidos en la población italiana (Espósito, 2004). En este estudio, 772 pacientes asintomáticos para la ECV fueron aleatorizados a dieta baja en grasa o DietMed. Después de sólo 3 meses de seguimiento, los sujetos que siguieron la dieta mediterránea habían logrado una reducción de la glucosa en sangre de 5,9 mg / dL (Estruch, 2006). Además, en un estudio de casos y controles obtenido de una población de aproximadamente 340.000 personas pertenecientes al estudio EPIC, los autores compararon 11.994 pacientes con un diagnóstico de diabetes con una población de 15.798 sujetos sin diagnóstico de diabetes, y demostrando que un mayor adherencia a la DietMed se asociaba con una reducción del 12% en la aparición de la diabetes (OR: 0.88, IC 95% 0,79-0,97) (Sofi, 2013). Salas-Salvadó y cols., observaron que la incidencia de diabetes se redujo en un 52% tras un seguimiento medio de 4 años (Salas-Salvadó, 2011).

4.2.3. Dieta Mediterránea y otras enfermedades

Varios estudios epidemiológicos han evaluado la relación entre el DietMed y el riesgo de cáncer. El estudio "*European Prospective Study and the Cancer and Nutrition (EPIC)"* para la cohorte griega de 23.349 hombres y mujeres, aparentemente sanos y con un tiempo de seguimiento medio de 8,5 años, pudo demostrar que una mayor adherencia a la DietMed se correlacionaba con un menor riesgo de mortalidad total y mortalidad por varias formas de cáncer (cuello, cabeza y mama) (Giugliano, 2006). Este mismo estudio pero con 142.605 hombres y 335.873 mujeres, mostró que el 4,7%

de los cánceres en los hombres y 2,4% en mujeres podrían haberse evitado si los pacientes hubiesen tenido una mayor adherencia al PDM (Gotsis, 2014; Sofi, 2013). Un reciente meta-análisis de 12 estudios prospectivos, señaló una reducción significativa del 6% por cada aumento de 2 puntos en la puntuación de adherencia a la DietMed para evaluar la mortalidad por cáncer (Sofi, 2010).

Por otro lado, una mayor adherencia a una DietMed se ha asociado con una reducción del deterioro cognitivo del 68% (Gotsis, 2014; Sofi, 2013) y del riesgo de padecer depresión en un 60% independientemente de la edad del paciente así como demencia o Alzheimer (Gotsis, 2014; Valls-Pedret, 2012; Sofi, 2013). En una reciente revisión, una mayor adherencia a la MedDiet se asoció con una mejor función cognitiva, una menor tasa de deterioro cognitivo, y reduce el riesgo de enfermedad de Alzheimer en nueve de 12 estudios analizados, mientras que los resultados para el deterioro cognitivo leve fueron inconsistentes (Lourida y col., 2013).

5. DIETA MEDITERRÁNEA EN LA PREVENCIÓN DE LA ATEROSCLEROSIS. BASES FISIOPATOLÓGICAS

Hay suficiente evidencia científica de que varios componentes fundamentales de la DietMed modulan favorablemente diferentes mecanismos fisiopatológicos implicados en la formación de la placa de ateroma (**Figura 5**).

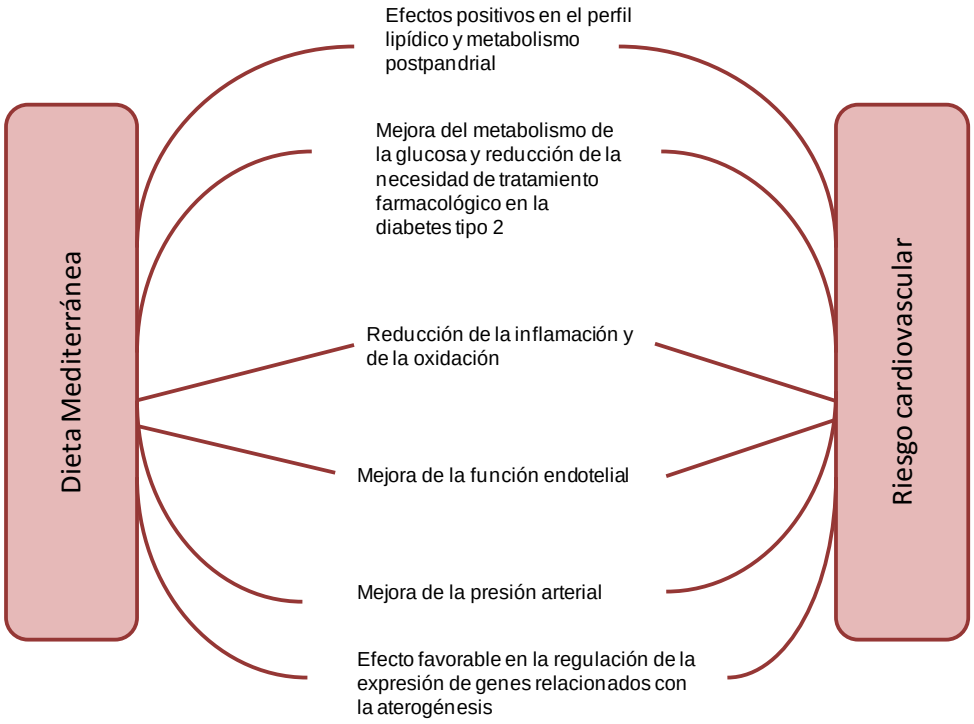

Figura 5. Mecanismos fisiopatológicos asociados a arteriosclerosis regulados por la dieta mediterránea. Adaptado de Delgado, 2011

Hasta ahora, el efecto beneficioso de la DietMed contra la enfermedad ECV se ha atribuido a sus efectos en el control de FRC clásicos. Así, varios estudios clínicos han demostrado que las dietas de estilo mediterráneo son protectoras contra el desarrollo de factores de riesgo prevalentes que promueven las enfermedades del corazón, tales como el síndrome metabólico (Kastorini, 2011; Babio, 2013), diabetes (Salas-Salvadó, 2011; Juanola-Falgarona, 2013), la hipertensión (Toledo, 2009; Doménech, 2014) o la dislipemia (Vincent-Baudry, 2005). Recientemente, algunos autores han sugerido que un efecto antiinflamatorio en la pared vascular puede ser otro mecanismo importante para explicar la relación entre el DietMed y baja mortalidad cardiovascular (Esposito, 2004). De hecho, la evidencia adicional de los ensayos clínicos han sugerido que la DietMed reduce la inflamación vascular (Esposito, 2004; Mena, 2008), el estrés

oxidativo (Marín y Yubero, 2013; Mitjavila, 2013), y la disfunción endotelial (Rallidis, 2009; Marín, 2011; Marín, 2013), que son factores que intervienen en el desarrollo de la aterosclerosis. Curiosamente, también se ha informado de que la DietMed puede modular favorablemente la expresión de genes pro-aterogénicos (Llorente-Cortés, 2010).

Por lo tanto, parece ser que los beneficios de la DietMed sobre la salud se podrían explicar mediante su actuación sobre los factores de riesgo clásico por un lado, y su efecto antiiflamatorio por el otro, habiendo diversos ejemplos que así lo corroboran. En este sentido, el estudio observacional ATTICA observó en 1.514 hombres griegos y 1.528 mujeres una mayor adherencia a una DietMed se correlacionaba con un descenso de los niveles de PCR (- 20%) y de IL-6 (-17%) (Sánchez-Taínta, 2008; Chrysohoou, 2004). En el *"Nurses' Health Study",* una mayor adherencia a la DietMed se asoció con menores concentraciones de los biomarcadores de inflamación y disfunción endotelial (PCR, IL-6, ICAM-1 y VCAM-1) (Athyros, 2011). Un patrón similar a un PDM se asoció inversamente con las concentraciones de PCR en plasma y E-selectina, mientras que un patrón occidental, con una mayor ingesta de carne roja, dulces, patatas fritas y cereales refinados, se asoció positivamente con concentraciones plasmáticas de PCR, IL-6, E -selectin, ICAM-1 y VCAM-1 (López-Garcia, 2004). Se ha referido que una dieta rica en nueces mejora la vasodilatación dependiente del endotelio y reduce la concentración sérica de VCAM-1 (Ros, 2004). Un reciente meta-análisis que incluyó un total de 17 ensayos informó que una mayor adherencia a la DietMed se asociaba con una mayor reducción de las concentraciones plasmáticas de IL-6 y de PCR, así como una mejora en la función endotelial (Schwingshackl , 2014) . Además, un grupo de pacientes con síndrome metabólico que siguieron un PDM mostraron una reducción de las concentraciones séricas de PCR, IL-6, IL-7 y IL-18, así como, una reducción de la resistencia a la insulina y mejora de la función endotelial (Esposito, 2004). Otros estudios han mostrado un aumento de

la expresión de la COX-2 y del receptor LDL-colesterol y de la expresión de MCP-1 en 49 individuos asintomáticos con alto riesgo de ECV después de una intervención de 3 meses con el DietMed (Llorente-Cortés, 2010). Además, la DietMed también puede ejercer, a corto plazo, un efecto modulador sobre la expresión de genes relacionados con la estabilidad de la placa, tales como la MMP-9, en una población de edad avanzada con alto riesgo de ECV (Camargo, 2012). La DietMed es capaz de mejorar de la disfunción endotelial en individuos sanos de edad avanzada después de una intervención de 4 semanas, aumentando la producción de las células progenitoras endoteliales y la disminución de la liberación de micropartículas endoteliales (Ruiz-Canela, 2014). En el estudio PREDIMED (Estruch, 2006), se analizaron los cambios observados en las concentraciones séricas de 4 moléculas solubles de adhesión (sICAM-1, sVCAM-1, IL-6 y PCR) sobre 772 participantes de alto RCV, y tras 3 meses de intervención dietética con DietMed suplementada con AOVE o frutos secos. Los resultados mostraron una disminución significativa de las concentraciones plasmáticas de IL6, sVCAM-1 e sICAM-1 para los 2 grupos de DietMed mientras que las concentraciones plasmáticas de PCR sólo disminuyeron en el grupo de DietMed suplementado con AOVE. En otro subestudio del ensayo PREDIMED, con 106 sujetos con alto RCV mostró una regulación sobre la expresión de monocitos CD49d y CD40 y una disminución de sICAM-1 para ambas DietMeds y una disminución de sVCAM-1, IL-6 y PCR para la DietMed suplementada con AOVE después de 3 meses con intervención dietética (Mena, 2008). Por otro lado, a nivel de inflamación, estudios experimentales y clínicos han demostrado que el aceite de oliva es capaz de regular la expresión endotelial de VCAM-1, ICAM-1 así como de E-selectina (Dell'Agli, 2006) y disminuir las concentraciones plasmáticas de sICAM-1, sVCAM-1, sE-selectina, IL-6, y la PCR en pacientes de alto RCV (Carluccio, 2003; Fitó, 2008).

El efecto protector de la DietMed puede ser debido a ciertos los componentes de la dieta. Así, la mayoría de los efectos beneficiosos del aceite de oliva sobre la salud son

atribuible a los AGMIs, concretamente, al ácido oleico, y a las propiedades antioxidantes de sus compuestos fenólicos como el hidroxitirosol y tirosol (Tripoli, 2005). Los AGMI son capaces de mejorar el perfil lipidico, reducir la concentración plasmática de colesterol total y LDL-colesterol, y mantener los niveles de HDL-colesterol. Además, las partículas de LDL enriquecidas con ácido oleico parecen ser más resistentes a la oxidación, lo que reduce el riesgo de aterosclerosis (Covas, 2006).

Los polifenoles del aceite de oliva ejercen su efecto beneficioso sobre el organismo disminuyendo la activación de mediadores proinflamatorios (como NF-κβ, VCAM-1), favoreciendo la producción ON y disminuyendo la activación leucocitaria (Fuentes, 2008; Serra-Majem, 2006; Covas, 2007; Pérez-Jiménez y Ruano, 2007; Leighton, 2007). El ácido oleico disminuye la activación endotelial y reduce la susceptibilidad de las lipoproteínas a ser oxidadas. Por su parte, el hidroxitirosol parece tener capacidad antiaterogénica al tener un efecto anti-inflamatorio, protector y prevenir la formación de placas de ateroma, entre otros. El mecanismo a través del cual actuaría sería inhibiendo la formación de especies reactivas de oxigeno y protegiendo del daño oxidativo a las LDL (Moreno, 2001; Visoli, 2002; Covas, 2006). Más recientemente se ha visto, que el hidroxitirosol es capaz de inhibir la peroxidación lipídica y mejorar el sistema de defensa antioxidante (Nakbi, 2010). También se ha mostrado que a mayor consumo de AGMI hay un menor riesgo de padecer hipertensión (Soriguer, 2003). También, varios estudios han demostrado que el AOV presenta mayores propiedades antiinflamatorias y una mayor protección sobre la peroxidación lipídica que el α-tocoferol de la dieta. En consecuencia, se cree que el AOV tiene mayores propiedades beneficiosas sobre el sistema cardiovascular que el aceite refinado (AR) (Puerta-Vázquez, 2004; Mataix, 2002).

Los estudios realizados sobre el efecto de los frutos secos y los niveles de HDL-colesterol han sido inconsistentes a pesar de que la relación colesterol total y HDL-colesterol tiende a disminuir. Parece ser que los frutos secos producirían un efecto neutro sobre las concentraciones de HDL-colesterol (Feldman, 2002).Varios estudios han mostrado que el consumo de nueces no afectan a la tendencia de las LDL a ser oxidadas (Ros, 2004). Por el contrario, Fitó y cols., observaron que una DietMed enriquecida con frutos secos durante 3 meses disminuía los niveles de LDL oxidada (Fitó y Cladellas, 2008). Así, los AGPI más concretamente, se les atribuyen propiedades tales como la reducción de presión arterial, la mejora de los parámetros lipídicos o de la función endotelial, prevención de la agregación plaquetaria o de la formación de la placa de ateroma e incluso propiedades antiarrítmicas.

Por otro lado, el efecto antiaterogénico atribuido a las bebidas alcohólicas y más concretamente al resveratrol (polifenol del vino) se asocia principalmente al cambio del perfil lipídico, principalmente, al incremento del HDL-colesterol, así como a la reducción de agregación plaquetaria, a un incremento de la fibrinólisis y/o a una reducción en la resistencia a la insulina. También éste puede tener un efecto protector sobre la oxidación de las LDL y estimulando la protección de sustancias vasodilatadoras. Al resveratrol se le ha reconocido su actuación como antioxidante, como antiinflamatorio y como fitoestrógeno, debido a su similitud estructural con el estrógeno sintético dietilestilbestrol (Lamuela, 2006; Cavaller, 2008). Más recientemente se han apuntado a nuevos mecanismos, como a su efecto antiinflamatorio sobre la pared arterial, una función endotelial más eficiente y una menor concentración plasmática de homocisteína (Sacanella, 2011; Lamuela, 2006).

La mejora en los factores de riesgo cardiovascular clásicos asociados a la intervención DietMed no pueden explicar por completo el efecto protector de la DietMed contra la ECV, quedando incógnitas por resolver como la duración del efecto anti-inflamatorio e

inmunomodulador de la DietMed y en qué etapas de la formación de la placa de ateroma actúa. Así, si este efecto aumenta, se mantiene o desaparece a medio-largo o largo plazo es hasta la fecha, desconocido. Al igual que el efecto de la DietMed sobre los biomarcadores que afectan a la vulnerabilidad de la placa o qué alimentos o nutrientes “clave” o si se trata del PDM podrían ser los responsables de los efectos ya observados. Para resolver algunos de estos interrogantes decidimos: evaluar los cambios a los 12 meses (medio plazo) y a los 3 y 5 años (largo plazo) de los marcadores inflamatorios de la aterosclerosis, así como de los marcadores de inestabilidad de la placa, y moléculas de adhesión leucocitaria en una población con alto riesgo de cardiovascular. Asimismo, pretendemos estudiar si el efecto observado se debe a un componente/nutriente específico de la DietMed o si por el contrario, se debe al PDM como tal, así como el papel anti-inflamatorio e inmunodulador de las adipocitocinas asociadas a las patologías cardiovasculares y el síndrome metabólico.

La población escogida para los estudios propuestos forma parte del mayor ensayo clínico llevado a cabo en prevención primaria, el estudio PREDIMED, donde se comparan los efectos de una DietMed suplementada con AOVE o frutos secos y una DBG en grasa en la ECV.

Los resultados de nuestro estudio podrían resultar de interés de Salud Pública ya que pretende demostrar la plausibilidad a la asociación epidemiológica entre DietMed y menor incidencia de ECV ya que parece ser que la DietMed podría modular la respuesta inflamatoria de la pared arterial y contribuir a estabilizar la placa de ateroma en aquellos sujetos de alto riesgo vascular sometidos a una DietMed suplementada con AOVE o FS además de una optimización en el control de los factores de riesgo vascular clásicos.

HIPÓTESIS

6. HIPOTÉSIS DE TRABAJO

6.1. HIPÓTESIS GENERAL

La DietMed reduce la morbi-mortalidad cardiovascular a largo plazo. Sin embargo, sus efectos sobre los factores de riesgo cardiovacular clásicos (HTA, diabetes tipo 2, obesidad, dislipemia, etc.) no explican la totalidad del efecto saludable y deben existir otros mecanismos probablemente antiinflamatorios, inmunomoduladores y antioxidantes que contribuyen a explicar el efecto preventivo de la DietMed contra la ECV. Así mismo queremos evaluar si esos potenciales efectos se mantienen a largo plazo.

6.2. HIPÓTESIS CONCRETAS

- Una intervención dietética con DietMed suplementada con aceite de oliva virgen extra o frutos secos, sobre individuos sin ECV pero con alto riesgo cardiovascular, producirá una mayor reducción de la expresión de moléculas de adhesión linfocitarias y monocitarias relacionadas con la aterosclerosis en comparación con aquellos individuos de similar riesgo cardiovascular pero que siguen una dieta baja en grasa.

- Una intervención dietética con DietMed suplementada con aceite de oliva virgen extra o frutos secos, sobre individuos sin ECV pero con alto riesgo cardiovascular, producirá un mayor efecto antiinflamatorio e inmunomodulador que se manifestará con unas concentraciones séricas de moléculas de adhesión endoteliales (sVCAM, sICAM, sP-Selectina y sE-Selectina) y de otros biomarcadores séricos inflamatorios (IL-6, PCR, TNF-α, IFN-γ, MCP-1, etc.) menores que en sujetos de similar riesgo cardiovascular pero que siguen una dieta baja en grasa.

- Una intervención dietética con DietMed suplementada con aceite de oliva virgen extra o frutos secos, sobre individuos sin ECV pero con alto riesgo cardiovascular, producirá una reducción en la concentración de marcadores de inestabilidad (IL-6, IL-18, MMP-9, TIMP-1) y un incremento en la concentración de marcadores estabilizadores (IL-10, TGF-β1) de la placa de ateroma que en aquellos individuos de similar riesgo cardiovascular pero que siguen una dieta baja en grasa.

- El efecto antiinflamatorio e inmunomodulador producido por la DietMed en una población con alto riesgo cardiovascular se mantiene a largo plazo (3 y 5 años) en paralelo a sus efectos clínicos ya demostrados.

- Una intervención dietética con DietMed suplementada con aceite de oliva virgen extra o frutos secos, sobre individuos sin ECV pero con alto riesgo cardiovascular, se asocia a menores concentraciones plasmáticas de otros marcadores inflamatorios como TNFR60 TNFR80 que en sujetos de similar riesgo cardiovascular pero que siguen una dieta baja en grasa.

- Existe una relación entre concentración de marcadores inflamatorios e ingesta de determinados alimentos considerados “clave” de una dieta mediterránea habitual (legumbres, pescado, verduras, etc.).

- Una intervención dietética con DietMed suplementada con aceite de oliva virgen extra o frutos secos, sobre individuos sin ECV pero con alto riesgo cardiovascular, producirá una disminución en las concentraciones séricas de ghrelina y adipocitocinas (leptina, adiponectina, adipsina, resistina, visfatina, y PAI-1), en comparación con aquellos sujetos de similar riesgo cardiovascular pero que siguen una dieta baja en grasa.

OBJETIVOS

7. OBJETIVOS

7.1. OBJETIVO GENERAL

El objetivo general del presente trabajo es evaluar si la DietMed suplementada con aceite de oliva virgen o frutos secos comparada con una dieta baja en grasas, en individuos sin ECV pero con alto riesgo cardiovascular, es capaz de modular la respuesta inflamatoria de la pared arterial implicada en la formación, progresión y ruptura de la placa de ateroma. Además analizaremos:

i) Si este efecto observado se mantiene a medio (1 año) y largo plazo (3 y 5 años).

ii) Si los cambios observados en algunos de los biomarcadores inflamatorios estudiados se correlacionan con la ingesta de determinados alimentos considerados “clave” (vino, frutas, verduras, etc.) en el patrón de dieta mediterránea.

iii) El papel de la visfatina y las principales adipocitocinas en las patologías cardiovasculares y el síndrome metabólico relacionándolo con los conceptos índice glicémico (IG) y carga glicémica (CG).

7.2. OBJETIVOS CONCRETOS

1) Evaluar el efecto de la dieta mediterránea suplementada con AOVE o FS sobre la expresión de moléculas de adhesión (CD11a, CD11b, CD49d) y CD40, en la superficie de linfocitos T y monocitos, que participan en la formación de la placa de ateroma en una cohorte de sujetos sin ECV pero con alto riesgo vascular a los 1, 3 y 5 años de iniciada la intervención dietética.

2) Analizar el efecto de la dieta mediterránea suplementada con AOVE o FS sobre la concentración sérica de moléculas de adhesión endotelial solubles (sVCAM-1, sICAM-1, sP-selectina y sE-selectina) y otros marcadores inflamatorios (IL-6, PCR, TNF-α, IFN-γ, MCP-1, etc.) predictoras de arteriosclerosis en una cohorte de sujetos sin ECV pero con alto riesgo vascular a los 1, 3 y 5 años de iniciada la intervención dietética.

3) Analizar el efecto de la dieta mediterránea suplementada con AOVE o FS sobre la concentración sérica de marcadores inflamatorios responsables de la inestabilidad (IL-6, IL-18, MMP-9, TIMP-1) y estabilidad (IL-10, TGF-β1) de la placa de ateroma en una cohorte de sujetos sin ECV pero alto riesgo enfermedad cardiovascular a los 1, 3 y 5 años de iniciada la intervención dietética.

4) Evaluar el efecto antiinflamatorio e inmunomodulador producido por la DietMed en una población sin ECV pero con alto riesgo cardiovascular se mantiene a largo plazo (3 y 5 años) en paralelo a sus efectos clínicos ya demostrados.

5) Analizar el efecto de la dieta mediterránea suplementada con AOVE o FS sobre los cambios de concentración sérica de marcadores inflamatorios (TNFR60 TNFR80) responsables del proceso aterosclerótico en una cohorte de sujetos sin ECV pero alto riesgo enfermedad cardiovascular a los 1, 3 y 5 años de iniciada la intervención dietética.

6) Evaluar si existe una relación entre concentración de marcadores inflamatorios e ingesta de determinados alimentos considerados “clave” de una dieta mediterránea habitual (legumbres, pescado, verduras, etc.).

7) Evaluar el efecto de la dieta mediterránea suplementada con AOVE o FS sobre los cambios de concentración sérica de ghrelina y adipocitocinas (adiponectina, adipsina, resistina, visfatina, y PAI-1) sobre individuos sin ECV pero con alto riesgo

cardiovascular en comparación con aquellos sujetos de similar riesgo cardiovascular pero que siguen una dieta baja en grasa.

RESUMEN DE LOS TRABAJOS

8. RESUMEN DE LOS TRABAJOS

TRABAJO 1:

Título: “The Mediterranean diet pattern and its main components are associated with lower plasma concentrations of tumor necrosis factor receptor 60 in patients at high risk for cardiovascular disease.”

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Antecedentes: La adherencia a una dieta mediterránea (DietMed) está asociada con un menor riesgo de enfermedad cardíaca coronaria. Sin embargo, los mecanismos moleculares implicados son poco conocidos hasta el momento.

Objetivos: El objetivo de este estudio fue comparar los efectos de dos DietMed con los de una dieta baja en grasa (DBG), sobre los niveles circulantes de biomarcadores inflamatorios relacionados con el proceso aterogénico.

Métodos: Un total de 516 participantes incluidos en el estudio de Prevención con Dieta Mediterránea (PREDIMED) fueron asignaron al azar en 3 grupos de intervención [DietMed suplementada con aceite de oliva virgen (AOVE); DietMed suplementada con frutos secos (FS); y DBG]. Al inicio del estudio y tras un año de intervención dietética, los participantes completaron los cuestionarios de frecuencia de alimentos (FFQ) y adherencia a la DietMed, y se midieron mediante ELISA las concentraciones plasmáticas de los siguientes marcadores inflamatorios: ICAM-1, IL-6, y dos receptores de TNF-α (TNFR60 y TNFR80).

Resultados: Después de un año de intervención dietética, los dos grupos de DietMed presentaron concentraciones plasmáticas más bajas de IL-6, TNFR60, y TNFR80 ($P< 0,05$), mientras que las concentraciones de ICAM-1, TNFR60, y TNFR80 aumentaron en el grupo de DBG ($P< 0,002$). Las diferencias entre los grupos mostraron que los x concentraciones plasmáticas más bajas de ICAM-1, IL-6, TNFR60, y TNFR80 en los participantes de los dos grupos de DietMed en comparación con los del grupo de DBG ($P \leq 0.028$). Cuando los participantes fueron clasificados en tertiles según los cambios observados al cabo de un año y según el consumo de alimentos seleccionados, se observa que aquellos participantes que se encuentran en el tertil para más alto, para el consumo de AOVE y verduras, presentaron una concentración plasmática TNFR60 menor que aquellos que se encontraban en el tertil más bajo ($P<0,002$). Además, los únicos cambios en el consumo de alimentos seleccionados que se asociaron con

cambios al cabo del año con la concentración media geométrica de TNFR60 fueron las de AOVE y verdura (P = 0,01).

Conclusiones: Este estudio sugiere que un MD reduce las concentraciones de TNF en pacientes con alto riesgo cardiovascular.

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The Mediterranean Diet Pattern and Its Main Components Are Associated with Lower Plasma Concentrations of Tumor Necrosis Factor Receptor 60 in Patients at High Risk for Cardiovascular Disease[1–4]

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Abstract

Adherence to a Mediterranean diet (MD) is associated with a reduced risk of coronary heart disease. However, the molecular mechanisms involved are not fully understood. The aim of this study was to compare the effects of 2 MD with those of a low-fat-diet (LFD) on circulating inflammatory biomarkers related to atherogenesis. A total of 516 participants included in the Prevention with Mediterranean Diet Study were randomized into 3 intervention groups [MD supplemented with virgin olive oil (MD-VOO); MD supplemented with mixed nuts (MD-Nuts); and LFD]. At baseline and after 1 y, participants completed FFQ and adherence to MD questionnaires, and plasma concentrations of inflammatory markers including intercellular adhesion molecule-1(ICAM-1), IL-6, and 2 TNF receptors (TNFR60 and TNFR80) were measured by ELISA. At 1 y, the MD groups had lower plasma concentrations of IL-6, TNFR60, and TNFR80 ($P < 0.05$), whereas ICAM-1, TNFR60, and TNFR80 concentrations increased in the LFD group ($P < 0.002$). Due to between-group differences, participants in the 2 MD groups had lower plasma concentrations of ICAM-1, IL-6, TNFR60, and TNFR80 compared to those in the LFD group ($P \leq 0.028$). When participants were categorized in tertiles of 1-y changes in the consumption of selected foods, those in the highest tertile of virgin olive oil (VOO) and vegetable consumption had a lower plasma TNFR60 concentration compared with those in tertile 1 ($P < 0.02$). Moreover, the only changes in consumption that were associated with 1-y changes in the geometric mean TNFR60 concentrations were those of VOO and vegetables ($P = 0.01$). This study suggests that a MD reduces TNFR concentrations in patients at high cardiovascular risk. J. Nutr. 142: 1019–1025, 2012.

Introduction

Coronary heart disease (CHD)[14] is the main cause of death worldwide, claiming 17.1 million lives in 2004 (1). Atherosclerosis is the main cause of CHD, and inflammation plays a key role from its onset to its progression to final lesions (2). In the earliest stages of CHD, vascular inflammation is activated by

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[3] Supplemental Tables 1–4 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[4] This trial was registered at www.controlled-trials.com as ISRCTN35739639.

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[14] Abbreviations used: CHD, coronary heart disease; ICAM-1, intercellular adhesion molecule-1; LFD, low-fat-diet; MD, Mediterranean diet; MD-Nuts, Mediterranean diet supplemented with mixed nuts; MD-VOO, Mediterranean diet supplemented with virgin olive oil; PREDIMED, Prevention with Mediterranean Diet; TNFR, TNF receptor; VCAM-1, vascular cell adhesion molecule-1; VOO, virgin olive oil.

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proinflammatory stimuli such as saturated fat intake, hypercholesterolemia, obesity, hyperglycemia, hypertension, and smoking, which induce the secretion of inflammatory cytokines that promotes the generation of endothelial adhesion molecules and other chemoattractants. These molecules are subsequently released to the circulation where they mediate the adhesion of circulating monocytes and lymphocytes to the vascular endothelium (2,3), leading to the formation of atherosclerotic lesions. New insight into the central role of inflammation in atherogenesis has linked inflammatory biomarkers such as vascular cell adhesion molecule-1 (VCAM-1), TNF, IL-1, IL-6, IL-18, and proteases (matrix metallopeptidase-9) to this disease (3). IL-6, IL-1β, and TNFα have also been associated with an increased risk of developing CHD (4). However, few studies have analyzed the effects of food interventions such as the Mediterranean diet (MD) on TNFα receptors. TNFα is a pleiotropic cytokine produced by activated monocytes and other cells (5) and has shown an ambivalent role in relation to CHD (5). TNF expresses its activity through 2 membrane receptors: TNF receptor (TNFR) 60, the 55–60 kDa TNFR 1, and the TNFR80, the 75–80 kDa TNFR 2. The activation of TNFR60 induces adhesion molecule expression and activates NF-κB and TNFR80 plays a role in T cell proliferation (6).

The prevention of atherosclerosis at early stages is based on healthy dietary and lifestyle habits that may diminish its progression. Epidemiological studies have suggested that the MD pattern and consumption of certain healthy foods such as legumes, grains, fruit and vegetables, olive oil, and wine may protect against CHD (7–9). Although the exact mechanisms of this protection are not fully understood, it has been suggested that functional compounds of some nutrients from the MD such as polyphenols from plant products (10–14) and fatty acids from vegetables or olive oil (15–18) may play a key role in these protective effects.

We therefore embarked on a study to evaluate the 1-y changes in plasma inflammatory markers [TNFR60, TNFR80, IL-6, and intercellular adhesion molecule-1 (ICAM-1)] in a free-living population with high risk of CHD following a MD and to study the relationship between these changes and modifications in their food intake. We studied a subpopulation from a larger feeding trial [the Prevention with Mediterranean Diet (PREDIMED) Study] designed to analyze the effects of 2 MD, one supplemented with virgin olive oil (MD-VOO), and one with mixed nuts (MD-Nuts), compared with a low-fat diet (LFD) control.

Methods

Participants and study design. The PREDIMED Study is a parallel-group, single-blind, multicenter, randomized, controlled, 5-y feeding trial assessing the effects of the Mediterranean diet (MD) supplemented with VOO (MD-VOO) or mixed nuts (MD-Nuts) on the primary prevention of CHD compared with a low-fat diet (LFD). Details of the study protocol were previously published (19,20). This substudy is a post hoc analysis using data already collected from 516 participants entering consecutively into the PREDIMED trial (Barcelona-Hospital Clinic, Navarra and Reus centers) in whom we determined plasma inflammatory biomarker concentrations in frozen stored samples. The study protocols were approved by the Institutional Review Boards of the centers and participants provided signed informed consent.

Eligible participants were community-dwelling men aged 54–79 y and women aged 58–79 y with no documented CHD who either had type 2 diabetes or at least 3 of the following risk factors: smoking, hypertension (blood pressure ≥140/90 mm Hg or treatment with antihypertensive drugs), LDL-cholesterol concentration ≥4.14 mmol/L (or treatment with hypolipidemic drugs), HDL-cholesterol concentration ≤1.03 mmol/L, BMI ≥25 kg/m^2, or a family history of early-onset CHD. Exclusion criteria were a history of previous CHD, any severe chronic illness, drug or alcohol abuse, history of allergy or intolerance to olive oil or nuts, or a low predicted likelihood of changing dietary habits according to the stages of change model.

Diets and physical activity. Participants were randomly assigned to 3 diet groups: LFD or 2 MD groups, one supplemented with VOO and the other with mixed nuts. For the LFD group, participants were advised to follow the AHA guidelines (21), which are oriented at reducing the intake of all types of fat. Participants in both MD groups were recommended to increase the intake of vegetables (≥2 servings/d), fresh fruit (≥3 servings/d), legumes, nuts, fish or seafood (≥3 servings/wk), and the use of olive oil for cooking and dressings as previously described (19,20,22,23). Participants assigned to the 2 MD groups received free provisions of 2 Mediterranean foods: participants assigned to the MD-VOO were provided with VOO (1 L/wk) and those assigned to the MD-Nuts were provided with mixed nuts (30 g/d, as 15 g walnuts, 7.5 g almonds, and 7.5 g hazelnuts). The fatty acid compositions of VOO and mixed nuts were previously reported (22). No specific recommendations for physical activity were given.

Measurements. At baseline and after 1 y of follow-up, participants completed a validated 14-item questionnaire assessing adherence to the MD (24), a validated 137-item validated FFQ (25), a validated version of the Minnesota Leisure Time Physical Activity Questionnaire for men (26) and women (27), and a 47-item questionnaire about education, lifestyle, history of illnesses, and medication use.

Trained personnel measured weight and height using calibrated scales and a wall-mounted stadiometer, respectively, waist circumference was determined midway between the lowest rib and the iliac crest using an anthropometric tape, and blood pressure was measured in triplicate with a validated semiautomatic oscillometer (Omron HEM-705CP) (19,20). In addition, fasting blood was collected and the plasma obtained was stored at −80°C until assay. Energy and nutrient intake estimates were obtained from Spanish food composition tables (20). All these procedures were repeated after 1 y of intervention.

The main outcome measurements were plasma concentrations of inflammatory biomarkers at baseline and after 1 y. ELISA assays were performed per participant in thawed plasma (kept at −80°C until analyzed) using commercial immunoassays kits for soluble ICAM-1, IL-6, TNFR60, and TNFR80 (Bender MedSystem). For the ELISA assays, the intra- and interassay CV ranged from 1.4 to 4.9% and from 2.0 to 8.6%, respectively.

Statistical methods. Statistical analyses were conducted using PASW Statistics 18 (version 18.0; SPSS). We estimated our sample size based on expected TNFR60 changes. Considering an expected decrease in TNFR60 of 0.15 μg/L in the intervention groups, an expected increase in TNFR60 of 0.15 μg/L in the LFD group, and an expected SD of 0.85 μg/L in all 3 groups, and assuming a 2-tailed α error of 0.05 and a statistical power of 0.8, our estimated sample size was 127 participants/group. Values for the baseline characteristics of the participants are expressed as means ± SD. Categorical variables are expressed as percentages. We transformed variables with a skewed distribution (ICAM-1, IL-6, TNFR60, and TNFR80) to their natural logarithm for analyses. Repeated-measures ANOVA was used to compare changes in inflammatory biomarkers and food variables, testing the effects of interaction of 2 factors: time as a within-participants factor with 2 levels (baseline and 1 y) and the groups of consumption (2 MD groups and LFD group), after adjustment for age, sex, BMI, smoking status, physical activity, research center, and drug use (aspirin and statins). To test the effects of individual factors, we calculated the differences between 1 y and baseline values for the molecules and then we applied an ANCOVA test after adjustment for the same variables as before. Participants from all groups were also categorized based on tertiles of 1-y changes in the consumption of 13 selected food groups (VOO, refined olive oil, nuts, vegetables, legumes, fruit, cereals, fish and seafood, meat and meat products, pastries, cakes or sweets, low-fat dairy products,

TABLE 1 Concentrations of circulating inflammatory molecules at baseline and after 1 y of intervention with MD-VOO, MD-Nuts, or LFD in patients at high risk for cardiovascular disease[1]

				Repeated-measures ANOVA[2]	*P* value for differences[3]		
	MD-VOO (*n* = 178)	MD-Nuts (*n* = 175)	LFD (*n* = 163)	Time x treatment	MD-VOO vs. MD-Nuts	MD-VOO vs. LFD	MD-Nuts vs. LFD
ICAM-1, *μg/L*							
Baseline	258 (245–271)	275 (261–290)	264 (251–279)				
1 y	248 (237–259)[b]	273 (261–285)[a]	288 (275–301)*,[a]	0.001	0.97	0.001	0.028
Change	−10 (−22 to −1)	−2 (−14 – 10)	24 (10–35)				
IL-6, *ng/L*							
Baseline	0.90 (0.76–1.07)	0.98 (0.84–1.14)	0.93 (0.78–1.10)				
1 y	0.67 (0.55–0.82)*,[b]	0.65 (0.54–0.77)*, [b]	1.06 (0.87–1.29)[a]	<0.001	1.00	0.004	<0.001
Change	−0.23 (−0.4 to −0.003)	−0.33 (−0.6 to −0.1)	0.13 (−0.1–0.4)				
TNFR60, *μg/L*							
Baseline	1.6 (1.5–1.8)	1.5 (1.3–1.6)	1.4 (1.3–1.6)				
1 y	1.4 (1.3–1.6)*,[b]	1.3 (1.2–1.4)*,[b]	1.8 (1.6–2.0)*,[a]	<0.001	1.00	<0.001	<0.001
Change	−0.2 (−0.4 to −0.1)	−0.2 (−0.3 to −0.1)	0.4 (0.2–0.5)				
TNFR80, *μg/L*							
Baseline	6.4 (6.0–6.8)	6.5 (6.1–6.9)	6.2 (5.8–6.6)				
1 y	5.8 (5.4–6.1)*,[b]	6.1 (5.8–6.5)*, [b]	6.8 (6.4–7.3)*,[a]	<0.001	0.81	<0.001	0.001
Change	−0.6 (−1.1 to −0.3)	−0.4 (−0.9 to −0.1)	0.6 (0.1–1.2)				

[1] Values are geometric means (95%CI). Means in a row with superscripts without a common letter differ, $P < 0.05$ (Bonferroni post hoc test). *Different from baseline, $P < 0.05$ (Bonferroni post hoc test). ICAM-1, intercellular adhesion molecule-1; LFD, low-fat diet; MD, Mediterranean diet; MD-VOO, Mediterranean diet supplemented with virgin olive oil; MD-Nuts, Mediterranean diet supplemented with mixed nuts; TNFR, TNF receptor.
[2] Data analyzed by repeated-measures 2-factor ANOVA ($P < 0.05$).
[3] Data analyzed by ANCOVA ($P < 0.05$). Repeated measures and ANCOVA were adjusted for age, sex, energy intake, BMI, smoking status, physical activity, research center, and drug use (aspirin and statins).

whole-fat dairy products, and wine), some nutrients (MUFA), and MD score. To study the interaction (time × treatment) between baseline and 1-y concentrations in plasma inflammatory molecules (TNFR60, TNFR80, ICAM-1, and IL-6) across tertiles, we used repeated-measures 2-factor ANOVA, and to study the effects of the individual factors we used an ANCOVA test, both performed after adjustment for age, sex, energy intake, BMI, smoking status, physical activity, research center, and drug use (aspirin and statins). In addition, to compare changes between baseline and 1-y concentrations of TNFR60 across tertiles in the 30 food groups, we fit a multivariate linear regression model obtaining the ratio of the geometric means of 1-y TNFR60 to baseline TNFR60 according to tertiles of changes in each of the 13 food groups after adjustment for the aforementioned variables. Then, we calculated the significance (*P*-trend) and the CI for the between-tertile differences. Significant interactions were analyzed by the simple-effect analysis. The multiple contrasts were adjusted by a Bonferroni post hoc test. Within- and between-group differences were expressed as estimated means and 95% CI. The significance level was set at $P < 0.05$.

Results

On average, participants were 66 y old and nearly one-half were men (**Supplemental Table 1**). Almost all the participants (>90%) were overweight or obese, 78.4% had hypertension, 62.0% had dyslipidemia, and 50.3% were diabetic. All these factors and characteristics were balanced among the 3 groups at baseline. We did not observe significant changes in medication treatments, body weight, or physical activity during the study period.

Food, energy, and nutrient intakes. The consumption of foods and nutrients in the participants of this substudy was similar to the overall PREDIMED population that followed a MD or a control LFD (23) (**Supplemental Tables 2** and **3**). We observed interactions between time and treatments ($P < 0.05$). The main dietary changes were the large increases in the consumption of VOO and mixed nuts in the corresponding MD groups ($P \leq 0.013$) and reciprocal decreases in the consumption of common olive oil in the MD-VOO and LFD groups ($P < 0.001$) (Supplemental Table 2). The increase in the intake of VOO was greater in the MD-VOO group than in the other 2 groups ($P < 0.001$). Compared with participants in the LFD group that decreased their consumption of nuts, those in the MD groups increased this intake ($P \leq 0.002$). Moreover, we observed higher increases in the MD-Nuts group than in the MD-VOO group ($P < 0.001$). The consumption of meat or meat products decreased after 1 y of intervention in the 3 groups ($P < 0.05$). The MD score increased by >2 points in the 2 MD groups after 1 y of intervention, whereas the increase in the LFD group was 0.4 points ($P \leq 0.013$). When we compared changes between groups, the MD score increased more in the MD groups than in the LFD group ($P < 0.001$). Energy intake increased in the MD-Nuts group ($P = 0.003$). In contrast, total energy and protein intakes decreased in the LFD group ($P < 0.001$) (Supplemental Table 3). Both MD groups also increased MUFA and PUFA intakes ($P < 0.001$), whereas the LFD group decreased their SFA, MUFA, and PUFA intakes ($P < 0.001$). α-Linolenic acid and marine (n-3) fatty acid intakes increased after 1 y of intervention in the 2 MD groups ($P \leq 0.03$) and decreased ($P = 0.013$) or did not change in the LFD group, respectively. The estimated energy expenditure from physical activity was similar in the 3 groups at baseline and after 1 y (data not shown).

Circulating inflammatory biomarkers. We observed interactions between time and treatment ($P \leq 0.001$) in the molecules

TABLE 2 Concentrations of circulating inflammatory molecules at baseline and after 1 y in all participants by tertile of change in consumption of selected foods and nutrients[1]

				Repeated-measures ANOVA[2]	*P* value for differences[3]		
	Δ Foods and Nutrients Tertiles			Time x treatment	1 vs. 2	1 vs. 3	2 vs. 3
	Δ VOO consumption tertiles, *g/d*						
	1 (*n* = 131) (≤ −0.3)	2 (*n* = 125) (−0.3–24)	3 (*n* = 128) (≥24)				
TNFR60, *μg/L*							
Baseline	1.4 (1.2–1.5)[b]	1.4 (1.3–1.6)[b]	1.8 (1.6–2.0)[a]				
1 y	1.4 (1.3–1.6)	1.5 (1.3–1.7)	1.5 (1.4–1.7)*	0.002	1.00	0.008	0.005
Change	0 (−0.1–0.2)	0.1 (−0.03–0.2)	−0.3 (−0.5 to −0.1)				
	Δ Nut consumption tertiles, *g/d*						
	1 (*n* = 132) (≤ −3.7)	2 (*n* = 127) (−3.7–9.8)	3 (*n* = 125) (≥9.8)				
TNFR60, *μg/L*							
Baseline	1.6 (1.4–1.8)	1.5 (1.3–1.7)	1.5 (1.3–1.6)				
1 y	1.6 (1.5–1.8)	1.5 (1.4–1.7)	1.3 (1.2–1.5)	0.08	1.00	0.14	0.18
Change	0 (−0.1–0.2)	0 (−0.2–0.1)	−0.2 (−0.3 to −0.02)				
	Δ Vegetable consumption tertiles, *g/d*						
	1 (≤ −24.5)	2 (−24.5–62.7)	3 (≥62.7)				
	(*n* = 101)	(*n* = 112)	(*n* = 121)				
TNFR60, *μg/L*							
Baseline	1.5 (1.3–1.7)	1.4 (1.3–1.6)	1.7 (1.5–1.9)				
1 y	1.6 (1.4–1.8)	1.4 (1.3–1.6)	1.5 (1.3–1.6)*	0.016	0.89	0.013	0.19
Change	0.1 (−0.1–0.2)	0 (−0.2–0.1)	−0.2 (−0.3 to −0.01)				
	Δ MUFA consumption tertiles, *g/d*						
	1 (*n* = 135) (≤22.5)	2 (*n* = 122) (22.5–38.1)	3 (*n* = 127) (≥38.1)				
TNFR60, *μg/L*							
Baseline	1.4 (1.2–1.6)	1.4 (1.3–1.6)	1.8 (1.6–2.1)				
1 y	1.5 (1.3–1.7)	1.4 (1.3–1.6)	1.6 (1.4–1.8)	0.10	0.57	0.10	0.54
Change	0.1 (−0.1–0.3)	0 (−0.2–0.1)	−0.2 (−0.4 to −0.04)				
	Δ MD score tertiles						
	1 (*n* = 124) (≤0.9)	2 (*n* = 127) (0.9–2.4)	3 (*n* = 134) (≥2.4)				
TNFR80, *μg/L*							
Baseline	6.6 (6.1–7.0)	6.1 (5.7–6.6)	6.5 (6.1–6.9)				
1 y	6.6 (6.2–7.0)[a]	6.4 (6.0–6.8)[ab]	5.9 (5.5–6.3)*,[b]	0.006	1.00	0.07	0.006
Change	0 (−0.5–0.7)	0.3 (−0.4–0.7)	−0.6 (−1.2 to −0.3)				

[1] Values are geometric means (95% CI). Means in a row with superscripts without a common letter differ, $P < 0.05$ (Bonferroni post hoc test). *Different from baseline, $P < 0.05$ (Bonferroni post hoc test). Dif, differences between 1 y and baseline; MD, Mediterranean Diet; TNFR, TNF receptor; VOO, virgin olive oil.
[2] Data analyzed by repeated-measures 2-factor ANOVA ($P < 0.05$).
[3] Data analyzed by ANCOVA ($P < 0.05$). Repeated measures and ANCOVA were adjusted for age, sex, energy intake, BMI, smoking status, physical activity, research center and drugs (aspirin and statins).

analyzed (**Table 1**). After the intervention in the MD groups, the plasma concentrations of IL-6, TNFR60, and TNFR80 decreased ($P < 0.05$), whereas that of ICAM-1 tended to decrease in the MD-VOO group ($P = 0.09$) and did not change in the MD-Nuts group ($P = 0.82$). In the LFD group, the plasma concentrations of ICAM-1, TNFR60, and TNFR80 increased ($P \leq 0.002$) and the concentration of IL-6 tended to increase ($P = 0.14$). Plasma concentrations of the molecules analyzed at baseline did not differ among the 3 intervention groups ($P > 0.29$). We compared the effects of between-group differences by the ANCOVA test (Table 1). Compared with the LFD group, the 2 MD groups had 1–34% lower plasma concentrations of ICAM-1, IL-6, TNFR60, and TNFR80 ($P \leq 0.028$).

Relationship among changes in food intake, body weight, and inflammatory markers. In this study, we mainly focused on the changes in plasma inflammatory molecules across tertiles of 1-y changes in the intake of selected foods and in the MD score (**Table 2**). We observed interactions between time and treatment ($P \leq 0.016$). Participants in the highest tertile of VOO and vegetable consumption had lower plasma TNFR60 concentrations after 1 y ($P < 0.009$) (Table 2). Participants in tertile 3 had a decrease of 17% in the plasma concentration of TNFR60 and this diminution was greater than in those in tertiles 1 and 2 ($P \leq 0.008$). The decrease in the TNFR60 concentration (−12%) in participants in tertile 3 of vegetable consumption was greater than in those in tertile 1 ($P = 0.013$). Again, the only significant 1-y change in food intake was in that of VOO and vegetables. A greater increase in the consumption of VOO and vegetables was associated with a lower plasma TNFR60 concentration after 1 y ($P = 0.01$) (**Table 3**). Participants in the lowest tertile of changes in alcohol intake, i.e., those who reduced their alcohol consumption, had a higher plasma ICAM-1 concentration ($P = 0.016$). In addition, participants who were more adherent to the MD according to the 14-point score had a lower plasma TNFR80 concentration ($P = 0.002$). Moreover, the decrease in plasma TNFR80 in tertile 3 of the MD score differed from the increase in tertile 2 ($P = 0.006$) and tended to differ from tertile 1, in which the concentration did not change ($P = 0.07$) (Table 2). Changes in the participants' body weight were

TABLE 3 Multivariate linear regression model describing the ratio of geometric means of 1-y TNFR60: baseline TNFR60 plasma concentrations in all participants by tertile of change in consumption of selected foods and nutrients[1]

	Tertile of change in consumption of each food group			
	1 (n = 128)	2 (n = 128)	3 (n = 128)	P-trend
VOO	1 (ref.)	0.99 (0.85–1.14)	0.80 (0.65–0.98)	0.010
Refined olive oil	1 (ref.)	0.98 (0.81–1.19)	0.99 (0.81–1.22)	0.63
Total nuts	1 (ref.)	1.07 (0.92–1.24)	0.94 (0.81–1.08)	0.36
Vegetables	1 (ref.)	0.99 (0.86–1.15)	0.79 (0.68–0.92)	0.010
Legumes	1 (ref.)	1.06 (0.92–1.22)	0.97 (0.84–1.12)	0.95
Fruits	1 (ref.)	0.92 (0.80–1.07)	1.02 (0.88–1.18)	0.67
Cereals	1 (ref.)	1.03 (0.89–1.20)	1.05 (0.90–1.23)	0.65
Fish and seafood	1 (ref.)	1.01 (0.87–1.16)	1.09 (0.94–1.26)	0.55
Meat and meat products	1 (ref.)	1.05 (0.91–1.21)	1.04 (0.90–1.21)	0.55
Pastries, cakes or sweets	1 (ref.)	1.16 (1.00–1.35)	1.04 (0.90–1.21)	0.45
Low-fat dairy products	1 (ref.)	1.06 (0.92–1.23)	0.94 (0.81–1.09)	0.33
Whole-fat dairy products	1 (ref.)	1.00 (0.86–1.16)	0.97 (0.83–1.13)	0.96
Wine	1 (ref.)	0.98 (0.84–1.16)	1.06 (0.92–1.23)	0.37

[1] Values are the ratio of geometric means (95% CI). The model was adjusted for age, sex, energy intake, BMI, smoking status, physical activity, research center, and drug use (aspirin and statins). Ref., reference; TNFR, TNF receptor; VOO, virgin olive oil.

not associated with changes in the plasma inflammatory biomarkers studied (data not shown).

Discussion

In the current study, we observed that the 2 MD interventions supplemented with either VOO or nuts had an antiinflammatory effect, inducing significant reductions in the plasma concentrations of TNFR, IL-6, and ICAM-1. The latter 2 have been widely related to cardiovascular disease (28) and TNFR signaling has been implicated in both the development and consequences of atherosclerosis (29,30). Results from controlled feeding trials or in free-living participant studies have suggested that consumption of VOO and nuts decreases plasma ICAM, VCAM, E-selectin, IL-6, and CRP concentrations (31–34). However, the most outstanding and novel result of our study is the effect observed with different dietary patterns on the concentrations of the TNFR, mainly in TNFR60 (6). To our knowledge, this is the first study in which TNFR60 and TNFR80 are linked to diet. We observed a close relationship between the consumption of certain foods (VOO and vegetables) and the plasma concentrations of TNFR60 ($P < 0.001$). Moreover, higher adherence to the MD was related to a reduction in the plasma TNFR80 concentration. Interestingly, the plasma concentrations of these receptors decreased after the 1-y intervention in the MD groups but increased after consumption of a LFD. In a previous study, we observed a significant increase in plasma VCAM-1 and ICAM-1 concentrations in the LFD group at 3 mo (19), possibly due to the increase in carbohydrate intake in the LFD participants to compensate for the reduction in energy intake from fat. In fact, high carbohydrate intake may promote an increase in insulin resistance, the underlying cause of metabolic syndrome, and an increase in the production of inflammatory cytokines (35).

VOO contains high amounts of polyphenols, α-tocopherol, and MUFA. The relationship between plasma TNFR concentrations and VOO consumption suggests a possible mechanism of action of certain foods. To our knowledge, there are no previous studies linking the consumption of MD, and thus some of its main components, with plasma TNFR concentrations.

Olive oil is the most remarkable food of the MD due to its high production and consumption in the Mediterranean area and its reported beneficial effects on a wide range of cardiovascular risk factors (7,36). The antiinflammatory properties of VOO have been attributed to its content of polyphenols such as tyrosol, hydroxytyrosol, and oleuropein (10,11,14,37,38) and a recently discovered phenolic compound with high antiinflammatory activity, oleocanthal (13). Moreover, other polyphenols in olive oil such as 1-phenyl-6,7-dihydroxy-isochroman inhibit the activity of COX-2 in vitro and thus inhibit TNFα production in LPS-primed human monocytes in a dose-dependent manner (39).

Although no studies to our knowledge have directly related the consumption of healthy diets and fatty acids to plasma concentrations of TNFR, several studies have shown healthy effects of fatty acids on various antiinflammatory markers, including TNF and IL (16,40). Thus, oleic acid reverses the in vitro inhibitory effect of the inflammatory cytokine TNFα on insulin production (18). Furthermore, type 2 diabetic mice fed an oleic acid diet derived from peanut oil had lower plasma glucose concentrations than those fed a high-fat diet without oleic acid (18). Recently, healthy humans receiving 50 mL of VOO and cod liver oil had significant reductions in plasma ICAM-1 and TNFα concentrations measured 3 h after the treatment, demonstrating the antiinflammatory effect of these oils (17). Mice treated with different oil-enriched diets such as fish oil, refined olive oil, and pomace olive oil for 8 wk showed that refined olive oil and fish oil diets reduced TNFα, IL-1, and IL-6 and PG E2 production (41). Chrysohoou et al. (42) studied the effect of adherence to a MD in a population from the Attica area of Greece and observed that the participants most adherent to this diet had lower plasma concentrations of CRP, IL-6, homocysteine, and fibrinogen as well as a lower white blood cell count and a borderline association with TNFα. Regular diets supplemented with olive oil (rich in MUFA) or with walnuts (rich in PUFA) induced a greater diminution in TNFα mRNA expression in peripheral blood cells than those diets rich in SFA such as butter (43). The intake of vegetable oils such as olive oil by healthy Tehran women was associated with lower plasma concentrations of TNFα, ICAM-1, and CRP (44). In a recent interventional study with VOO in humans, the expression of genes related to atherosclerosis was downregulated, with

polyphenols of VOO having a significant impact on the changes in the genetic expression of the disease (12). All these mechanisms may help to explain the observation that a dietary intervention to enhance a MD rich in VOO can contribute to a reduction in the risk of type 2 diabetes mellitus (45).

On the other hand, participants who reduced alcohol consumption had a significant increase in the plasma ICAM-1 concentration. Several studies have shown the antiinflammatory effects of moderate alcohol consumption (46). Finally, variations in the body weight of the participants did not mediate changes in the plasma inflammatory biomarkers studied.

The main limitations of our study are the higher age of the participants and their high cardiovascular risk factors, which do not allow extrapolation of the results to the general population. Ensuring adherence to dietary instructions is difficult in a diet trial. However, adherence to recommended dietary patterns and supplemental foods was good, as judged by self-report and objective measurements (23). On the other hand, the strengths of our study are the robust epidemiological design (randomized, controlled feeding trial), the reproduction of real-life conditions with home-prepared foods that reflect usual practice, the high completion rates, the adherence to the MD, and the compliance with supplemented foods.

In conclusion, this is the first time, to our knowledge, that a diminution in TNFR concentrations has been related to a MD pattern and, concretely, to the consumption of some of its main foods, such as VOO and vegetables. However, further investigations should be performed to identify the molecular mechanisms underlying these relationships.

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TRABAJO 2:

Título: “Dietary glycemic index/load and peripheral adipokines and inflammatory markers in elderly subjects at high cardiovascular risk.”

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Antecedentes: Los estudios epidemiológicos y clínicos sugieren que las dietas de índice glucémico bajo podrían tener un efecto protector contra el aumento de peso. Sin embargo, la relación entre estas dietas y adipoquinas o marcadores inflamatorios no está clara.

Objetivo: En el presente estudio se analiza la influencia del índice glucémico de la (IG) y la carga glucémica (CG) de la dieta sobre diversas adipoquinas y marcadores de riesgo metabólicos relacionados con la obesidad y la diabetes de una manera transversal y longitudinal.

Métodos: 511 hombres y mujeres de edad avanzada con alto riesgo cardiovascular fueron reclutados para el estudio PREDIMED. Se recogieron datos de la dieta al inicio del estudio y después de 1 año de seguimiento. Se calculó el IG y la CG. Las concentraciones plasmáticas de leptina, adiponectina y otros marcadores de riesgo metabólico se midieron al inicio del estudio y después de 1 año de intervención.

Resultados: Al inicio del estudio, los sujetos en los cuartiles más altos de IG mostraron niveles significativamente más altos de TNF e IL-6 que los de los cuartiles más bajos. El IG de la dieta se correlacionó negativamente con los niveles plasmáticos de leptina y adiponectina. Después de 1 año de seguimiento, los sujetos con un mayor aumento del IG y CG de la dieta mostraron una mayor reducción de los niveles de leptina y adiponectina en plasma. No hubo asociación entre el IG o CG y los otros marcadores metabólicos medidos.

Conclusiones: Nuestros resultados sugieren que un consumo de IG alto o dieta con CG altas pueden modular las concentraciones plasmáticas de leptina y adiponectina, ambas moléculas adipostáticas implicadas en el balance de energía y el riesgo cardiometabólico.

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Dietary glycemic index/load and peripheral adipokines and inflammatory markers in elderly subjects at high cardiovascular risk

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KEYWORDS
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Abstract *Background and Aims:* Epidemiological and clinical studies suggest that low-glycemic index diets could protect against weight gain. However, the relationship between these diets and adipokines or inflammatory markers is unclear. In the present study we examine how the dietary glycemic index (GI) and dietary glycemic load (GL) are associated with several adipokines and related metabolic risk markers of obesity and diabetes in a cross-sectional and longitudinal manner.
Methods and Results: 511 elderly community-dwelling men and women at high cardiovascular risk were recruited for the PREDIMED trial. Dietary data were collected at baseline and after 1 year of follow-up. The GI and GL were calculated. Plasma leptin, adiponectin and other metabolic risk markers were measured at baseline and after 1 year. At baseline, subjects in the highest quartiles of GI showed significantly higher levels of TNF and IL-6 than those in the lowest quartiles. Dietary GI index was negatively related to plasma leptin and adiponectin levels. After 1 year of follow-up, subjects with a higher increase in dietary GI or GL showed a greater reduction in leptin and adiponectin plasma levels. There was no association between GI or GL and the other metabolic markers measured.

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Conclusion: Our results suggest that the consumption of high-GI or high-GL diets may modulate plasma concentrations of leptin and adiponectin, both adipostatic molecules implicated in energy balance and cardiometabolic risk.

Introduction

Chronic low-grade inflammation associated with increased adipocytokine production from adipose tissue is recognized as a central mechanism underlying obesity and its comorbidities. Two major adipocytokines, leptin and adiponectin, are involved in the regulation of energy balance and cardiovascular homeostasis. Leptin acts on the hypothalamus and regulates satiety, food intake and energy expenditure. Leptin also induces insulin resistance through its role in the phosphorylation of the insulin receptor [1]. Adiponectin increases fat oxidation, which reduces peripheral levels of fatty acids and increases insulin sensitivity [2]. The adiponectin/leptin ratio is suggested to be a useful parameter for assessing insulin resistance and atherogenic risk, and is even more sensitive and reliable than the homeostasis model assessment-insulin resistance (HOMAIR).

Since diet is the first line of intervention for preventing and treating obesity and cardiovascular risk factors, in the last years there has been growing interest in the role that different types of carbohydrates play in the modulation of postprandial glucose/insulin response, inflammatory markers and related molecules. The consumption of diets containing high amounts of whole grains and/or dietary fiber has been associated with low serum inflammatory markers. However, the many types of carbohydrates and fiber, have a different effect on postprandial glucose and insulin responses. In this regard, Jenkins introduced the concept of the glycemic index (GI), which ranks carbohydrate-rich foods in accordance with how much they raise blood glucose levels in comparison to standard foods [3]. The concept of glycemic load (GL) was subsequently developed to take into account the amount of food consumed [4]. Hypothetically, repeated postprandial hyperglycemia and hyperinsulinemia induced by foods with a high-glycemic index may cause insulin resistance, beta cell dysfunction and inflammation by many mechanisms. However, the results of epidemiological and interventional studies on this issue have been controversial. Although epidemiological studies have linked dietary GI and GL with obesity, type 2 diabetes and high risk of cardiovascular disease [5—8], they have not been able to consistently link them with inflammatory biomarkers [9—12]. Nevertheless, the few clinical trials that have evaluated the effect of dietary GI or GL on inflammation have reported an inconsistent reduction in circulating protein-C reactive levels and no effect on tumor necrosis factor (TNF) or interleukin-6 (IL-6) [13—15].

The scarcity and the inconsistency of the evidence available on the effect of dietary GI and GL on adipokines led us to examine the changes in dietary GI and GL and changes in several adipokines and related metabolic risk markers of obesity and diabetes in a cohort of elderly subjects at high cardiovascular risk.

Methods

Study population

We assessed 568 consecutively admitted participants recruited from the PREDIMED trial centers in Reus and Barcelona. The PREDIMED study is a large, parallel group, multicenter, controlled, randomized, 6-year clinical trial designed to evaluate the effects of the Mediterranean diet (MeDiet) on the primary prevention of cardiovascular disease. Candidates were community-dwelling men and women aged 55—80 years and 60—80 years, respectively, who had no previously documented cardiovascular disease and met at least one of the two following criteria: type 2 diabetes mellitus, or three or more cardiovascular risk factors [current smoking, hypertension (blood pressure $\geq$ 140/90 mmHg or treatment with antihypertensive drugs), low-density lipoprotein cholesterol level $\geq$ 160 mg/dL (or treatment with hypolipidemic drugs), high-density lipoprotein cholesterol level $\leq$ 40 mg/dL, BMI $\geq$ 25 kg/m^2, or family history of premature cardiovascular disease]. Exclusion criteria included any severe chronic illness, drug or alcohol addiction, history of allergy or intolerance to olive oil or nuts, or a low predicted likelihood of changing dietary habits according to the Proschaska and DiClemente stages-of-change model. Participants were randomly assigned to three interventions: MeDiet with virgin olive oil (VOO), MeDiet with mixed nuts and control group (low-fat diet). Both MeDiet groups received intensive education to follow the MeDiet and VOO or mixed nuts (walnuts, hazelnuts, almonds) were provided by the study. In the control group, participants were given advice to follow a low-fat diet. Full details of the study protocol have been published elsewhere [16]. The protocol was approved by the institutional review board of both Institutions and all participants provided written informed consent.

Dietary assessment

At baseline (before randomization) and after 1 year of follow-up participants were assessed by means of a 137-item FFQ to estimate average daily nutrient intake over the previous 12-month period. Detailed information regarding the development of FFQ and the reproducibility and validity of the questionnaire has been previously reported [17]. We estimated energy and nutrient intakes by multiplying the frequency of consumption of each food by the nutrient content estimated using Spanish food composition tables. The GI was determined using the Brand-Miller tables [18].

The average daily dietary GI was calculated by multiplying the GI of individual foods by the percentage of total energy contributed by carbohydrate ($\sum$[GI food item $\times$ (g carbohydrate per serving food item $\times$ servings consumed per day/g carbohydrate consumed per day)]) [19,20]. Dietary GL was calculated by multiplying the daily GI of each food by the amount of carbohydrate consumed and dividing the product by 100((daily GI $\times$ g carbohydrate consumed per day)/100), and then adding up the values for all foods.

Other measurements

All measurements were performed at baseline and after 1 year of follow-up using the same procedures. Information was collected on the subjects' medical history and use of medication. Leisure-time physical activity (LTPA) was evaluated using the validated Spanish version of the Minnesota LTPA Questionnaire. Height and weight were measured wearing light clothing and no shoes. Waist circumference was measured midway between the lowest rib and the iliac crest. Blood pressure was measured, using a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, Netherlands). Fasting plasma glucose, serum cholesterol, HDL-c and triglyceride levels were measured using standard enzymatic automated methods in a centralized laboratory. In patients whose triglyceride levels were less than 400 mg/dL, LDL-c concentrations were estimated using the Friedewald formula. Plasma adiponectin, adipsin, ghrelin, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), IL-6, leptin, plasminogen activator inhibitor-1 (PAI-1), resistin, TNF-α and visfatin were determined using the Bio-Plex cytokine assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to manufacturer's instructions.

Statistical analysis

Subjects were categorized according to quartiles of dietary GI, GL or quartiles of changes in both cases. Chi-square tests and ANOVA were used to compare the qualitative traits and means of quantitative variables, respectively, across dietary GI quartiles. Inflammatory and metabolic risk markers were logarithmically transformed to achieve a normal distribution and the geometric mean and 95% confidence interval were used to describe these variables. Mean differences in inflammatory and metabolic risk markers were normally distributed. Analysis of covariance (ANCOVA), adjusted for potential confounding variables (sex, age, BMI, baseline waist circumference, baseline LTPA, smoking, insulin medication, presence of T2DM, w-3-fatty acids and fiber intake), was used to test for any differences in inflammatory and metabolic risk markers across GI or GL quartiles. Smoking and insulin medication were considered as a dichotomic variables (yes/no). Multiple logistic regression models were fitted to estimate the adjusted differences in the changes in inflammatory and metabolic risk markers between each of the three upper quartiles of GI or GL and the lower quartile at 1 year after adjusting for potential confounders (sex, age, LTPA, smoking, insulin medication, presence of T2DM, intervention group, and changes in BMI, waist circumference, and total w-3-fatty acid and fiber intake). The covariates selected were those that were clinically plausible and statistically significant correlated with more than one biochemical metabolic risk marker. Interaction tests for sex (product-terms, sex $\times$ glycemic index) and age (product-terms, age $\times$ glycemic index) showed that there were no statistically significant sex or age differences in the association between inflammatory markers and GI or GL quartiles. All statistical tests were two-tailed, and the significance level was $p < 0.05$. Statistical analysis was performed using SPSS 17.0.

Results

Of the 568 consecutively admitted participants recruited for the PREDIMED trial center in Reus and Barcelona with dietary data and blood samples at baseline and after 1 year of follow-up, 57 were excluded because of anti-inflammatory medication use at baseline, or showed leukocytosis (11.0×10^9/L). No under- and over-reporters of energy intake, considered as those whose energy intake was $\leq$800 or $\geq$4000 kcal/d in men and $\leq$500 or $\geq$3500 kcal/d in women were identified [21]. The general characteristics of the subjects are summarized in Table 1. Proportionally, more men were located in the highest quartile of GI than the reference quartile. The subjects in the highest quartile of GI showed a greater baseline waist circumference and higher total energy intake than those in lower quartiles. Moreover, an increasing linear trend was observed in total energy intake across GI quartiles. No significant differences were observed in baseline energy expenditure or LTPA between groups.

Tables 2 and 3, respectively, show the baseline inflammatory and metabolic risk markers levels in relation to the quartiles of dietary GI and GL, respectively. Significantly higher levels of TNF and IL-6 were observed in those subjects in the highest GI quartile. However, at baseline, no significant differences were observed in any inflammatory or metabolic risk markers analyzed in any of the quartiles of dietary GL. Plasma adiponectin levels decreased across both the GI and GL quartiles, although the differences did not reach statistical significance ($p = 0.114$, $p = 0.106$, respectively). The adiponectin/leptin ratio was lower as the GL quartile increased, although the difference did not reach the conventional level for statistical significance ($p = 0.059$).

In a longitudinal analysis, after 1 year of follow-up, no significant relationship was observed between changes in GI or GL and most of the metabolic risk markers measured (Tables 4 and 5, respectively). However, subjects in the highest quartile of changes in GI and GL showed a greater reduction in leptin and adiponectin plasma levels, even after adjusting for potential confounders. Additionally, those subjects in the highest quartile of changes in GL showed a significant increase in GIP ($p = 0.029$). A non-significantly higher decrease of BMI in those subjects allocated in the lowest quartile of change in GI (Q1: -0.49 ± 0.11, Q4: -0.11 ± 0.87, p for trend $= 0.071$) and a significantly higher decrease in the case of quartiles of change in GL (Q1: -0.49 ± 1.34, Q4: -0.36 ± 0.08, p for trend ± 0.021) were observed. In agreement with these data, a significant increase in total energy intake was

Table 1 Baseline characteristics of study subjects according to glycemic index quartiles.

	Q1	Q2	Q3	Q4	*p* for trend
	(*n* = 126)	(*n* = 129)	(*n* = 128)	(*n* = 128)	
Glycemic index	61.27 ± 0.39	69.02 ± 0.13	73.99 ± 0.13	80.09 ± 0.23	<0.001
Glycemic load	110.05 ± 1.74	149.92 ± 0.90	190.29 ± 1.23	263.79 ± 4.37	<0.001
Men/women (*n*)	43/83	55/74	54/74	75/53	<0.001
Age (y)	67.0 ± 0.5	67.2 ± 0.5	68.0 ± 0.5	66.4 ± 0.3	0.203
BMI (kg/m^2)	29.5 ± 0.3	29.2 ± 0.3	29.0 ± 0.2	29.2 ± 0.3	0.729
Waist circumference (cm)	99.4 ± 0.8	99.4 ± 0.8	99.2 ± 0.7	102.2 ± 0.8	0.029
Diabetic subjects (%)	60.3	58.1	56.2	45.3	0.076
Hypertensive subjects (%)	78.2	78.3	79.6	81.2	0.935
Dyslipidemic subjects (%)	69.0	77.5	71.1	75.8	0.382
Total energy intake (kcal/d)	2234 ± 48	2367 ± 47	2417 ± 45	2528 ± 50	<0.001
Carbohydrates (energy %)	39.61 ± 0.60	40.02 ± 0.49	41.91 ± 0.62	44.91 ± 0.59	<0.001
Fat (energy %)	40.14 ± 0.55	39.55 ± 0.45	38.74 ± 0.59	36.74 ± 0.51	<0.001
Protein (energy %)	17.51 ± 0.24	17.18 ± 0.22	16.72 ± 0.22	15.94 ± 0.22	<0.001
Alcohol (energy %)	2.76 ± 0.44	3.24 ± 0.37	2.61 ± 0.31	2.41 ± 0.26	0.401
Fiber (g/d)	27.4 ± 0.8	27.9 ± 0.7	28.1 ± 0.7	26.8 ± 0.9	0.704
EE in PA (kcal/d)	270.0 ± 20.7	281.5 ± 24.9	283.4 ± 21.5	310.2 ± 29.1	0.696

Data are mean ± SE, number (*n*) or percentage (%).
Abbreviations: BMI = body mass index; EE = energy expenditure; PA = physical activity.

observed across the quartiles of change in GI (Q1: −26.18 ± 55.79 kcal/d, Q4: 169.22 ± 49.64 kcal/d, *p* for trend = 0.039) and GL (Q1: −422.22 ± 39.3, Q4: 554.49 ± 42.06, *p* for trend < 0.001).

Discussion

The results of the cross-sectional analysis conducted in 511 elderly subjects show an inverse association between plasma leptin and adiponectin concentrations, and dietary GI and GL. Furthermore, in a prospective longitudinal assessment after a 1-year follow-up we demonstrated an inverse association between an increased dietary GI or GL and changes in both plasma leptin and adiponectin levels, independently of potential dietary and non-dietary confounders. However, no significant relationships were observed between dietary GI or GL and other adipokine metabolic markers analyzed.

High dietary GI and GL have been related with increased incidence of obesity, T2DM [5], and cardiovascular disease [5,6,22]. The most direct effect of a diet with high-GI and GL is the fast increase in postprandial glycemia and insulinemia. The induced hyperinsulinemia promotes glucose uptake by liver and muscle, while suppressing lipolysis in

Table 2 Inflammatory and obesity or diabetes risk markers according to glycemic index quartiles at baseline.

	Glycemic index quartiles				
	Q1	Q2	Q3	Q4	*p* ANOVA
	61.27 (60.49–62.05)	69.02 (68.75–69.28)	73.99 (73.72–74.26)	80.09 (79.63–80.54)	
Ghrelin (pg/mL)	12.42 (11.24–13.60)	12.66 (11.55–13.87)	13.30 (12.10–14.58)	13.63 (12.40–15.00)	0.509
GIP (pg/mL)	91.74 (82.68–101.90)	88.23 (79.83–97.91)	91.10 (82.18–100.98)	96.54 (82.18–107.77)	0.670
GLP (ng/mL)	1.29 (1.07–1.41)	1.11 (0.97–1.26)	1.20 (1.04–1.36)	1.40 (1.30–1.60)	0.127
IL-6 (pg/mL)	9.48 (8.23–10.94)	8.81 (7.68–10.12)	9.97 (8.67–11.47)	11.63 (10.08–13.40)	0.050
Leptin (ng/mL)	2.98 (2.74–3.25)	3.03 (2.80–3.30)	2.95 (2.73–3.22)	2.98 (2.74–3.24)	0.986
PAI-1 (ng/mL)	3.38 (3.18–3.56)	3.37 (3.18–3.56)	3.15 (2.95–3.32)	3.22 (3.04–3.42)	0.278
Resistin (pg/mL)	1.05 (0.97–1.14)	1.04 (0.96–1.13)	0.94 (0.88–1.04)	0.98 (0.91–1.06)	0.318
TNF (pg/mL)	11.82 (10.06–14.09)	11.25 (9.56–13.19)	12.89 (10.91–15.18)	15.56 (13.14–18.41)	0.046
Visfatin (ng/mL)	4.03 (3.32–4.88)	3.77 (3.14–4.54)	4.29 (3.53–5.16)	4.40 (3.64–5.41)	0.677
Adiponectin (μg/mL)	53.22 (44.90–63.08)	50.62 (42.71–59.41)	43.14 (36.39–51.13)	41.02 (34.62–48.64)	0.114
Adipsin (μg/mL)	1.11 (0.95–1.28)	1.12 (0.97–1.11)	0.96 (0.83–1.11)	1.02 (0.88–1.19)	0.411
A/L ratio	28.42 (23.82–33.20)	26.67 (22.13–31.22)	25.35 (20.64–30.06)	22.25 (17.56–26.95)	0.320

Metabolic markers values are expressed as geometric mean (CI 95%). Values were adjusted for sex, age, body mass index, waist circumference, physical activity in leisure time, smoking, insulin use, presence of type 2 diabetes mellitus, w-3 fatty-acid intake and fiber intake.
A/L ratio = adiponectin/leptin ratio.

Table 3 Inflammatory and obesity or diabetes risk markers according to glycemic load quartiles at baseline.

	Glycemic load quartiles				
	Q1	Q2	Q3	Q4	*p* ANOVA
	135.43 (128.61–142.26)	163.11 (155.70–170.53)	188.5 (178.91–198.22)	229.58 (217.02–242.13)	
Ghrelin (pg/mL)	12.42 (11.24–13.73)	12.67 (11.58–13.87)	12.67 (11.47–13.87)	12.67 (11.47–13.87)	0.292
GIP (pg/mL)	62.28 (73.69–92.57)	89.83 (80.04–9.68)	95.77 (86.31–106.27)	100.48 (89.12–112.28)	0.137
GLP (ng/mL)	1.17 (1.01–1.35)	1.16 (1.01–1.32)	1.23 (1.07–1.41)	1.36 (1.36–1.57)	0.447
IL-6 (pg/mL)	9.58 (8.21–11.20)	9.17 (7.97–10.56)	9.58 (8.37–11.11)	11.39 (9.79–13.25)	0.220
Leptin (ng/mL)	2.73 (2.49–2.98)	3.03 (2.80–3.30)	3.02 (2.78–3.28)	2.08 (2.92–3.49)	0.139
PAI-1 (ng/mL)	3.35 (3.15–3.58)	3.24 (3.06–3.44)	3.23 (3.05–3.42)	3.30 (3.05–3.51)	0.809
Resistin (pg/mL)	1.03 (0.94–1.13)	1.00 (0.92–1.08)	1.01 (0.94–1.09)	0.99 (0.90–1.07)	0.925
TNF (pg/mL)	12.55 (10.38–15.02)	11.47 (9.74–13.62)	12.21 (10.32–14.43)	15.16 (12.69–18.13)	0.161
Visfatin (ng/mL)	3.68 (2.98–4.54)	3.80 (3.15–4.60)	4.22 (3.50–5.10)	4.82 (3.95–5.90)	0.313
Adiponectin (μg/mL)	48.64 (39.82–60.55)	49.62 (39.82–58.82)	50.93 (42.92–60.36)	38.41 (31.99–46.13)	0.106
Adipsin (μg/mL)	1.03 (0.88–1.22)	1.04 (0.90–1.22)	1.12 (0.70–1.30)	1.01 (0.86–1.17)	0.792
A/L ratio	29.23 (24.16–34.31)	26.71 (22.08–31.34)	27.41 (22.76–31.05)	19.66 (14.69–24.63)	0.059

Metabolic markers values are expressed as geometric mean (CI 95%). Values were adjusted for sex, age, body mass index, waist circumference, physical activity in leisure time, smoking, insulin use, presence of type 2 diabetes mellitus, w-3 fatty-acid intake, fiber intake.
A/L ratio = adiponectin/leptin ratio.

adipoctyes and reducing the release of glucose from the liver into the circulation. As a result, blood glucose decreases rapidly, and hunger response occurs faster with a high-GI or GL than with a low-GI or GL diet [23].

It is quite clear that energy homeostasis requires a fine regulation of food intake, nutrient absorption, energy expenditure and storage. These processes are coordinated by the central nervous system after controlling the

Table 4 Mean changes in inflammatory and obesity or diabetes markers after 1 year in subjects in the 4 quartiles of glycemic index at baseline relative to the change in quartile 1.

	Quartiles of changes in glycemic index				
	Q1	Q2	Q3	Q4	*p*[a]
	−11.62 (−12.29 to −10.95)	−3.67 (−6.89 to −1.54)	0.78 (−1.49 to 3.46)	8.92 (3.59–27.60)	
Ghrelin (pg/mL)	0	0.31 (−1.91 to 2.54)	−1.17 (−3.40 to 1.05)	−1.69 (−3.91 to 0.54)	0.069
GIP (pg/mL)	0	−4.66 (−19.93 to 10.59)	−1.16 (−16.46 to 14.12)	−2.43 (−12.84 to 17.71)	0.662
GLP (ng/mL)	0	0.10 (−0.17 to 0.38)	0.01 (−0.29 to 0.26)	−0.09 (−0.37 to 0.19)	0.384
IL-6 (pg/mL)	0	1.48 (−1.75 to 4.71)	−0.19 (−3.45 to 3.05)	−1.14 (−4.39 to 2.10)	0.329
Leptin (ng/mL)	0	−0.34 (−0.71 to 0.15)	−0.55 (−0.92 to −0.19)	−0.39 (−0.87 to −0.26)	0.019
PAI-1 (ng/mL)	0	−0.39 (−0.66 to −0.12)	−0.36 (−0.63 to −0.09)	−0.16 (−0.43 to 0.10)	0.293
Resistin (ng/mL)	0	−0.09 (−0.20 to 0.01)	−0.09 (−0.20 to 0.02)	−0.08 (−0.21 to −0.03)	0.166
TNF (pg/mL)	0	2.84 (−2.43 to 8.12)	−0.62 (−5.92 to 4.67)	−1.42 (6.72–3.87)	0.367
Visfatin (ng/mL)	0	0.61 (−0.80 to 2.03)	0.16 (−1.27 to 1.60)	0.19 (−1.23 to 1.62)	0.959
Adiponectin (μg/mL)	0	−2.46 (−14.73 to 0.97)	−0.96 (−13.04 to 11.12)	−15.14 (−27.32 to −0.29)	0.027
Adipsin (μg/mL)	0	0.06 (−0.11 to 0.27)	0.11 (−0.06 to 0.28)	−0.12 (−0.29 to 0.03)	0.233
A/L ratio	0	1.34 (−7.39 to 10.08)	2.23 (−6.33 to 10.80)	−3.87 (−12.59 to 479)	0.461

Associations were calculated using a linear regression model. Values are mean differences (CI 95%) compared to Q1. Values were adjusted for sex, age, changes in waist circumference, changes in body mass index, intervention group, physical activity in leisure time, smoking, insulin use, presence of type 2 diabetes mellitus, w-3 fatty-acid intake and fiber.
[a] *p* for linear trend. A/L ratio = adiponectin/leptin ratio.

Table 5 Mean changes in inflammatory and obesity or diabetes markers after 1 year in subjects in the 4 quartiles of glycemic load at baseline relative to the change in quartile 1.

	Quartiles of changes in glycemic load				
	Q1	Q2	Q3	Q4	*p* for linear trend
	−87.21 (−235.9 to −44.26)	−23.07 (−43.71 to −6.00)	10.13 (−5.97 to 29.40)	68.52 (29.44−245.89)	
Ghrelin (pg/mL)	0	0.97 (−1.30 to 3.26)	−0.28 (−2.57 to 2.00)	−0.30 (−2.67 to 2.08)	0.548
GIP (pg/mL)	0	−6.35 (−21.79 to 9.08)	−2.88 (−18.40 to 12.63)	17.61 (1.52−33.69)	0.029
GLP (ng/mL)	0	0.025 (−0.26 to 0.31)	−0.10 (−0.38 to 0.18)	0.10 (−0.19 to 0.39)	0.713
IL-6 (pg/mL)	0	−0.61 (−3.92 to 2.70)	−1.80 (−5.13 to 1.53)	0.33 (−3.11 to 3.78)	0.969
Leptin (ng/mL)	0	−0.28 (−0.62 to 0.05)	−0.37 (−0.71 to −0.03)	−0.35 (−0.71 to 0.005)	0.030
PAI-1 (ng/mL)	0	−0.14 (−0.42 to 0.13)	−0.28 (−0.56 to −0.006)	−0.09 (−0.37 to 0.19)	0.368
Resistin (ng/mL)	0	0.01 (−0.05 to 0.17)	−0.07 (−0.18 to 0.04)	0.004 (−0.11 to 0.12)	0.483
TNF (pg/mL)	0	−0.04 (−5.45 to 5.36)	−2.49 (−7.93 to 2.94)	1.63 (−3.99 to 7.25)	0.798
Visfatin (ng/mL)	0	0.24 (−1.21 to 1.70)	−0.16 (1.63−1.30)	1.00 (−0.49 to 2.51)	0.282
Adiponectin (μg/mL)	0	−4.00 (−16.40 to 8.47)	−8.53 (−21.35 to 4.28)	−12.06 (−25.23 to 1.11)	0.054
Adipsin (μg/mL)	0	−0.01 (−0.18 to 0.15)	−0.06 (−0.23 to 0.11)	−1.43 (−0.32 to 0.03)	0.101
A/L ratio	0	2.37 (−6.42 to 11.57)	0.095 (−8.93 to 9.12)	−5.41 (−14.73 to 3.90)	0.206

Associations were calculated using a linear regression model. Values are mean differences (CI 95%) compared to Q1. Values were adjusted for sex, age, changes in waist circumference, changes in body mass index, intervention group, physical activity in leisure time, smoking, insulin use, presence of type 2 diabetes mellitus, w-3 fatty-acid intake and fiber.
A/L ratio = adiponectin/leptin ratio.

homeostatic signals derived from peripheral tissues. Since leptin discovered, the secretory activities of adipose tissue have increased exponentially with more than 50 adipocyte-derived products that make different contributions to obesity and its pathophysiological features [22]. Therefore, because a low grade of chronic inflammation is now recognized as one of the central mechanisms underlying obesity and associated comorbidities, the potential effect of dietary GI and GL on inflammatory modulation seems relevant. However, the few studies carried out to date are controversial because they focus on the plasma C-reactive protein and do not evaluate the long-term effects of GI or GL on adipostats or other adipokines related to obesity and comorbidities [9,13].

The results of our 1-year prospective longitudinal study conducted in a large sample of subjects at high cardiovascular risk are in agreement with the adipostatic theory. The adipokines, leptin and adiponectin, are considered to be the two major adipostats in humans because of their role in the central nervous system and in peripheral tissues [1,2]. In our study, we have demonstrated that an increase in the dietary glycemic index and glycemic load is associated with a decrease in leptin and adiponectin plasma concentrations. Our results are in agreement with those obtained using an intervention study conducted in rats fed with a high-glycemic index starch diet for 12 weeks [23] and those reported in a postprandial state [24]. Therefore, if we consider that higher leptin levels are associated with a decrease in food intake and an increase in energy expenditure acting at the hypothalamic central level [25], the down-regulation of leptin induced by an increase in GI or GL observed in our study could be considered as a mechanism favoring the weight gain and obesity attributed to high-GI diets. Moreover, because leptin also exerts autocrine or paracrine actions increasing lipolysis and decreasing lipogenesis, the decrease in circulating plasma leptin levels observed in our study may lead to a decrease in fatty-acid oxidation and an increase in glucose oxidation, which favors fat deposition. Finally, because leptin is primarily known as a satiety factor, the decrease in plasma leptin after a high-GI diet sustained the concept that these diets are less satiating than low-GI diets [26].

Adiponectin is the most abundant adipocytokine in humans. A low level of circulating adiponectin results in insulin resistance, glucose intolerance, dyslipidemia and atherosclerosis [27]. Recently, adiponectin has been identified in the cerebrospinal fluid of rodents suggesting that it has an important role in the central regulation of energy intake and energy expenditure [28]. However, although the central effects of adiponectin on energy balance are still unclear and controversial in humans [2] the results of our study support the hypothesis that high-GI-induced hypoadiponectinemia could be to the detriment of obesity. Moreover, the hypoadiponectinemia induced by the increase in dietary GI that we observed and reported in a previous epidemiologic study [12] could partly explain the relationship between the GI of the diet and the increased risk of T2DM and cardiovascular disease associated with the consumption of this type of diet.

In our study, we failed to show that the GI and GL had any relationship with incretins, other adipokines or related molecules. We only observed significantly higher levels of TNF and a trend to higher levels of IL-6 in those subjects in the higher GI quartiles, after adjusting for confounders, suggesting that pro-inflammatory cytokines worsened in

those subjects consuming high-GI foods. In a longitudinal manner, we also reported a positive relationship between an increase in GL and an increase in GIP, but not GLP-1, thus suggesting that a high dietary GL contributes to fat deposition and obesity.

The major strengths of the current study are that it carried out a longitudinal analysis of a large sample of individuals and measured a panel of adipokines and related molecules involved in energy metabolism, glucose metabolism and cardiovascular risk. However, we recognize several limitations. First, the study has been conducted in elderly subjects who are at high cardiovascular risk, and thus the results cannot be generalized to other populations. Second, potential dietary measurement error is a significant limitation. The FFQ used for dietary data collection was not designed to assess dietary GI and dietary GL, therefore both measurements are likely to have substantial errors. Moreover, due to scarcity of data, it was necessary to use GI values derived from studies conducted in different countries where the food or its properties may differ from that consumed in Spain. Nevertheless, these limitations would also apply to some other epidemiologic studies and clinical trials involving glycemic index and glycemic load measurements. Finally, our study was conducted in a cohort that was undergoing nutritional interventions, which might have had differential effects on peripheral adipocytokines. However, to address this limitation and to minimize the effect, we have adjusted all longitudinal analyses for the intervention group.

In conclusion, this study adds to the growing evidence that consumption of diets with high-GI foods or high dietary GL may modulate plasma concentrations of some cardiometabolic markers thus contributing to the promotion of obesity and cardiovascular disease. Additional research is necessary to evaluate these associations or effects on other populations and to explore the mechanisms leading to the interactions observed between dietary GI and GL, body weight and cardiovascular risk.

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TRABAJO 3:

Título: “The effects of the mediterranean diet on biomarkers of vascular wall inflammation and plaque vulnerability in subjects with high risk for cardiovascular disease. A randomized trial.”

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Antecedentes: La adherencia a la dieta mediterránea (DietMed) se asocia con la reducción de la morbilidad y mortalidad por ECV. Los mecanismos celulares y/o moleculares através de los cuales la DietMed ejerce sus efectos no se conocen totalmente.

Objetivo: Evaluar los cambios de biomarcadores inflamatorios relacionados con la estabilidad de la placa después de un año de intervención dietética con DietMed suplementada con AOVE o FS en comparación a una DBG sobre una subcohorte del estudio PREDIMED (Prevención con Dieta Mediterránea).

Métodos: Un total de 164 participantes con alto riesgo de enfermedad cardiovascular fueron distribuidos aleatoriamente en tres grupos de dieta: DietMed suplementada con 50 ml / día de aceite extra virgen de oliva (DietMed + AOVE) o 30 g / d de frutos secos (DietMed + FS) y una dieta baja en grasa. Se midieron los cambios de los factores clásicos de riesgo cardiovascular, biomarcadores inflamatorios de la aterosclerosis y la placa de la vulnerabilidad después de 12 meses de intervención.

Resultados: En comparación con los participantes en el grupo de dieta baja en grasas, los que recibieron DietMed+ AOVE y DietMed+FS mostraron una mayor disminución (P = 0.02; ambos) de la presión arterial sistólica (6 mmHg) y diastólica (3 mmHg), así como una reducción del 10% y 8% del LDL-colesterol (P = 0.04; ambos), respectivamente. Además, los pacientes en el grupo DietMed + FS mostraron una reducción significativa de 34% en la expresión de CD40 en la superficie de monocitos comparado con los voluntarios de DBG (P = 0.03). Además, los biomarcadores inflamatorios relacionados con la inestabilidad de la placa tales como la proteína C reactiva y la interleucina-6 se redujeron en un 45% y el 35% y el 95% y el 90% en los grupos de DietMed + AOVE y DietMed+FS, respectivamente (P<0.05; todos) en comparación con el grupo de DBG. Del mismo modo, para el grupo de DietMed + AOVE, sICAM-1 y P-selectina también disminuyeron en un 50% y 27%,

respectivamente (P = 0.04), mientras que sP-selectina se redujo en un 19% para el grupo de DietMed + FS (P = 0.04) en comparación con el grupo de DBG.

Conclusiones: La adherencia a la DietMed se asocia con un aumento en los marcadores séricos de estabilidad de la placa de ateroma lo que puede explicar, al menos en parte, la función protectora de la DietMed contra la enfermedad isquémica del corazón.

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PLOS ONE

The Effects of the Mediterranean Diet on Biomarkers of Vascular Wall Inflammation and Plaque Vulnerability in Subjects with High Risk for Cardiovascular Disease. A Randomized Trial

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Abstract

Background: Adherence to the Mediterranean diet (MD) is associated with reduced morbidity and mortality due to cardiovascular disease. However, how the MD exerts its effects is not fully known.

Aim: To assess the 12-month effects of two enhanced MDs compared to a low-fat diet on inflammatory biomarkers related to atherosclerosis and plaque vulnerability in a subcohort of the PREDIMED (Prevención con Dieta Mediterránea) study.

Methods: A total of 164 participants at high risk for cardiovascular disease were randomized into three diet groups: MD supplemented with 50 mL/d of extra virgin olive oil (MD+EVOO) or 30 g/d of nuts (MD+Nuts) and a low-fat diet. Changes in classical cardiovascular risk factors, inflammatory biomarkers of atherosclerosis and plaque vulnerability were measured after 12 months of intervention.

Results: Compared to participants in the low-fat diet group, those receiving MD+EVOO and MD+Nuts showed a higher decrease in systolic (6 mmHg) and diastolic (3 mmHg) blood pressure ($P = 0.02$; both), as well as a reduction of 10% and 8% in LDL-cholesterol ($P = 0.04$), respectively. Patients in the MD+Nuts group showed a significant reduction of 34% in CD40 expression on monocyte surface compared to low-fat diet patients ($P = 0.03$). In addition, inflammatory biomarkers related to plaque instability such as C-reactive protein and interleukin-6 were reduced by 45% and 35% and 95% and 90% in the MD+EVOO and MD+Nuts groups, respectively ($P<0.05$; all) compared to the low-fat diet group. Likewise, sICAM and P-selectin were also reduced by 50% and 27%, respectively in the MD+EVOO group ($P = 0.04$) and P-selectin by 19% in MD+Nuts group ($P = 0.04$) compared to the low-fat diet group.

Conclusions: Adherence to the MD is associated with an increase in serum markers of atheroma plaque stability which may explain, at least in part, the protective role of MD against ischemic heart disease.

Trial Registration: www.controlled-trials.com ISRCTN35739639

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

Atherosclerosis, the pathological substratum of coronary heart disease (CHD), is a low-grade chronic inflammatory disease of the vascular wall initiated by the accumulation of cholesterol-laden inflammatory cells (monocytes and T-lymphocytes) in the subendothelial space in a self-perpetuating process that leads to the formation of atheroma plaques, the hallmark of the disease [1]. Inflammatory mediators such as adhesion molecules (selectins, integrins) and interleukins (e.g., IL-6, IL-1β, IL-18) participate in this process. In some instances, the atheroma plaque becomes unstable, leading to cap rupture and ensuing thrombosis that occludes the artery and finally, induces cardiovascular events such myocardial infarction or stroke [2]. Some of these inflammatory mediators (e.g., C-reactive protein [CRP], IL-6, intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1]) have been considered as useful predictive markers of atherosclerosis [3], whereas other biomarkers (matrix metalloprotease-9 and IL-18) have been associated with plaque vulnerability [4].

The PREDIMED (*Prevención con Dieta Mediterránea*) study is the first randomized clinical trial designed to assess the beneficial effects of the MD on the primary prevention of cardiovascular diseases in elderly subjects at high cardiovascular risk. Up to now the PREDIMED study has demonstrated that adherence to the MD is associated with a reduced incidence of diabetes [5–6], the metabolic syndrome [7], hypertension [8], and better control of other cardiovascular risk factors [9–10]. In fact, Estruch et al [11] have recently reported that a MD intervention reduces the incidence of cardiovascular events by 30% in subjects at high cardiovascular risk. However, improvement in classical cardiovascular risk factors associated with the MD intervention could not fully explain the protective effect of the MD against CHD [11]. Some authors have suggested that, at least in the short term, MD reduces oxidative stress [12], vascular inflammation [13–14], and endothelial dysfunction [15], all of which are related to atheroma plaque formation. Thus, in a previous study we have observed lower serum concentrations of VCAM-1, ICAM-1, E- and P-selectin, CRP and IL-6, as well as a down-regulation of adhesion molecules on T-lymphocyte and monocyte surfaces after 3 months of MD intervention [10,14,16]. However, whether this effect is maintained in the long run is unknown. Moreover, little is known about the effect of the MD on markers of plaque vulnerability. Therefore, we embarked on a study to assess 1-y changes in inflammatory biomarkers of atherosclerosis as well as markers of plaque instability in a free-living population with high risk of CHD participating in the PREDIMED study.

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Subjects and Design

The PREDIMED study is a parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the MD on the primary prevention of cardiovascular disease (CVD) (http://www.predimed.es) [17]. Recruitment took place between October 2003 and January 2009, and the 7447 participants were randomly assigned to one of three interventions (two Mediterranean diets enriched with extra virgin olive oil (EVOO) or mixed nuts, and a control low-fat diet). The design, methodology and eligibility criteria for the PREDIMED study have been previously described [10–11,17]. Briefly, we recruited men aged 55 to 80 years and women aged 60 to 80 years with no previously documented CVD. They were eligible if they had type 2 diabetes, or 3 or more major cardiovascular risk factors (hypertension, high plasma LDL-cholesterol, low plasma HDL-cholesterol, overweight or obesity, current smoking, or a family history of premature coronary heart disease). At baseline and after 12 months of follow-up, the participants filled out a 137-item validated food frequency questionnaire (FFQ), a 14-item questionnaire assessing adherence to the MD and the Minnesota leisure-time physical activity questionnaire. We also recorded medication use, measured anthropometric parameters and blood pressure, and collected fasting blood and a spot urine samples, as described previously [14].

In the current study we screened 193 consecutive potential participants from October 2003 to November 2004 in a primary care center associated with the Hospital Clínic of Barcelona, Spain, but 29 did not fulfill the inclusion criteria. Thus, 164 were finnaly included in this substudy. Main outcome measures were 12-month changes in classical cardiovascular risk factors, and inflammatory markers predictive of atherosclerosis or related to plaque instability.

Diets

After two screening visits with the dietician, participants who fullilled inclusion criteria signed an informed consent and were randomily assigned in a 1:1:1 ratio to one of three dietary interventions (MD+EVOO, MD+Nuts or low-fat diet). Randomization was performed centrally by means of a computer-generated random-number sequence. All of the participants had a face-to-face interview with the dietician and a group session at the baseline visit and quarterly thereafter. The dietary intervention in the three treatment groups was delivered by the same dietician as described [11]. The group sessions were organized separately for each of the three intervention groups. In each session the 14-point score of adherence to the MD was the main tool to change dietary habits for the two MDs, and a similar 9-point score was used in the participants of the low-fat diet group. The focus in the MD groups was to change portion sizes and the frequency of intake of the different foods and to modify cooking methods towards the traditional MD of Mediterranean countries in the sixties. Thus, participants in both MD groups were recommended to increase the intake of vegetables (≥2 servings/d), fresh fruit (≥3 servings/d), legumes, nuts, fish or seafood (≥3 servings/wk), and the use of olive oil for cooking and dressings, as previously described [11]. The focus in the control group was to reduce all types of fat, with particular emphasis on recommending the consumption of lean meats, low-fat dairy products, cereals, potatoes, pasta, rice, fruits and vegetables. All participants were provided with descriptions of seasonal foods, shopping lists, weekly meal plans and cooking recipes, according to the their intervention group. Olive oil and nut industry companies supplied EVOO (50 mL/d) or 30 g/d of walnuts, almonds and halzelnuts free of charge to the respective MD groups, whereas those in the control group received small nonfood gifts. The fatty acid composition of the EVOO and nuts used in the trial is described elsewhere [10]. No total calorie restriction was advised, nor was physical activity promoted. From the beginning of the study all participants were recommended to not use multivitamin or antioxidant supplements.

Ethics Statement

All participants provided informed consent. Participants had signed the informed Consent. The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the

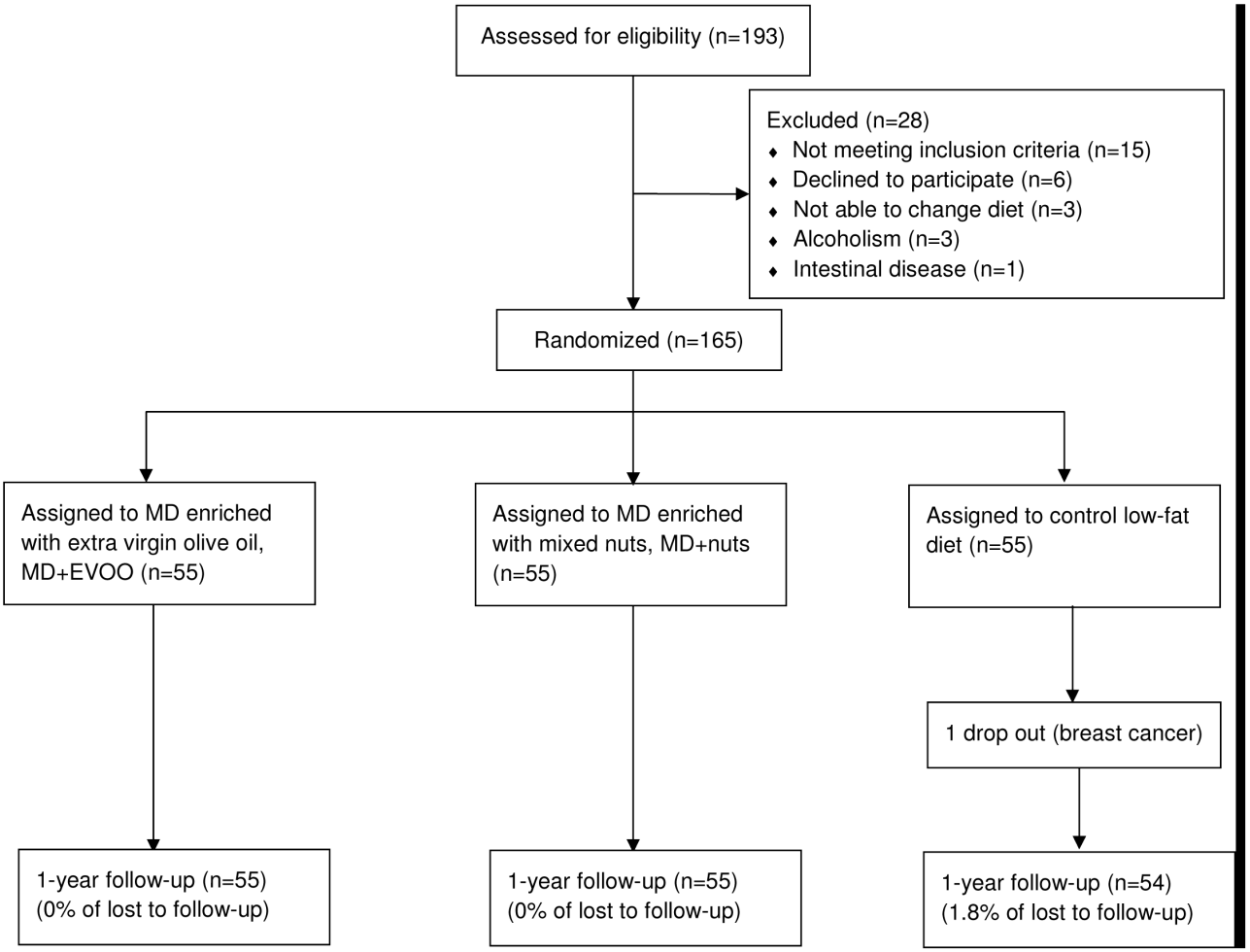

Figure 1. Flowchart of study participants. The diagram includes detailed information on the excluded participants. Abbreviations: MD, Mediterranean diet.
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US Department of Health and Human Services (DHHS) update for Federal wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July, 16, 2002. The protocol was also approved by the ethical review board of our hospital.

Clinical and Laboratory Measurements

Trained personnel measured weight and height using calibrated scales and a wall-mounted stadiometer, respectively; waist circumference was determined midway between the lowest rib and the iliac crest using an anthropometric tape, and blood pressure (BP) was measured in triplicate with a validated semiautomatic oscillometer (Omron HEM-705CP) [10–11]. Samples of serum, EDTA-plasma, and urine were coded and stored at $-80^{\circ}C$ until assay. A technician blinded to group allocation processed peripheral blood mononuclear cells (PBMCs) on the same day of blood extraction. PBMCs were isolated from whole blood by Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AC) density-gradient. The expression of adhesion molecules on the surface of PBMCs was analyzed via double direct immunofluorescence with the use of commercial monoclonal antibodies following the manufacturer's instructions. The adhesion molecules analyzed were: anti-CD49d (Cytogmos), anti-CD11a and anti-CD11b (Bender Medsystems), anti-CD40, anti-CD14 and anti-CD2 monoclonal antibodies (Caltag). Cell counts (5000 events for T-lymphocytes and 2000 for monocytes) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton-Dickinson) using CellQuest software. Results are expressed as mean fluorescence intensity (MFI) in arbitrary units.

ELISAs were performed in baseline and 1-year samples at the end of the study period in thawed plasma with commercial kits for soluble (s) E- and P-selectin, sVCAM-1, sICAM-1, IL-18, IL-6 (BLK and PelkinElmer Elast Amplification System), IL-10 and tissular inhibitor of metalloproteases-1 (TIMP-1) (R&D Systems), MMP-9 (Amersham), and transforming growth factor beta 1 (TGF-β1) (R&D Systems). A technician blinded to group allocation processed the ELISA kits.

Additional serum analytes determined included fasting glucose and immunoreactive insulin, total cholesterol, triglycerides, HDL and LDL-cholesterol, and high-sensitivity CRP, as described elsewhere [10–11]. In a random sample of 56 participants (34%) we measured urinary tyrosol and hydroxytyrosol levels (as a measure of adherence to EVOO consumption recommendations) and the plasma α-linolenic acid (ALA) proportion (as a measure of adherence to walnut consumption recommendations), as reported previously [10]. For all laboratory methods, the intra- and inter-assay variation coefficients ranged from 1.8 to 8.9% and from 0.9 to 9.9%, respectively.

Table 1. Baseline characteristics of the participants.[1]

	MD+EVOO (n = 55)	MD+nuts (n = 55)	Low-fat diet (n = 54)
Age (years)	68.1±6	67.6±6	67.4±6
Men (%)	24 (43.6)	31 (56.4)	22 (40.7)
Family history of CHD (%)	16 (29.1)	9 (16.4)	13 (24.1)
Current smokers (%)	10 (18.2)	11 (20)	11 (20.4)
BMI (kg/m^2)	27.9±3.4	27.8±3.1	28.5±3.7
BMI≥25 kg/m (%)	47 (85.5)	45 (81.8)	44 (81.5)
Type 2 diabetes (%)	46 (83.6)	44 (80)	37 (68.5)
Hypertension (%)	39 (70.9)	29 (52.7)	37 (68.5)
Dyslipidemia (%)	32 (58.2)	34 (61.8)	38 (70.4)
Medications (%)			
ACE inhibitors	11 (20)	12 (21.8)	13 (24.1)
Diuretics	13 (23.6)	6 (10.9)	14 (25.9)
Other antihypertensive agents	10 (18.2)	8 (14.5)	9 (16.7)
Statins	17 (31)	14 (25.5)	10 (18.5)
Other-lipid-lowering agents	4 (7.3)	2 (3.6)	6 (11.1)
Insulin	3 (5.5)	7 (12.7)	3 (5.6)
Oral hypoglycemic drugs	30 (54.5)	25 (45.5)	29 (53.7)
Aspirin or antiplatelet drugs	10 (18.2)	8 (14.5)	5 (9.3)
NSAIDS	6 (10.9)	10 (18.2)	8 (14.8)

[1]Values are mean ± SD or n (%). ACE, angiotensin converting enzyme; BMI, body mass index; CHD, coronary heart disease; EVOO, extra virgin olive oil; MD+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MD+Nuts, Mediterranean diet supplemented with nuts; NSAIDS, Non-steroidal antiinflammatory drugs.
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Statistical Analyses

For a parallel design, the sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a maximum loss of 10% of participants. To detect a mean difference of 10 MFI units on the expression of monocyte CD49d with a conservative standard deviation (SD) of 10, 20 subjects would be needed to complete the study (α risk = 0.05, power = 0.9). The monocyte expression of CD49d was considered the primary outcome and used to determine the sample size, but changes in all endpoints were of equal interest in this study.

We used descriptive statistics with the mean ± SD for the baseline characteristics of the participants. Categorical variables are expressed as percentages. Variables with a skewed distribution (Kolmogorov) were transformed to their natural logarithm for analysis. One-factor analysis of variance or chi-square tests, as appropriate, were used to determine differences in baseline characteristics among the three study groups. Changes in all outcomes were assessed with repeated-measures analysis of variance for the two factors, diet and time, and their interactions. Significant interactions were analyzed by the simple effects test with multiple contrasts of Bonferroni. Within- and between-group differences are expressed as means and 95% confidence intervals (CI).

The relationship between monounsaturated acid (MUFA) intake and inflammatory markers was determined by partial correlation analysis. All statistical tests were two-tailed, with significance set at $P<0.05$. Analyses were performed using the SPSS, version 18.0 (SPSS Inc., Chicago, IL).

Results

Participant Characteristics

Of the 164 participants finnally included, 55, 55 and 54 were randomized to a MD supplemented with virgin olive oil, a MD supplemented with nuts, and a low-fat control diet, respectively. The retention rates for 1 year follow-up were 100%, 100% and 98.2%, respectively (**Figure 1**). **Table 1** shows the baseline characteristics of these 164 participants, of whom none were lost to follow-up. The groups were well balanced regarding demographic characteristics, adiposity and cardiovascular risk factors. The medication taken and occupation levels were also similar in the three groups. Drug regimens did not appreciably change during the 12-month follow-up.

Cardiovascular Risk Factors

The baseline and 12-month values for the classical cardiovascular risk factors are shown in **Table 2**. The MD+EVOO and MD+Nuts groups showed a mean reduction in systolic BP of 6 mmHg (P = 0.02; both) and in diastolic BP of around 3 mmHg (P = 0.02; both), of 6% and 7% (P = 0.04), respectively, in total-cholesterol, of 10% and 8% (P = 0.04), respectively, in LDL-cholesterol, and of 9% and 5% (P = 0.01), respectively in the cholesterol/HDL-cholesterol ratio. Both MDs showed a decrease in the waist perimeter ($P<0.05$; both) of the participants from baseline.

Adhesion Molecule and CD40 Expression in PBMC

As shown in **Table 3**, after 12 months of intervention the MD+EVOO group showed a decrease in CD11a ($P<0.001$), CD49d ($P<0.004$) and CD40 ($P<0.001$) in peripheral T-lymphocytes. In

Table 2. Changes in adiposity, blood pressure and cardiovascular risk factors.

		MD+EVOO (n = 55)		MD+Nuts (n = 55)		Low-fat diet (n = 54)		
		Mean	P[3]	Mean	P[3]	Mean	P[3]	*Pint*[4]
Weight (Kg)	Baseline[1]	75.5±1.6		76.9±1.6		75.3±1.7		0.70
	1y.[1]	74.9±1.6		76.7±1.7		75.0±1.7		
	Mean changes[2]	−0.6 (−1.4 to 0.1)	0.09	−0.2 (−1.0 to 0.5)	0.54	−0.3 (−1.1 to 0.5)	0.44	
BMI (Kg/m^2)	Baseline	29.2±0.5		28.9±0.5		29.3±0.5		0.85
	1y.	29.1±0.5		28.8±0.5		29.2±0.6		
	Mean changes	−0.1 (−0.4 to 0.2)	0.99	−0.1 (−0.4 to 0.2)	0.90	−0.1 (−0.5 to 0.2)	0.44	
Waist circumference (cm)	Baseline	102±1.3		102±1.3		100±1.4		0.09
	1y.	98.6±1.4		99.2±1.4		99.4±1.5		
	Mean changes	−3.2 (−4.6 to −1.7)	<0.001	−2.8 (−4.3 to −1.4)	<0.001	−0.6 (−2.1 to 0.9)	0.42	
Systolic blood pressure (mmHg)	Baseline	152±2.6		148±2.6		153±2.7		0.02
	1y.	146±2.6		141±2.5		155±2.7		
	Mean changes	−6.0 (−10.1 to −2.0)[a]	0.004	−6.4 (−10.5 to −2.4)[a]	0.002	2.2 (−2.1 to 6.5)	0.32	
Diastolic blood pressure (mmHg)	Baseline	85.0±1.3		85.1±1.3		86.8±1.4		0.02
	1y.	81.8±1.2		82.5±1.2		88.4±1.3		
	Mean changes	−3.2 (−5.4 to −0.9)[a]	0.07	−2.6 (−4.9 to −0.4)[a]	0.02	1.6 (−0.8 to 4.0)	0.20	
Glucose (mg/dL)	Baseline	130±5.5		127±5.6		132±5.6		0.83
	1y.	131±5.5		127±5.5		129±5.5		
	Mean changes	1.2 (−6.8 to 9.2)	0.77	−0.2 (−8.3 to 7.9)	0.96	−2.9 (−11.1 to 5.3)	0.49	
Glycated hemoglobin (mg/dL)	Baseline	6.1±0.2		6.0±0.2		6.1±0.3		0.90
	1y.	6.3±0.2		6.1±0.2		6.0±0.2		
	Mean changes	0.2 (−0.1 to 0.5)	0.23	0.1 (−0.2 to 0.3)	0.66	−0.1 (−0.4 to 0.2)	0.45	
Triglycerides (mg/dL)	Baseline	147±11.1		138±11.1		148±11.5		0.80
	1y.	143±8.9		135±8.9		133±9.2		
	Mean changes	−4.2 (−24.0 to 15.5)	0.67	−2.9 (−22.6 to 16.9)	0.77	−15.5 (−36.0 to 5.0)	0.14	
Total-cholesterol (mg/dL)	Baseline	228±4.6		225±4.6		208±4.7		0.04
	1y.	214±4.5		209±4.5		207±4.6		
	Mean changes	−13.5 (−23.0 to −4.1)[a]	0.005	−15.7 (−25.1 to −6.3)[a]	0.001	0.1 (−9.4 to 9.6)	0.98	
HDL-Cholesterol (mg/dL)	Baseline	54.5±1.6		53.8±1.7		55.6±1.7		0.47
	1y.	56.6±1.7		52.6±1.7		53.2±1.8		
	Mean changes	2.1 (−0.2 to 4.4)	0.07	−1.2 (−3.5 to 1.1)	0.31	−2.4 (−2.8 to −2.0)	0.74	
LDL-Cholesterol (mg/dL)	Baseline	145±3.9		144±3.9		128±4.0		0.04
	1y.	130±4.0		132±4.0		124±4.0		
	Mean changes	−14.4 (−21.1 to −7.7)[a]	<0.001	−11.7 (−18.4 to −5.0)[a]	0.001	−3.6 (−10.4 to 3.2)	0.56	
Cholesterol: HDL-Cholesterol ratio	Baseline	4.4±0.1		4.3±0.1		3.8±0.1		0.01

Table 2. Cont.

		MD+EVOO (n = 55)		MD+Nuts (n = 55)		Low-fat diet (n = 54)		
		Mean	P[3]	Mean	P[3]	Mean	P[3]	Pint[4]
	1y.	4.0±0.1		4.1±0.1		3.9±0.1		
	Mean changes	−0.4 (−0.6 to −0.2)[a]	<0.001	−0.2 (−0.5 to −0.03)[a]	0.03	0.05 (−0.2 to 0.3)	0.83	

Data analyzed by repeated-measures 2-factor ANOVA (simple-effect analysis by Bonferroni's multiple contrast).
[1]Values are mean ± SD.
[2]Mean differences (95% CI).
[3]P: Significant differences (P<0.05) between before and after the intervention.
[4]Pint: comparison between measures obtained before and after intervention and among the 3 diet groups, P<0.05.
[a]MD+EVOO or MD+nuts vs. low fat-diet are significantly different, P<0.05. EVOO, extra virgin olive oil; MD+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MD+Nuts, Mediterranean diet supplemented with nuts.
BMI, body mass index.
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addition, MD+EVOO showed decreased CD11a, CD11b, CD49d and CD40 (P<0.001; all) in circulating monocytes.

On the other hand, the MD+Nuts group showed a significant decrease in CD11a and CD40 of (P<0.00; all) in T- lymphocytes. For monocytes, the MD+Nuts group showed a significant decrease in CD11a, CD11b, CD49d and CD40 (P<0.001; all).

Finally, the low-fat diet only showed a significant decrease in CD11a in T-lymphocytes and a decrease in CD11a and CD11b expression in circulating monocytes (P<0.001; all).

Circulating Markers of Plaque Instability and other Inflammatory Biomarkers

Baseline plasma concentrations of inflammatory biomarkers were similar among groups. As can be observed in **Table 4**, after a 12-month intervention the participants allocated to the MD+ EVOO showed a decrease in sVCAM-1 (P<0.02), sICAM-1 (P< 0.001) and sP-selectin (P<0.001) concentrations. Furthermore, the MD+Nuts group showed a decrease in sVCAM-1 (P<0.001) and sE-selectin (P<0.002) and sP-selectin of (P<0.007). By contrast, the serum concentration of sICAM-1 was significantly increased (P<0.02) in the control group.

Other molecules related to plaque instability (**Table 4**), such as CRP and IL-6 (P<0.001; both) and the IL-18/IL-10 ratio (P≤ 0.04) decreased in the MD+EVOO and MD+nuts groups. Furthermore, in the MD+nuts group, IL-18 concentration also decreased (P<0.003). Finally, the control group showed a significant increase in IL-6 (P<0.001), MMP-9 (P<0.003) and TGF-β1 (P<0.02) levels. The same group, showed an increase, albeit no significant, in the MMP-9/TIMP-1 ratio (P<0.003) and TIMP-1, although the changes were not significant.

None of the groups showed significant Pearson correlation coefficients between MUFA intake and any of the inflammatory marker concentrations.

Changes in Food and Nutrient Intake during Follow-up

The self-reported dietary habits of the participants prior to starting the study were similar among the three groups. Baseline diets were high in fiber, total fat and MUFA because of a high baseline consumption of olive oil and marine n3 fatty acids due to frequent fish intake (**Appendix S1 and S2**). The saturated fatty acid (SFA) and ω3-polyunsaturated fatty acid (PUFA) content of the diets was relatively low.

Dietary intervention resulted in favorable changes in food consumption, mainly in the MD groups (**Appendix S1**). Accordimgly, after 12 months these subjects showed a significant increase in adherence to the MD pattern (P<0.001).

Appendix S2 shows changes in baseline energy and nutrient intake after the 12-month intervention. At 12 months, the participants in the MD+EVOO group increased the average urine concentration of the phenolic compound tyrosol 8.0 ng/mL (P<0.02) from a baseline value of 61.0 ng/mL, and hydroxytyrosol increased 39.0 ng/mL (P<0.04) from a baseline value of 205 ng/mL, while the plasma content of ALA increased 0.14 mol% (P<0.01) from a baseline value of 0.3 mol% in subjects assigned to the MD+Nuts group. These three parameters were used as an objective measure of compliance in the MD intervention diet groups.

Discussion

Higher adherence to a MD intervention supplemented with EVOO or nuts, for at least 12 months, were associated with a significant decrease in inflammatory markers related to atheroma plaque formation and plaque instability, in addition to a reduction

Table 3. Changes in adhesion molecule expression in circulating T- lymphocytes and monocytes.

		MD+EVOO (n = 55)		MD+Nuts (n = 55)		Low-fat diet (n = 54)		
		Mean	**P[3]**	**Mean**	**P[3]**	**Mean**	**P[3]**	***Pint*[4]**
T-LYMPHOCYTES								
CD11a	Baseline[1]	132±4.7		137±5.2		121±5.2		0.26
	1y.[1]	107±5.3		107±6.0		103±6.0		
	Mean changes[2]	−24.4 (−36.0 to −13.0)	<0.001	−29.9 (−43.1 to −16.7)	<0.001	−18.3 (−31.2 to −5.2)	0.006	
CD49d	Baseline	48.3±1.1		39.0±1.1		34.8±1.1		0.33
	1y.	36.7±1.1		41.0±1.1		39.9±1.1		
	Mean changes	−11.7 (−16.1 to −7.2)	0.004	2.0 (−0.7 to 4.7)	0.54	5.1 (3.1 to 7.1)	0.14	
CD40	Baseline	47.5±1.1		55.3±1.1		44.2±1.1		0.20
	1y.	36.7±1.1		42.0±1.1		38.6±1.1		
	Mean changes	−11.0 (−12.6 to −9.3)	0.001	−13.5 (−15.5 to −11.4)	0.001	−5.6 (−6.5 to −4.8)	0.09	
MONOCYTES								
CD11a	Baseline	85.0±4.2		80.1±4.4		78.8±4.2		0.41
	1y.	57.3±2.0		56.8±2.1		53.9±2.1		
	Mean changes	−27.7 (−36.1 to −19.5)	<0.001	−23.3 (−32.0 to −14.6)	<0.001	−24.9 (−33.3 to −6.6)	<0.001	
CD11b	Baseline	44.7±2.1		47.3±2.2		43.9±2.2		0.38
	1y.	34.6±1.3		36.5±1.3		35.1±1.3		
	Mean changes	−10.1 (−14.6 to −5.5)	<0.001	−10.8 (−15.6 to −6.1)	<0.001	−8.8 (−13.6 to −4.1)	<0.001	
CD49d	Baseline	35.2±1.1		39.0±1.1		35.0±1.1		0.50
	1y.	27.2±1.1		29.2±1.1		30.7±1.1		
	Mean changes	−8.0 (−9.4 to −6.5)	<0.001	−9.8 (−11.6 to −8.1)	<0.001	−4.3 (−5.3 to −3.2)	0.06	
CD40	Baseline	36.1±2.9		46.5±2.9		35.2±2.9		0.03
	1y.	26.3±1.9		30.9±1.9		31.1±1.9		
	Mean changes	−9.8 (−15.0 to −4.6)[b]	<0.001	−15.6 (−20.9 to −10.2)[a,b]	<0.001	−4.1 (−9.4 to 1.2)	0.13	

Data analyzed by repeated-measures 2-factor ANOVA (simple-effect analysis by Bonferroni's multiple contrast).
[1]Values are mean ± SD.
[2]Mean differences (95% CI).
[3]P: Significant differences (P<0.05) between before and after the intervention.
[4]Pint: comparison between measures obtained before and after intervention and among the 3 diet groups, P<0.05.
[a]MD+EVOO or MD+nuts vs. low fat-diet and [b]MD+EVOO vs. MD+nuts are significantly different, P<0.05. EVOO, extra virgin olive oil; MD+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MD+Nuts, Mediterranean diet supplemented with nuts.
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Table 4. Changes in the expression of circulating markers of plaque instability and other inflammatory biomarkers.

		MD+EVOO (n = 55)		MD+Nuts (n = 55)		Low-fat diet (n = 54)		
		Mean	P[3]	Mean	P[3]	Mean	P[3]	*Pint*[4]
sVCAM (ng/mL)	Baseline[1]	872±47.0		935±49.2		776±48.6		0.30
	1y.[1]	734±44.9		727±47.1		720±46.5		
	Mean changes[2]	−138 (−251 to −25.2)	0.02	−208 (−327 to −89.6)	0.001	−55.6 (−173 to 61.5)	0.35	
sICAM (ng/mL)	Baseline	437±27.3		394±23.3		369±24.0		0.04
	1y.	217±22.0		364±18.8		431±19.2		
	Mean changes	−220 (−273 to −166)[b]	<0.001	−30.3 (−76.1 to 15.5)[b]	0.20	62.3 (15.5 to 109)[b]	0.01	
sE-SEL (ng/mL)	Baseline	28.6±2.5		33.0±2.6		32.3±2.6		0.55
	1y.	26.9±2.4		28.3±2.5		30.1±2.5		
	Mean changes	−1.7 (−4.5 to 1.2)	0.26	−4.7 (−7.7 to −1.7)	0.003	−2.2 (−5.3 to 0.9)	0.16	
sP-SEL (ng/mL)	Baseline	91.4±9.3		87.6±9.4		50.0±10.5		0.04
	1y.	66.5±8.3		70.8±8.4		51.1±9.3		
	Mean changes	−25.0 (−32.3 to −17.6)[a]	<0.001	−16.8 (−24.3 to −9.4)[a]	<0.001	1.1 (−7.1 to 9.4)	0.78	
IL-6 (pg/mL)	Baseline	0.7±0.1		0.9±0.1		0.7±0.1		0.04
	1y.	0.4±0.1		0.5±0.1		1.0±0.1		
	Mean changes	−0.3 (−0.9 to 0.3)[a]	<0.001	−0.4 (−1.0 to 0.2)[a]	<0.001	0.3 (−1.1 to 1.7)	<0.001	
CRP (mg/mL)	Baseline	3.8±1.1		3.5±1.1		3.6±1.1		0.04
	1y.	1.9±1.1		2.1±1.1		3.3±1.1		
	Mean changes	−1.9 (−2.4 to −1.6)[a]	<0.001	−1.4 (−2.1 to −0.7)[a]	<0.001	−0.3 (−1.3 to 0.8)	0.46	
IL-18 (pg/mL)	Baseline	139±14.3		131±14.5		103±14.6		0.18
	1y.	137±13.1		112±13.2		101±13.4		
	Mean changes	−1.8 (−13.8 to 10.2)	0.76	−18.6 (6.4 to 30.7)	0.003	−1.3 (−13.5 to 11.0)	0.84	
IL-10 (pg/mL)	Baseline	1.4±1.1		1.3±1.1		1.2±1.1		0.40
	1y.	1.5±1.1		1.4±1.1		1.3±1.1		
	Mean changes	0.05 (−0.2 to 0.3)	0.62	0.05 (−0.2 to 0.3)	0.60	0.1 (−0.1 to 0.3)	0.29	
IL-18/IL-10 ratio	Baseline	31.9±4.0		17.0±4.1		20.6±4.0		0.02
	1y.	17.2±3.4		7.9±3.5		19.0±3.4		
	Mean changes	−14.7 (−23.1 to −6.2)	0.001	−9.1 (−18.0 to −0.3)	0.04	−1.6 (−10.1 to 6.9)	0.71	
MMP-9 (ng/mL)	Baseline	7.7±1.2		7.9±1.2		6.2±1.2		0.78
	1y.	10.0±1.2		10.4±1.2		10.5±1.2		
	Mean changes	2.3 (0.9 to 3.8)	0.13	2.5 (1.1 to 3.8)	0.11	4.3 (1.2 to 7.3)	0.003	
TIMP-1 (ng/mL)	Baseline	143±6.7		146±7.3		144±7.2		0.94
	1y.	146±7.4		144±8.2		152±8.2		
	Mean changes	2.7 (−8.7 to 14.0)	0.64	−2.4 (−14.9 to 10.1)	0.71	7.5 (−4.7 to 19.8)	0.23	
MMP-9/TIMP-1 ratio	Baseline	0.06±1.2		0.06±1.2		0.04±1.2		0.60

Table 4. Cont.

		MD+EVOO (n = 55)		MD+Nuts (n = 55)		Low-fat diet (n = 54)		
		Mean	P[3]	Mean	P[3]	Mean	P[3]	Pint[4]
	1y.	0.08±1.2		0.08±1.2		0.07±1.2		
	Mean changes	0.02 (0.01 to 0.03)	0.125	0.02 (0.01 to 0.03)	0.15	0.03 (0.01 to 0.06)	0.03	
TGF-β1 (pg/mL)	Baseline	40.2±2.3		46.7±2.4		43.0±2.5		0.11
	1y.	44.5±2.1		49.3±2.2		49.0±2.3		
	Mean changes	4.3 (−0.4 to 9.0)	0.08	2.6 (−2.2 to 7.5)	0.30	5.9 (0.9 to 11.0)	0.02	

Data analyzed by repeated-measures 2-factor ANOVA (simple-effect analysis by Bonferroni's multiple contrast).
[1]Values are mean ± SD.
[2]Mean differences (95% CI).
[3]P: Significant differences (P<0.05) between before and after the intervention.
[4]Pint: comparison between measures obtained before and after intervention and among the 3 diet groups, P<0.05.
[a]MD+EVOO or MD+nuts vs. low fat-diet are significantly different, P<0.05.
[b]All the groups differed, P<0.05. EVOO, extra virgin olive oil; MD+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MD+Nuts, Mediterranean diet supplemented with nuts.
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in BP and plasma LDL-cholesterol concentration [18–19]. All of these mechanisms may explain, at least in part, the lower incidence of CHD, stroke and mortality in subjects at high risk of CVD following a Mediterranean diet as has been recently demonstrated by the PREDIMED study [11].

In the long term, a decrease of 10 or 5 mmHg in systolic or diastolic BP, respectively, is associated with a 40% and 30% reduction in the risk of stroke or myocardial infarction, respectively [20]. Moreover, a 10% reduction in plasma cholesterol concentrations has been associated with a 20% reduction in CHD risk [21].

In the current paper, we observed a significant decrease of 6 mmHg in systolic BP and 3 mmHg in diastolic BP in both MD groups [10]. This same trend was observed in plasma total-cholesterol concentrations, with a decrease of 5% for the MD+EVOO and 8% for the MD+Nuts compared to the control group. These declines were also similar to those already observed at 3 months for both MDs [10]. Nonetheless, improvements in BP and the lipid profile can not explain the whole protective action of the MD against atherosclerosis, thereby suggesting the presence of alternative effects.

It is well known that atherosclerosis is a chronic low-grade inflammatory disease of the arterial wall [2]. Thus, modulation of this inflammatory reaction may be another potential way by which the MD protects against atherosclerosis. In the current study, we detected several antiinflammatory effects in the three diets studied, although they were more intense in subjects allocated to the two MD interventions. These subjects showed a higher down-regulation of adhesion molecules in T-lymphocytes and monocytes compared to those in the control group. Moreover, serum concentrations of endothelial soluble cell adhesion molecules, CRP and IL-6 were also lower in subjects following the two MDs compared to control subjects. These results agree with those previously published by Castaner et al [28] and Estruch et al [10], both based in data from PREDIMED trial, in which a sustained traditional MD supplemented with EVOO or nuts may exert health benefits through changes in the transcriptomic response of genes related to cardiovascular risk. Moreover, the IL-18 and IL-18/IL-10 ratio, which are related to ischemic events in the heart and brain [29–33], were decreased after the MD+nuts and both MD interventions, respectively, suggesting a greater stability of atheroma plaque in patients following a MD diet.

This antiinflammatory effect has already been associated with different interventions related to a lower incidence of cardiovascular disease such as a diet rich in fruit, vegetables and olive oil [22], statins [23], moderate alcohol intake [24] or physical activity [25]. In fact, previous studies have reported that some components of the MD such as EVOO or nuts may down-regulate inflammatory markers related to atherosclerosis such as VCAM-1, ICAM-1, E- and P-Selectin, CRP and IL-6 [26–27].

When we analyzed the possible mechanisms responsible for the antiinflammatory effect observed in both MD groups, the role of exercise on immunomodulation is probably residual since no changes in physical activity were observed in the three groups. In fact, we only observed a significant increase in physical activity in the low-fat diet group compared to both MD groups, but paradoxically this situation was not associated with a greater antiinflammatory effect as would be expected in this group [25]. Finally, the antiinflammatory effect may be due to a synergistic action among nutrients from key foods of the MD such as EVOO or nuts.

Interestingly, the antiinflammatory effect of the MD seems to be greater and more intense in the mid-term compared to the short-term [10,14], while the effect on classical cardiovascular risk

factors was similar, thereby suggesting that the MD exerts its effects on lipids and blood pressure relatively quickly (at 3 mo), with the maximum effect on systemic inflammatory biomarkers being achieved later (at 1 y). Thus, in the short-term the effect on BP and the lipid profile is higher, whereas in the mid-term the effect on chronic inflammatory response in the arterial wall is more pronounced.

The strengths of our study are its design as a randomized controlled clinical trial, excellent completion rates, good compliance, and the specific inflammatory biomarkers studied, which are involved in different phases of atheroma plaque formation. Finally, there is the question as to the clinical relevance of the antiinflammatory effects of MD. However, the recently published results of PREDIMED [11] showing lower cardiovascular morbility and mortality are sufficiently consistent to reject any doubt about the antiinflammatory effect of the MD. Regarding the limitations, this study was performed in subjects at high cardiovascular risk and therefore, the results may not be generalized to the overall population. Moreover, this study was limited to classical cardiovascular risk factors and inflammatory parameters. Thus, we can not exclude other protective effects on other clinical or biological parameters related to cardiovascular risk such as markers of arterial structure and function or oxidative stress.

In summary, the results of our study suggest that the Mediterranean diet supplemented with EVOO or nuts has a dual effect on the prevention of cardiovascular disease improving classical cardiovascular risk factors and also has an intense antiinflammatory effect. Both of these effects could partially explain the overall beneficial effect of the MD on the primary prevention of cardiovascular disease observed in high risk subjects.

Supporting Information

Appendix S1 Consumption of key food items, physical ctivity and 14-point Mediterranean diet score.
(PDF)

Appendix S2 Changes in baseline energy and nutrient intake.
(PDF)

Protocol S1 Predimed Study: Mediterranean diet in the primary prevention of cardiovascular disease.
(PDF)

Checklist S1 CONSORT checklist.
(DOC)

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Author Contributions

Conceived and designed the experiments: RE RC ES MU-S ER JS-S M-AM-G RML-R. Performed the experiments: RC MU-S GC-B. Analyzed the data: RC ES RE. Contributed reagents/materials/analysis tools: RC MU-S GC-B M-AM-G MF FA MC RML-R. Wrote the paper: RC ES RE. Read and approved the final manuscript: ES RC MU-S GC-B ER M-AM-G MC RML-R JS-S MF FA RE.

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TRABAJO 4:

Título: “Long-term immunomodulatory effect of the Mediterranean diet. A randomized nutrition intervention trial with subjects at high-risk for cardiovascular disease.”

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Antecedentes: La dieta mediterránea (DietMed) ha demostrado a corto-medio plazo efectos anti-inflamatorios, pero se sabe poco acerca de sus propiedades antiinflamatorias e inmunomoduladoras a largo plazo.

Objetivo: Evaluar los efectos a largo plazo de la DietMed sobre los marcadores inflamatorios relacionados con la aterogénesis en sujetos con alto riesgo de enfermedad cardiovascular (ECV) en comparación con los efectos de una dieta baja en grasa (DBG).

Métodos: Se asignaron al azar 165 participantes sin ECV (casi la mitad son hombres, con una edad media de 66 años) pero con alto riesgo cardiovascular, a una de tres dietas: DietMed suplementada con aceite de oliva virgen extra (AOVE), un DietMed suplementada con frutos secos, o DBG. Los participantes recibieron sesiones educativas individuales y grupales trimestrales y, en función del grupo asignado recibieron AOVE o frutos secos gratuitos para ser consumidos diariamente, o bien, pequeños regalos (no alimenticios). Se utilizó ANOVA de medidas repetidas ajustando por posibles factores de confusión para evaluar los cambios en la adherencia a la dieta, los factores de riesgo cardiovascular clásicos, y los marcadores inflamatorios.

Resultados: Se observaron reducciones plasmáticas significativas de PCR, IL-6, TNF-α, y MCP-1 en ambos grupos de DietMed en comparación con el grupo DBG (P≤0.04; todos). Las comparaciones entre grupos también mostraron reducciones significativas en la expresión linfocitaria de CD49d (≥16%) y de CD40 (≥27%) a los 3 y 5 años y en ambas DietMed. Además, ambos grupos de DietMed mostraron una reducción en la expresión monocitaria de CD49d y CD40 (≥49%, ambos) a los 3 y 5 años (P <0,001; ambos). Los cambios dentro de los grupos mostraron una mayor adherencia de los dos grupos de DietMed a la DietMed, así como un aumento en las concentraciones de HDL- colesterol (P≤0.04; todos) y una reducción en las concentraciones de colesterol,

LDL-colesterol total, y relación [colesterol- total / HDL-colesterol], y triglicéridos (P≤0.03; todos) en comparación con el grupo DBG.

Conclusiones: La adherencia a la DietMed a largo plazo se asocia con niveles séricos disminuidos de los marcadores inflamatorios relacionados con la aterosclerosis. Este papel antiinflamatorio de la DietMed podría explicar, en parte, el efecto cardioprotector de la MedDiet contra las enfermedades cardiovasculares.

Long-term immunomodulatory effect of the Mediterranean diet. A randomized nutrition intervention trial with subjects at high-risk for cardiovascular disease.

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RUNNING TITLE: Immunoregulatory effects of Mediterranean diet

Footnotes to the title disclosing:

i) Supplemental Table 1 and Supplemental Table 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

ii) Abbreviations used: BP: blood pressure; CHD: coronary heart disease (CHD); CRF: cardiovascular risk factors; CRP: C-reactive protein; CVD: cardiovascular disease; EVOO: extra-virgin olive oil; FQQ: food frequency questionnaire; hs-CRP: ultra-sensitive C-reactive protein; ICAM-1: intercellular adhesion molecule-1; IL-6: Interleukin 6; LFD: low-fat diet; MCP-1: monocyte chemoattractant protein-1; MeDiet: Mediterranean diet; MMP-9: matrix metalloproteinase-9; MUFA: monounsaturated fat; LDL: low-density lipoprotein; oxLDL: oxidized low-density lipoprotein; HDL: high-density lipoprotein; PBMCs: peripheral blood mononuclear cells; PUFA: polyunsaturated fatty acid; PREDIMED: Prevention with Mediterranean Diet; ROO: refined olive oil; SFA: saturated fatty acids; TGF-β1: transforming growth factor beta 1; TNF-α: tumor necrosis factor alpha; VCAM-1: vascular cell adhesion molecule-1; VOO: virgin olive oil.

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iv) Disclosures: RE reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. ER reports to be a non paid member of the Scientific Advisory Committee of the California Walnut Commission, and he has received grants from the same through his institution. JS-S serves on the board of and has received grant support from the International Nut and Dried Fruit Council; he has received consulting fees from Danone and has received grant support from Eroski and Nestlé. RML-R reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from PepsiCo. No other potential conflict of interest relevant to this article was reported.

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ABSTRACT

Background: The Mediterranean Diet (MeDiet) has demonstrated short-term anti-inflammatory effects, but little is known about its long-term immunomodulatory properties.

Objective: To assess the long-term effects of the MeDiet on inflammatory parameters related to atherogenesis in subjects at high risk of cardiovascular disease (CVD) compared to the effects of a low-fat diet (LFD).

Methods: We randomized 165 participants (nearly half men, on average 66 years old) who were at high cardiovascular risk, but without overt CVD at enrollment, to one of three diets: a MeDiet supplemented with extra-virgin olive oil (EVOO), a MeDiet supplemented with nuts, or a LFD. Participants received quarterly individual and group educational sessions and, depending on group assignment, free provision of EVOO or nuts to be consumed daily, or small nonfood gifts. Repeated-measures ANOVA adjusting for potential confounding variables was used to evaluate changes in diet adherence, classical cardiovascular risk factors, and inflammatory parameters.

Results: Significant reductions in high-sensitivity C-reactive protein, interleukine-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1 in plasma were observed in both MeDiet groups compared to LFD ($P \leqq 0.04$;all). Comparisons between groups also showed significant reductions of ≥16% in CD49d and ≥27% in CD40 expressions on T- lymphocytes at 3 and 5 years, respectively, for both MeDiets. Moroever, both MeDiet groups showed a lower reduction in the monocyte expression of CD49d and CD40 (≥49%) at 3 and 5 years ($P<0.001$;both). Changes within groups showed that both MeDiet groups exhibited greater adherence to the MeDiet, an increase in HDL-cholesterol concentrations ($P \leq 0.04$;all) and a reduction in concentrations of total cholesterol, LDL-cholesterol, [total-cholesterol/HDL-cholesterol] ratio, and triglycerides ($P \leq 0.03$; all) compared to LFD.

Conclusions: Long-term adherence to the MeDiet is assosciated with decreased serum levels of inflammatory parameters related to atherosclerosis. This antiinflammatory role of the MeDiet could explain, in part ,the cardioprotective effect of the MeDiet against CVD.

Clinical trial registration: The trial is registered in the London-based Current Controlled Trials register with ISRCTN number 35739639.

Keywords: Mediterranean diet, adhesion molecules, cardiovascular disease, peripheral blood mononuclear cells, inflammation, long-term.

Word count: 300

Introduction

The Mediterranean diet (MeDiet) is recognized as one of the healthiest dietary patterns. Several epidemiological studies have shown that high adherence to the MeDiet is associated with a reduced risk of developing metabolic syndrome, hypertension, type 2 diabetes and some neurodegenerative diseases and cancers, as well as, a lower mortality and incidence of cardiovascular disease (CVD) (1,2,3). There is also consistent evidence demonstrating that the MeDiet improves classical cardiovascular risk factors (4,5). Accordingly, intervention studies such as the PREDIMED (PREvención con DIeta MEDiterránea) study (6,7) and the Lyon Diet Heart study (8) have demonstrated the beneficial effect of the MeDiet in the primary and secondary prevention of CVD, respectively.

Atherosclerosis is a complex degenerative process in which monocytes and T-cells play a key role. The cells migrate from the circulation to the subendothelial space where they differentiate into macrophages and later into foam cells after taking up oxidized low-density lipoprotein (oxLDL)(9,10,11). In parallel, the endothelium is activated due to the accumulation of modified LDL and upregulates the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin and other chemotactic agents, such as monocyte chemoattractant protein-1 (MCP-1)(12,13), which perpetuate the activation, recruitment and transmigration of monocytes, lymphocytes and other inflammatory cells across the endothelial layer into the subendothelial space, whereby initiating the formation of atheroma plaque (10,12).

Clinical and epidemiological studies have shown that adherence to the MeDiet is associated with antiatherogenic effects (14) such as reduced blood pressure (15,16), improved lipid profile (17,18), and diminished vascular inflammation (19,20), oxidative stress (21,22) and endothelial dysfunction (23,24).

Previous sub-studies of the PREDIMED trial revealed that a MeDiet supplemented with extra-virgin olive oil (EVOO) or nuts reduced systemic inflammatory biomarkers related to atherosclerosis [tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and C-reactive protein (CRP)] after 3 months (19) and 1 year (14,20) of intervention. In addition, at 3 and 12 months, monocyte expression of CD49d, an adhesion molecule crucial for leukocyte homing, and CD40, a proinflammatory ligand, decreased after both MeDiets (19,20).

Whether this anti-inflammatory effect of the MeDiet is maintained in the long-term remains to be elucidated. The aim of this study was to investigate the long-term anti-inflammatory effect of two MeDiets supplemented with either EVOO or nuts. Thus, we assessed changes in the expression of adhesion molecules related to atheroma plaque formation and changes in the plasma levels of the main and more representative immunomodulatory biomarkers (hs-CRP, IL-6, TNF-α and MCP-1) related to atherosclerosis after 3 and 5 years of intervention in a sub-cohort of the PREDIMED study. These are secondary outcomes of our randomized controlled trial.

MATERIALS AND METHODS

Design

The PREDIMED study is a parallel-group, single-blind, multicenter, randomized, controlled 5-year clinical trial conducted in Spain to assess the effects of the MeDiet on the primary prevention of CVD (www.predimed.es) (5,6). The design, methodology and eligibility criteria for the PREDIMED study have been described previously (5,6).

Setting and participants

From October 2003 to November 2004 we screened 193 consecutive candidates to the PREDIMED study recruited in primary care centers associated with the Hospital Clínic of Barcelona, Spain. Twenty-nine of these candidates did not fulfill the inclusion criteria. Four participants withdrew before 5 years (1 from the MeDiet+EVOO group, 1 from the MeDiet+nuts group and 2 from the control group). Thus, 160 subjects completed the study; 74 men (55 to 80 years of age) and 86 women (60 to 80 years of age) who were free of CVD at inclusion but had either type-2 diabetes mellitus or at least three of the following cardiovascular risk factors: current smoking, hypertension, high levels of LDL cholesterol, low levels of high-density lipoprotein (HDL) cholesterol, overweight/obesity, or family history of premature coronary heart disease (CHD). Further details of the inclusion and exclusion criteria can be found elsewhere (5,6).

Diets, physical activity and clinical measurements

All the participants were randomly assigned to one of three intervention groups: MeDiet supplemented with EVOO, MeDiet supplemented with mixed nuts (walnuts, almonds, and hazelnuts), or a control low-fat diet (LFD), as described elsewhere (5,6).

Randomization was performed centrally by means of a computer-generated random-number sequence. The baseline examinations included the administration of a 14-item and 9-item questionnaires to assess the adherence to the MeDiet and LFD, respectively, a 137-item validated food frequency questionnaire (FFQ), and the Minnesota leisure-time physical activity questionnaire, as previously described in detail (5,6). In addition, the study nurse administered a 47-item questionnaire about education, lifestyle, chronic illness and medication used, performed anthropometrical and blood pressure (BP) measurements (Omron HEM-705CP, Hoofddorp, the Netherlands), and obtained pre-specified biological samples that were stored at -80 ºC until assay (4-6). These examinations were repited at years 3 and 5 of follow-up.

The same dietitian perfomed the interventions in the 3 study groups. All the participants received quarterly individual and group educational sessions, that included a face-to-face interview and a group session that was specific for each intervention group and included no more than 20 participants per group. In the individual session, the dietitian gave personal recommendations directed to improve adherence to the MedDiet or LFD, depending on the intervention assigned. In the group sessions, participants were provided with descriptions of seasonal foods, shopping lists, weekly meal plans and cooking recipes according to the intervention group assigned. Participants allocated to the LFD group were advised to reduce all types of fat and were given written recommendations according to the American Heart Association guidelines. In the 2 MeDiet groups, participants were encouraged to increase the intake of vegetables (>=2 servings/d), fresh fruit (>=3 servings/d), legumes, nuts, fish or seafood (>=3 servings/wk), and to use olive oil for cooking and dressings. The detailed protocol including the study design, rationale, and organization has been published previously (5,6).

Participants in the two MeDiet groups were given supplementary foods at no cost. These foods included either EVOO (1 liter/week for the participants and their families) or mixed nuts (30 g/day: 15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds) according to the intervention group. The composition of the olive oil and nuts used in the study was measured by standard methods in a reference laboratory and is shown in **Table 1** (5). Energy restriction was not specifically advised nor was physical activity promoted in any of the three groups.

Ethics Statement

All participants provided signed informed consent. The Institutional Review Board of the Hospital Clinic (Barcelona, Spain), accredited by the US Department of Health and Human Services (DHHS) update for Federal wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738, approved the study protocol July 16, 2002. The trial was registered (ISRCTN35739639).

Laboratory measurements

The main outcome measurements were changes in circulating adhesion molecules involved in the first stages of atherosclerosis development at baseline and after 3 and 5 years of intervention.

First, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque (Lymphoprep™, Axis-Shield PoC AC) density-gradient. The expression of adhesion molecules on the surface of PBMCs was analyzed via double direct immunofluorescence using commercial monoclonal antibodies following the manufacturer's instructions. The adhesion molecules analyzed were: anti-CD14 and anti-CD2 monoclonal antibodies (Caltag) as markers of monocytes and T-lymphocytes, anti-CD11a and anti-CD11b (Bender Medsystems), anti-CD49d (Cytogmos), anti-CD40 (Caltag). Cell counts (5000 events for T-lymphocytes and 2000 for monocytes) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton-Dickinson) using CellQuest software. The results are expressed as mean fluorescence intensity (MFI) in arbitrary units.

Plasma was obtained after centrifugation of blood. Plasma and PBMC were stored at -80 ºC until assay. Plasma concentrations of four inflammatory biomarkers related to different stages of the atherosclerotic process were measured. Ultra-sensitive (hs) CRP was determined by standard enzyme-linked immunosorbent assays (5). IL-6, TNF-α, and MCP-1 were determined using the Bio-Plex Pro™ cytokine, adhesion molecules and chemokine assays (Bio-Rad Laboratories Inc., Hercules, CA, USA), which are based on magnetic bead-based multiplex assays designed to measure multiple cytokines, adhesion molecules and chemokines in matrices of plasma. Data from reactions are acquired using the Luminex system. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity and target concentrations on the Luminex® 200™ System. Thereafter, the data are processed and analyzed with the Bio-plex Manager 6.1™. We performed all analyses in duplicate.

The analytes determined for each participant in frozen samples of whole serum or plasma as appropriate were: blood glucose levels using the glucose– oxidase method; serum insulin level by radioimmunoassay; cholesterol and triglyceride levels by enzymatic procedures; HDL cholesterol levels after precipitation with phosphotungstic acid and magnesium chloride; and apolipoproteins A1 and B levels using turbidimetry. In a random sample of 90 participants (56%), we measured urinary tyrosol and hydroxytyrosol levels by gas chromatography–mass spectrometry as markers of adherence to extra virgin olive oil intake and the α-linolenic acid plasma content by gas chromatography as a measure of adherence to nut (walnut) intake (5,6).

Diagnostic criteria for new cases of diabetes

We considered new cases of type 2 diabetes mellitus as all those patients without a previous diagnosis of the disease who fulfilled the diagnostic criteria of the American Diabetes Association (ADA) for type-2 diabetes mellitus(25) (plasma glycemia $\geq$ 124 mg/dL and/or glycated hemoglobin ≥ 6.5%) during the follow-up period of the PREDIMED trial.

Statistical analyses

For a parallel design, the sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a maximum loss of 10% of participants. To detect a mean difference of 10 MFI units in the expression ofmonocyte CD49d with a conservative standard deviation (SD) of 10, 20 subjects would be needed to complete the study (a risk = 0.05, power = 0.9). Monocyte expression of CD49d was considered the primary outcome and was used to determine the sample size. Nonetheless, changes in all the endpoints were of equal interest in this study.

We used descriptive statistics with the mean ± SD for the baseline characteristics of the participants. We transformed variables with a skewed distribution (CD49d for T-lymphocytes and monocytes and hs-CRP) to their natural logarithm for analysis. We used descriptive statistics with the mean ± SD for the baseline characteristics of the participants. Categorical variables are expressed as percentages. Differences in food and nutrient intake, adiposity, and cardiovascular risk factors at baseline and at 3 and 5 years were assessed by the Student's t test. One-factor analysis of variance was used, as appropriate, to determine differences in the baseline characteristics among the 3 study groups. Repeated-measures ANOVA was used to compare changes in food and nutrient intake, adiposity parameters and cardiovascular risk factors, testing the effects of interaction of 2 factors: time as a within-participants factor with 2 levels (first, at baseline and at 3 years, second, at baseline and at 5 years, and third at 3 and 5 years) and the 3 intervention groups, adjusting for potential confounding variables as age, sex, body mass index (BMI), waist circumference, antihypertensive drugs, oral hypoglycemic agents and lipid-lowering agents. Changes in adhesion molecules and other inflammatory biomarkers were mesured using repeated-measures ANOVA testing the effects of interaction of 2 factors: time as a within-participants factor with 3 levels (at baseline, at 3 years, and at 5 years) and the 3 intervention groups, adjusting for potential confounding variables as age, sex, BMI, waist circumference, aspirin, oral hypoglycemic agents and statins. To test the effects of individual factors, we calculated the differences between 3 years and baseline and 5 years and

baseline values for the adhesion molecules and inflammatory molecules and then applied an ANOVA test, with the intervention group as fixed factor. Significant interactions were assessed by the simple-effect analysis. All the multiple contrasts were adjusted by a Bonferroni post hoc test. Within- and between-group differences were expressed as estimated means and 95% CI. The significance level was set at $P< 0.05$. All analyses were performed using SPSS v. 20.0 (SPSS Inc, Chicago, IL).

RESULTS

Study population

Of the 165 participants included, equal numbers (*n*=55) were randomized into each of the three intervention groups. **Figure 1** shows the retention rates (≥ 96% for all) for the 3- and 5-year follow-ups. One participant was lost to follow-up in each of the 2 MeDiet groups and three in the control group.

All participants in this sub-study were selected at random and had similar characteristics to those of the whole PREDIMED cohort. **Table 2** shows the characteristics of the study subjects by intervention group. On average, the participants were 66 years old and nearly half were men. Most participants (85%) were overweight or obese, 64% had hypertension, 64% had dyslipidemia, and 77% were diabetic. The numbers of participants who changed the medication increased in the 3 intervention groups throughout, but only aspirin use significantly increased in the 3 groups (P<0.001;all). However, the differences between groups in aspirin use did not attain statitistical significance (*P*=0.210).

Food, energy balance and dietary adherence

Adherence to the supplemental foods was good in the two MeDiet groups. Compared to baseline, urinary concentration of tyrosol and hydroxytyrosol increased in the MeDiet+EVOO group at 3 and 5 years of intervention (*P*<0.001;both), while the MeDiet+nuts group showed an increase in α-linolenic acid (*P*≤0.003) which was greater than in the other diet groups at both 3 and 5 years of intervention. A reduction in energy (*P*≤0.01;all), protein (*P*≤0.04;all), carbohydrate (*P*≤0.006;all) and cholesterol (*P*≤0.04;all) intake was observed in the 3 groups at 3 and 5 years compared to baseline **(Supplemental Table 1).** In both assessment periods total fat and MUFA intake significantly increased in the participants in the MeDiet+EVOO group while polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA) intake decreased. In the MeDiet+nuts group we observed an increase in total fat and PUFA and a decrease in SFA intake. Finally, the LFD group showed a significant decrease in the intake of fiber, total fat, SFA and PUFA.

As shown in **Supplemental Table 2,** participants in the MeDiet+EVOO group significantly increased EVOO consumption and decreased the refined olive oil (ROO) consumption the consumption of pastries, cakes and sweets at 3 and 5 years. Nut consumption increased in the MeDiet+nuts group but decreased in the other two groups. At 3 and 5 years, the consumption of vegetables and legumes increased in the two MeDiet groups , while the consumption of cereals and meat and meat products decreased in the three groups. Fruit consumption increased in the two MeDiet groups at 3 years, while fish consumption increased after 5 years only in the MeDiet+nuts group. Physical activity was maintained in all the treatment

groups throughout the intervention. Adherence to the MeDiet increased in all the groups, with between-group differences in favor of the two MeDiet arms.

Classical cardiovascular risk factors

As shown in **Table 3**, systolic and diastolic BP significantly decreased in the 2 MeDiet groups at 3 and 5 years. Compared to the LFD group, the MeDiet+EVOO and MeDiet+nuts groups showed a mean reduction of 6-7 and 10-11 mmHg in systolic BP and of 5 and 7-8 mmHg in diastolic BP at 3 and 5 years. On the other hand, weight and the body mass index (BMI) decreased by ≥1% in the MeDiet+EVOO group at 3 and 5 years of intervention. Waist circumference reduced by ≥1.2% in the 3 intervention groups at 3 years, but only the MeDiet+nuts group showed a significant reduction at 5 years of intervention compared to baseline. Finally, at 3 and 5 years, the MeDiet+EVOO and MeDiet+nuts groups showed a reduction in triglyceride concentrations, LDL-cholesterol, [total-cholesterol/HDL-cholesterol] ratio and total-cholesterol as well as an increase in HDL-cholesterol concentrations. The LFD group showed a significant increase in glucose and glycated hemoglobin levels at 5 years.

Compared to the LFD group at both 3 and 5 years the MeDiet+EVOO group showed a reduction (P<0.001) of 10% in the body mass index (BMI), while the MeDiet+nuts group reduced LDL-cholesterol 31 %.

The number of new cases of diabetes (plasma glucose $\geq$ 124 mg/dL and glycated hemoglobin ≥ 6.5%) was greater in patients in the LFD group (7 cases) than the two MeDiet groups (one in each group) (P<0.001;both).

Adhesion molecules and CD40 expression in PBMC at 3 and 5 years

Table 4 shows that CD11a expression on lymphocyte and monocyte surfaces was down-regulated in the three intervention groups at the two time points. After 3 and 5 years, CD49d and CD40 expression in peripheral T-lymphocytes was down-regulated in both MeDiet groups while CD49d expression in T cells was increased in the LFD group. Participants in the control group also showed up-regulation of CD40 in T-lymphocytes at 5 years.

At 3 and 5 years, circulating monocytes showed a significant decrease in CD11b, CD49d and CD40 in the two MeDiet groups compared to baseline.

Comparisons among the 3 intervention groups showed a greater reduction of CD49d (≥ 16%) and CD40 (≥ 27%) expression in T- lymphocytes in the MeDiet+EVOO and MeDiet+nut groups than the LFD group after 3 and 5 years intervention.

In relation to monocytes, we observed a greater reduction in CD11b expression (≥40%) in the MeDiet+nut group after 5 years, while the expression of CD49d and CD40 (≥49%; both) was lower in both MeDiet groups, compared to the LFD.

Plasma Inflammatory Biomarkers

At 3 and 5 years, participants of the 2 MeDiets also showed significant reductions of ≥30% in plasma concentrations of hs-CRP (*P*≤0.02; both), ≥35% IL-6 (*P*≤0.005;both), ≥21% TNF-α (*P*≤0.04; both) and ≥16% MCP-1 (*P*≤0.009; both), whereas the changes in LFD did not achieve statistical significance (**Table 5**). However, plasma concentrations of inflammatory biomarkers were compared between the 3 groups, participants in the MeDiet+EVOO group showed highly significant reductions of all inflammatory parameters evaluated (*P*<0.001; all), whereas those allocated in the MeDiet+nut group only showed significant reduction in MCP-1 and IL-6 (*P*≤0.002;both).

DISCUSSION

Adherence to the MeDiet down-regulates the expression of adhesion molecules on circulating T-lymphocyte (CD11a, CD49d and CD40) and monocyte (CD11a, CD11b, CD49d, CD40) surfaces as well as inflammatory biomarkers (TNF-α, IL-6, MCP-1, hs-CRP) in serum. These molecules play an essential role in the recruitment of monocytes from the bloodstream to the subendothelial space in the initial stages of atherogenesis and throughout its course. This anti-inflammatory effect of the MeDiet was maintained in the long-term and was also associated with an improvement in classical cardiovascular risk factors, including reduced blood pressure and waist circumference and a shift of the lipid profile towards less atherogenicity. A large body of scientific evidence supports the cardioprotective effect of the MeDiet (5,6,19,20,26). The best proof of the health effects of the MeDiet has been provided by the results of the PREDIMED study showing that a MeDiet supplemented with EVOO or nuts reduces the incidence of CVD events by 30% in subjects at high cardiovascular risk(6). In addition, the PREDIMED study has also investigated the mechanisms involved in this salutary effect. The results of the present study suggest that the MeDiet has a dual effect against CVD. First, it improves the classical cardiovascular risk factors(5,19,20) and, second, it has a significant anti-inflammatory effect(14,19,20) in the short- and long-term. Thus, the MeDiet reduces systolic and diastolic BP(5,17,18) and fasting glucose levels(17,27), improves insulin resistance(27,28), and decreases abdominal fat(27,28,29,30). The lipid profile(5) also improved with a decrease in LDL cholesterol and an increase in HDL cholesterol in both MeDiet groups. On the other hand, the MeDiet seems to exert its effects on classical risk factors at an early stage (3 months)(19). Experimental and clinical studies have shown that the MeDiet exerts its anti-inflammatory and immunomodulating effects through down-regulation of the expression of leukocyte adhesion molecules(19,20), decreasing pro-inflammatory interleukins (IL-1, IL-6), hs-CRP, TNF-α and its receptors, chemoattractant molecules (MCP-1), and soluble endothelial adhesion molecules (sVCAM-1, sICAM-1, sE- and sP-Selectin)(5,14,19,20). Moreover, the MeDiet also down-regulates the expression of molecules related to plaque instability, such as IL-18, MMP-9 or TGF-β1(20). The results of the present study confirm the long-term anti-inflammatory effects of the MeDiet.

An important question is whether it is the MeDiet pattern itself or specific food components that are responsible for these effects. Olive oil is one of the main components of the MeDiet. Besides MUFA, EVOO contains α-tocopherol and phenolic compounds with strong antioxidant and anti-inflammatory properties(31,32). *In vitro* and *ex vivo* studies with EVOO have shown down-regulation of the expression of systemic VCAM-1, ICAM-1, and E-selectin in circulating lymphocytes and monocytes(32) and decreases of plasma concentrations of IL-6, and CRP in patients with stable CHD(33). In addition, cross-sectional

studies(34) have shown low concentrations of VCAM-1, ICAM-1, IL-6 and CRP in subjects with the highest consumption of EVOO.

In a study using a nutrigenomic approach, the 3-week intake of EVOO reduced the gene expression on PBMNCs of CD40L, its downstream products, and related genes involved in atherogenic and inflammatory processes in humans(35). These results are in accordance with the reduction of the expression of CD40 on T-lymphocytes and monocytes in a short- (3 and 12 months)(19,20) and long-term follow-up of 3 and 5 years.

On the other hand, nuts, another key component of the MeDiet, are rich in unsaturated fatty acids (α-linolenic acid in the case of walnuts), fiber, phytosterols, folic acid and vitamin E and polyphenols(36). Nut consumption has also been associated with decreased levels of IL-6, CRP and fibrinogen in cross-sectional studies(34,37), as well as lower plasma concentrations of sVCAM-1, sICAM-1 and sE-selectin in hypercholesterolemic patients in interventional studies(38). On the other hand, several studies have associated the immunomodulatory and anti-inflammatory effects of the MeDiet with the dietary pattern itself and not to specific foods(23,39,40,41) showing reductions in the concentrations of biomarkers of inflammation and endothelial dysfunction (CRP, IL6, ICAM-1 and VCAM-1) in subjects with higher adherence to the MeDiet. However, these former studies all of these studies evaluated the effects of the MeDiet at only 3 to 12 months after intervention.

After 3 and 5 years of intervention, the two MeDiet groups in the current study showed increased adherence to the MeDiet assessed by food questionnaires and to the supplemental foods assessed by changes in objective biomarkers such as plasma urinary tyrosol and hydroxytyrosol levels (as a measure of adherence to EVOO consumption recommendations) and the plasma α-linolenic acid proportion (as a measure of adherence to walnut consumption recommendations). Concomitantly, we observed a down-regulation of the expression of T-lymphocyte and monocyte adhesion molecules. Therefore, according to these results, the composition of the diet could lead to a modification in the expression of leukocyte adhesion molecules in participants assigned to the 2 MeDiet groups and could modify the expression of these adhesion molecules not only in the short- and medium-term but could also maintain or even increase these effects in the long-term, for up to at least 5 years of follow-up.

Our study has several strengths, one of which is its randomized design and reproduction of real life conditions, such as home-prepared foods, excellent completion rates, and good compliance, which were assessed with serum biomarkers and close monitoring of the participants, the number of inflammatory leukocyte adhesion molecules evaluated, and, importantly, the long duration of the follow-up.

Nonetheless, there are also limitations to our study. The results cannot be generalized to other populations because the participants were older subjects at high risk for CHD. Other limitation of the study could be that a great proportion of our patients had type 2 diabetes which may have a great effect on the development of atherogenesis (inflammation and inmune cell activation); therefore, these data should be replicated in another cohort with lower inicidence of type 2 diabetes.

On the other hand, the outcomes of the study were changes in classical cardiovascular risk factors and inflammatory molecules, while the effects on other variables related to arterial structure and function or oxidative stress were not studied.

CONCLUSION

The current study supports the recommendation of the MeDiet as a useful tool against CVD. This healthy effect seems to be reached achieved through several mechanisms, including modulating inflammatory response and improving classical cardiovascular risk factors which are maintained in the long-term.

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The authors' responsibilities were as follows -RE, RC, MU-S, ES: study conception and design; RC, OC: laboratory and clinical data; RC, ES, MU-S, ER and RE: analysis and interpretation of the data; RC, ES, RML-R and RE: draft of the article; and RC, ES, MU-S, ES, DC, OC, RML-R, JS-S, M-AM-G, ER and RE: critical revision and final approval. RC, MU-S, ES and RE wrote the paper. RE had primary responsibility for the final content. All the authors have read and approved the final manuscript. None of the authors declare a conflict of interest related to the study.

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TABLES

TABLE 1. Fatty acid, tocopherol, and sterol composition of the extra-virgin olive oil and nuts used in the trial*.

Constituents	Extra Virgin Olive Oil	Walnuts	Almonds	Hazelnuts
Total fat, *%*	100	62.9 (0.3)	50.2 (0.2)	53.2 (0.3)
Palmitic acid, *%*	8.2 (0.2)	6.3 (0.0)	7.4 (0.1)	7.4 (0.1)
Stearic acid, *%*	3.2 (0.1)	2.6 (0.0)	1.8 (0.0)	1.9 (0.1)
Oleic acid, *%*	75.0 (0.8)	14.0 (0.3)	61.2 (0.4)	72.1 (0.2)
Linoleic acid, *%*	6.8 (0.2)	61.3 (0.4)	26.7 (0.2)	13.3 (0.2)
α-Linolenic acid, *%*	0.4 (0.0)	14.3 (0.1)	0.1 (0.0)	0.8 (0.0)
α-Tocopherol, *mg/100 g*	14.7 (0.0)	4.9 (0.1)	48.4 (0.9)	38.8 (1.5)
β-Tocopherol, *mg/100 g*	4.3 (0.0)	2.0 (0.1)	5.4 (0.9)	8.8 (1.5)
γ-Tocopherol, *mg/100 g*	0.4 (0.0)	50.2 (1.3)	6.0 (0.2)	20.7 (0.4)
Total sterols, *mg/100 g*	155.8 (0.0)	198.5 (7.8)	224.2 (25.4)	174.6 (8.6)
β-Sitosterol, *%*	95.5 (0.1)	84.0 (0.8)	79.1 (0.5)	82.8 (1.1)
Campesterol, *%*	3.2 (0.0)	5.3 (0.0)	3.3 (0.0)	5.2 (0.1)
Δ-5-Avenasterol, *%*	<0.1	7.6 (0.9)	6.3 (1.2)	11.1 (0.2)

* Values are means (SD) of 6 measurements of random samples from different lots.

TABLE 2. Baseline characteristics of the participants at high risk for cardiovascular disease included in the trial and classified according to the dietary intervention administered.

	MeDiet+EVOO	MeDiet+nuts	Low-fat diet	P^2
Age, *years*	66.7 ± 6.0[1]	65.8 ± 5.6	66.3 ± 6.3	0.72
Men, *n (%)*	23 (42.6) [1]	31 (57.4)	20 (38.5)	0.20
Family history of early-onset CHD, *n*	15 (27.8)	9 (16.7)	11 (21.2)	1.00
Smoking status, *n (%)*				
Current smokers	9 (16.7)	11 (20.4)	9 (17.3)	0.15
BMI, *kg/m²*	29.4 ± 4.0	28.7 ± 3.1	29.1 ± 3.8	0.60
BMI ≥ 25 kg/m², *n (%)*	47 (87.0)	45 (83.3)	44 (84.6)	0.41
Waist circumference, *cm*	100 ± 9.7	101 ± 7.8	100 ± 10.4	0.83
Waist-to-height ratio	0.47 ± 0.06	0.47 ± 0.05	0.47 ± 0.06	0.97
Glucose, *mg/dL*	133 ± 53.1	136 ± 54.7	130 ± 42.3	0.86
Glycated hemoglobin, *mg/dL*	6.3 ± 2.1	6.0 ± 1.6	6.0 ± 1.3	0.61
Type 2 diabetes, *n (%)*	45 (83.3)	43 (79.6)	35 (67.3)	0.23
Years of diagnosis				
1-5y	18 (32.7)	21 (38.2)	12 (22.2)	0.21
> 5y	27 (50.0)	22 (40.7)	23 (44.2)	0.10
Hypertension, *n (%)*	38 (70.4)	29 (53.7)	35 (67.3)	0.10
Dyslipidemia, *n (%)*	32 (59.3)	34 (63.0)	36 (69.2)	0.40
Medications, *n (%)*				
ACE inhibitors	10 (18.5)	12 (22.2)	13 (25.0)	0.41
Diuretics	12 (22.2)	6 (11.1)	12 (23.1)	0.22
Other antihypertensive agents	10 (18.5)	8 (14.8)	9 (17.3)	0.84
Statins	17 (31.5)	14 (25.9)	10 (19.2)	0.56
Other-lipid-lowering agents	4 (7.4)	2 (3.7)	4 (7.7)	0.27
Insulin	3 (5.5)	7 (13.0)	3 (5.8)	0.51
Oral hypoglycemic drugs	29 (53.7)	24 (44.4)	27 (51.9)	0.87
Biguanides	11 (20.4)	14 (24.6)	17 (37)	0.44
Increase insulin secretion	14 (25.5)	13 (23.6)	16 (29.6)	0.43
Others	4 (7.3)	5 (9.0)	3 (5.8)	0.19
Aspirin or antiplatelet drugs	9 (16.7)	8 (14.8)	5 (9.6)	0.93
NSAIDS	5 (9.3)	9 (16.7)	6 (11.5)	0.52

[1]Values are mean ± SD or n (%) as appropriate, *n*=54 in both MeDiet and in 52 LFD groups unless noted otherwise. [2]From Pearson's chi-square test for categorical variables and one-factor ANOVA for continuous variables. ACE, angiotensin converting enzyme; BMI, body mass index; CHD, coronary heart disease; EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; NSAIDS, Non-steroidal anti-inflammatory drugs.

TABLE 3. Changes in cardiovascular risk factors and adiposity at baseline, and after 3 and 5 years of follow-up in the 3 intervention groups.

		Within-group mean changes			
		MeDiet + EVOO	MeDiet + Nuts	Low-fat diet	*Pint* [3]
Systolic blood pressure, *mmHg*	Baseline[1]	152 ± 15.4	148 ± 13.7	147 ± 16.0	
	3y[2]	-6.2 (-10.0, -2.3)*	-7.2 (-10.9, -3.6)*	-0.5 (-4.6, 3.5)	0.04
	5y[2]	-9.7 (-13.9, -5.5)*[, b]	-10.9 (-15.0, -6.9)*[, b]	-1.1 (-5.5, 3.3) [γ]	0.03
Diastolic blood pressure, *mmHg*	Baseline	85.1 ± 8.7	84.7 ± 9.1	81.0 ± 10.5	
	3y	-5.3 (-7.6, -3.0)*	-5.5 (-7.8, -3.3)*	0.1 (-2.4, 2.5)	0.002
	5y	-7.2 (-9.7, -4.6)*	-7.8 (-10.3, -5.3)*[, a, ,γ]	0.5 (-2.2, 3.3) [γ]	<0.001
Triglycerides, *mg/dL*	Baseline	135 ± 65.9	144 ± 73.6	137 ± 69.1	
	3y	-19.0 (-36.1, -1.8)*	-21.6 (-37.8, -5.4)*	-10.2 (-28.9, 8.6)	0.65
	5y	-22.2 (-42.1, -2.3)*	-24.4 (-43.2, -5.7)*	-13.7 (-35.4, 8.1)	0.75
Total-cholesterol, *mg/dL*	Baseline	228 ± 30.9	219 ± 36.0	213 ± 31.3	
	3y	-19.2 (-28.7, -9.8)*	-18.4 (-27.5, -9.4)*	-7.6 (-18.0, 2.8)	0.20
	5y	-31.1 (-41.2, -21.0)*[, b, γ]	-39.1 (-48.9, -29.4)*[, b, γ]	-22.7 (-33.9, -11.5)*, [γ]	0.10
HDL-Cholesterol, *mg/dL*	Baseline	51.4 ± 12.3	47.6 ± 9.4	51.7 ± 15.0	
	3y	7.5 (4.9, 10.0)*	6.5 (4.1, 8.9)*	3.9 (1.2, 6.7)*	0.16
	5y	4.4 (0.2, 8.5)*	7.4 (3.5, 11.3)*	2.8 (-1.7, 7.3)	0.30
LDL-Cholesterol, *mg/dL*	Baseline	144 ± 27.8	141 ± 34.0	130 ± 21.0	
	3y	-11.7 (-20.0, -3.6)*	-16.5 (-24.5, -8.5)*	-0.05 (-9.3, 9.2)	0.03
	5y	-23.8 (-33.8, -13.7)*[, b, γ]	-44.2 (-54.0, -34.4)*[, a, b, γ]	-7.7 (-19.0, 3.7) [γ]	<0.001
[Total-Cholesterol: HDL-Cholesterol] ratio	Baseline	4.7 ± 1.1	4.7 ± 1.1	4.2 ± 1.2	
	3y	-0.9 (-1.2, -0.6)*	-0.9 (-1.2, -0.6)*	-0.4 (-0.7, -0.2)*	0.02
	5y	-1.0 (-1.3, -0.6)*	-1.2 (-1.5, -0.8)*	-0.5 (-0.9, -0.08)*	0.12
Glucose, *mg/dL*	Baseline	133 ± 53.1	136 ± 54.7	130 ± 42.3	
	3y	0.8 (-11.6, 13.1)	2.1 (-9.5, 13.7)	1.4 (-12.0, 14.8)	0.99

	5y	-2.6 (-15.5, 10.2)	0.6 (-11.4, 12.7)	16.5 (2.7, 30.4)*, ϒ	0.11
Glycated hemoglobin, *mg/dL*	Baseline	6.3 ± 2.1	6.0 ± 1.6	6.0 ± 1.3	
	3y	0.2 (-0.2, 0.6)	0.3 (-0.09, 0.6)	0.3 (-0.07, 0.7)	0.92
	5y	0.05 (-0.3, 0.4)	0.2 (-0.2, 0.5)	0.5 (0.1, 0.9)*, ϒ	0.22
Weight, *Kg*	Baseline	76.3 ± 18.2	77.1 ± 14.5	75.7 ± 16.7	
	3y[2]	-0.8 (-0.8, -0.7)*	-0.03 (-0.08, 0.02)	0.03 (-0.02, 0.09)	<0.001
	5y[2]	-1.3 (-1.4, -1.2)*	-0.05 (-0.2, 0.08)	0.05 (-0.09, 0.2)	<0.001
BMI, *kg/m2*	Baseline	29.4 ± 4.0	28.7 ± 3.1	29.1 ± 3.8	
	3y	-0.3 (-0.3, -0.2)*	-0.02 (-0.03, 0.001)	0.01 (-0.01, 0.03)	<0.001
	5y	-0.5 (-0.6, -0.5)*	-0.02 (-0.07, 0.03)	0.02 (-0.03, 0.07)	<0.001
Waist circumference, *cm*	Baseline	100 ± 9.8	101 ± 7.8	101 ± 9.4	
	3y	-4.0 (-5.2, -2.8)*	-2.8 (-4.0, -1.6)*	-2.1 (-3.4, -0.8)*	0.08
	5y	-1.2 (-2.5, 0.2) ϒ	-1.6 (-2.9, -0.3)*	-1.5 (-3.0, 0.04)	0.90

[1]Values are mean ± SD, *n*=54 in both MeDiet and in 52 LFD groups unless noted otherwise. [2]Mean differences (95% CI). *P: Significant differences (*P*<0.05) between before and after the interventions. ϒP: Significant differences (*P*<0.05) between 3 and 5 y of intervention. [3]*P*int: comparison between measures obtained before and after intervention and among the 3 diet groups, *P*<0.05. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. LFD, low-fat diet; BMI, body mass index.

TABLE 4. Changes in adhesion molecule expression in circulating T- lymphocytes and monocytes at baseline and at 3 and 5 years of follow-up in the 3 intervention groups.

		Within-group mean changes			Between-group changes		
		MedDiet + EVOO	MedDiet + Nuts	LFD	MeDiet+ EVOO	MeDiet+	MeDiet+Nuts vs.
T-LYMPHOCYTES							
CD11a	Basel	130 ± 33.1	126 ± 24.7	115 ± 31.8			
	3y	-66.9 (-81.5, -52.3)*, a	-58.8 (-76.0, -4167)*	-33.5 (-51.1, -	0.03	0.31	0.92
	5y	-71.8 (-88.5, -55.0)*	-55.6 (-75.4, - 35.8)*	-40.3 (-60.5, -	0.03	0.01	1.00
CD49d	Basel	46.2 ± 1.7	44.4 ± 1.7	35.7 ± 1.7			
	3y	-10.8 (-16.6, -6.1)*,a	-9.0 (-15.8, -3.9)*,a	18.5 (16.0, 20.0)*	<0.001	1.00	<0.001
	5y	-13.3 (-18.5, -9.1)*,a	-10.6 (-16.5, -6.1)*,a	15.3 (16.0, 14.0)*	<0.001	0.93	<0.001
CD40	Basel	47.8±1.8	51.5±1.8	38.6±1.4			
	3y	-13.7 (-18.8, -9.4)*	-14.5 (-20.4, -15.1)*	0.4 (-3.5, 3.2)	0.01	1.00	0.02
	5y	-15.6 (-19.1, -12.7)*, a	-18.3 (-22.6, -14.8)*, a	17.4 (15.4, 19.5)*, γ	<0.001	1.00	<0.001
MONOCYTES							
CD11a	Basel	82.3±26.4	80.7±35.1	74.2±22.8			
	3y	-50.1 (-60.3, -39.9)*	-48.2 (-61.1, -35.4)*	-41.9 (-55.2, -	0.34	1.00	1.00
	5y	-60.5 (-71.4, -49.6)*, a, γ	-54.4 (-68.2, -40.7)*, γ	-41.2 (-55.5, -	0.03	0.33	1.00
CD11b	Basel	45.5±16.0	43.6±13.1	42.4±15.2			
	3y	-10.0 (-17.4, -2.7)*	-7.5 (-15.1, 0.09)*	-4.3 (-12.9, 4.4)	0.85	1.00	1.00
	5y	-22.9 (-31.4, -14.4)*, γ, a	-17.3 (-26.0, -8.5)*, γ, a	-3.2 (-132, 6.9)	<0.001	0.82	0.01
CD49d	Basel	35.8±1.7	40.8±1.6	33.6±1.4			
	3y	-18.9 (-22.7, -15.7)*, a	-24.3 (-29.9, -19.7)*, a	-4.5 (-7.7, -2.0)	<0.001	1.00	<0.001
	5y	-19.6 (-23.2, -16.6)*, a	-23.6 (-28.6, -19.4)*, a	0.3 (-1.2, 1.4)γ	<0.001	1.00	<0.001
CD40	Basel	34.2±1.5	40.7±1.7	33.9±1.5			
	3y	-17.2 (-20.4, -14.4)*, a	-21.5 (-26.4, -17.5)*, a	-0.4 (-2.2, 0.9)	<0.001	1.00	<0.001
	5y	-18.5 (-21.9, -15.6)*, a	-22.7 (-27.7, -18.5)*, a	-2.2 (-4.1, -0.6)	<0.001	1.00	<0.001

[1]Values are mean ± SD, *n*= *n*=54 in both MeDiet and in 52 LFD groups unless noted otherwise. [2]Mean differences (95% CI). [*]*P*: Significant differences (*P*<0.05) between before and after the interventions. [γ]*P*: Significant differences (*P*<0.05) between 3 and 5 y of intervention. [3]*P* value: Significant differences (*P*<0.05) in changes between groups. [4]*P*int: comparison between measures obtained before and after intervention and among the 3 diet groups, *P*<0.05. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. LFD, low-fat diet.

TABLE 5. Changes in inflammatory serum biomarkers at baseline and at 3 and 5 years of follow-up in the 3 intervention groups.

		Within-group mean changes			Between-group changes		
		MedDiet + EVOO	MedDiet + Nuts	LFD	MeDiet+ EVOO	MeDiet+	MeDiet+ Nuts vs.
MCP-1,	Basel	4.3 ± 2.3	4.6 ± 2.2	3.8 ± 1.2			
	3y [2]	-1.4 (-1.9, -0.9)*, [a]	-0.7 (-1.3, -0.1)*	-0.3 (-1.0, 0.4)	0.001	0.04	0.50
	5y [2]	-1.2 (-1.9, -0.6)*	-1.4 (-2.1, -0.7)*, [γ]	-0.1 (-0.9, 0.7)	0.003	1.00	0.002
IL-6,	Basel	1.3 ± 1.2	1.4 ± 1.3	1.0 ± 0.8			
	3y	-0.5 (-0.9, -0.2)*	-0.4 (-0.8, -0.1)*	0.07 (-0.3, 0.5)	0.006	1.00	0.08
	5y	-0.6 (-0.9, -0.3)*	-0.6 (-0.9, -0.2)*	0.02 (-0.3,	0.003	1.00	0.001
TNF-α,	Basel	3.6 ± 2.8	3.6 ± 4.2	2.3 ± 1.8			
	3y	-1.6 (-2.5, -0.7)*	-1.0 (-1.9, -0.04)*	0.3 (-0.8, 1.5)	<0.001	0.91	0.02
	5y	-1.9 (-2.7, -1.1)*	-1.2 (-2.0, -0.3)*	-0.4 (-1.4, 0.6)	0.006	0.82	0.10
hs-CRP,	Basel	3.7 ± 1.7	3.5 ± 1.8	3.4 ± 1.7			
	3y	-1.8 (-2.4, -1.4) *, [b]	-1.3 (-1.8, -1.0) *, [b]	1.4 (0.9, 1.7)	<0.001	0.16	0.003
	5y	-2.0 (-2.7, -1.4)*, [b]	-1.5 (-2.0, -1.1)*	1.1 (0.7, 1.7)	0.001	0.31	0.08

[1]Values are mean ± SD, *n*=54 in both MeDiet and in 52 LFD per treatment group unless noted otherwise. [2]Mean differences (95% CI). [*]*P*: Significant differences (*P*<0.05) between before and after the intervention. [γ]*P*: Significant differences (*P*<0.05) between 3 and 5 y of intervention. [3]*P* value: Significant differences (*P*<0.05) in changes between groups. [a]MeDiet+EVOO vs. MeDiet+nuts and [b]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet are significantly different, *P*<0.05. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. LFD, low-fat diet.

LEGENDS

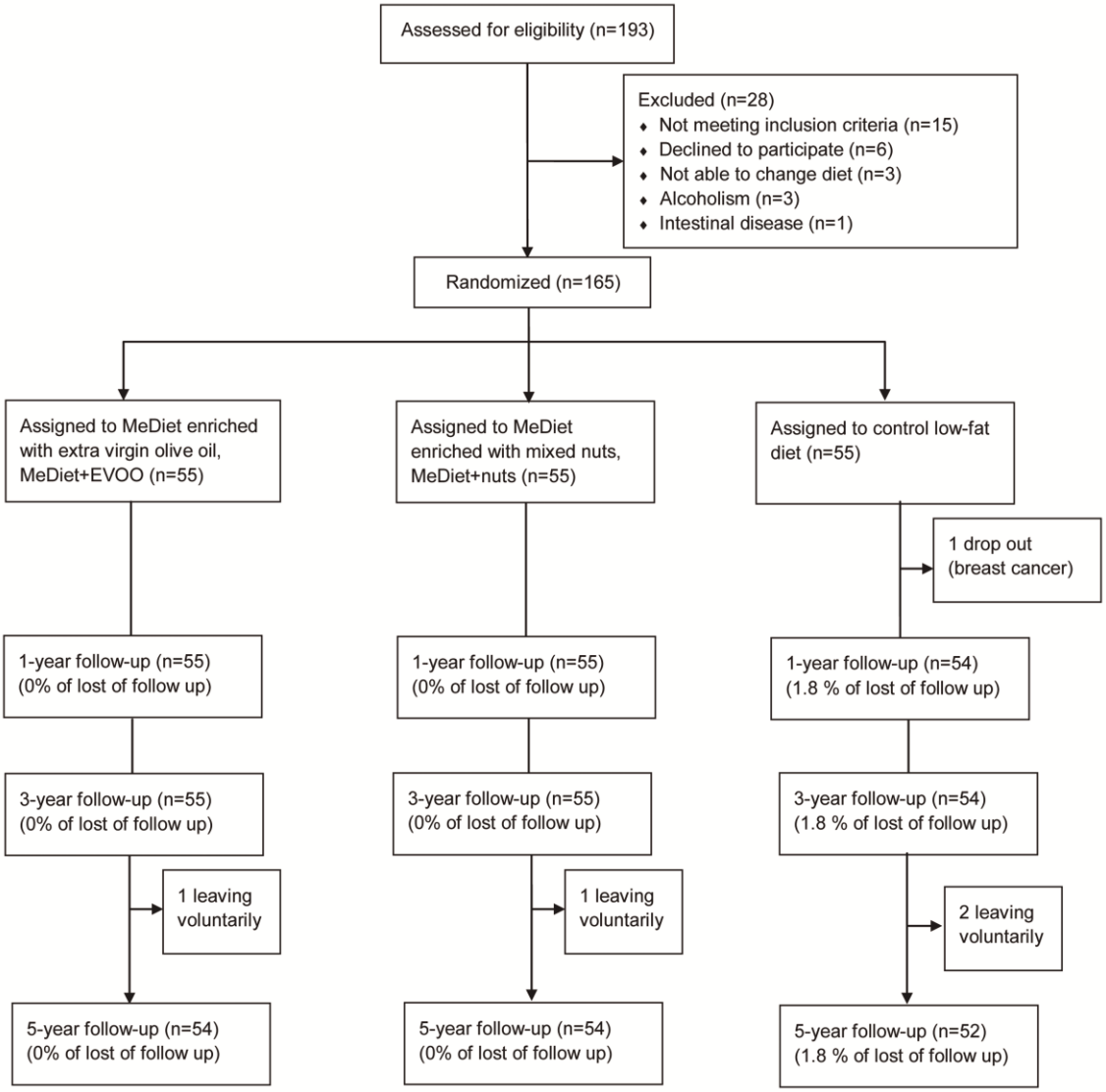

FIGURE 1. Flowchart of the study participants. The diagram includes detailed information on the participants excluded. Abbreviations: EVOO, extra virgin olive oil and MeDiet, Mediterranean diet.

Supplemental TABLE 1. **Changes in energy and nutrient intake at baseline and at 3 and 5 years of intervention in subjects at high-risk for cardiovascular disease.**

		Within-group mean changes			
		MedDiet + EVOO	MedDiet + Nuts	LFD	*Pint* [3]
Energy, *kcal/d*	Baseline[1]	2681 ± 634	2640± 562	2432 ± 746	
	3y[2]	-268 (-420, -116)*	-207 (-352, -62.8)*, [a]	-235 (-399, -71.7)*	0.85
	5y[2]	-633 (-794, -472)*, [γ]	-383 (-533, -233)*, [a, γ]	-415 (-585, -244)*, [γ]	0.06
Protein, *g/d*	Baseline	113 ± 29.1	105 ± 22.1	103 ± 21.3	
	3y	-11.8 (-18.4, -5.2)*	-6.5 (-12.9, -0.1)*, [a]	-14.6 (-21.7, -7.5)*	0.23
	5y	-20.5 (-27.2, 13.8)*, [γ]	-11.1 (-17.3, -5.0)*, [a, γ]	-19.3 (-26.3, -12.2)*, [γ]	0.09
Carbohydrate, *g/d*	Baseline	306 ± 115	277 ± 83.5	257 ± 257	
	3y	-60.2 (-85.5, -34.9)*	-40.5 (-64.4, -16.5)*	-38.6 (-66.0, -11.3)*	0.43
	5y	-108 (-136, -80.7)*, [γ]	-71.6 (-97.7, -45.5)*, [γ]	-64.0 (-93.8, -34.2)*, [γ]	0.06
Fiber, *g/d*	Baseline	32.0 ± 10.4	30.5 ± 8.5	27.8 ± 6.6	
	3y	0.5 (-2.3, 3.3) [a]	1.8 (-0.9, 4.4) [a]	-2.3 (-5.4, 0.7)	0.13
	5y	0.7 (-2.1, 3.6) [a]	1.9 (-0.7, 4.6) [a]	-4.6 (-7.6, -1.5)*, [γ]	0.002
Total fat, *g/d*	Baseline	107 ± 27.0	105 ± 24.6	101 ± 29.3	
	3y	11.2 (4.1, 18.3)*, [a]	13.6 (6.9, 20.3)*, [a]	-2.1 (-9.8, 5.6)	0.007
	5y	7.3 (0.4, 14.2)*, [a]	7.2 (0.5, 13.8)*, [a]	-13.2 (-22.6, -3.8)*, [γ]	0.001
SFA, *g/d*	Baseline	31.2 ± 10.4	29.3 ± 7.9	27.3 ± 9.5	
	3y	-5.6 (-7.9, -3.3)*	-2.3 (-4.5, -0.06)*	-1.7 (-4.1, 0.8)	0.04
	5y	-7.6 (-10.0, -5.3)*, [γ]	-4.3 (-6.8, -1.7)*, [γ]	-4.7 (-7.9, -1.5)*, [γ]	0.12
MUFA, *g/d*	Baseline	49.4 ± 12.0	52.7 ± 12.3	49.2 ± 14.6	
	3y	8.5 (4.5, 12.6)*, [a, b]	0.1 (-3.7, 3.9)	-0.7 (-5.1, 3.7)	0.003
	5y	7.9 (4.3, 11.5)*, [a, b]	1.0 (-2.5, 4.5)	-1.7 (-6.6, 3.3)	0.001
PUFA, *g/d*	Baseline	18.8 ± 7.0	18.6 ± 6.5	17.0 ± 6.8	
	3y	-3.1 (-5.5, -0.7)*, [b]	3.2 (1.0, 5.4)*, [a]	-1.9 (-4.4, 0.6)	<0.001
	5y	-5.3 (-7.5, -3.1)*, [b, γ]	2.7 (0.6, 4.8)*, [a]	-4.0 (-7.0, -1.0)*	<0.001
Linoleic acid, *g/d*	Baseline	14.9 ± 5.1	16.1 ± 7.1	13.8 ± 6.3	
	3y	-0.8 (-3.0, 1.4) [b]	1.6 (-0.4, 3.5) [a]	-1.2 (-3.5, 1.1)	0.13
	5y	-1.6 (-3.6, 0.5) [a, b]	-0.4 (-2.4, 1.5) [a, γ]	-3.4 (-5.7, -1.2)*, [γ]	0.14
α-linolenic acid, *g/d*	Baseline	1.8 ± 0.8	1.8 ± 0.8	1.7 ± 0.8	
	3y	-0.5 (-0.7, -0.2)*, [b]	0.4 (0.1, 0.6)*, [a]	-0.6 (-0.8, -0.3)*	<0.001
	5y	-0.7 (-0.9, -0.4)*, [b, γ]	0.3 (0.08, 0.6)*, [a]	-0.6 (-0.9, -0.3)*	<0.001
Marine n-3 fatty acids, *g/d*	Baseline	1.0 ± 0.6	0.9 ± 0.5	0.8 ± 0.4	
	3y	0.01 (-0.1, 0.2) [a]	0.09 (-0.05, 0.2) [a]	-0.07 (-0.2, 0.09)	0.33
	5y	0.01 (-0.1, 0.2)	0.2 (0.03, 0.4)*	-0.02 (-0.3, 0.2)	0.17
Cholesterol, *mg/d*	Baseline	423 ± 120	418 ± 112	396 ± 111	
	3y	-57.6 (-85.3, -29.8)*	-28.1 (-55.3, -0.9)*	-44.7 (-74.6, -14.8)*	0.33
	5y	-83.7 (-117, -50.5)*	-43.8 (-79.0, -8.7)*	-69.5 (-113, -26.1)*	0.26

[1]Values are mean ± SD. *n*=54 in both MeDiet and in 52 LFD groups unless noted otherwise. [2]Mean differences (95% CI). **P*: Significant differences (*P*<0.05) between before and after the interventions. [γ]*P*: Significant differences (*P*<0.05) between 3 and 5 y of intervention. [3]*P*int: comparison between measures obtained before and after intervention and among the 3 diet groups, *P*<0.05. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, *P*<0.05. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. LFD, low-fat diet; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; Refined OO, refined olive oil; SFA, Saturated fatty acids.

Supplemental TABLE 2. Changes in consumption of key food items, 14-point Mediterranean diet score and physical activity at baseline and at 3 and 5 years of intervention in subjects at high-risk for cardiovascular disease.

		Within-group mean changes			
		MeDiet + EVOO	MeDiet + Nuts	LFD	*Pint* [3]
EVOO, *g/d*	Baseline[1]	12.4 ± 17.6	11.6 ± 13.9	12.5 ± 16.8	
	3y[2]	39.8 (35.0, 44.7)*,a, b	3.7 (-1.1, 8.4)	4.4 (-0.8, 9.7)	<0.001
	5y[2]	39.4 (34.6, 44.3)*, a, b	4.7 (-0.2, 9.6)	2.7 (-2.7, 8.1)ᵞ	<0.001
Refined OO, *g/d*	Baseline	21.6 ± 16.1	19.5 ± 15.2	23.4 ± 17.8	
	3y	-21.1 (-26.6, -15.6)*,a,b	2.8 (-2.5, 8.2)	0.6 (-5.4, 6.5)	<0.001
	5y	-18.3 (-23.8, -12.9)*,a, b	0.6 (-4.8, 5.9)	-1.8 (-7.7, 4.1)	<0.001
Total nuts, *g/d*	Baseline	17.1 ± 17.7	21.1 ± 21.2	15.6 ± 15.9	
	3y	-10.0 (-15.6, -4.3)*,b	8.3 (2.9, 13.6)*,a	-10.1 (-16.2, -4.0)*	<0.001
	5y	-13.0 (-18.9, -7.2)*,b, ᵞ	6.4 (1.0, 11.7)*,a	-11.1 (-19.0, -3.3)*	<0.001
Vegetables, *g/d*	Baseline	400 ± 163	377 ± 190	363 ± 140	
	3y	118 (63.7, 172.2)*,a	89.5 (38.3, 140.8)*	16.4 (-42.1, 75.0)	0.04
	5y	72.6 (11.3, 134)*,a, ᵞ	34.4 (-21.9, 90.6)*,a, ᵞ	-41.7 (-104, 20.9)ᵞ	0.04
Legumes, *g/d*	Baseline	20.9 ± 14.7	18.9 ± 8.0	19.0 ± 9.0	
	3y	7.5 (3.3, 11.6)*,a	8.1 (4.1, 12.0)*	2.8 (-1.7, 7.2)	0.17
	5y	7.3 (3.9, 10.8)*,a	9.1 (5.3, 13.0)*,a	0.2 (-4.4, 4.8)	0.01
Fruits, *g/d*	Baseline	421 ± 183	458 ± 202	409 ± 217	
	3y	146 (80.5, 212)*	62.3 (0.2, 124)*	77.7 (6.8, 149)*	0.16
	5y	-0.3 (-74.1, 73.4) ᵞ	-10.8 (-80.5, 58.8) ᵞ	-7.3 (-86.8, 72.2) ᵞ	0.98
Cereals, *g/d*	Baseline	313 ± 122	281 ± 98.8	267 ± 113.6	
	3y	-86.0 (-118, -54.0)*	-54.8 (-84.1, -25.5)*	-51.7 (-85.1, -18.2)*	0.25
	5y	-122 (-158, -85.7)*, ᵞ	-96.4 (-135, -57.4)*, ᵞ	-109 (-156, -63.0)*, ᵞ	0.64
Fish or seafood, *g/d*	Baseline	117 ± 60.4	119 ± 49.3	102 ± 33.8	
	3y	3.9 (-9.9, 17.7)	6.5 (-6.5, 19.5)	0.7 (-14.1, 15.5)	0.85
	5y	6.9 (-9.4, 23.2)	16.4 (1.0, 31.8)*,a	4.2 (-13.4, 21.8)	0.54
Meat or meat products, *g/d*	Baseline	154 ± 67.8	152 ± 64.9	153 ± 53.7	
	3y	-17.0 (-33.2, -0.7)*	-17.0 (-32.4, -1.4)*	-31.5 (-49.0, -14.0)*	0.39
	5y	-19.8 (-36.3, -3.3)*	-22.1 (-40.1, -4.1)*	-36.7 (-58.1, -15.4)*	0.44
Pastries, cakes or sweets, *g/d*	Baseline	16.7 ± 16.5	15.0 ± 16.2	16.9 ± 21.5	
	3y	-6.1 (-11.3, -1.0)*	-1.0 (-5.8, 3.8)	-2.9 (-8.5, 2.6)	0.35
	5y	-10.3 (-15.7, -4.8)*, ᵞ	-2.2 (-7.5, 3.0)	-1.9 (-7.7, 3.8)	0.06
Dairy products, *g/d*	Baseline	419 ± 197	366 ± 250	408 ± 225	
	3y	-15.5 (-89.2, 58.1)	-54.6 (-123, 14.3)	-7.8 (-86.5, 70.8)	0.62
	5y	-34.2 (-101, 32.7)	-36.8 (-98.6, 25.1)	-37.1 (-109, 35.2)	0..99
Alcohol, *g/d*	Baseline	11.1 ± 14.2	15.0 ± 26.3	11.5 ± 15.6	
	3y	-0.7 (-5.7, 4.2)	-1.2 (-5.8, 3.5)	-0.5 (-5.8, 4.9)	0.98
	5y	-1.9 (-5.0, 1.2)	-1.1 (-4.5, 2.3)	-3.4 (-7.4, 0.7)	0.70
Wine, *mL/d*	Baseline	67.6 ± 98.4	62.1 ± 90.1	66.9 ± 112	
	3y	7.3 (-12.3, 26.9)	2.9 (-15.9, 21.6)	11.8 (-9.4, 33.0)	0.82
	5y	5.5 (-16.7, 27.8)	5.2 (-19.2, 29.6)	-4.6 (-34.0, 24.8)ᵞ	0.84
Physical Activity, *kcal/d*	Baseline	285 ± 220	260 ± 207	238 ± 211	
	3y	12.8 (-49.4, 75)	1.9 (-57.0, 60.6)	6.3 (-58.6, 71.1)	0.97
	5y	35.7 (-28.3, 99.7)	2.5 (-56.7, 61.7)	-1.3 (-66.7, 64.0)	0.67
MeDiet Score	Baseline1	8.4 ± 1.6	8.2 ± 1.6	8.0 ± 1.6	
	3y[2]	1.8 (1.5, 2.0)*,a	1.4 (1.2, 1.7)*,a	0.4 (0.08, 0.7)*	<0.001
	5y[2]	1.8 (1.4, 2.1)*,a	1.7 (1.4, 2.1)*,a	0.4 (0.1, 0.8)*	<0.001

[1]Values are mean ± SD, *n*=54 in both MeDiet and in 52 LFD groups unless noted otherwise. [2]Mean differences (95% CI). [*]*P*: Significant differences (*P*<0.05) between before and after the interventions. [ᵞ]*P*: Significant differences (*P*<0.05) between 3 and 5 y of intervention. [3]*P*int: comparison between measures

obtained before and after intervention and among the 3 diet groups, $P<0.05$. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, $P<0.05$**.** MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; LFD, low-fat diet.

TRABAJO 5:

Título: “Role of Mediterranean Diet in early and late stages of atheroma plaque development”

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Antecedentes: Varios estudios epidemiológicos han demostrado que una mayor adherencia a la dieta mediterránea (DietMed) se asocia con una menor incidencia de enfermedad coronaria y la mortalidad. La mayoría de estos datos provienen de estudios a corto plazo de intervención (3-12 semanas) evaluándose sólo una pequeña proporción de los posibles biomarcadores implicados en el proceso.

Objectivos: Evaluar los efectos a largo plazo de una intervención con DietMed de los niveles plasmáticos de los marcadores inflamatorios y moléculas relacionadas con la estabilidad de la placa en sujetos con alto riesgo de enfermedad cardiovascular (ECV) en comparación con los efectos de una dieta baja en grasa (DBG).

Métodos: 65 participantes de centros de atención primaria afiliados con el Hospital Clínic de Barcelona se asignaron al azar en 3 grupos: DietMed suplementada con aceite de oliva virgen extra (AOVE) o frutos secos, y una dieta baja en grasa (DBG). Se evaluaron los cambios en 24 biomarcadores inflamatorios relacionados con las diferentes etapas del proceso aterosclerótico (sVCAM-1, sICAM-1, E- y P-selectina, IL-1β, lL-4, IL-5 , IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-18, IFNg-γ, TNF-α, lP-10, GCSF-1, GMCSF-1, ENA78, ITAC, MCP -1, MIP-1β y RANTES) por Luminex®, tanto en el basal, 3 y 5 años después de la intervención dietética.

Resultados: Después de 3 y 5 años, ambos grupos de DietMed mostraron una reducción significativa en la concentración plasmática de IL-6, IL-8, MCP-1 y MIP-1β (P <0,05; todos). Las concentraciones de IL-1β, IL-5, IL-7, IL-12p70, IL-18, TNF-α, IFN-γ, GCSF, GMCSF y ENA 78 (P <0,05; todos) también disminuyeron en el grupo DietMed con AOVE. Mientras que, E-selectina (P <0,02) sólo disminuyó en el grupo de DietMed con frutos secos. El grupo de DBG mostró un aumento en las concentraciones de ITAC, IP-10, G-CSF, 78 y ENA (P <0,05; todos).

Conclusiones: Los efectos de la ingesta de DietMed a largo plazo reducen las concentraciones plasmáticas de moléculas inflamatorias que están implicadas en las diferentes etapas del desarrollo de la placa de ateroma (reclutamiento, rodamiento y transmigración de monocitos y linfocitos T a la pared arterial) en las personas de edad avanzada con alto riesgo cardiovascular.

Role of Mediterranean Diet in early and late stages of atheroma plaque development

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Footnotes to the title disclosing:

i) Supplemental Table 1, Supplemental Table 2 and Supplemental Table 3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

ii) Abbreviations used: BP: blood pressure; CHD: coronary heart disease (CHD); CRF: cardiovascular risk factors; CRP: C-reactive protein; CVD: cardiovascular disease; EVOO: extra-virgin olive oil; FQQ: food frequency questionnaire; hs-CRP: ultra-sensitive C-reactive protein; ICAM-1: intercellular adhesion molecule-1; IL-6: Interleukin 6; LFD: low-fat diet; MCP-1: monocyte chemoattractant protein-1; MeDiet: Mediterranean diet; MMP-9: matrix metalloproteinase-9; MUFA: monounsaturated fat; LDL: low-density lipoprotein; oxLDL: oxidized low-density lipoprotein; HDL: high-density lipoprotein; PBMCs: peripheral blood mononuclear cells; PUFA: polyunsaturated fatty acid; PREDIMED: Prevention with Mediterranean

Diet; ROO: refined olive oil; SFA: saturated fatty acids; TGF-β1: transforming growth factor beta 1; TNF-α: tumor necrosis factor alpha; VCAM-1: vascular cell adhesion molecule-1; VOO: virgin olive oil.

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iv) Disclosures: RE reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. JS-S serves on the board of and has received grant support from the International Nut and Dried Fruit Council; he has received consulting fees from Danone and has received grant support from Eroski and Nestlé. RML-R reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from PepsiCo. No other potential conflict of interest relevant to this article was reported.

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ABSTRACT

Background: Several epidemiological studies have shown that greater adherence to the Mediterranean Diet (MeDiet) is associated with a lower incidence of coronary heart disease and mortality.

Objectives: To evaluate the long-term effects of a MeDiet intervention on the expression of plasma inflammatory and plaque stability-related molecules in healthy elderly people at high risk for cardiovascular disease.

Methods: 65 participants from Primary Care Centers affiliated with the Hospital Clínic of Barcelona were randomized in 3 groups: MeDiet plus extra-virgin olive oil (EVOO) or nuts, and a low-fat diet (LFD). At baseline and after 3 and 5y, we evaluated changes on 24 inflammatory biomarkers related to different stages of the atherosclerotic process (sVCAM-1, sICAM-1, E- and P-selectin, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-18, IFNg-γ, TNF-α, IP-10, GCSF, GMCSF, ENA78, ITAC, MCP-1, MIP-1β and RANTES) by Luminex®.

Results: At 3 and 5y, both MEDIET groups showed a significant reduction in the plasma concentration of IL-6, IL-8, MCP-1 and MIP-1β (P<0.05;all). IL-1β, IL-5, IL-7, IL-12p70, IL-18, TNF-α, IFN-γ, GCSF, GMCSF and ENA78 (P<0.05;all) concentrations decreased in the MEDIET+EVOO group. E-selectin (P<0.02) decreased only in the MeDiet+nut group. The LFD showed an increase in IP-10, GCSF, ENA78 and ITAC concentrations (P<0.05;all).

Conclusions: The effects of long-term intake of MeDiet lead to decrease in plasma concentrations of inflammatory molecules in the different steps of atheroma plaque development in elderly persons at high cardiovascular risk, being involved in the recruitment, rolling and transmigration of monocytes and T-lymphocytes into the arterial wall.

Introduction

Atherosclerosis is a chronic inflammatory disorder of the vessel wall which leads to the development of atheroma plaques (1). In the initial steps of the process (rolling, adhesion and migration) several endothelial adhesion molecules (EAM), endothelial-expressed chemokines (EACh) and cytokines participate in this process (2,3,4). Once monocytes are in the subendothelial space they will differentiate to macrophages mediated by M-CSF (3,5,6), proinflammatory cytokines and chemokines (CD40 ligand, IL-1, IL-6, IL-8, and IL-18, IFN-γ, TNF-α, IP-10, IL-10,IL-13) released by atheroma plaque cells (2,3,4) influencing its size and stability (6,7).

Several dietary patterns such as the Mediterranean diet (MeDiet), or specific elements of diet (SFA, MUFA, n-3 PUFA) can modulate these inflammatory biomarkers (8-10). It is already known that MeDiet can decrease plasmatic levels of some of these molecules such as MCP-1, CRP, E and P-Selectin, VCAM-1, ICAM-1, TNF-α, IL-6, IL-7, IL-8, IL-10 and IL-18. However most of these data comes from short-term intervention studies (3-12 weeks) and only a small proportion of biomarkers were assessed (11). Thus, in the current study we have evaluated long-term effects of MeDiet in all biomarkers of vascular wall inflammation in a cohort of elderly subjects at high cardiovascular risk

METHODS

Design overview

An expanded Methods section is available in the online Supplementary data.

RESULTS

Study population

As shown Supplementary Table 1, all participants (22 per group) were selected at random and with similar characteristics to those of the whole group (demographic characteristics, medication taken, adiposity and risk factors). We did not observe significant changes in medication treatments during years of intervention. Food, energy balance and dietary adherence are online avaible-only in supplementary data.

Plasmatic levels of Colony stimulating factors and Soluble Endothelial Molecules

Colony stimulating factors GCSF and GMCSF (P≤0.02, both) were decreased for the MeDiet+EVOO at 3 and 5 years of dietary intervention. MeDiet+nuts showed a reduced serum levels of GCSF (P=0.03) after 3 years of intervention and a reduced serum levels of GMCSF (P=0.04) after 5 years of intervention. The LFD showed an increased of CGSF at 3 and 5 years (P≤0.03) (Figure 1).In addition, the MeDiet+nuts group showed lower serum levels for E-selectin (P≤0.006) in both evaluations while the LFD group showed higher levels of P-selectin (P=0.03) after 5 years of dietary intervention.

Comparisons among the three intervention groups showed significant increase ≥ 40% for CGSF at 3 and 5 years in LFD group compared to both MeDiets groups while MeDiet+nuts reduced E-Selectin concentrations by 30% compared to LFD group after 5 years of intervention (P<0.05; all).

Plasmatic levels of Inflammatory Chemokines

As shown in Figure 2, serum levels of MCP-1 (P≤0.03) and MIP-1β (P≤0.02) decreased in both MeDiets at 3 and 5 years after intervention. ENA78 (P≤0.01) and RANTES (P≤0.008) decreased in the MeDiet+EVOO group after 3 and 5 years of intervention. RANTES also decreased in the MeDiet+nuts group (P=0.008) at 5 years of intervention. On the other hand, the LFD group showed an increase in the RANTES (P≤0.02), ENA78 and ITAC (P≤0.04) and IP-10 (P≤0.004) levels after 3 and 5 years of intervention. Comparasions

among groups showed significant reductions of 20% for MCP-1 and 15% for MIP-1β in both MeDiets groups after 3 and 5 years intervention compared to LFD group. On the other hand, RANTES and ENA78 were increased between 25-50% in the LFD group compared to both MeDiets groups after 3 and 5 years of nutritional intervention.

Plasmatic levels of inflammatory cytokines

After 3 and 5 years of intervention (**Figure 3**), both MeDiet groups showed lower serum concentrations of IL-6 and IL-8 (P≤0.04; both). Furthermore, MeDiet+EVOO group has also lower levels of IL-1β, IL-5, IL-12p70 and TNF-α (P≤0.03, all), IL-7 (P≤0.02), IFN-γ (P=0.005) for both assessments. After 5 years of intervention, MeDiet+nuts group showed an improving in the serum levels of IL-1β (P=0.03), IL-7 (P=0.002), IL-5 and IL-12p70 (P=0.03), TNF-α (P=0.02) and IFN-γ (P=0.008). Besides, LFD showed an increase after 5 years in the serum concentration of IL-7 (P≤0.04). When we compared the three intervention groups we found significant reductions between 30-50% for IL-5, IL-12p70, TNF-α and IFN-γ and between 35-40% for IL-6 and IL-8 for both MeDiets groups after 5 years intervention compared to LFD group. Also, MeDiet+EVOO group showed significant reductions greater than 30% of IL-1β after 3 and 5 years of intervention while the LFD cohort showed an increase of 39% after 5 years of intervention compared to both MeDiets groups (P<0.05; all).

Biomarkers related to Plaque Stability

MeDiet+EVOO group showed lower levels of IL-18 (P≤0.03), a instability marker, 3 and 5 years after beginning the intervention, and higher levels of stability markers
IL-13 (P=0.02) and IL-10 (P=0.04) after 5 years of intervention (Figure 4). Compared to LFD group, MeDiet+EVOO levels of IL-18 were reduced more than 20% after 3 and 5 years of intervention.

DISCUSSION

Our current results suggest that long-term adherence to MeDiet could reduce rolling, adhesion and migration processes of circulatory mononuclear cells and as a result delay atheroma plaque development. In addition, the same diet lowers molecules related to plaque unstability (IL-18) while increases those related with stability (IL-10 and IL-13).
Several mechanisms have been proposed to know how MeDiet may exert its anti-inflammatory propierties. Some evidences show that MeDiet could modulate the expression of genes related to plaque stability (such as MMP-9 (12) or diminish plasmatic levels of IL-18 (15), EAM (sSelectin-1, sICAM-1, and sVCAM-1) or other inflammation biomarkers (TNFR-60 and 80, IL-6 and CRP) (13-16). Thus, consumption of a tomato-based drink for 26 days lowered TNF-α secretion by 34% (17), while the consumption of EVOO and vegetables were associated with a reduction of circulating TNFR-60 (15). In the Multi-Ethnic Study of Atherosclerosis (MESA) trial, “Vegetables and Fish Pattern” were inversely related to CRP and sSelectin (10) and a second interventional study with supplements of DHA, the concentration of IL-6, GMC-SF and MMP-2 were decreased after 91 days of intervention (10). Several studies with olive oil (OO), rich in antioxidant polyphenols and MUFA, have found reduced levels of IL- 6, sVCAM-1 and sICAM-1 (9) beside others biomarkers as CRP, IL-7 or IL-18 (8). Finnally, whole grains (18), fiber (19) or wine (20) also have been associated with an improving in the inflammatory process improving biomarkers as hs-CRP, IL-6, IL-1α, TNFα-R2, MCP-1, sICAM-1or sVCAM-1.

Therefore, according to our results, a MeDiet pattern seems to be implicated in early and late stages of atheroma plaque development, it could lead to an improvement in the levels of different biomarkers implicated in all stages of atherosclerosis in participants assigned to the two MeDiet groups at long-term. These findings may be explained by synergy of specific foods (fruits, vegetables, olive oil and fish) or their specific nutrients as flavonoids, α-tocopherol, ascorbic acid, β-carotene and ω-3 PUFA.
Although similar findings have been reported by our group (9, 15, 16, and 17) at medium and short-term, it is the first time, to our knowledge, that a high number of biomarkers have been studied at long-term. Moreover, the fact MeDiet could modulate the expression as biomarkers implicated in the first stages (EAM, cytokines, chemokines, etc.) and on those implicated in the late stage (molecules related with stability of the plaque) of the disease, leads to think MeDiet could be used a good tool against ECV not only in primary prevention whether also when the disease has appeared.
Strength of our study is that can be used not only in primary prevention whether also in secondary prevention, excellent completion rates, we used a prospective follow-up design and a close monitoring of participants and the specific inflammatory biomarkers studied. Additional strengths are the time of follow-up (5 years) and good compliance of all participants. Some limitations are also acknowledged. Results may not be generalized to the overall population because of participants were older subjects at high risk for CVD and lived in a Mediterranean country. The sample studied is little and we didn't examine the effects of the diet on other variables as oxidative stress or endothelial dysfunction.
In conclusion, the results of the present study support the recommendation of the MeDiet as key in the primary prevention of CVD. The anti-inflammatory and anti-atherosclerotic effects of MeDiet seem to act through several mechanisms modulating inflammatory responses in the arterial wall, being maintained these effects in the long-term.

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The authors' responsibilities were as follows -RE, RC, MU-S, ES: study conception and design; RC, OC: laboratory and clinical data; RC, ES, MU-S and RE: analysis and interpretation of the data; RC, ES, RML-R and RE: draft of the article; and RC, ES, MU-S, ES, DC, OC, RML-R, JS-S, MP-P, JL, ER and RE: critical revision and final approval. RC, MU-S, ES and RE wrote the paper. RE had primary responsibility for the final content. All the authors have read and approved the final manuscript. None of the authors declare a conflict of interest related to the study.

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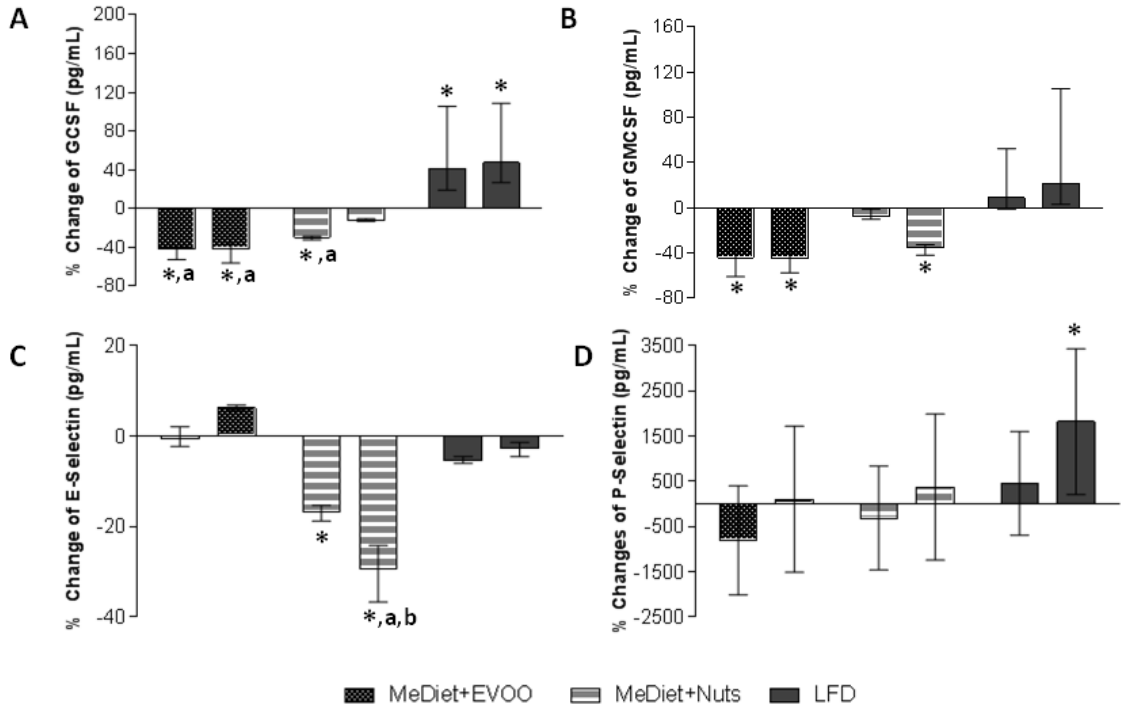

Figure 1. Percentage of change in plasma concentration of colony stimulating factors [GCSF (A) and GMCSF (B)] and soluble adhesion molecules [E-selectin (C) and P-selectin (D)] at baseline, 3 and 5 years of dietetic intervention. Error bars are 95% CIs. *P< 0.05 for difference from baseline to 3 or 5 years based on a simple-effect analysis by Bonferroni's multiple contrasts. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, P<0.05 EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.

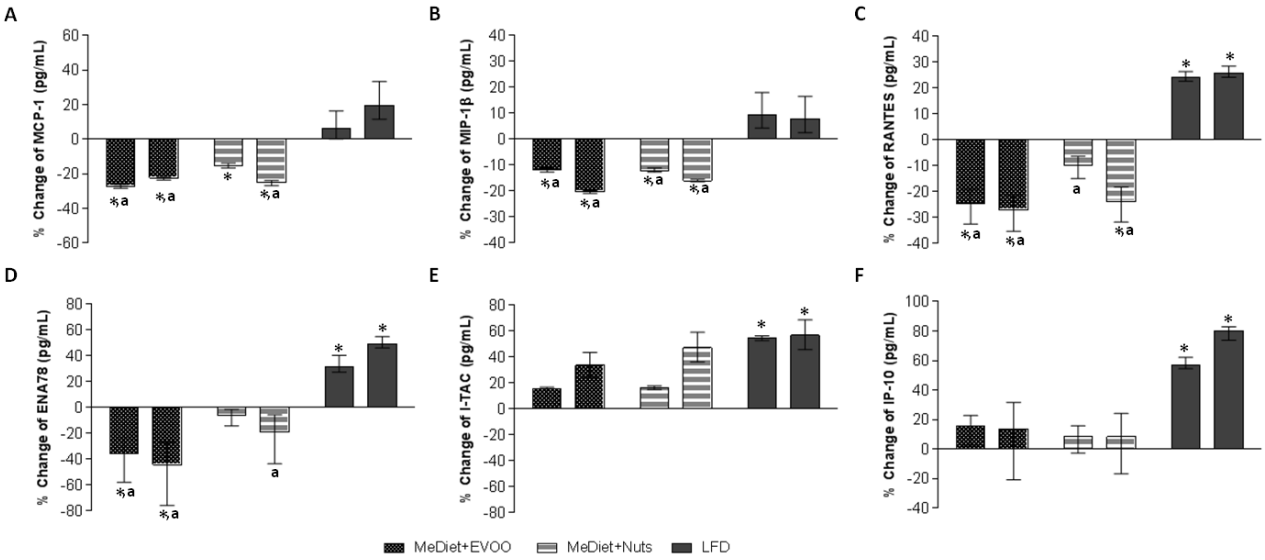

Figure 2. Percentage of change in plasma concentration of chemokines [MCP-1 (A), MIP-1β (B), RANTES (C), ENA78 (D), I-TAC (E) and IP-10 (F)] at baseline, 3 and 5 years of dietetic intervention. Error bars are 95% CIs. *P< 0.05 for difference from baseline to 3 or 5 years based on a simple-effect analysis by Bonferroni's multiple contrasts. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, P<0.05 EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.

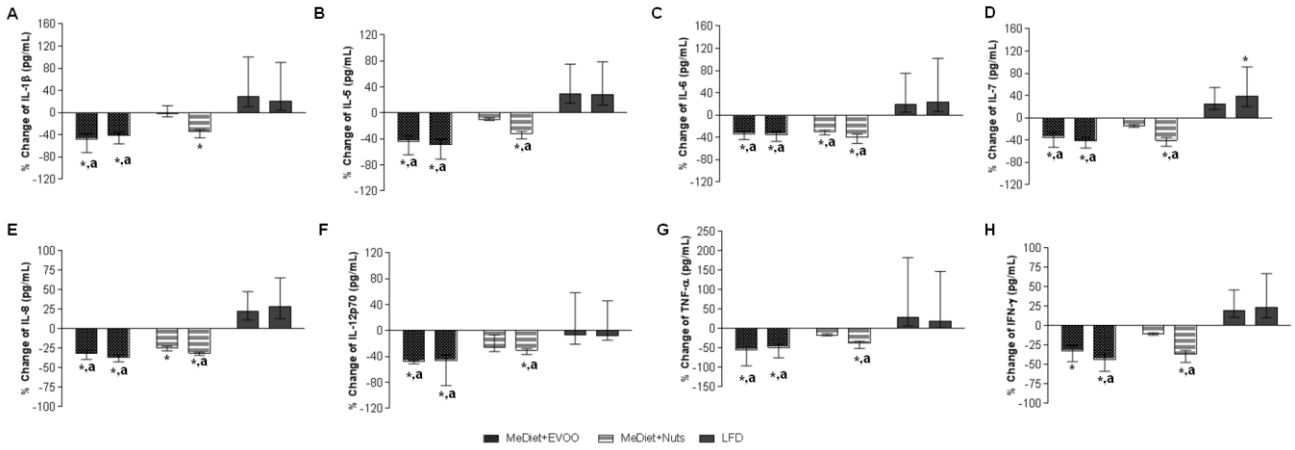

Figure 3. Percentage of change in plasma concentration of cytokines [IL-1β (A), IL-5(B), IL-6 (C), IL-7 (D), IL-8 (E), IL-12p70 (F), TNF-α (G) and IFN-γ (H)] at baseline, 3 and 5 years of dietetic intervention. Error bars are 95% CIs. *P< 0.05 for difference from baseline to 3 or 5 years based on a simple-effect analysis by Bonferroni's multiple contrasts. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, P<0.05 EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.

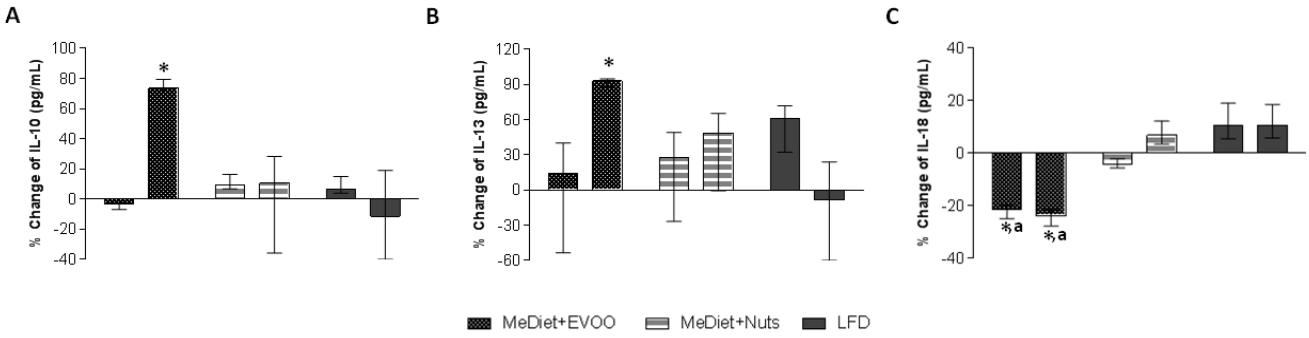

Figure 4. Percentage of change in plasma concentration of molecules related with the vulnerability of the atheroma plaque [IL-10 (A), IL-13 (B) and IL-18 (C)] at baseline, 3 and 5 years of dietetic intervention. Error bars are 95% CIs. *P< 0.05 for difference from baseline to 3 or 5 years based on a simple-effect analysis by Bonferroni's multiple contrasts. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, P<0.05 EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts.

MATERIAL SUPLEMENTARIO

DETAILED AND EXPANDED METHODS

Design overview

The PREDIMED (Prevención con Dieta Mediterránea) study is a parallel-group, single-blind, multicenter, randomized, controlled 5-year clinical trial conducted in Spain which aimed to assess the effects of the MeDiet on the primary prevention of cardiovascular diseases (www.predimed.es) (4,23). The design, methodology and eligibility criteria for the PREDIMED study have been previously described (4, 23-24).

Setting and participants

Recruitment took place between October 2003 and January 2009 and the 7447 participants were randomly assigned to one of three interventions: a MEDIETsupplemented with extra-virgin olive oil (MeDiet-EVOO), a MeDiet supplemented with nuts (MeDiet-Nuts), or a control low-fat diet (LFD). Randomization was performed centrally by means of a computer-generated random-number sequence.

The select participants were men (55 to 80 years of age) and women (60 to 80 years of age) who were free of CVD at beginning but with a high cardiovascular risk because of the presence of either type 2 diabetes mellitus or at least three of the following major risk factors: current smoking, hypertension, high levels of low-density lipoprotein cholesterol, low levels of high-density lipoprotein cholesterol, overweight/obesity, or family history of premature coronary heart disease (CHD). Further details of the inclusion and exclusion criteria can be found in our previously published report (4,22-23). Eligible participants were selected in primary care centers affiliated with the Hospital Clínic of Barcelona and all participants provided written informed consent.

In the current study we screened 80 consecutive potential participants in primary care centers associated with the Hospital Clínic of Barcelona, Spain, but 10 did not fulfill the inclusion criteria. After 5 years, 4 volunteers left the study voluntarily (1 from MeDiet+EVOO group, 1 from MeDiet+Nuts and 2 from the control group). Thus, 66 were finnaly included in this substudy.

Diets and physical activity

All participants randomly assigned to one of three intervention groups: a Mediterranean diet supplemented with extra-virgin olive oil (MeDiet+EVOO), a Mediterranean diet supplemented with mixed nuts (MeDiet+nuts: walnuts, almonds, and hazelnuts), or a low-fat diet (LFD) or control, as described elsewhere (4, 23-24). Randomized participants had an annual face-to-face interview with the dietitian.

Group sessions had place every 3 months and were specific for each intervention group, with no more than 20 participants by group and where participants were provided with descriptions of seasonal foods, shopping lists, weekly meal plans and cooking recipes. In the individual sessions, a 14-item dietary screening questionnaire was used to assess for adherence to either of the MEDIETs, and a 9-item dietary screening questionnaire was used to check for adherence to the control low-fat diet (4,23-24). Also, individual motivational interview included: a 137-item validated food frequency questionnaire (FFQ), the Minnesota leisure-time physical activity questionnaire and personal individual recommendations for changes to be introduced in the participant's diet in order to achieve a personalized goal and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use. Participants allocated to a low-fat diet were advised to reduce all types of fat, and were given written recommendations according to the American Heart Association guidelines. For the 2 MedDiets, the focus was shifted from to increase the intake of vegetables (>=2 servings/d), fresh fruit (>=3 servings/d), legumes, nuts, fish or seafood (>=3 servings/wk), and the use of olive oil for cooking and dressings. The detailed protocol including study design, rationale, and organization has been previously published (4, 23-24).

Participants in the two intervention groups were given supplementary foods at no cost: either EVOO (1 liter/week for the participant and their families) or mixed nuts (30 g/day: 15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds) according to their randomization group.

Energy restriction was not specifically advised nor was physical activity promoted in any of the three groups.

Laboratory measurements

The main outcome measurements were to study changes of 24 inflammatory biomarkers all of them related with differents stages of atherosclerotic process at baseline and after 3 and 5-years dietary intervention: Plasma Interleukin-1β (IL-1β), Plasma Interleukin-4 (IL-4), Plasma Interleukin-5 (IL-5), Plasma Interleukin-6 (IL-6), Plasma Interleukin-7 (IL-7), Plasma Interleukin-8 (IL-8), Plasma Interleukin-10 (IL-10), Plasma Interleukin-12p70 (IL-12p70), Plasma Interleukin-13 (IL-13), Plasma Interleukin-18 (IL-18), Tumor Necrosis Factor Alpha (TNF-α), Monocyte Chemotactic Protein-1 (MCP-1), Regulated on Activation, Normal T cell Expressed and Secreted (RANTES/CCL5), Macrophage inflammatory protein (MIP-1β/CCL4), Interferon gamma-induced protein 10 (IP-10/CXCL10), Interferon gamma (IFN-γ), Granulocyte colony-stimulating factor (GCSF) and Granulocyte-macrophage colony-stimulating factor (GMCSF) were determined by using the Bio-Plex Pro™ cytokine, adhesion molecules and chemokine, assays (Bio-Rad Laboratories Inc., Hercules, CA, USA) which is based in magnetic bead-based multiplex

assays designed to measure multiple cytokines, adhesion molecules and chemokines in a matrices of plasma.

On the other hand, Epithelial neutrophil-activating protein 78 (ENA78/CXCL5), inducible T-cell alpha chemoattractant (ITAC/CXCL11s), sVCAM-1 (Soluble Vascular Cell Adhesion Molecule 1), sICAM-1 (Soluble Intercellular Adhesion Molecule 1) and E- and P-selectin, were determined by using the VersaMAP™ Human custom multi-Analyte Profiling Development system (R&D Systems, Abingdon, UK) which is also based in multiplex assays designed to measure analytes in a matrices of plasma. Data from reaction are acquired using the Luminex system. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity and target concentration on Luminex® 200™ System. Thereafter, the data are processed and analyzed with Bio-plex Manager 6.1™. Plasma samples were diluted 1:3 with the diluients provided for each assay. Data from reaction were acquired using the Luminex® 100™ System (Luminex, Austin, TX) and the Bio-plex Manager 6.1 Software (Bio-Rad, Hercules, CA). Concentrations were obtained by standard calibration curves. Results were shown in pg/mL. We performed all analyses in duplicate.

Statistical analyses

Analyses were performed using SPSS, version 20.0 (SPSS Inc., Chicago, IL). Variables were presented as mean and standard deviation (SD) or standard error of the mean (SEM) as appropriate. Categorical variables are expressed as percentages. Plasma inflammatroy biomarkers had a skewed distribution (Kolmogorov and Levene tests), thus, they were transformed to their natural logarithm for analysis. Repeated-measures ANOVA was used to compare changes in the inflammatory biomarkers, testing the effects of interaction of 2 factors: time as a within-participants factor with 2 levels (first, baseline and 3 y, and after, baseline and 5y) and the groups of consumption (2 MeDiet groups and control group). To test the effects of individual factors, we calculated the changes between 3 years and baseline and 5 y and baseline values for the inflammatory biomarkers and clinical, food and nutrient variables and then the ANOVA test was applied. Significant interactions were analyzed by the simple-effect analysis. The multiple contrasts were adjusted by a Bonferroni post hoc test. Within- and between-group differences were expressed as estimated means and 95% CI. The significance level was set at $P< 0.05$. Pearson's correlation was used for univariate association among changes in variables. The significance level was set at $P< 0.05$.

Food, energy balance and dietary adherence

In according to supplemental table 2, we observed a significant increase in the EVOO consumption (P=0.001) and a decreased in the ROO consumption (P≤0.003) for the MeDiet+EVOO at the 3 and 5 years. In a similar way the MeDiet+nuts group increased nuts consumption (P≤0.01) at 3 and 5 years after of intervention, contrary to the other two groups. At 3 and 5 years after the intervention, both MeDiet showed an increase in the consumption of vegetables (P≤0.02), legumes (P≤0.04), fruit (P<0.05) and fish (P≤0.02) and a reduction in the intake of cereals (P≤0.02) and meat or meat products (P<0.05). Furthermore, the low-fat diet group decreased its consumption of cereals (P≤0.04) and meat or meat products (P≤0.007) at 3 and 5 years intervention and of vegetables after 5 years intervention. Both MeDiets groups increased their adherence to the MeDiet (P<0.001) at 3 and 5years of intervention. Adherence to supplemental foods was good for two MEDIETs. MUFA levels increased from baseline in the MeDiet+EVOO group (P<0.001) and α- linolenic acid levels increased in the MeDiet+nuts group (P≤0.009) relative to other diets after 3 and 5 years of intervention. A reduction in energy (P≤0.03; both), carbohydrate (P≤0.01; both) and cholesterol (P≤0.03; both) intake, was observed in the 3 groups and in the two intervention periods (Supplemental table 3). For both periods of intervention, participants in the two MeDiets groups increased their intake of total of fiber (P≤0.02; both), total fat (P≤0.04; both) and marine ω3 (P≤0.007; both) and reduced their saturated fat (P≤0.02; both) intake. The three groups showed a reduced consumption of protein (P≤0.04;all) after 5 years although the control group also showed a reduction (P=0.02) at 3 years of intervention. In both assessment periods the participants in the MeDiet+nuts group significantly increased polyunsaturated fatty acid (PUFA) (P<0.001).

Supplementary Table 1. Baseline characteristics of participants groups

	MeDiet+EVOO (n=22)	MeDiet+nuts (n=22)	Low-fat diet (n=22)	P[2]
Age, *years*	67.8 ± 4.8[1]	66.0 ± 5.8	66.0 ± 7.1	0.50
Men, *n (%)*	10 (45.5) [1]	12 (54.5)	9 (41)	0.66
Family history of CHD, *n (%)*	5 (22.7)	4 (18.2)	7 (31.8)	0.57
Smoking status, *n (%)*				0.24
Never smoked	17 (72.3)	12 (54.5)	14 (63.6)	
Former smoker	4 (18.2)	6 (27.3)	13.6 (18.2)	
Current smokers	1 (22.8)	4 (18.2)	5 (22.7)	
BMI, *kg/m2*	29.7 ± 3.7	30.4 ± 3.2	28.5 ± 3.6	0.20
BMI > 25 kg/m2 *n (%)*	22 (100)	21 (95.5)	20 (90.0)	0.29
Waist circumference, *cm*	101 ± 9.7	106 ± 6.9	101.5 ± 7.4	0.11
Type 2 diabetes, *n (%)*	16 (72.7)	15 (68.2)	13 (59.1)	0.63
Hypertension, *n (%)*	18 (81.8)	18 (81.8)	14 (63.6)	0.28
Dyslipidemia, *n (%)*	10 (45.5)	13 (63.6)	13 (63.6)	0.59
Medications, *n (%)*				
ACE inhibitors	6 (27.3)	9 (40.9)	7 (31.8)	0.63
Diuretics	4 (18.2)	5 (22.7)	4 (18.2)	0.91
Other antihypertensive agents	1 (4.5)	2 (9.0)	1 (4.5)	0.77
Statins	4 (18.2)	5 (22.7)	8 (36.4)	0.37
Other-lipid-lowering agents	1 (4.5)	0 (0)	2 (9.1)	0.36
Insulin	1 (4.5)	3 (13.6)	3 (13.6)	0.54
Oral hypoglycemic drugs	9 (40.9)	8 (36.4)	9 (40.9)	0.94
Aspirin or antiplatelet drugs	1 (4.5)	6 (27.3)	3 (13.6)	0.11
NSAIDS	3 (13.6)	6 (27.3)	3 (13.6)	0.41

[1] Values are mean ± SD or n (%) as appropriate. [2] From Pearson's chi-square test for categorical variables and one-factor ANOVA for continuous variables.

ACE, angiotensin converting enzyme; BMI, body mass index; CHD, coronary heart disease; EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; NSAIDS, Non-steroidal antiinflammatory drug.

Supplementary Table 2. Consumption of key food items, physical activity and 14-point Mediterranean diet score.

		Within-group mean changes				Between-group changes[4]		
		MeDiet + EVOO (n=22)	MeDiet + Nuts (n=22)	Low-fat diet (n=22)		MeDiet+EVOO vs. LFD	MeDiet+EVOO vs. MeDiet+Nuts	MeDiet+Nuts vs. LFD
		Mean	Mean	Mean	*Pint*[3]	*P*	*P*	*P*
EVOO, *g/d*	Baseline[1]	24.7 ± 22.7	18.0 ± 17.2	22.3 ± 16.5				
	3y.[2]	20.9 (9.3, 32.5)*,a,b	-0.8 (-12.3, 10.8)	-4.5 (-16.1, 7.1)	0.006	<0.001	0.001	1.00
	5y.[2]	20.7 (9.1, 32.3)*,a,b	0.8 (-10.8, 12.4)	-3.0 (-14.5, 8.7)	0.01	0.001	0.003	0.96
Refined OO, *g/d*	Baseline	22.7 ± 14.7	19.9 ± 18.2	25.0 ± 14.8				
	3y.	-14.3 (-23.5, -5.0)*,a,b	4.0 (-5.3, 13.2)	1.6 (-7.6, 10.9)	0.01	<0.001	0.001	1.00
	5y.	-17.7 (-26.9, -	0.6 (-8.6, 9.7)	-0.6 (-9.8, 8.5)	0.01	0.001	0.003	1.00
Total nuts, *g/d*	Baseline	14.6 ± 13.3	13.4 ± 19.6	8.7 ± 10.1				
	3y.	-5.6 (-15.2, 3.9)	13.5 (4.0, 23.1)*,a,b	4.2 (-5.4, 13.8)	0.02	0.45	0.02	0.52
	5y.	-9.0 (-19.3, 1.4)	13.6 (3.3, 24.0)*,a,b	2.4 (-8.0, 12.8)	0.01	0.32	0.02	0.75
Vegetables, *g/d*	Baseline	419 ± 146	330 ± 133	379 ± 132				
	3y.	144 (65.0, 223)*	101 (22.5, 180)*,a	20.4 (-58.5, 99.3)	0.09	0.09	1.00	0.46
	5y.	75.4 (13.3, 138)*	103 (40.4, 165)*,a	-36.4 (-100, 27.2)	0.007	0.04	1.00	0.008
Legumes, *g/d*	Baseline	18.1 ± 5.5	21.1 ± 6.8	19.3 ± 11.1				
	3y.	6.7 (0.5, 13.0)*	6.6 (0.4, 12.8)*	5.1 (-1.1, 11.4)	0.93	1.00	1.00	1.00
	5y.	7.0 (1.2, 12.8)*	6.3 (0.5, 12.1)*	3.2 (-2.6, 9.0)	0.61	1.00	1.00	1.00
Fruits, *g/d*	Baseline	494 ± 227	372 ± 187	488 ± 223				
	3y.	140 (32.4, 247)*,a	180 (72.7, 287)*	48.7 (-58.5, 156)	0.22	0.71	1.00	0.27
	5y.	109 (1.0, 217)*,a	135 (27.0, 243)*	-89.8 (-198, 18.1)	0.008	0.04	1.00	0.01
Cereals, *g/d*	Baseline	274 ± 98.2	260 ± 107	275 ± 115				
	3y.	-78.1 (-123, -33.3)*	-53.4 (-98.2, -8.5)*	-58.9 (-104, -14.1)*	0.72	0.34	1.00	1.00
	5y.	-97.6 (-143, -52.0)*	-88.7 (-134, -43.1)*	-92.2 (-138, -46.6)*	0.96	1.00	1.00	1.00
Fish or seafood, *g/d*	Baseline	93.8 ± 44.1	94.9 ± 44.2	114 ± 32.7				
	3y.	23.8 (7.3, 40.4)*	20.2 (3.6, 36.7)*	10.8 (-5.7, 27.3)	0.52	0.81	1.00	1.00
	5y.	30.7 (13.2, 48.2)*	45.0 (27.4, 62.5)*	2.7 (-14.9, 20.2)	0.004	0.08	0.76	0.003
Meat or meat products, *g/d*	Baseline	141 ± 66.9	172 ± 51.5	159 ± 51.1				
	3y.	-35.4 (-60.8, -9.9)*,b	-26.5 (-52.5, -0.4)*	-31.7 (-57.2, -6.2)*	0.89	1.00	1.00	1.00
	5y.	-31.9 (-56.1, -7.6)*	-45.3 (-69.0, -21.6)*	-40.4 (-64.1, -16.7)*	0.73	1.00	1.00	1.00
Pastries, cakes or sweets, *g/d*	Baseline	20.3 ± 18.0	22.1 ± 25.7	23.0 ± 60.8				
	3y.	-5.7 (-19.9, 8.5)	-4.2 (-18.4, 10.0)	-8.0 (-22.2, 6.2)	0.93	1.00	1.00	1.00
	5y.	-3.6 (-21.5, 14.2)	-5.8 (-23.6, 12.0)	-12.3 (-30.2, 5.5)	0.77	1.00	1.00	1.00
Dairy products, *g/d*	Baseline	374 ± 128	340 ± 196	372 ± 271				
	3y.	17.1 (-100, 135)	-25.5 (-140, 89.2)	1.6 (-116, 119)	0.87	1.00	1.00	1.00
	5y.	-50.2 (-144, 43.3)	-32.1 (-145, 81.0)	-25.6 (-128, 76.3)	0.93	1.00	1.00	1.00
Alcohol, *g/d*	Baseline	10.7 ± 12.9	6.5 ± 6.4	7.5 ± 11.0				
	3y.	1.1 (-1.2, 3.5)	1.2 (-1.2, 3.5)	0.7 (-1.6, 3.1)	0.96	1.00	1.00	1.00
	5y.	2.3 (-0.6, 5.2)	1.3 (-1.6, 4.2)	-0.4 (-3.3, 2.4)	0.41	0.56	1.00	1.00
Wine, *mL/d*	Baseline	30.0 ± 61.4	25.0 ± 29.7	35.1 ± 55.7				
	3y.	11.6 (-8.1, 31.2)	7.1 (-12.6, 26.8)	4.7 (-15.5, 24.8)	0.88	1.00	1.00	1.00
	5y.	5.1 (-15.2, 25.4)	11.0 (-9.4, 31.3)	-5.2 (-25.6, 15.1)	0.53	1.00	1.00	0.79
Physical Activity, *kcal/d*	Baseline	317 ± 205	236 ± 267	282 ± 215				
	3y.	8.7 (-89.6, 107)	4.5 (-93.8, 103)	43.7 (-54.6, 142)	0.83	1.00	1.00	1.00
	5y.	11.7 (-81.4, 105)	12.8 (-80.2, 106)	-9.7 (-103, 83.3)	0.93	1.00	1.00	1.00
MeDiet Score	Baseline	9.0 ± 1.5	8.0 ± 1.9	8.1 ± 1.4				
	3y.	1.7 (1.3, 2.2)*,a	1.5 (1.1, 1.9)*,a	0.05 (-0.4, 0.5)	<0.001	<0.001	1.00	<0.001
	5y.	1.5 (1.0, 2.1)*,a	1.7 (1.2, 2.2)*,a	0.1 (-0.4, 0.7)*	<0.001	0.001	1.00	<0.001

Data analyzed by repeated-measures 2-factor ANOVA (simple-effect analysis by Bonferroni's multiple contrast).[1]Values are mean ± SD. [2]Mean differences (95% CI). [*]P: Significant differences (P<0.05)

between before and after the intervention. [3]Pint: comparison between measures obtained before and after intervention and among the 3 diet groups, P<0.05. [4]Pvalue: Significant differences (P<0.05) between-group changes (Data analyzed by ANCOVA test, with the intervention group as fixed factor. [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, P<0.05. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; Refined OO, refined olive oil. LFD, low-fat diet.

Supplementary Table 3. Changes in baseline energy and nutrient intake.

		Within-group mean changes				Between-group changes[4]		
		MeDiet + EVOO (n=22)	MeDiet + Nuts (n=22)	Low-fat diet (n=22)		MeDiet+EVOO vs. LFD	MeDiet+EVOO vs.	MeDiet+Nuts vs. LFD
		Mean	Mean	Mean	*Pint*[3]	*P*	*P*	*P*
Energy, *kcal/d*	Baseline[1]	2484 ± 529	2609 ± 591	2430 ± 794				
	3y.[2]	-246 (-466, -25.9)*	-241 (-456, -26.6)*	-257 (-477, -36.7)*	0.99	1.00	1.00	1.00
	5y.[2]	-552 (-795, -309)*	-392 (-685, -99.0)*	-612 (-877, -348)*	0.52	1.00	1.00	0.80
Protein, *g*	Baseline	101 ± 20.8	107 ± 23.0	102 ± 23.1				
	3y.	-8.9 (-18.8, 1.1)	-5.4 (-15.1, 4.4)	-12.5 (-22.5, -2.5)*	0.59	1.00	1.00	0.93
	5y.	-19.9 (-29.5, -10.3)*	-12.6 (-24.2, -0.9)*	-19.1 (-29.6, -8.6)*	0.59	1.00	1.00	1.00
Carbohydrate, *g*	Baseline	267 ± 64.9	275 ± 92.1	270 ± 125				
	3y.	-45.2 (-80.6, -9.8)*	-55.0 (-90.0, -20.4)*	-53.3 (-88.7, -18.0)*	0.92	1.00	1.00	1.00
	5y.	-83.2 (-124, -42.9)*	-93.9 (-143, -45.2)*	-108 (-152, -63.8)*	0.71	1.00	1.00	1.00
Fiber, *g/d*	Baseline	28.2 ± 4.1	27.0 ± 7.3	26.5 ± 6.3				
	3y.	3.4 (0.5, 6.2)*	4.6 (1.8, 7.5)*	0.3 (-2.5, 3.2)	0.10	0.42	1.00	0.12
	5y.	3.0 (0.8, 5.3)*	3.5 (1.2, 5.8)*	-0.6 (-3.0, 1.7)	0.03	0.80	1.00	0.04
Total fat, *g*	Baseline	101 ± 24.5	104 ± 21.0	97.3 ± 31.7				
	3y.	13.0 (1.6, 24.4)*	17.3 (6.0, 28.7)*[,b]	2.6 (-8.8, 14.0)	0.18	0.61	1.00	0.22
	5y.	8.9 (0.03, 17.8)*	11.2 (2.3, 20.1)*	1.2 (-7.7, 10.1)	0.26	0.68	1.00	0.35
SFA, *g*	Baseline	29.8 ± 9.5	30.1 ± 8.3	28.3 ± 7.2				
	3y.	-5.3 (-8.4, -2.2)*	-3.7 (-6.8, -0.6)*	-1.8 (-5.0, 1.3)	0.30	0.37	1.00	1.00
	5y.	-6.6 (-10.2, -3.0)*	-5.7 (-9.3, -2.1)*	-2.4 (-6.0, 1.2)	0.24	0.32	1.00	0.63
MUFA, *g*	Baseline	46.3 ± 15.0	51.6 ± 13.9	51.3 ± 11.8				
	3y.	11.6 (5.7, 17.4)*[,a]	1.5 (-4.3, 7.3)	0.06 (-5.8, 5.9)	0.01	0.02	0.04	1.00
	5y.	11.0 (5.4, 16.5)*[,a]	-1.7 (-7.2, 3.9)	-5.2 (-10.7, 0.4)	<0.001	<0.001	0.006	1.00
ω3 PUFA, *g*	Baseline	16.7 ± 5.4	16.5 ± 5.5	15.9 ± 4.6				
	3y.	-0.5 (-3.4, 2.4)[b]	5.8 (2.9, 8.7)*	2.2 (-0.7, 5.1)	0.01	0.60	0.01	0.25
	5y.	-2.5 (-5.1, 0.2)[b]	4.4 (1.8, 7.0)*	0.9 (-1.8, 3.5)	0.002	0.23	0.001	0.19
Linoleic acid, *g/d*	Baseline	12.8 ± 3.8	14.1 ± 5.9	13.4 ± 7.3				
	3y.	-0.3 (-2.8, 2.2)	1.9 (-0.6, 4.5) [a,b]	-1.7 (-4.3, 0.8)	0.13	1.00	0.68	0.14
	5y.	-2.1 (-4.6, 0.4)	0.4 (-2.1, 2.9) [a,b]	-2.1 (-4.6, 0.4)	0.27	1.00	0.50	0.46
α- linolenic acid, *g*	Baseline	1.8 ± 0.7	1.8 ± 0.7	1.7 ± 0.8				
	3y.	-0.3 (-0.7, 0.2)	0.7 (0.2, 1.1)*[, a,b]	-0.2 (-0.7, 0.2)	0.004	1.00	0.009	0.01
	5y.	-0.3 (-0.8, 0.2)	0.8 (0.3, 1.2)*[, a,b]	-0.1 (-0.6, 0.4)	0.005	1.00	0.007	0.04
Marine n-3 fatty acids, *g/d*	Baseline	0.8 ± 0.5	0.9 ± 0.5	0.7 ± 0.4				
	3y.	0.2 (0.06, 0.4)*	0.4 (0.2, 0.6)*[,b]	0.05 (-0.1, 0.2)	0.01	0.40	0.35	0.008
	5y.	0.3 (0.1, 0.4)*	0.4 (0.3, 0.6)*	0.2 (-0.002, 0.3)	0.09	1.00	0.50	0.09
Cholesterol, m*g/d*	Baseline	452 ± 128	439 ± 117	421 ± 130				
	3y.	-62.4 (-111, -14.1)*	-54.6 (-103, -6.3)*	-70.5 (-119, -22.1)*	0.90	1.00	1.00	1.00
	5y.	-94.3 (-141, -48.0)*	-90.5 (-137, -44.1)*	-95.7 (-142, -49.3)*	0.99	1.00	1.00	1.00

Data analyzed by repeated-measures 2-factor ANOVA (simple-effect analysis by Bonferroni's multiple contrast).[1]Values are mean ± SD. [2]Mean differences (95% CI). [*]P: Significant differences (P<0.05) between before and after the intervention. [3]Pint: comparison between measures obtained before and after intervention and among the 3 diet groups, P<0.05. [4]Pvalue: Significant differences (P<0.05) between-group changes (Data analyzed by ANCOVA test, with the intervention group as fixed factor). [a]MeDiet+EVOO or MeDiet+nuts vs. low fat-diet and [b]MeDiet+EVOO vs. MeDiet+nuts are significantly different, P<0.05. EVOO, extra virgin olive oil; MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts. EVOO, extra virgin olive oil;

MeDiet+EVOO, Mediterranean diet supplemented with extra virgin olive oil; MeDiet+Nuts, Mediterranean diet supplemented with nuts; LFD, low-fat diet; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; Refined OO, refined olive oil; SFA, Saturated fatty acids.

DISCUSIÓN CONJUNTA

9. DISCUSIÓN CONJUNTA

En nuestro **primer estudio**, observamos una reducción significativa de la presión sistólica y diastólica, de 3 y 6 mm Hg respectivamente, así como una reducción de los niveles plasmáticos de colesterol total (5-8%). Estas mejoras significativas ya habían sido observadas en estudios previos, a corto plazo (Estruch, 2006; Mena 2009). No obstante, la acción protectora de la DietMed frente a la aterosclerosis no se puede explicar exclusivamente por las mejoras observadas los factores de riesgo clásico, lo que sugiere la presencia de efectos alternativos. Es bien sabido que la aterosclerosis es una enfermedad inflamatoria crónica de bajo grado que ocurre en la pared arterial (Finn, 2010) por lo que, la modulación de este proceso inflamatorio podría ser otro de los mecanismos por el cual la DietMed ejercería su efecto protector contra la aterosclerosis. Así, los sujetos que siguieron una DietMed suplementada con AOVE o FS mostraron una mayor regulación de la expresión de las moléculas de adhesión leucocitarias y una disminución de las concentraciones séricas de PCR e IL-6 respecto al grupo control. Los resultados obtenidos, coinciden con los ya publicados con anterioridad por el grupo de Castañer et cols, (Castañer, 2013) o Estruch y cols, (Estruch, 2006), ambos basados en datos del estudio PREDIMED, en el que el mantenimiento de una DietMed tradicional suplementada con AOVE o FS podría ejercer beneficios sobre la salud a través de cambios en la respuesta de los genes relacionados con el RCV. Además, para ambas DietMeds se observó también una disminución de los niveles séricos de IL-18 así como de la ratio IL-18 / IL-10, lo que sugiere una mayor estabilidad de placa de ateroma en pacientes que siguen una DietMed. Es sabido que, IL-18 induce la producción de IFNγ por linfocitos T y además aumenta la expresión de MMPs contribuyendo por estas dos vías a la vulnerabilidad de la placa (Koenig, 2007). Algunos estudios previos ya habían reportado que algunos componentes específicos de la DietMed como AOVE o los FS podían disminuir los

marcadores inflamatorios asociados a la aterosclerosis como VCAM-1, ICAM-1, E y P-selectina, PCR e IL-6 (López-Miranda, 2008; Ros, 2009).

Además, cabe destacar, que el efecto antiinflamatorio ejercido por la DietMed parece ser mayor y más intenso a medio plazo (12 meses) que no a corto plazo (3 meses) (Estruch, 2006; Mena, 2009), mientras que el efecto sobre los factores de RCV clásicos parecen ser similares, lo que sugiere que la DietMed ejerce sus efectos sobre estos factores de forma relativamente rápida (a los 3 meses), pero sin embargo, parece ejercer un efecto más tardío y más intenso (un año) sobre los biomarcadores inflamatorios.

En resumen, los resultados de nuestro estudio sugieren que la DietMed suplementada con AOVE o frutos secos podría tener un efecto dual sobre la prevención de la ECV, ya que por un lado mejora los factores de RCV clásicos y también tiene un intenso efecto anti-inflamatorio, lo que explicaría parcialmente el efecto beneficioso y global de la DietMed en la prevención primaria de la ECV observada en los sujetos sanos pero de alto riesgo.

En nuestro **segundo estudio** (under review), observamos que una mayor adherencia a la MedDiet conlleva una mayor regulación de la expresión de las moléculas de adhesión de linfocitos T circulantes (CD11a, CD49d y CD40) y monocitos (CD11a, CD11b, CD49d, CD40), así como de los biomarcadores inflamatorios (TNF-α, IL-6, MCP-1, hs-CRP). Estas moléculas desempeñan un papel esencial en el reclutamiento de monocitos del torrente sanguíneo al espacio subendotelial en las etapas iniciales y durante todo el curso del proceso aterogénico. Este efecto antiinflamatorio observado de la DietMed se mantiene a largo plazo y se asocia con una mejora en los factores clásicos de riesgo cardiovascular: reducción de la presión arterial reducida y cintura, así como un cambio del perfil lipídico hacia una menor aterogenicidad. Nuestros resultados sugieren que la DietMed tiene un efecto dual frente a la ECV. En primer

lugar, mejora los factores de riesgo cardiovascular clásicos (Estruch, 2006; Mena, 2009; Casas, 2014) y, en segundo lugar, tiene un efecto antiinflamatorio significativo (Urpí-Sardà, 2012; Mena, 2009; Casas, 2014) tanto a corto como a largo plazo. La DietMed parece ejercer sus efectos sobre los factores de riesgo clásicos en una etapa temprana (3 meses) (Mena, 2009) mientras que su efecto sobre la expresión de las moléculas de adhesión y biomarcadores inflamatorios podría ser modificada no sólo a corto y medio plazo (Mena, 2009; Casas, 2014), sino que también podría mantener o incluso aumentar estos efectos a largo plazo, por lo menos hasta los 5 años de seguimiento. Los resultados de este estudio apoyan la recomendación de usar la DietMed como herramnienta dietética útil para minimizar la enfermedad crónica inflamatoria como la enfermedad cardiovascular. Este efecto saludable parece ser alcanzado a través de varios mecanismos, la modulación de la respuesta inflamatoria y la mejora de los factores de riesgo cardiovascular clásicos que se mantienen e incluso intensifican a largo plazo.

La regulación de la expresión en moléculas adhesión leucocitaria (Linfocitos-T y monocitos) así como una reducción de los niveles séricos de las moléculas proinflamatorias, se podrían explicar por la composición de la dieta: ya sea por un alimento o un nutriente “específico”, o la sinergia de varios de ellos (*Patrón de Dieta Mediterránea*). Así, con este **tercer** decidimos estudiar el efecto observado con diferentes patrones dietéticos en las concentraciones de la TNFR, principalmente en TNFR60 (Tartaglia, 1991). Los resultados de este estudio mostraron una correlación negativa entre los niveles plasmáticos de TNFR80 y adherencia a la DietMed ($P=0.002$), así como una relación inversa entre el consumo de ciertos alimentos (AOVE y verduras) y las concentraciones plasmáticas de TNFR60 ($P< 0.001$). Por otra parte, una mayor adherencia a la DietMed se correlacionó con una mayor reducción de la concentración plasmática de TNFR80. Estudios previos han mostrado aumentos significativos en las concentraciones plasmáticas de VCAM-1 e ICAM-1 en el grupo de

DBG a los 3 meses (Mena, 2009), posiblemente debido al aumento en la ingesta de carbohidratos en los participantes de DBG para compensar la reducción de la energía ingesta de grasas. De hecho, el alto consumo de hidratos de carbono puede promover un aumento de la resistencia a la insulina, la causa subyacente del síndrome metabólico, y un aumento en la producción de citoquinas inflamatorias (Gallagher, 2010). El AOVE se caracteriza por su alto contenido en polifenoles, α-tocoferol, y ácidos grasos monoinsaturados. La relación entre las concentraciones plasmáticas de TNF-α y el consumo de AOVE sugiere un posible mecanismo de acción de ciertos alimentos. Son muchos los estudios que han mostrado las propiedades anti-inflamatorias del AOVE debido a su contenido en polifenoles tales como tirosol, hidroxitirosol, oleuropeína (Abe, 2011; Covas, 2006; Vivancos, 2008) y un compuesto fenólico recientemente descubierto con alta actividad antiinflamatoria, oleocantal (Lucas, 2011). El estudio Attica (Chrysohoou, 2004), en Grecia, mostró que una mayor adherencia a la DietMed se correlacionaba con concentraciones plasmáticas más bajas de PCR, IL-6, la homocisteína, fibrinógeno, y TNF-α. Así, dietas suplementadas con AOVE (rico en ácidos grasos monoinsaturados) o con frutos secos (ricos en AGPI) inducen una mayor disminución en la expresión génica de TNF-α en comparación a una dieta rica en grasa saturada (Jiménez-Gómez, 2009). La ingesta de aceites vegetales tales como el AOVE en mujeres sanas de Teherán se asoció con una menor concentración plasmática de TNF-α, ICAM-1, y PCR (Esmaillzadeh, 2008).

Por otro lado, también se observó que aquellos participantes que disminuyeron la ingesta de alcohol (tertil más bajo) presentaron niveles plasmáticos en la concentración de ICAM-1 mayores. Varios estudios han demostrado los efectos anti-inflamatorios de consumo moderado de alcohol (Estruch y Sacanella, 2004). Finalmente, las variaciones en el peso corporal de los participantes no parecen mediar en los cambios plasmáticos de los biomarcadores inflamatorios estudiados.
Hasta donde sabemos, este es el primer estudio en el que TNFR60 y TNFR80 se han

vinculado con la dieta, DietMed, y más concretamente con AOVE y verduras. Sin embargo, faltan estudios que nos ayuden a identificar y a estudiar los posibles mecanismos moleculares implicados y que subyacen a estas relaciones.

Las dietas con alto IG y CG se han relacionado con una mayor incidencia de obesidad, diabetes tipo 2 (Sluijs, 2010), y la ECV (Sluijs, 2010; Hardy, 2010; Lago, 2009). El efecto más directo de una dieta con un alto IG y una elevada CG es el rápido aumento de la glucemia postprandial e insulinemia. Dado que la obesidad, al igual que la aterosclerosis, se puede considerar como una inflamación crónica de bajo grado, el IG o la CG de una dieta podrían modular la respuesta inflamatoria.

En nuestro **cuarto** estudio, los resultados del análisis transversal realizado en 511 sujetos de edad avanzada mostraron una asociación inversa entre los niveles plasmáticos de leptina y adiponectina, con el IG y la CG de la dieta. Nuestros resultados están de acuerdo con los obtenidos mediante un estudio de intervención realizado en ratas alimentadas con una dieta rica en almidón índice glucémico durante 12 semanas (Kabir, 2000) y los reportados en estado postprandial (Barkoukis, 2007). Además, en el estudio prospectivo y longitudinal después de un año de seguimiento se observó una asociación inversa entre el aumento del IG y CG de la dieta y los cambios en los niveles plasmáticos de leptina y adiponectina. Los resultados obtenidos para la leptina, la cual se asocia con una disminución en la ingesta de alimentos y un aumento en el gasto de energía que actúa a nivel central hipotálamo (Bulló, 2002), podría ser considerado como un mecanismo que favorece el aumento de peso y la obesidad atribuido a las dietas de IG alto. Además, la leptina también ejerce acciones autocrinas o paracrinas aumentando la lipólisis y disminuyendo la lipogénesis, por lo que la disminución en los niveles circulantes de leptina en plasma observados en nuestro estudio pueden conducir a una disminución en la oxidación de ácidos grasos y un aumento en la oxidación de la glucosa, lo que favorece la deposición de grasa.

Finalmente, debido a que la leptina es conocida principalmente como un factor de saciedad, la disminución de la leptina en plasma después de una dieta de IG alto es coherente con el concepto de que estas dietas son menos saciantes que las dietas de bajo índice glucémico (Holt, 1992). Por otro lado, niveles bajos de adiponectina circulante se asocian con resistencia a la insulina, intolerancia a la glucosa, dislipidemia y aterosclerosis (Ouchi, 2001). En este sentido, nuestros resultados apoyan la hipótesis de que hipoadiponectinemia inducida por dietas con alto IG podría explicar, en parte, la relación entre el IG de la dieta y el aumento del riesgo de diabetes tipo 2 y ECV asociada con el consumo de este tipo de dieta.

No se observaron relaciones significativas entre el IG o CG de la dieta y otras adipoquinas o incretinas analizadas. Sí se observaron niveles significativamente más altos de TNF-α y una tendencia a mayores niveles de IL-6 en los sujetos con IG mayor (los del cuartil superior). De manera longitudinal, también se informó de una relación positiva entre el aumento de CG y un aumento de GIP, pero no de GLP-1, lo que sugiere que una alta carga glucémica dietética contribuye a la deposición de grasa y la obesidad.

Las conclusiones de este estudio se suman a la creciente evidencia que el consumo de dietas con alimentos de alto IG alto o una dieta de alta CG puede modular las concentraciones plasmáticas de algunos marcadores cardiometabólicos contribuyendo así a la promoción de la obesidad y enfermedad cardiovascular.

En el **quinto estudio** (submitted), observamos nuevamente un efecto antiinflamatorio de la DietMed que es mantenido o incluso mejorado a largo plazo y una mejora de los biomarcadores inflamatorios (Factores estimulantes de colonias, moléculas solubles endoteliales, quimiocinas, citoquinas y moléculas relacionadas con la estabilidad de la placa de ateroma), así como de los factores de riesgo clásicos a los 3 y 5 años de intervención. Una gran cantidad de evidencia científica apoya el efecto cardioprotector

de la DietMed (Estruch R, 2006; Estruch R, 2013; Mena, 2009; Casas, 2014, Fitó, 2014). Los estudios experimentales y clínicos han demostrado que la DietMed ejerce sus efectos antiinflamatorios e inmunomoduladores, regulando la expresión de moléculas de adhesión leucocitaria (Mena, 2009; Casas, 2014), disminuyendo la concentración de interleuquinas proinflamatorias (IL-1, IL-6), hs -CRP, TNF-α y sus receptores, moléculas quimioatrayentes (MCP-1, RANTES, ENA-78, etc.), así como de moléculas de adhesión endoteliales solubles (VCAM-1, ICAM-1, sE- y SP-Selectina) (Mena, 2009; Casas, 2014; Urpí-Sardà, 2012; Estruch, 2006). Por otra parte, la DietMed es capaz de regular la expresión de moléculas relacionadas con la inestabilidad de la placa, tales como IL-18, MMP-9 o TGF-β1 (Casas, 2014).

Los resultados de este estudio confirman nuevamente los efectos antiinflamatorios a largo plazo de la DietMed. Con este estudio, además, demostramos que la DietMed a largo plazo podría ejercer un efecto protector en cualquiera de los estadios del desarrollo de la placa de ateroma, es decir, tanto en las etapas iniciales de la enfermedad (rodamiento, adhesión y migración de las células mononucleares) así como en etapas más tardías y relacionadas con la estabilidad de la placa. Este efecto protector se manifiesta con una disminución de los niveles séricos de las citoquinas (TNF-α), interlequinas (IL-1, IL-6, etc.) y moléculas quimioatrayentes (MCP-1, RANTES, etc.) que participan en las primeras etapas de la lesión aterosclerótica como de aquellas relacionadas con la placa inestabilidad (IL-18), mientras que aumenta las relacionadas con la estabilidad (IL-10, IL-13).

Aunque hallazgos similares han sido reportados por nuestro grupo (Mena, 2009; Casas, 2014; Urpí-Sardà, 2012; Estruch, 2006) a corto y medio plazo, es la primera vez, para nuestro conocimiento, que se han estudiado este número tan alto de biomarcadores a largo plazo, algunos de ellos considerados como nuevos marcadores inflamatorios emergentes (RANTES, MIP-1β, entre otros). Por otra parte, el hecho de

DietMed podría modular la expresión como biomarcadores implicados en las primeras etapas (moléculas de adhesión circulantes, citoquinas, quimioquinas, etc.) y sobre los implicados en la fase tardía (moléculas relacionadas con la estabilidad de la placa) de la enfermedad, lleva a pensar que la DietMed podría usarse como una buena herramienta contra la ECV no sólo en la prevención primaria de si también en prevención secundaria.

Todos los trabajos presentados, en conjunto, en esta memoria aportan diversas evidencias que sugieren por un lado, que la DietMed tiene un efecto dual frente a la ECV aunque, la mejor prueba de los efectos beneficiosos de la DietMed sobre la salud viene proporcionada por los resultados del estudio PREDIMED, que muestran que una DietMed complementada con AOVE o FS reduce la incidencia de eventos cardiovasculares en un 30% en sujetos con alto RCV (Estruch, 2013). En primer lugar, mejora los factores de riesgo cardiovascular clásicos y, en segundo lugar, tiene un efecto antiinflamatorio significativo (reduce las moléculas de adhesión leucocitaria y moléculas inflamatorias de la pared arterial así como las relacionadas con la estabilidad de la placa) tanto a corto (3 meses), medio (1 año) como a largo plazo (5 años). Por lo tanto, la DietMed reduce la PA sistólica y diastólica y los niveles de glucosa en ayunas, mejora la resistencia a la insulina, reduce los niveles plasmáticos de LDL-colesterol aumentando los del HDL-colesterol, y disminuye la grasa abdominal. La DietMed parece ejercer sus efectos sobre los factores de riesgo clásicos muy rápidamente (3 meses). Por otro lado, la DietMed parece ejercer sus efectos antiinflamatorios e inmunomoduladores a través de la regulación por disminución de la expresión de moléculas de adhesión leucocitarias, la disminución de interleuquinas pro-inflamatorias (IL-1, IL-6), hs -CRP, TNF-α y sus receptores, moléculas quimioatrayentes (MCP-1), y las moléculas de adhesión endoteliales solubles (VCAM-1, ICAM-1, sE- y SP-Selectina. Este efecto parece ser más tardío, aparece a corto plazo (3 meses) pero se hace más patente a medio plazo (1 año) y es mantenido o se

intensifica a largo plazo (5 años). Además, la DietMed es capaz de disminuir las concentraciones séricas de aquellas moléculas que contribuyen a la estabilidad o inestabilidad de la placa, tales como IL-18, MMP-9, IL-10, IL-13 o TGF-β1. Por otro lado, a parte del efecto inmunomodulador y antiinflamatorio de la DietMed a largo plazo el hecho de que la DietMed pueda actuar sobre la expresión de los marcadores inflamatorios implicados en las primeras etapas del proceso aterosclerótico y modular también la expresión de aquellos marcadores de inestabilidad de placa (última etapa de la aterosclerosis), nos hace pensar que la DietMed podría no solo tener un efecto protector sobre aquellas personas sanas pero con RCV (*Prevención primaria*) sino también, un efecto protector sobre aquellos individuos que se encuentran en una etapa más avanzada de la enfermedad (*Prevención* secundaria). Estos resultados estarían acorde con los ya publicados por Aleix y cols. (Sala-Vila, 2014), quienes observaron, después de 2.4 años, un retraso en la progresión de ICA-IMT y de la placa de ateroma.

Una cuestión importante es saber si el efecto ejercido por la DietMed se debe a un alimento o nutriente “específico”, o a la sinergia de varios de ellos, es decir, al Patrón de DietMed. Nuestro cuarto estudio, puso de manifiesto la capacidad antiinflamatoria e immunomoduladora de la DietMed sobre los receptores de los marcadores inflamatorios TNFR60 y TNFR80. Más concretamente se observó una correlación negativa entre las concentraciones plasmáticas de TNFR60 y con ciertos alimentos (AOVE y verduras), mientras que el consumo de bajo de alcohol se asoció con mayores concentraciones de ICAM-1. En este contexto, sabemos que el AOVE es uno de los principales componentes de la DietMed. Además de ácidos grasos monoinsaturados, el AOVE contiene α-tocoferol y compuestos fenólicos con fuertes propiedades antioxidantes y anti-inflamatorias. Estudios in vitro y ex vivo con AOVE han demostrado una mayor regulación en la expresión de VCAM-1, ICAM-1 y E-selectina (Dell'Agli, 2006) y una disminución de las concentraciones plasmáticas de IL-

6 y PCR en pacientes con enfermedad coronaria estable (Fitó, 2008). Además, los estudios transversales (Salas-Salvadó, 2008) han asociado bajas concentraciones de VCAM-1, ICAM-1, IL-6 y PCR en sujetos con un mayor consumo de AOVE.

Por otro lado, los frutos secos, otro alimento clave de la DietMed, son ricos en ácidos grasos poliinsaturados (ácido α-linolénico en el caso de las nueces), fibra, fitoesteroles, ácido fólico y la vitamina E y polifenoles (Ros, 2010). El consumo de frutos secos también se ha asociado con la disminución de los niveles de IL-6, PCR y fibrinógeno en estudios transversales (Salas-Salvadó, 2008; Jiang, 2006), así como concentraciones plasmáticas disminuidas de VCAM-1, ICAM-1 y sE-selectina en pacientes hipercolesterolémicos en estudios de intervención (Cortés, 2006). Por otra parte, varios estudios han asociado los efectos inmunomoduladores y anti-inflamatorias de la DietMed con el patrón de dieta en sí y no a alimentos específicos (Esposito, 2004; Sánchez-Taínta, 2008; Schwingshackl, 2014), mostrando concentraciones disminuidas de los marcadores biológicos de la inflamación y de disfunción endotelial (PCR, IL6, ICAM-1 y VCAM-1) en aquellos sujetos con una mayor adherencia a la DietMed. Dado que la obesidad, al igual que la aterosclerosis, se puede considerar como una inflamación crónica de bajo grado, el IG o la CG de una dieta podrían modular la respuesta inflamatoria. Así, dietas con alto IG o alta CG se han relacionado con una mayor incidencia de obesidad, diabetes tipo 2 (Sluijs, 2010) y ECV (Sluijs, 2010; Hardy, 2010; Lago, 2009). Por lo tanto, debido a que la inflamación crónica de baja intensidad se considera como uno de los mecanismos centrales subyacentes de la obesidad y comorbilidades asociadas, el efecto potencial del IG y CG de la dieta en la modulación inflamatoria parece pertinente. Sin embargo, son pocos los estudios realizados hasta la fecha además de controvertidos porque se centran en la PCR en plasma y no evalúan los efectos a largo plazo del IG y CG así como de los adipocitos u otras adipoquinas relacionadas con la obesidad y comorbilidades (Wolever, 2008; Qi, 2006).

CONCLUSIONES

10. CONCLUSIONES

1. Una mayor adherencia a la DietMed suplementada con AOVE o FS se correlaciona con una reducción de los niveles plasmáticos de TNFR60 yTNFR80, y otros marcadores pro-inflamatorios como IL-6, PCR, VCAM-1 o ICAM-1 que participan en la formación y progresión de la placa de ateroma.

2. Una mayor adherencia a la DietMed suplementada con AOVE o FS se correlaciona con una reducción de la expresión de las moléculas de adhesión leucocitarias (linfocitos T y monocitos) relacionadas con la formación de la placa de ateroma.

3. Una mayor adherencia a la DietMed suplementada con AOVE o FS se asocia a incremento de los biomarcadores séricos (IL-10, IL-13) de placa de ateroma estable en sujetos con alto riesgo vascular.

4. La DietMed suplementada con AOVE o FS y caracterizada por ser una dieta baja en IG y CG, puede modular las concentraciones plasmáticas de algunos marcadores cardiometabólicos (IL-6, TNF-α, leptina y adiponectina), directamente relacionados con obesidad y enfermedades cardiovasculares.

5. El consumo de un patrón de DietMed y, especialmente de algunos de sus principales alimentos, como el AOVE y verduras se asocia a disminución de las concentraciones de TNFR60 que se relaciona con el desarrollo de la aterosclerosis.

6. El patrón de DietMed parece retrasar el desarrollo de la placa de ateroma actuando tanto en etapas precoces (rodamiento, adhesión firme y migración transendotelial de células mononucleares circulantes) como tardías (crecimiento y ruptura de placa).

7. La DietMed suplementada con AOVE o frutos secos podría tener un efecto dual sobre la prevención de la ECV, ya que por un lado mejora los factores de de riesgo clásicos y también tiene un intenso efecto anti-inflamatorio e inmunomodulador, lo que explicaría parcialmente el efecto beneficioso y global de la DietMed en la prevención primaria de la ECV.

8. El efecto antiinflamatorio ejercido por la DietMed parece ser mayor y más intenso a medio (1 año) y largo plazo (5 años) que a corto plazo (3 meses) donde predominan los efectos sobre los factores de riesgo cardiovascular clásicos

9. El efecto anti-inflamatorio e inmunomodulador de la DietMed no es transitorio, sino que persiste al menos después de 5 años de iniciada la intervención y confirma que los efectos saludables de la DMed van más allá del control de los FRC.

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