Balanç benefici/risc del contingut en polifenols i alcohol del vi: bases científiques dels efectes del consum moderat de vi sobre el sistema cardiovascular

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UNIVERSITAT DE BARCELONA
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BALANÇ BENEFICI / RISC DEL CONTINGUT EN POLIFENOLS I ALCOHOL DEL VI: BASES CIENTÍFIQUES DELS EFECTES DEL CONSUM MODERAT DE VI SOBRE EL SISTEMA CARDIOVASCULAR

Memòria de Tesi per a optar al grau de Doctora per la Universitat de Barcelona

Presentada per: Gemma Chiva Blanch

Dirigida per: Ramon Estruch i Riba

Programa de Doctorat: Medicina

Universitat de Barcelona

Ramon ESTRUCH RIBA, Professor Associat de Medicina de la Facultat de Medicina de la Universitat de Barcelona.

Certifica:

Que **Gemma CHIVA BLANCH**

ha realitzat sota la meva direcció la Tesi Doctoral sobre que porta per títol: "**BALANÇ BENEFICI / RISC DEL CONTINGUT EN POLIFENOLS I ALCOHOL DEL VI: BASES CIENTÍFIQUES DELS EFECTES DEL CONSUM MODERAT DE VI SOBRE EL SISTEMA CARDIOVASCULAR**", que està en condicions de ser presentada per a l'obtenció del Grau de Doctor.

El que certifico a Barcelona a 03 de desembre de 2012.

Ramon ESTRUCH RIBA

Al meu pare,

que em va transmetre la passió per La Ciència.

“*Quasi tot el que faig serà insignificant, però és molt important que ho faci.*”
Gandhi

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AGRAÏMENTS

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ANNEX III

Impacte mediàtic dels resultats de la Tesi Doctoral a la premsa científica i a la premsa en general

ABREVIACIONS

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ACE	*Angiotensin-Converting Enzyme*
AHA	*American Heart Association*
ALAT	Alanina Aminotransferasa
ANCOVA	*Analysis of Covariance*
ANOVA	*Analysis of Variance*
APO	Apolipoproteïna
ASAT	Aspartat Aminotransferasa
BMI	*Body Mass Index*
BP	*Blood Pressure*
CCR2	*C-C Chemokine Receptor Type 2*
CD	*Cluster of Differentiation*
CD40A	*CD40 Antigen*
CD40L	*CD40 Ligand*
cGMP	*cyclic Guanosine Monophosphate*
CHD	*Coronary Heart Disease*
CI	*Confidence Interval*
CRP	*C-Reactive Protein*
CVD	*Cardiovascular Disease*
DBP	*Diastolic Blood Pressure*
DRW	*Dealcoholized Red Wine*
EAG/L	Equivalents d'Àcid Gàl·lic/L
FMD	*Flow Mediated Dilation*
G	Ginebra
GGT	Gamma Glutamil Transpeptidasa
GH	*Growth Hormone*

HDL-C	*High Density Lipoprotein-Cholesterol*
HOMA-IR	*Homeostasis Model Assessment of Insulin Resistance*
HPLC-DAD	*High Performance Liquid Chromatography- Diode Array Detector*
HTA	Hipertensió Arterial
ICAM-1	*Intercellular Adhesion Molecule-1*
IL	*Interleukin*
IMC	Índex de Massa Corporal
IP-10	*Interferon Gamma-Induced Protein-10*
IR	*Insulin Resistance*
IS	*Insulin Sensitivity*
ISCIII	Instituto de Salud Carlos III
ISRCTN	*International Standard Randomised Controlled Trial Number*
JAM	*Junction Adhesion Molecule*
LDL-C	*Low Density Lipoprotein-Cholesterol*
LFA-1	*Lymphocyte Function-Associated Antigen-1*
Lp(a)	Lipoproteïna(a)
LPL	*Lipoprotein Lipase*
Mac-1	*Macrophage Adhesion Ligand-1*
MCP	*Monocyte Chemotactic Protein*
MCV	Malalties Cardiovasculars
MDA	Malondialdehid
MDC	*Macrophage-Derived Chemokine*
MFI	*Mean Fluorescence Intensity*
MIP-1α	*Macrophage Inflammatory Protein-1 Alpha*
MPIF-1	*Myeloid Progenitor Inhibitory Factor-1*
n.d.	no detectat

NF-κB	*Nuclear Factor κB*
NO	*Nitric Oxide*
NSAID	*Non-Steroidal Anti-Inflammatory Drug*
OMS	Organització Mundial de la Salut
PAI-1/tPA	Plasminogen-1/Activador del Plasminogen
PCR	Proteïna C Reactiva
PCRus	Proteïna C Reactiva Ultra Sensible
PDGF	*Platelet-Derived Growth Factor*
Qm	Quilomicró
RANTES	*Regulated Upon Activation, Normal T-Cell Expressed and Secreted*
RW	*Red Wine*
SBP	*Systolic Blood Pressure*
SD	*Standard Deviation*
SMC	*Smooth Muscle Cells*
SLe^x	*Sialyl-Lewis X*
TGF-B	*Transforming Growth Factor Beta*
T_H1	Limfòcits T *Helper* Tipus 1
TNF-α	*Tumor Necrosis Factor Alpha*
TOF	*Time-Of-Flight*
Tr.	Traces
Treg	Limfòcits T Reguladors
VCAM-1	*Vascular Cell Adhesion Molecule-1*
VLA-4	*Very Late Activation Antigen-4*
VLDL	*Very Low Density Lipoprotein*
vWF	*von Willebrand Factor*
WHO	*World Health Organization*

INTRODUCCIÓ

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1. Introducció general

Segons dades del Centre Nacional d'Epidemiologia (ISCIII), les malalties cardiovasculars (MCV) són la principal causa de mort al nostre país. Dades del Sistema Nacional de Salut del 2009 revelen que les morts per malalties cardiovasculars van suposar el 28.4% del total de morts a Espanya (el 20.3% de malalties cardíaques i el 8.1% de malalties cerebrovasculars), ocupant el segon i tercer lloc en el rànking de causes de mort al nostre país (el primer lloc l'ocupa el càncer) i essent un 60% més elevada en homes que en dones. A més a més, quatre de cada cinc morts per MCV es produeixen en persones majors de 65 anys. Dades de la OMS (Organització Mundial de la Salud) de 2011 (WHO, 2011) apunten a que la mortalitat per MCV és el 33% del total de morts a Espanya. Les MCV són, així mateix, responsables d'uns 17.3 milions de morts a l'any i són la primera causa de mort al món. La seva incidència no ha disminuït apreciablement en les últimes dècades, i per aconseguir-ho és necessari reforçar-ne la prevenció primària.

Els factors de risc tradicionals de les MCV són l'edat, el sexe, el tabaquisme, la hipertensió arterial (HTA), el sobrepès o obesitat, la diabetis tipus 2, les dislipèmies (augment del colesterol LDL i/o disminució del colesterol HDL), els antecedents familiars de MCV prematura, el sedentarisme i la dieta (Terrados, 2010). Donat que aquest factors de risc només expliquen el 50% de les complicacions cardiovasculars de les MCV, hi ha força interès en determinar nous factors de risc que permetin estratificar més acuradament el risc cardiovascular de la població i identificar aquelles persones a qui dirigir les campanyes de prevenció cardiovascular. Els factors de risc CV emergents són el quocient colesterol LDL/colesterol HDL, les apolipoproteïnes, les subclasses de les HDL, els triglicèrids, les partícules d'LDL "petites i denses", les lipoproteïnes residuals o romanents, els marcadors d'inflamació i adhesió

cel·lular, l'homocisteïnèmia, la glucèmia en dejú alterada i els factors trombogènics/hemostàtics.

Dins les MCV, l'arteriosclerosi és una malaltia progressiva caracteritzada per la rigidesa i estenosi arterial, que provoca una mala circulació sanguínia. L'aterosclerosi és la forma més comú d'arteriosclerosi i comença amb una disfunció endotelial on les cèl·lules endotelials, activades per factors com les LDL oxidades, expressen molècules d'adhesió i quimioatractives que capten leucòcits a l'endoteli. En aquest moment els lípids plasmàtics s'acumulen a la íntima provocant reaccions inflamatòries i oxidatives i això desemboca en la migració i proliferació de les cèl·lules musculars arterials. Per tant, l'aterosclerosi és una malaltia crònica inflamatòria de baix grau mitjançada per l'expressió de molècules d'adhesió i quimiocines que implica el reclutament de leucòcits circulants a l'endoteli vascular i la subsegüent migració a l'espai subendotelial, conduint a la formació de les lesions ateroscleròtiques.

En el desenvolupament de les MCV en general i l'aterosclerosi en particular, la dieta té un paper molt important, ja que pot modificar molts factors de risc tradicionals i emergents. Molts estudis epidemiològics han trobat que el consum moderat d'alcohol (fins a 30 g/dia en homes i fins a 20 g/dia en dones) té un efecte beneficiós en les MCV (Brien, 2011; Ronksley, 2011). No obstant, no totes les begudes alcohòliques tenen la mateixa composició. Les begudes alcohòliques destil·lades es composen pràcticament només d'alcohol i aigua, mentre que les begudes fermentades es composen d'aigua, alcohol, macro i micronutrients i polifenols. Diversos estudis on han distingit l'acció de diferents begudes alcohòliques, han observat que el consum moderat de vi negre té uns efectes protectors enfront a les MCV majors a altres begudes alcohòliques, probablement degut al seu alt contingut en polifenols (Estruch, 2004; Sacanella, 2007; Covas, 2010). Els efectes cardiovasculars dels polifenols del vi negre han estat estudiats en models experimentals (Vinson, 2001; Pal, 2003; Stocker, 2004), però pocs estudis en humans s'han focalitzat en estudiar els efectes dels compostos fenòlics del vi negre independents de l'alcohol. Per tant, els

diferents efectes de les diferents fraccions del vi negre (la alcohòlica i la no alcohòlica –principalment polifenòlica-) encara són desconeguts, així com també es desconeix si aquestes dues fraccions tenen un efecte sinèrgic.

Per tot això, l'objectiu de la present tesi doctoral és avaluar i comparar els efectes del consum moderat de vi negre i les seves fraccions (alcohol i polifenols) en persones d'alt risc cardiovascular, sobre els factors de risc vasculars següents: expressió de molècules solubles i leucocitàries d'adhesió i de citocines proinflamatòries relacionades amb l'aparició i progressió de l'aterosclerosi, perfil lipídic, metabolisme de la glucosa i pressió arterial.

2. Malalties del sistema cardiovascular: L'Aterosclerosi

Segons la definició de l'organització mundial de la salut (WHO, 2011), les MCV inclouen diferents cardiopaties, malalties cerebrovasculars i patologies dels vasos sanguinis, com les següents:

1. MCV de causa ateroscleròtica:
    1. Malaltia cardíaca isquèmica o coronària (per exemple, l'infart de miocardi)
    2. Malaltia cerebrovascular (per exemple, l'accident vascular cerebral)
    3. Malalties de l'aorta o arteries (incloent la HTA i la malaltia vascular perifèrica)
2. Altres MCV
    1. Malaltia cardíaca congènita
    2. Malaltia cardíaca reumàtica
    3. Miocardiopaties
    4. Arítmies cardíaques

2.1. L'Aterosclerosi

L'aterosclerosi és una malaltia crònica progressiva de les artèries mitjanes i de gran grandària caracteritzada per la formació de plaques d'ateroma (Libby, 2011), que es manifesta clínicament quan causa una trombosi. Durant molts anys es considerava l'aterosclerosi com una simple acumulació de colesterol a la paret vascular, però avui se sap que és una autèntica malaltia inflamatòria crònica dels vasos sanguinis (Hansson, 2011). L'aterosclerosi dóna lloc a la malaltia cerebrovascular i la malaltia coronària a través d'una lenta progressió de les lesions ateroscleròtiques, la formació de plaques d'ateroma i l'estretament de la llum arterial (Weber, 2011; WHO, 2011). Aquestes plaques es poden trencar i desenvolupar una trombosi aguda que

acaba taponant la llum del vas, donant lloc a un síndrome coronari agut, infart de miocardi o accident vascular cerebral. La patologia subjacent inicial es caracteritza per un procés inflamatori crònic de la paret arterial focalitzat en determinats punts on hi ha un flux sanguini laminar pertorbat com els punts de bifurcació. S'inicia a través d'una disfunció endotelial i d'alteracions estructurals que permeten l'acumulació subendotelial de partícules de LDL (Weber, 2011) i progressivament es van desenvolupant les lesions arterioscleròtiques com s'explica més detalladament a continuació.

2.1.1. Endoteli vascular i aterosclerosi

L'endoteli vascular (**Figura 1**) no només és una barrera física que separa la sang dels teixits, sinó que també és una membrana semipermeable que controla el pas de molècules a l'interior de la paret arterial i a través de les parets dels capil·lars i vènules. En condicions normals, l'endoteli controla el to vascular, manté l'equilibri entre trombosi i fibrinòlisi i regula el reclutament de cèl·lules inflamatòries a la paret vascular. Aquests efectes són provocats per l'alliberament de diverses molècules com l'òxid nítric (NO), la prostaciclina (PGI2), el factor d'hiperpolarització derivat de l'endoteli i la endotelina 1. El NO juga un paper central ateroprotector a través de la regulació del to vascular, la inhibició de l'agregació plaquetària, la supressió de la proliferació de cèl·lules vascular del múscul llis i el bloqueig de l'adhesió i transmigració de leucòcits (Badimon, 2012).

La disfunció endotelial és deguda a una disfunció de la NO sintasa endotelial (Badimon, 2011) i es caracteritza per un deteriorament de la vasodilatació dependent de l'endoteli, ja que es trenca l'equilibri entre vasodilatadors i vasoconstrictors, acompanyat d'un estat protrombòtic i proinflamatori de les cèl·lules endotelials. La disfunció endotelial és un marcador precoç i independent de mal pronòstic en la majoria de les formes de MCV, ja que s'ha trobat consistentment en la HTA, l'aterosclerosi, la malaltia cardíaca coronària, la diabetis, l'obesitat i l'envelliment (Perez-Vizcaino, 2006).

Al contrari que les partícules de colesterol LDL, sembla que el colesterol HDL podria disminuir la disfunció endotelial disminuint l'expressió de molècules d'adhesió i restablint la biodisponibilitat del NO (Badimon, 2012).

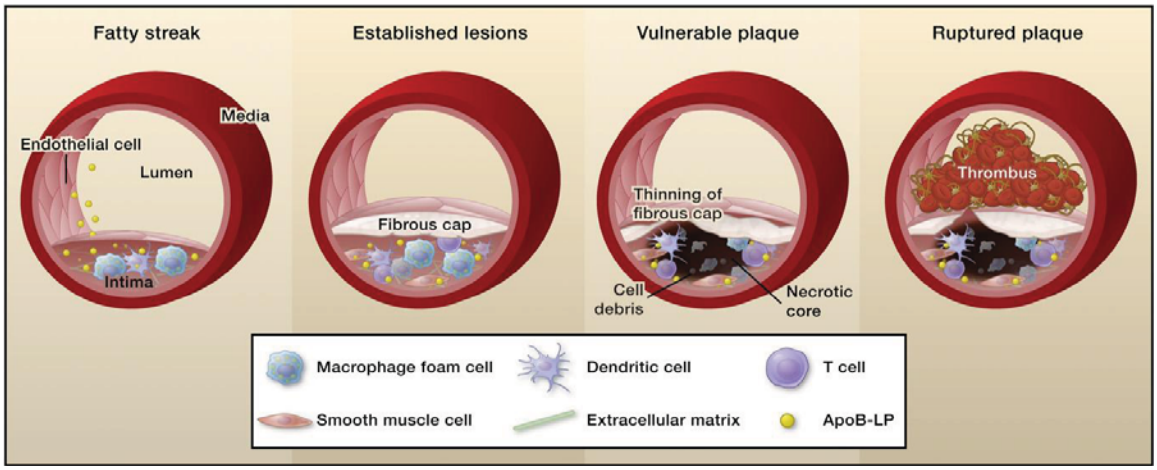

Figura 1. Progressió de la lesió ateroscleròtica a l'endoteli vascular (Moore, 2011).

2.1.2. Iniciació de la placa d'ateroma

Diversos estudis experimentals han demostrat que una alta concentració de colesterol plasmàtic promou la formació de la placa d'ateroma. El colesterol és transportat per la sang a través de les LDL, que són unes partícules clau en el desenvolupament i progressió de l'aterosclerosi (Badimon, 2012). Aquestes partícules contenen colesterol esterificat i triglicèrids envoltats per una capa de fosfolípids, colesterol lliure i l'apolipoproteïna B (ApoB), així com epítops de limfòcits T i B (Hansson, 2011). Les partícules LDL circulants (unides a l'ApoB) es poden acumular a la íntima, la capa més interna de l'artèria (**Figura 1**). En aquesta capa, l'ApoB es pot unir a proteoglicans de la matriu extracel·lular a través d'interaccions iòniques. Això és un factor iniciant molt important en l'aterogènesi primerenca. Alts nivells plasmàtics de LDL disminueixen la biodisponibilitat del NO, produint una disfunció endotelial que és l'anterior pas a l'entrada de les LDL a la íntima arterial (Badimon, 2012). Com a conseqüència d'aquesta retenció subendotelial, les LDL queden atrapades a la íntima, on són susceptibles a modificacions oxidatives d'origen enzimàtic o a través d'espècies

reactives d'oxigen generades a la íntima a causa de l'inflamació produïda per l'aterosclerosi mateixa (Badimon, 2011; Hansson 2011). Aquestes LDL oxidades llavors activen les cèl·lules endotelials i els macròfags per a produir molècules d'adhesió i quimiocines com la Interleucina (IL)-1β o el *Tumor Necrosis Factor* (TNF)-α (Libby, 2011) i atraure leucòcits a l'espai subendotelial. Dins la íntima, els monòcits procedents de la sang es diferencien a macròfags, procés necessari pel desenvolupament de l'aterosclerosi. Aquests macròfags capten anormalment una gran quantitat de LDL oxidades, mitjançant la sobreexpressió de receptors d'epítops específics de l'oxidació de les LDL tals com el *Cluster of Differentiation* (CD)36 o els *toll-like receptors* (Badimon, 2011), transformant-se llavors a cèl·lules espumoses, característiques de les lesions ateroscleròtiques (Hansson, 2011; Libby, 2011; Weber 2011). Els *toll-like receptors* expressats a l'endoteli i als monòcits també es poden unir a les LDL oxidades i provocar reaccions vascular proateroscleròtiques (Hansson, 2011). Els macròfags i les cèl·lules dendrítiques ràpidament capten LDL oxidada, no només per a degradar-la, sinó també per processar els antígens i presentar-los a les cèl·lules T.

2.1.3. Progressió de la placa d'ateroma

Les LDL, especialment les formes modificades, activen les cèl·lules endotelials (possiblement també activades pel flux sanguini turbulent als punts de bifurcació arterial), cosa que condueix a un augment de l'expressió i la secreció de compostos quimiotàctics solubles com la *Monocyte Chemotactic Protein* (MCP)-1, la IL-8 o la *Vascular Cell Adhesion Molecule* (VCAM)-1 i augmenten l'expressió de molècules d'adhesió com ara integrines i selectines com per exemple l'E-selectina, que estan exposades a la superfície de les cèl·lules de l'endoteli activat i afavoreixen el reclutament, l'adhesió i la transmigració dels monòcits i limfòcits T del torrent circulatori (**Figura 1** i **2**). Això actua sinèrgicament amb quimiocines com el *Regulated Upon Activation, Normal T-cell Expressed and Secreted* (RANTES) o l'*Interferon gamma-*

induced protein (IP)-10 per atraure més monòcits, cèl·lules dendrítiques i limfòcits T a la íntima arterial (Badimon, 2012; Hansson, 2011).

La transmigració dels monòcits es produeix preferentment en les zones endotelials on la làmina basal està més enriquida amb partícules LDL modificades i té lloc principalment a través de les unions entre les cèl·lules endotelials. Les *junction adhesion molecule* (JAM) -A i -C participen en el control de la permeabilitat vascular i la transmigració de leucòcits a través de la superfície de les cèl·lules endotelials (Badimon, 2011).

Els limfòcits T no són tan abundants com els macròfags (amb una *ratio* macròfags/ cèl·lules T entre 10 i 4 a 1 a les lesions ateroscleròtiques humanes). No obstant, les cèl·lules T són activades a les lesions, produeixen mediadors aterogènics i contribueixen al creixement de la lesió i a l'agreujament de la malaltia. Els limfòcits B i els mastòcits són presents només ocasionalment a les lesions però abundants a la part adventícia de l'arteria ateroscleròtica, encara que hi ha indicis que certes subpoblacions de limfòcits B exerceixen efectes contraris en la malaltia (Hansson, 2011).

L'aterosclerosi, en part, és conduïda per la resposta dels limfòcits T *helper* tipus 1 (TH1). L'interferó-γ, la citocina per excel·lència dels TH1, i també la IL-18, una citocina promotora dels TH1 (Hansson, 2011) o el TNF-α (Libby, 2011), són presents a les plaques d'ateroma humanes, i tenen efectes patogènics tals com la disminució de la formació de fibres de col·lagen, l'augment de l'expressió del complex major d'histocompatibilitat de classe II, l'increment de la secreció de proteases i quimiocines, la sobreexpressió de molècules d'adhesió, l'inducció de citocines proinflamatòries, així com l'activació de macròfags i cèl·lules endotelials. D'altra banda, diverses subpoblacions de limfòcits T reguladors (Treg), sembla que tenen un efecte protector en models d'aterosclerosi. Les citocines produïdes per les Treg, el *Transforming growth factor* (TGF)-β i la IL-10, tenen un efectes ateroprotectors molt profunds en models de ratolins (Hansson, 2011; Libby 2011).

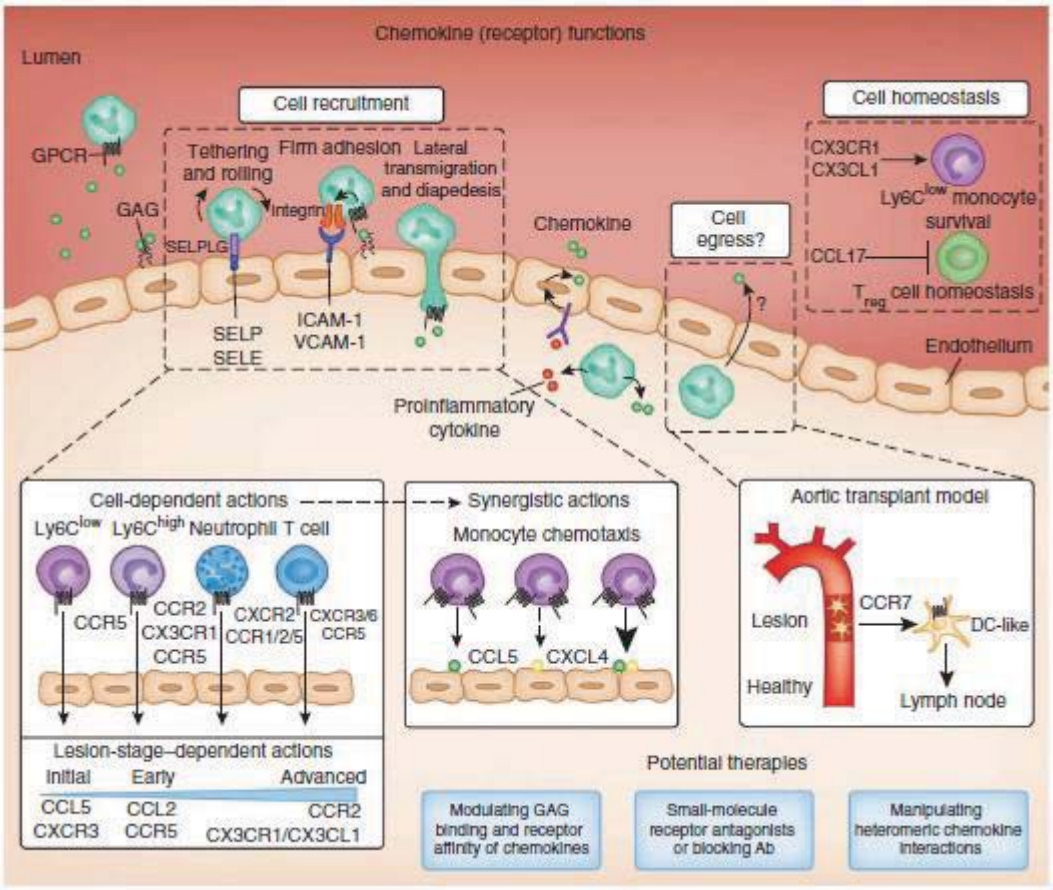

Figura 2. Paper de les quimiocines i els seus receptors en l'aterogènesi. El reclutament de leucòcits implica el seu rodament, adherència, migració lateral i diapedesis transendotelial, controlat per quimiocines (Weber, 2011).

2.1.4. Molècules d'adhesió, quimiocines i citocines relacionades amb l'iniciació i progressió de la placa d'ateroma

El reclutament aterogènic de leucòcits involucra una seqüència composta per un rodament lent per la superfície endotelial, una adhesió ferma, una migració lateral i la diapedesi transendotelial. Aquest procés és controlat per quimiocines (**Figura 2**), que són citocines quimiotàctiques. Els leucòcits reclutats durant la cascada inflamatòria inclouen neutròfils, monòcits, limfòcits T i B, cèl·lules dendrítiques i mastòcits (Weber, 2011). Per tant, el sistema de quimiocines involucrat és molt extens i específic (**Taula 1**).

A més a més, la formació de la placa d'ateroma també involucra el reclutament de les cèl·lules musculars llises (SMC) des de la capa mediana a la

íntima arterial i la seva proliferació en resposta a mediadors com el *Platelet-Derived Growth Factor* (PDGF). A la íntima, les SMC produeixen molècules de la matriu extracel·lular, com col·lagen i elastina, i formen una capa fibrosa que cobreix la placa, normalment recoberta a la seva vegada amb cèl·lules espumoses que alliberen lípids que s'acumulen extracel·lularment quan moren. Si les cèl·lules mortes no s'evacuen d'una manera eficient, s'acumulen restes cel·lulars i lípids extracel·lulars, formant el centre necròtic de la placa. Els trombes s'originen per la disrupció física de la placa, normalment a través d'una fractura de la capa fibrosa que exposa les substàncies procoagulants al nucli de la placa per afavorir la coagulació proteica a la sang, desencadenant una trombosi. Les plaques que són més propenses a trencar-se tenen una capa fibrosa de col·lagen prima i poques SMC, però abundants macròfags que secreten enzims col·lagenolítics, mediadors de la mort de les SMC i factors tissulars procoagulants que transformen el nucli lipídic de la placa trombogènic. Per tant, les cèl·lules inflamatòries infiltrades interaccionen amb les cèl·lules arterials intrínseques (endotelials i les SMC), promovent la formació de la lesió i les seves complicacions (Libby, 2011).

Les plaquetes també intervenen en aquest procés. Les cèl·lules endotelials inflamades expressen molècules com la fibronectina, la *Intercellular Adhesion Molecule* (ICAM)-1, la P-selectina, la E-selectina, la integrina αvß3, i el factor de von Willebrand (vWF) en la seva superfície i tot això promou l'adhesió i activació plaquetes, que majoritàriament intervé en la progressió de la lesió ateroscleròtica (i no a una complicació trombòtica). En canvi, l'erosió endotelial i el trencament de la placa d'ateroma exposa components de la matriu vascular (per exemple, diferents tipus de col·lagen, vWF, fibronectina, laminina, fibulina i trombospondina) al corrent sanguini que activa profundament l'adhesió i l'activació de plaquetes que eventualment condueix a l'agregació i la formació de trombes (Badimon, 2011). A més, cada vegada més s'estan observant noves funcions de les plaquetes en el reclutament leucocitari a l'endoteli.

Taula 1. Característiques i funcions de les diferents quimiocines i citocines leucocitàries i endotelials involucrades en la formació i progressió de la placa d'ateroma estudiades en aquesta tesi doctoral.

MOLÈCULA	CLASSIFICACIÓ	EXPRESSIÓ*	FUNCIONS
CD40a	Superfamília del TNF	Endotelial i leucocitària	Proinflamatori i procoagulant.
CD40L	Superfamília del TNF	Endotelial, en cèl·lules vasculars de múscul lis, plaquetària i leucocitària	Pro-inflamatori i factor independent de risc cardiovascular. Activador de cèl·lules endotelials, que promou la secreció de E-Selectina, VCAM-1 i ICAM-1 afavorint el procés de reclutament constant de leucòcits a l'endoteli (sobretot a les zones amb lesió ateroscleròtica).
E-Selectina	Selectina; Molècula d'adhesió cel·lular	Endotelial	Reclutament de leucòcits a l'endoteli (durant la fase de rodament), a través de la unió amb el SLex. Molècula proinflamatòria.
ICAM-1	Superfamília de les immunoglobulines	Endotelial i leucocitària	Lligand d'integrines (LFA-1, Mac-1) per activar la transmigració endotelial dels leucòcits.
IL-1α	Superfamília de les interleucines-1	Monocitària i dendrítica	Proinflamatòria, estimula l'expressió de factors d'adhesió endotelials que condueixen a una transmigració leucocitària com la VCAM-1.
IL-10	Citocina de classe 2	Monocitària i limfocitària	Antiinflamatòria: bloqueja el NF-κB i inhibeix la síntesi de TNF-α i altres citocines.
IL-16	Superfamília de les interleucines-1	Limfocitària	Quimioatractiva per limfòcits T, macròfags, cèl·lules dendrítiques i eosinòfils.
IL-18	Superfamília de les interleucines-1	En macròfags	Citocina pro-inflamatòria: indueix l'interferó gamma, que activa els macròfags.
IL-6	Interleucina	Leucocitària i en cèl·lules vasculars de múscul lis	Proinflamatòria.
MCP-1	Família de les citocines CC	Monocitària i dendrítica	Recluta monòcits, limfòcits i cèl·lules dendrítiques a les zones ateroscleròtiques. Receptor del CCR2.
MCP-2	Família de les citocines CC	Monocitària i dendrítica	Recluta leucòcits a les zones ateroscleròtiques.
MCP-3	Família de les citocines CC	En macròfags	Recluta monòcits a les zones ateroscleròtiques. Receptor del CCR2.
MDC	Família de les citocines CC	En macròfags i cèl·lules dendrítiques	Quimioatractiu per monòcits, cèl·lules dendrítiques i limfòcits.
MIP-1α	Família de les citocines CC	En macròfags	Inductora de la síntesi de IL-1, IL-6 i TNF-α. Recluta leucòcits a l'endoteli.
MPIF-1	Família de les citocines CC	En macròfags	Altament quimiotàctica per limfòcits.
PCR	Pentraxina	Hepàtica en resposta a factors secretats per macròfags i adipòcits.	Molècula inflamatòria de fase aguda que activa el sistema del complement. Promotora d'adhesió plaquetària a l'endoteli Marcador del risc d'infart de miocardi i embòlia.
TNF-α	Superfamília del TNF	Monòcits, limfòcits i neutròfils.	Activa el NF-κB i per tant, estimula l'expressió de l'E-Selectina, ICAM-1, VCAM-1, així com l'adhesió i transmigració monocitària i de neutrpofils a l'endoteli. Inhibidor de la NOS. Involucrat en la inflamació sistèmica.
VCAM-1	Superfamília de les immunoglobulines	Endotelial i en cèl·lules vasculars de múscul lis	Receptor del VLA-4, que quan s'uneixen provoquen l'adherència ferma i la transmigració endotelial de leucòcits, que llavors alliberen citocines inflamatòries com el CD40L.
LFA-1	Integrina de la subfamília β2	Limfòcitària (T i B) i en macròfags	Molècula d'adhesió que s'uneix a l'ICAM-1 per a la transmigració endotelial.
Mac-1	Integrina de la subfamília β2	Leucocitària	Molècula d'adhesió que s'uneix a l'ICAM-1 per a la transmigració endotelial dels leucòcits.
VLA-4	Integrina de la subfamília β1	Leucocitària	S'uneix a la VCAM-1 provocant l'adherència ferma i la transmigració endotelial de leucòcits.

Taula 1 (continuació). Característiques i funcions de les diferents quimiocines i citocines leucocitàries i endotelials involucrades en la formació i progressió de la placa d'ateroma estudiades en aquesta tesi doctoral.

MOLÈCULA	CLASSIFICACIÓ	EXPRESSIÓ*	FUNCIONS
SLex	Glicolípid d'adhesió molecular	Leucocitària	S'uneix a la E-Selectina pel rodament leucocitari i l'adhesió ferma a l'endoteli.
CD40	Superfamília dels receptors de TNF	Leucocitària	S'uneix al CD40L provocant l'adhesió endotelial.
CD36	Família dels *scavenger receptors* classe B	En macròfags	S'uneix a la LDL oxidada integrant-la en la cèl·lula i promovent els pas a cèl·lula espumosa. Proinflamatori.
CCR2	Receptor de quimiocines CC tipus 2	Monocitària	Mediador de la quimiotaxis monocitària (s'uneix a les MCP).

*Expressió monocitària implica en macròfags també, però expressió en macròfags no inclou l'expressió monocitària. CD40 antigen (CD40a), CD40 Lligand (CD40L), CRP (Proteïna C reactiva), Intercellular Adhesion Molecule-1 (ICAM-1), Interleukin-1 alpha (IL-1α), Interleukin-10 (IL-10), Interleukin-16 (IL-16), Interleukin-18 (IL-18), Interleukin-6 (IL-6), Monocyte Chemotactic Protein-1 (MCP-1), Monocyte Chemotactic Protein2 (MCP-2), Monocyte Chemotactic Protein-3 (MCP-3), Macrophage-Derived Chemokine (MDC), Macrophage Inflammatory Protein-1 alpha (MIP-1α), Myeloid Progenitor Inhibitory Factor-1 (MPIF-1), Tumor Necrosis Factor alpha (TNF-α) and Vascular Cell Adhesion Molecule-1 (VCAM-1), Very Late Activation Antigen-4 (VLA-4), Lymphocyte Function-Associated Antigen-1 (LFA-1), Macrophage adhesion ligand-1 (Mac-1), Sialyl-Lewis X (SLe^x) and C-C chemokine receptor type 2 (CCR2).

Per tant, podríem concloure que l'aparició i progressió de l'aterosclerosi és una complexa orquestra de diferents tipus cel·lulars i quimiocines, que actuen a diferents nivells i estadis. Així és lògic pensar que la modul·lació de l'expressió d'aquestes quimiocines podria frenar o revertir la lesió escleròtica.

2.2. Factors de risc de l'aterosclerosi

Existeix una àmplia evidència científica demostrant que els factors de risc ambientals i metabòlics tenen un paper clau en l'etiologia de l'aterosclerosi (de Lorgeril M, 2006; WHO, 2011). Els principals factors de risc de l'aterosclerosi són:

2.2.1. Factors de risc ambientals o comportamentals

2.2.1.1. Ús del tabac

La OMS atribueix a l'ús del tabac el 9% de les morts globals i causa el 10% de MCV (WHO, 2011). Hi ha nombrosa evidència científica d'estudis prospectius de cohorts referents a l'efecte beneficiós d'abandonar l'ús del tabac en la mortalitat cardiovascular (WHO, 2007).

2.2.1.2. Inactivitat física

L'activitat física insuficient es defineix com a menys de 5 vegades 30 minuts d'activitat moderada a la setmana, o menys de 3 vegades 20 minuts d'activitat física intensa a la setmana, o l'equivalent. L'inactivitat física és el quart factor de risc de mortalitat i provoca el 6% de les morts globals (WHO, 2011).

En adults, 150 minuts d'activitat física moderada a la setmana (o l'equivalent) redueix un 30% el risc de malaltia cardíaca isquèmica i un 27% el risc de diabetis (WHO, 2007).

L'activitat física és un dels determinants clau de la despesa energètica i, per tant, fonamental pel balanç energètic i el control de pes. A més, millora la funció endotelial, que a la seva vegada augmenta la vasodilatació i la funció vasomotora als vasos sanguinis, millorant la pressió arterial. També

contribueix a la pèrdua de pes, al control glicèmic i la sensibilitat a la insulina, i a la millora del perfil lipídic. Per tant, els efectes beneficiosos de l'activitat física en el risc cardiovascular són mitjançats, almenys en part, a través d'aquests efectes en els factors de risc intermediaris.

2.2.1.3. Dieta no saludable

Actualment existeix un alt grau d'evidència científica del rerefons dietètic de l'aterosclerosi i la malaltia coronària. Encara que aquest aspecte serà comentat més extensament en l'apartat 3, una ingesta alta de greix saturat, àcids grassos *trans*-, colesterol i sal, i una baixa ingesta de fruites i verdures, així com de peix està vinculada a un augment del risc cardiovascular (Renaud, 1992). Només destacarem com a exemple que aproximadament el 3% del total de morts al món és atribuïble a un baix consum de fruites i verdures (WHO, 2011).

2.2.1.4. Ús nociu de l'alcohol

L'ús nociu i/o excessiu de l'alcohol és un factor de risc per múltiples malalties, tant físiques com socials, tals com HTA, infart agut de miocardi, miocardiopaties, arítmia cardíaca, cirrosi hepàtica, pancreatitis, neuropaties, encefalopaties, síndrome de la mort sobtada, violència, suïcidi i lesions inintencionades.

El consum perillós i nociu de l'alcohol va ser responsable del 3.8% de morts globals al 2004, més del 50% de les quals van ser causades per MCV, cirrosi hepàtica i càncer (WHO, 2011). No obstant això, la relació entre el consum d'alcohol i les MCV és complexa. Tal com serà desenvolupat a l'apartat 4, segons el grau, patró de consum i tipus de beguda alcohòlica existeix un risc o una protecció enfront a les MCV.

2.2.2. Factors de risc metabòlics

2.2.2.1. Augment de la pressió arterial

La pressió arterial és la pressió exercida per la circulació de la sang a les parets dels vasos sanguinis i és un dels principals signes vitals. En cada batec del cor, la pressió arterial varia entre un màxim (pressió sistòlica) i un mínim (pressió diastòlica) de pressió. La pressió arterial mitjana disminueix a mesura que la circulació sanguínia va des del cor per les artèries, i té el seu major descens en les petites artèries i arterioles, i segueix disminuint a mesura que la sang es mou a través dels capil·lars i torna al cor per les venes.

La OMS defineix la hipertensió arterial (HTA) com l'elevació de les xifres de pressió arterial sistòlica ≥ 140 mmHg i/o pressió arterial diastòlica ≥ 90 mmHg.

La HTA és uns dels factors de risc principals de l'aterosclerosi, ja que incrementa la tensió de la paret arterial, conduint a un procés de reparació arterial defectuós i a la formació d'aneurismes. A més, també és un dels factors de risc d'accident vascular cerebral, infart de miocardi, insuficiència cardíaca i aneurisma arterial, i és la principal causa d'insuficiència renal crònica. La OMS atribueix a la HTA el 13% de les morts globals (WHO, 2011).

Els factors que controlen la pressió arterial són endocrins i vasculars. Els factors endocrins són les catecolamines (dopamina, norepinefrina i epinefrina), l'aldosterona, la renina i l'angiotensina, la hormona antidiürètica i les hormones tiroïdals. Els factors endocrins i vasculars estan interrelacionats, com s'ha vist amb l'angiotensina II, una de les principals hormones reguladores de la pressió arterial, que pot alterar la funció endotelial incitant l'adhesió leucocitària (Libby, 2011). No obstant,

aquesta tesi està focalitzada en el sistema vascular, així que es tractaran amb més profunditat els factors vasculars.

Els factors vasculars poden actuar com a vasoconstrictors o com a vasodilatadors. Un dels principals factors vasoconstrictors és l'endotelina. Els factors vasodilatadors inclouen el NO, les prostaglandines i els factors d'hiperpolarització derivats de l'endoteli (ions de potassi, metabòlits de l'àcid araquidònic –àcids epoxieicosatrienoics-, peròxid d'hidrogen, monòxid de carboni, sulfit d'hidrogen i el pèptid C-natriurètic) que vasodilaten per relaxació de les cèl·lules vasculars de múscul llis per activació dels diferents canals de potassi. Per tant, la pèrdua de les funcions normals endotelials (la disfunció endotelial) acaba impedint la vasodilatació i augmenta la pressió arterial, cosa que pot ser un mecanisme freqüent de la HTA (Giles, 2012).

El NO endotelial és un dels principals reguladors de la vasodilatació. La força de fricció exercida a la paret vascular secundària al flux sanguini obre els canals de calci de les cèl·lules endotelials, promovent l'activació de la NO sintasa endotelial, que a la seva vegada, indueix la secreció de NO. Llavors el NO difon a la capa muscular arterial, on activa la guanilat ciclasa, causant un increment de la guanosina monofosfat cíclica que provoca una relaxació muscular. Per tant, l'òxid nítric juga un paper molt important en la regulació de la resistència vascular sistèmica, el relaxament arterial i la capacitat de distensibilitat, és a dir, en el manteniment i regulació de la pressió arterial (Giles, 2012). A més també té efectes antiagregants en plaquetes i per tant també juga un paper fonamental en la fluïdesa de la sang (Perez-Vizcaino, 2006).

2.2.2.2. Augment de la glucèmia (diabetis)

Es consideren alteracions de la glucosa o prediabetis, la intolerància a la glucosa [glucèmia aleatòria entre ≥ 140 mg/dL (7,8 mmol/L) i < 200 mg/dL

(11,1 mmol/L)] i la glucosa basal alterada [glucèmia en dejú entre ≥ 110 mg/dL (6,1 mmol/L) i < 126 mg/dL (7 mmol/L)]. La diabetis mellitus és diagnosticada quan la glucèmia basal és ≥ 126 mg/dL (7 mmol/L) en dues ocasions, o quan la glucèmia a l'atzar és ≥ 200 mg/dL (11,1 mmol/L) amb símptomes típics de la malaltia (Mata, 2009).

La OMS atribueix a l'augment de la glicèmia el 6% de les morts globals, i el 60% de les morts de persones amb diabetis són atribuïbles a les MCV. El risc de MCV és de 2 a 3 vegades major en persones amb diabetis tipus 1 o tipus 2 que en persones sense diabetis, i també tenen pitjor pronòstic després d'un esdeveniment cardiovascular (WHO, 2011; Rydén, 2012). La detecció i tractament precoç de la diabetis és molt important alhora d'evitar complicacions severes com l'infart de miocardi, l'accident vascular cerebral, insuficiència renal, amputacions i ceguera.

Normalment, la desregulació de la glucosa és simultània amb altres factors de risc cardiovascular com l'obesitat central, la pressió arterial elevada, nivells baixos d'HDL i alts nivells de triglicèrids plasmàtics (Zhou, 2012). La patogènesi de la diabetis tipus 2 està composta per diversos mecanismes moleculars que involucren a diferents vies de senyalització que condueixen en última instància a la hiperglucèmia. L'augment dels nivells de glucosa en dejú són causats per una disfunció en l'acció de la insulina i la secreció d'insulina per les cèl·lules β pancreàtiques (resistència a la insulina) (Gong, 2012). La resistència a la insulina compensada caracteritza un estat prediabètic, on es veu afectada l'acció de la insulina, però encara és suficient per mantenir una condició euglucèmica a través d'un augment en els nivells circulants d'insulina. La resistència a la insulina és una condició patològica que es troba generalment en els pacients obesos, en què el teixit adipós visceral blanc en proporcions anormals segrega una sèrie de citocines anomenades adipocines. Algunes d'aquestes molècules tenen efectes antiinflamatoris com l'adiponectina, que és una citocina sensibilitzant a la insulina,

notablement reduïda en l'obesitat, mentre que altres tenen propietats proinflamatòries com la leptina, el TNF-α, la IL-6, la IL-1β el *C-C chemokine receptor type 2* (CCR2) i la MCP-1, entre d'altres molècules com el CD36 o la PCR-us, que està vinculada a un major risc cardiovascular en pacients amb diabetis mellitus tipus 2. De fet, l'augment dels nivells de PCR-us estan associats amb els indicadors de risc cardiovascular relacionats amb la diabetis com l'índex *Homeostasis Model Assessment of Insulin Resistance* (HOMA-IR), els nivells d'insulina, l'índex de massa corporal (IMC) i la disfunció de les cèl·lules β, però no es correlaciona amb la durada de la malaltia o el control de la glucèmia. No obstant, el paper de la PCR-us no està gaire clar en els estats prediabètics (Dali-Youcef, 2012).

A més, una disminució de l'activitat del NO, així com una disfunció endotelial s'han associat amb la resistència a la insulina i la diabetis mellitus. La disfunció que s'observa en el sistema endotelial de NOsintasa/ guanilat ciclasa soluble/ guanosina monofosfat cíclica (cGMP) és un mecanisme comú pel qual factors de risc cardiovascular com la diabetis o la hipertensió deterioren la paret vascular (Giles, 2012).

2.2.2.3. Augment de la lipèmia

Globalment, un terç de la malaltia cardíaca isquèmica és atribuïble a alts nivells de colesterol. En general, el nivell elevat de colesterol s'estima que causa 2,6 milions de morts a nivell mundial. Una reducció del 10% en el colesterol sèric en una persona de 40 anys s'ha associat a una reducció del 50% del risc de malaltia cardíaca i en una persona de 70 anys a una reducció del 20% (WHO, 2011).

En termes generals, el perfil lipídic inclou: el colesterol associat a les LDL, el colesterol associat a les HDL i els triglicèrids, encara que cada cop agafen més importància algunes proteïnes de les partícules de

lipoproteïnes com la ApoA-I, la ApoA-II, la ApoB i ApoE, així com la lipoproteïna(a) [lp(a)].

Com s'ha comentat en apartats anteriors, els nivells circulants de partícules LDL es correlacionen amb el risc d'esdeveniments CV i amb un augment de la susceptibilitat individual d'aterosclerosis i les seves complicacions (Libby, 2011), ja que el colesterol LDL es diposita a les parets de les artèries. El colesterol HDL protegeix contra les malalties vasculars mitjançant l'eliminació del colesterol LDL de les parets de les artèries, encara que alts nivells d'HDL només retarden (no eviten) l'aparició de la MCV (Sbrana, 2012). En general, però, i a nivell de prevenció primària, la dislipèmia, en termes de nivells elevats de colesterol i triglicèrids, així com la disminució de les concentracions d'HDL, és un factor independent de risc cardiovascular (Tognon, 2012).

Recentment s'ha suggerit que les apolipoproteïnes (Apo) poden ser marcadors de risc més informatius que les lipoproteïnes (LDL i HDL, per exemple), sobretot la relació entre l'apolipoproteïna B i apolipoproteïna A-I (Apo B / ApoA-I) (Tognon, 2012). L'ApoB és l'apolipoliproteïna majoritària de les partícules LDL i la més aterogènica. L'ApoA-I és l'apolipoliproteïna majoritària de les partícules HDL i la més antiaterogènica (McQueen, 2000), mentre que l'ApoA-II n'és la segona majoritària, però sembla que no té un paper protector gaire clar enfront a les MCV (Blanco-Vaca, 2002).

Els nivells postprandials elevats de lipoproteïnes riques en triglicèrids com els quilomicrons (Qm) i les lipoproteïnes de molt baixa densitat (VLDL), s'han associat a l'aterosclerosi coronària i caròtida. L'eliminació hepàtica de Qm i VLDL de la circulació està intervinguda per l'ApoB100 i ApoE; de fet, la falta d'ApoE condueix a l'acumulació plasmàtica de Qm remanents. Els triglicèrids al nucli dels Qm i les VLDL en sang circulant són hidrolitzats per l'acció endotelial de la lipoproteïna lipasa (LPL). Una baixa activitat de la LPL pot ésser causada per una baixa expressió de la

LPL, així com per una baixa expressió o activitat de l'ApoC-II, que activa la LPL, i s'associa amb nivells plasmàtics elevats de Qm, aterosclerosi prematura i a una progressió accelerada de l'aterogènesi. Els membres de la família de les Apo-C són de gran importància per al metabolisme de les lipoproteïnes riques en triglicèrids postprandials. Tant l'ApoC-I com l'ApoC-III inhibeixen la unió de l'ApoE als receptors de les lipoproteïnes riques en triglicèrids, augmentant la vida mitja d'aquestes partícules al torrent sanguini. No obstant, l'ApoC-II promou la hidròlisi d'aquestes lipoproteïnes per part de la LPL. El nombre d'ApoC-I per partícula de VLDL és major en persones amb aterosclerosi carotídia i podria servir com a marcador independent d'aterosclerosi i, fins i tot, podria jugar un paper decisiu en la iniciació i progressió de l'aterosclerosi (Notø, 2008). L'Apo-CIII, a més, actua com a mediadora proinflamatòria i com a lligand endogen de la via de senyalització de la *toll-like receptor 2*, que està implicat en l'agreujament de l'aterosclerosi, almenys en models animals (Libby 2011). La lp(a) és una lipoproteïna complexa que consisteix en una molècula d'ApoB-100 unida covalentment a una molècula d'Apo(a), una glicoproteïna que confereix propietats aterogèniques i trombòtiques a la partícula, ja que posseeix una estructura homòloga al plasminogen i la plasmina sense activitat fibrinolítica (Nordestgaard, 2010). Així, la lp(a) ha estat identificada com un nou marcador de risc cardiovascular (Gurdasani, 2012; Konerman, 2012; Virani, 2012).

2.2.2.4. Sobrepès i obesitat

La obesitat és un problema creixent al món, tant als països industrialitzats com als països en vies de desenvolupament. La OMS atribueix al sobrepès i l'obesitat el 5% de les morts globals (WHO, 2011).

El risc de malaltia coronària, isquèmica, resistència a la insulina i de diabetis tipus 2, HTA i el risc de morbimortalitat cardiovascular incrementa de manera constant quan augmenta l'IMC (WHO, 2011; Zhou, 2012). El

sobrepès i la obesitat causen efectes metabòlics adversos en la pressió arterial, el colesterol, els triglicèrids i la resistència a la insulina, així com en la disfunció endotelial, ja que la obesitat també és en sí mateix un estat crònic inflamatori (Sell, 2012). No obstant, més que el sobrepès o obesitat (mesurat amb l'IMC) el factor de risc cardiovascular és l'obesitat abdominal, ja que està relacionat amb un augment de la diabetis tipus 2, la dislipèmia aterogènica, la hipertensió i la disfunció endotelial, així com un estat procoagulant i inflamatori crònic (Després, 2001).

En els darrers anys, el teixit adipós no es considera únicament un dipòsit de greix, sinó també un dels òrgans endocrins més grans, que produeix molts factors bioactius denominats adipocines, com certes citocines (el TNF-α, la IL-6, l'inhibidor de l'activador del plasminògen-1, el fibrinogen, la visfatina i l'omentina) i hormones (l'angiotensinògen, la leptina, la resistina i l'adiponectina). Les adipocines actuen en qualsevol forma (paracrina o endocrina) per regular l'homeòstasi de l'energia, la glucosa i el metabolisme lipídic i la funció cardiovascular. El teixit adipós obès es caracteritza per la hipertròfia i hiperplàsia de l'adipòcit i la infiltració excessiva dels macròfags i els limfòcits, donant lloc a una elevada producció d'adipocines proinflamatòries i vasoactives que resulta en una disfunció endotelial, proliferació i migració de les cèl·lules musculars llises vasculars, i en una inflamació vascular. A diferència de la majoria de les adipocines, que tenen efectes perjudicials sobre el sistema vascular, l'adiponectina exerceix efectes beneficiosos en els trastorns vasculars a través del seus efectes vasodilatador, antiinflamatori i antioxidant en les cèl·lules vasculars (Zhou, 2012).

2.2.3. Altres factors de risc

Altres factors de risc cardiovascular són la hiperhomocisteïnèmia, l'edat avançada, que és factor de risc de moltes malalties; el sexe, ja que les hormones sexuals femenines confereixen protecció enfront les MCV; la predisposició genètica, és a dir, els antecedents familiars de MCV precoç (en homes <55 anys i en dones <65 anys); factors psicològics tals com l'estrès o la depressió i també el baix nivell d'educació i l'edat on es comencen a agafar mals hàbits dietètics i es fa un mal ús de l'alcohol i el tabac (WHO, 2011).

L'homocisteïna elevada en plasma (>15 μmol/L) indueix a una disfunció endotelial i per tant, afavoreix el desenvolupament de MCV, ja que indueix a l'estrès oxidatiu i està molt relacionada a l'acumulació de la dimetilarginina asimètrica, que és un potent inhibidor de la NO sintasa, impedint la secreció endotelial de NO (Perez-Vizcaino, 2006; Tyagi, 2005). De fet, l'homocisteïna elevada està relacionada amb un augment del risc d'accident vascular cerebral, aterosclerosi, malaltia perifèrica vascular i malaltia cardíaca isquèmica. Tant l'àcid fòlic com la vitamina B_{12} actuen com a coenzims en el metabolisme de l'homocisteïna i per tant, en disminueixen els seus nivells quan estan elevats, cosa que podria millorar la funció endotelial (Sudchada, 2012).

3. Dieta i malaltia cardiovascular

Des de fa moltes dècades s'estudia la relació entre els hàbits dietètics, és a dir el consum de determinats aliments i nutrients, i les malalties cardiovasculars. Al 1986, en el marc de l'Estudi dels 7 països, s'observà que la prevalença de malaltia coronaria era molt més alta als països del Nord d'Europa (Finlàndia) i als Estats Units que als països del Sud com Itàlia o Grècia. De fet, el lloc amb la prevalença més baixa de malaltia coronaria era la Illa de Creta, malgrat de ser el lloc amb el nivell socioeconòmic i sanitari més baix. Així mateix, es va veure que existia una correlació positiva entre la prevalença de malaltia coronaria i el consum d'energia derivada del greix saturat, i també amb la disminució del percentatge d'energia consumida provinent d'àcids grassos monoinsaturats (especialment l'oleic). No es va trobar cap associació entre la taxa de mortalitat i el percentatge d'energia derivada dels àcids grassos poliinsaturats, proteïnes, hidrats de carboni i alcohol (Keys, 1986). Així, es va considerar que el fet que millor explicava les diferències entre la prevalença de malaltia coronaria entre els països del Nord i Sud d'Europa era la dieta dels països mediterranis. De fet, la dieta òptima per prevenir MCV es considera que era la de Creta de les dècades dels 50 i 60. A aquesta dieta se l'anomenà dieta mediterrània i estava caracteritzada per ésser fonamentalment vegetariana (amb un alt consum de verdures i hortalisses, fruites, llegums i fruits secs, llavors i cereals no refinats), amb l'oli d'oliva com a font primària de greix, un consum baix de carn i làctics i un consum raonable de peix (Covas, 2002; Yngve, 2009). Posteriorment, nombrosos estudis ecològics i epidemiològics han analitzat les diferències entre incidència de diferents formes de malaltia cardiovascular entre països i en gran grups de població, i tots ells han confirmat el possible paper protector de la dieta mediterrània. No obstant, els estudis que permeten obtenir conclusions amb el grau més alt d'evidència científica són els assajos clínics aleatoritzats d'intervenció. Entre aquestos, mereix destacar-se el *Lyon Diet Heart Study* (de Lorgueril, 1999), on es van assignar 605 persones a un grup control (seguint les recomanacions dietètiques de l'*American Heart Association -AHA-*) o a una intervenció amb

dieta mediterrània, que també incloïa un consum moderat de vi. L'estudi va mostrar un 72% de reducció en la mortalitat cardiovascular i en els infarts aguts de miocardi no fatals en el grup d'intervenció amb dieta mediterrània comparat amb el grup control, així com una reducció del 65% de mortalitat coronària i del 55% la mortalitat per qualsevol causa.

Han estat forces els estudis que han analitzat el paper de la dieta mediterrània i els seus principals aliments, com l'oli d'oliva, el vi i d'altres, en la prevenció de la malaltia cardiovascular i els seus principals factors de risc. Així, en un estudi creuat i aleatoritzat a nivell europeu, 200 participants es van assignar aleatòriament a 3 seqüències d'administració diària de 25 mL de 3 olis d'oliva, amb el mateix contingut d'àcids grassos, però diferent concentració de polifenols. Després de 3 setmanes d'intervenció s'observà un augment lineal en les HDL a mesura que augmentà el contingut en fenols de l'oli. El *ratio* colesterol total/HDL disminuí linealment amb el contingut fenòlic de l'oli d'oliva. Els nivells de triglicèrids disminuïren després de la ingesta dels 3 olis d'oliva. Els marcadors d'estrès oxidatiu també disminuïren linealment amb l'augment del contingut de polifenols (Covas, 2006). Aquests resultats posen de manifest que el consum d'oli d'oliva verge (que és el que té el contingut més alt en polifenols) indueix efectes cardioprotectors a l'augmentar la concentració del colesterol protector, l'HDL.

Un altre exemple de la relació entre dieta i MCV és el realitzat en dones holandeses per Beulens *et al* (2007), que va demostrar que els aliments amb alta càrrega glicèmica dietètica i alt índex glicèmic incrementaven el risc de MCV en dones de mitjana edat.

Finalment, a l'estudi PREDIMED (Prevenció amb Dieta Mediterrània) dut a terme pel nostre grup, en col·laboració amb altres grups de l'Estat Espanyol, persones d'alt risc cardiovascular van ésser assignades aleatòriament a seguir una dieta baixa en greix (segons les recomanacions de l'AHA), una dieta mediterrània complementada amb oli d'oliva o una dieta mediterrània

complementada amb fruits secs. Els primers resultats de l'estudi mostren que les dietes mediterrànies comparades amb la dieta baixa en greix tenen efectes beneficiosos a nivell cardiovascular, ja que disminuïren els nivells de glucosa circulants, la pressió sistòlica i el *ratio* colesterol/HDL, així com la PCR circulant en el cas de la dieta mediterrània complementada amb oli d'oliva (Estruch, 2006).

En conclusió, aquests només són alguns exemples dels nombrosos estudis que demostren que la dieta és un factor clau en el desenvolupament i progressió de les MCV.

4. El vi i la salut cardiovascular

En l'apartat anterior d'aquesta introducció s'ha posat de manifest que la dieta mediterrània és un patró dietètic protector enfront a les malalties cardiovasculars. La dieta mediterrània, a més de caracteritzar-se pel consum de vegetals, oli d'oliva i fruits secs entre d'altres coses, també es caracteritza per un consum moderat de vi.

El consum d'alcohol a Espanya el 2009 va ser de 11.37 litres per càpita i any (tenint en compte només els adults majors de 15 anys), dels quals 3.91 L provenien del consum de vi (WHO, 2011). Per tant, el consum de vi representa el 34.4% del consum d'alcohol al nostre país.

Existeix força evidència científica que el consum moderat d'alcohol té un paper protector enfront a les MCV. No obstant, poc a poc s'està estudiant quines begudes alcohòliques podrien ésser les més beneficioses per a la prevenció de les MCV, ja que sembla que el vi, especialment el negre, a causa de la seva composició (és molt ric en polifenols) podria conferir efectes addicionals al consum moderat d'alcohol (**Figura 3**). Per tant, aquesta tesi està centrada en l'estudi dels efectes del consum moderat de vi negre i les seves fraccions (alcohòlica i no alcohòlica).

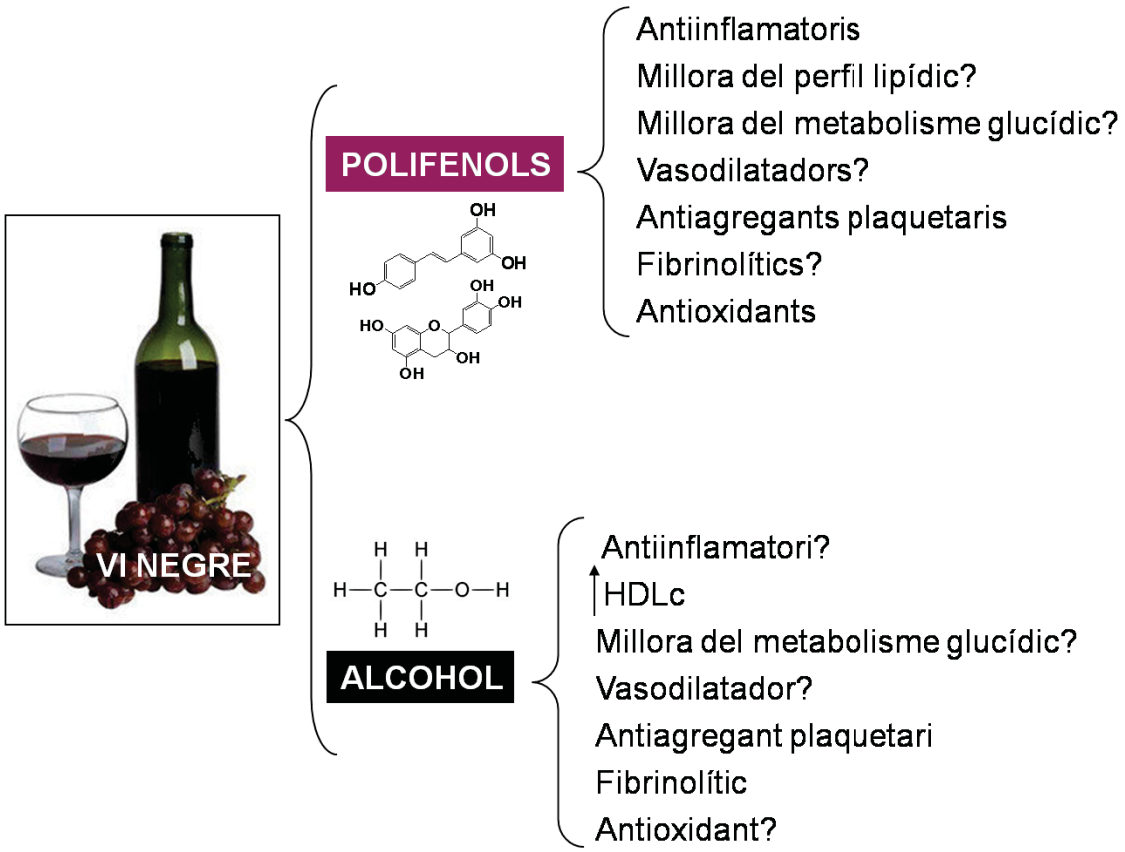

Figura 3. Potencials efectes del consum moderat de vi negre.

4.1. Composició del vi

El vi és una beguda alcohòlica provinent de la fermentació del most de raïm (*Vitis vinifera*). La fermentació es produeix per l'acció metabòlica del llevat que transforma els sucres del fruit en alcohol etílic i gas en forma de diòxid de carboni. El sucre i els àcids que posseeix la fruita fa que siguin suficients per al desenvolupament de la fermentació. No obstant el vi és la suma d'un conjunt de factors ambientals on s'ha cultivat el raïm (clima, latitud, altitud, hores de llum, etc.), així com del procés de fermentació.

El raïm conté al seu interior tots els elements necessaris per a l'elaboració del vi. La seva morfologia és com una divisió concèntrica de zones

que comença per les llavors que ocupen la posició més interior. A la zona més propera a les llavors es troba la concentració més alta de sucres i àcid màlic (àcid que també es pot convertir en un sucre mitjançant gluconeogènesi). En la següent zona, concèntrica a l'anterior, la concentració de sucres disminueix progressivament i augmenta la presència d'àcid tartàric. De fet, el segon i tercer components químics en el raïm, després dels sucres, són l'àcid màlic i el tartàric. Ambdós àcids tenen un paper important en l'elaboració dels vins. A les capes més externes és on es troben les sals minerals, principalment potassi, les aromes i els polifenols, dels quals en parlarem amb més profunditat l'apartat 4.3.

La manera en la qual s'aixafa el raïm pot afectar les propietats organolèptiques i nutritives del most. Així, el vi negre és l'elaborat a partir de raïm negre i com el color està en la pellofa, normalment la fermentació s'ha de realitzar amb el most sense filtrar (amb pellofes) i només una vegada acabada la fermentació (uns 20 dies) es procedeix al filtrat. Per tant el vi negre és el vi més ric en polifenols (aproximadament un 0.2% de la massa total del vi).

El vi rosat s'elabora amb raïm negre en què es permet una certa maceració del raïm després del seu premsat, d'aquesta manera el most pren una mica de color i hi ha una extracció parcial dels polifenols de la pell. Després es fermenta el most filtrat. Per tant, el vi rosat té un contingut intermedi de polifenols.

El vi blanc es pot elaborar amb raïm blanc o negre, en aquest segon cas separant el most de la pellofa immediatament, perquè no li doni color. En general, la fermentació es realitza amb most filtrat (separat de pellofes, llavors, raspons, etc.), i encara que no és freqüent envellir-ho, hi ha vins blancs amb criança o amb una segona fermentació alcohòlica en un envàs tancat (llavors s'anomena cava). El vi blanc és el vi amb més baix contingut en polifenols, aproximadament d'un 0,01% (Landrault, 2001; Lippi, 2010).

Encara que els ingredients principals de vi són l'aigua i l'alcohol (**Taula 2**), s'han identificat més de 500 compostos en el vi, 160 dels quals són esters. L'aigua, que representa del 80% al 85% de la massa total del vi, deriva del suc de raïm i per tant és biològicament pura. El contingut d'alcohol varia àmpliament entre els vins (de 10% a 17%) i és principalment aconseguit pel llevat de conversió dels sucres. A més d'afegir els seus propis sabors i olors característics, els alcohols són els principals portadors d'aroma o bouquet. L'alcohol més abundant és l'etanol; sota condicions estàndard de fermentació es forma prop d'un 14-15%, però en general les concentracions d'etanol estan en un rang d'entre 10% i 13%, segons el contingut de sucre del raïm, la temperatura i la soca de llevat. El metanol, que és predominantment generat a partir de la descomposició enzimàtica de les pectines, és un constituent menor del vi (0,1 a 0,2 g/L). Altres alcohols presents al vi són els alcohols de cadena lineal (1-propanol, 2-metil-1-propanol, 2-metil-1-butanol i 3-metil-1-butanol). El glicerol també hi és present i normalment afegeix un grau de dolçor.

Taula 2. Composició mitjana del vi (per 100mL).

	VI BLANC	VI ROSAT	VI NEGRE
Energia [kcal]	61	66,9	70,72
Proteïna [g]	0,1	0,1	0,23
Hidrats de carboni [g]	0,1	1,4	0,3
Fibra [g]	0	0	0
Greix total [g]	0	0	0
Àcids grassos saturats [g]	0	0	0
Àcids grassos monoinsaturats [g]	0	0	0
Àcids grassos poliinsaturats [g]	0	0	0
Colesterol [mg]	0	0	0
Alcohol [g]	8,58	8,7	9,82
Aigua [g]	91,2	89,8	89,7
Calci [mg]	9	12	7,6
Ferro [mg]	0,6	0,95	0,9
Iode [µg]	0	0	0
Magnesi [mg]	10	7	11
Zinc [mg]	0,07	0,04	0,05
Seleni [µg]	0,3	0,2	0,2
Sodi [mg]	2	4	4
Potassi [mg]	82	75	93
Fòsfor [mg]	15	6	14
Vit. B1 (Tiamina) [mg]	0,01	0,004	0,005
Vit. B2 (Riboflavina) [mg]	0,05	0,01	0,02
Eq. niacina [mg]	0,08	0,07	0,09
Vit. B6 (Piridoxina) [mg]	0,02	0,02	0,02
Àcid Fòlic [µg]	tr.	tr.	1
Vit. B12 (Cianocobalamina) [µg]	0	0,01	0,01
Vit. C (Àcid ascòrbic) [mg]	0	0	0
Retinol [µg]	0	0	0
Carotenoides (Equivalents ß carotens) [µg]	n.d.	n.d.	n.d.
Vit. A (Equivalents Retinol) [µg]	tr.	tr.	tr.
Vit. D [µg]	0	0	0
Vit. E (Tocoferols) [µg]	0	0	0
Polifenols totals (EAG/L)	32	82	215

n.d.: no detectat; tr.: traces; Vit.: vitamina.

(Ortega, 2005; Pérez-Jiménez, 2010)

Els àcids (tartàric, màlic i cítric, principalment), representen el 0.4-1% de la massa total i donen l'aspecte agre o sec al vi que realça el sabor quan estan en equilibri amb altres components. Altres àcids com el succínic, làctic i acètic (font d'acidesa volàtil) són produïts per fermentació.

Encara que el raïm conté un 15%-25% de glucosa i fructosa, aquests sucres es transformen en alcohol principalment a través de la fermentació. No obstant, es poden trobar restes de sucre residual (0,1%) als vins secs, mentre que els vins dolços poden contenir fins a un 10% de sucres.

Les sals minerals presents al vi (de 0,2% a 0,4%), que principalment són potassi, sodi, magnesi, calci i ferro, deriven d'àcids minerals o àcids orgànics.

Les substàncies que donen color (de 0,01% a 0,5%) estan representats principalment per polifenols. Els compostos fenòlics poden afectar l'aparença, sabor, sensació a la boca, aroma i les propietats antimicrobianes del vi. Un cop més, aquests compostos són presents en una quantitat més gran en el vi negre que en els vins blancs. Els vins negres contenen fins 1060 equivalents d'àcid gàl·lic/L (EAG/L) de flavonoids i fins a 235 EAG/L de no flavonoids, i al vi blanc hi són presents a nivells de 175 i 30 EAG/L, respectivament. Els flavonols, és a dir, tanins i antocianines, són exclusivament presents en els vins negres, a una concentració de 450 i 20 EAG/L, respectivament. La concentració de compostos fenòlics del vi augmenta durant la fermentació de la pell, però durant l'envelliment els fenols precipiten amb proteïnes i amb la superfície dels llevats, disminuint-ne la seva concentració final. No obstant, el vins envellits en barriques poden agafar polifenols de la fusta i augmentar-ne la seva concentració (Sanz, 2012). Traces de diversos compostos químics volàtils (alcohols, aldehids, esters, àcids i cetones) determinen certes olors dels vins.

Els sulfits s'utilitzen per protegir el raïm i esterilitzar i conservar els vins. La presència de sulfits és d'entre 10 a 200 ppm, segons la legislació de cada país (Lippi, 2010).

En conclusió, el vi és una beguda amb un contingut alcohòlic que oscil·la entre el 10 i el 15% (v/v) i amb un alt contingut en polifenols, sobretot en el cas del vi negre.

4.2. Polifenols del vi i salut cardiovascular

Els polifenols són metabòlits secundaris de plantes i també són els antioxidants més abundants en la dieta humana. Aquests compostos estan compostos per un anell aromàtic amb un o més grups hidroxil. S'han establert dos grups principals de polifenols, denominats flavonoids i no-flavonoids segons el nombre d'anells fenòlics i d'elements estructurals que s'uneixen a aquests anells. Com es veu a la **Figura 4**, el grup dels flavonoids es conforma pels compostos amb una estructura C6-C3-C6: les flavanones, les flavones, els dihidroflavonols, els flavonols, els flavan-3-ols, les antocianidines, les isoflavones i les proantocianidines (Andres-Lacueva, 2009).

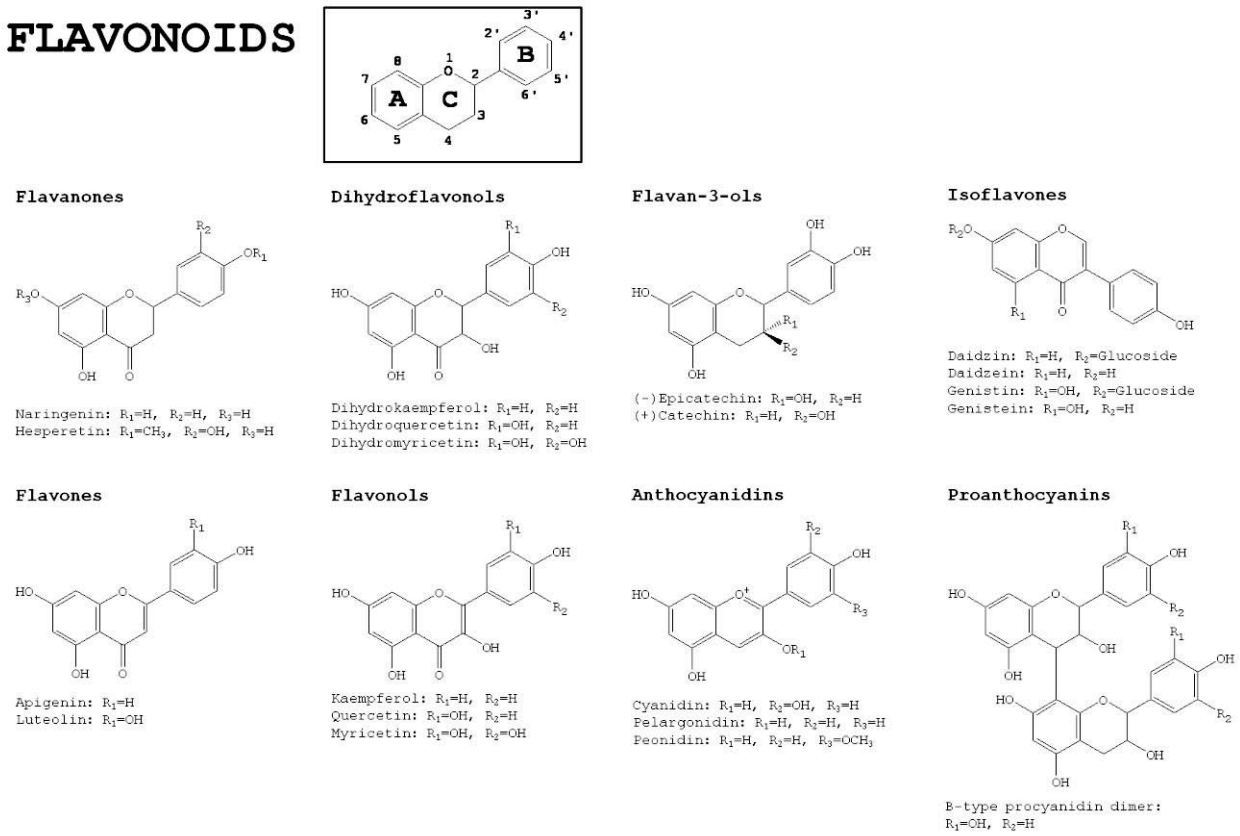

Figura 4. Estructura química dels flavonoids (Andres-Lacueva, 2009).

El grup dels no-flavonoids es classifica segons el nombre de carbonis que tenen (**Figura 5**) i comprèn els següents subgrups: fenols simples, àcids fenòlics i aldehids, tanins hidrolitzables, acetofenones i àcids fenilacètics, àcids hidroxicinàmics, cumarines, benzofenones, xantones, estilbens, xalcones, lignans i secoiridoids (Andres-Lacueva, 2009).

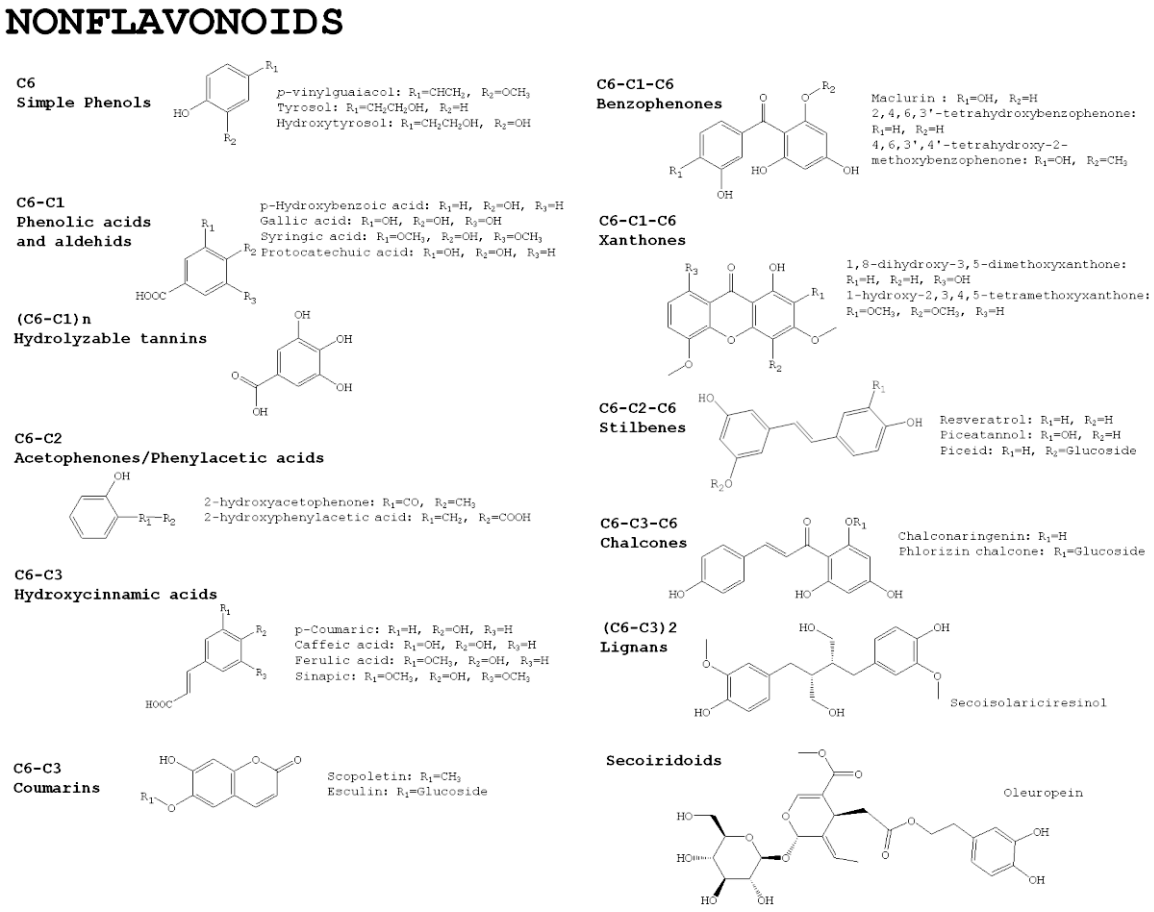

Figura 5. Estructura química dels no-flavonoids (Andres-Lacueva, 2009).

Tal com es mostra a la **Taula 3**, el vi negre conté nombrosos polifenols provinents del raïm, especialment de la seva pell (és per això que el vi blanc i rosat en contenen en menor grau). Els polifenols del vi negre són una barreja complexa de flavonoids (sobretot antocians i flavan-3-ols) i no-flavonoids (com el resveratrol i l'àcid gàl·lic). Els flavan-3-ols són els més abundants (Corder, 2006), junt amb les procianidines oligomèriques i polimèriques (tanins condensats), que sovint representen un 25-50% dels constituents fenòlics totals (**Taula 3**).

Taula 3. Composició fenòlica mitja del vi negre.

Polifenols	(mg/L)	Polifenols	(mg/L)
Antocianidines		**Flavonols (cont.)**	
Cianidina 3-*O*-(6′-acetil-glucòsid)	0.8	Kaempferol	2.3
Cianidina 3-*O*-glucòsid	2.1	Kaempferol 3-O-glucòsid	7.9
Delfinidina 3-*O*-(6′-acetil-glucòsid)	4.2	Miricetina	8.3
Delfinidina 3-*O*-(6′-*p*-coumaroyl-glucòsid)	1.8	Quercetina	8.3
Delfinidina 3-*O*-glucòsid	10.6	Quercetina 3-O-arabinòsid	4.9
Malvidina 3-*O*-(6′-acetil-glucòsid)	35.2	Quercetina 3-O-glucòsid	11.4
Malvidina 3-*O*-(6′-caffeoyl-glucòsid)	1.8	Quercetina 3-O-ramnòsid	11.5
Malvidina 3-*O*-(6′-*p*-coumaroyl-glucòsid)	19.5	Quercetina 3-O-rutinòsid	8.1
Malvidina 3-O-glucòsid	99.7	**Àcids fenòlics**	
Peonidina 3-O-(6"-acetil-glucòsid)	4.7	Àcid 2,3-dihidroxibenzoic	0.8
Peonidina 3-*O*-(6′-*p*-coumaroyl-glucòsid)	5.2	Àcid 2-hidroxibenzoic	0.4
Peonidina 3-*O*-glucòsid	8.2	Àcid 4-Hidroxibenzoic	5.5
Petunidina 3-*O*-(6′-acetil-glucòsid)	5.7	Àcid Gàl·lic	35.9
Petunidina 3-*O*-(6′-*p*-coumaroyl-glucòsid)	3.9	Àcid Gàl·lic etil éster	15.3
Petunidina 3-*O*-glucòsid	14.0	Àcid Gentísic	4.6
Pigment A	0.7	Àcid Protocatèquic	1.7
Pinotina A	2.2	Àcid Siríngic	2.7
Vitisina A	3.1	Àcid Vaníl·lic	3.2
Dihidroflavonols		**Àcids hidroxicinàmics**	
Dihidromiricetina 3-*O*-ramnòsid	44.7	Àcid 2,5-di-S-Glutathionil caftàric	28.6
Dihidroquercetina 3-*O*-ramnòsid	9.7	Àcid cafeic	18.8
Flavan-3-ols		Àcid caffeoil tartàric	33.5
(+)-Catequina	68.1	Àcid férulic	0.8
(+)-Gal·locatequina	0.8	Àcid o-cumàric	0.3
(−)-Epicatequina	37.8	Àcid p-cumàric	5.5
(−)-Epicatequina 3-*O*-gal·lat	7.7	Àcid p-cumaroil tartàric	11.8
(−)-Epigal·locatequina	0.6	Àcid sinàpic	0.7
Proantocianidines		**Àcids fenilacètics**	
Procianidina dímer B1	41.4	Àcid 4-hidroxifenilacètic	1.6
Procianidina dímer B2	49.7	**Estilbens**	
Procianidina dímer B3	94.7	d-Viniferina	6.4
Procianidina dímer B4	72.9	e-Viniferina	1.5
Procianidina dímer B7	2.7	Palidol	2.0
Procianidina trímer C1	25.6	Picetanol	5.8
Procianidina trímer T2	67.1	Picetanol 3-O-glucòsid	9.5
Prodelfinidina dímer B3	1.1	Resveratrol	2.7
Flavanones		Resveratrol 3-O-glucòsid	6.2

Taula 3 (continuació). Composició fenòlica mitja del vi negre.

Polifenols	(mg/L)		Polifenols	(mg/L)
Hesperetina	0.5		**Aldehids fenòlics**	
Naringenina	0.5		Aldehid protocatèquic	0.5
Naringina	7.5		Siringaldehid	6.6
Flavonols			**Fenols simples**	
Isorhamnetina	3.3		Hidroxitirosol	5.3
Isorhamnetina 3-*O*-glucòsid	2.6		Tirosol	31.2

(Arranz, 2012)

També són nombrosos els estudis en models cel·lulars o animals que determinen possibles mecanismes d'acció dels polifenols individualment (substancies pures) o en conjunt (extractes de fruites), que és una aproximació més realista a la ingesta dietètica. No obstant, donada la seva baixa biodisponibilitat (entre el 10 i el 50%), sovint no s'observen en els estudis clínics els efectes observats en models experimentals perquè les concentracions finals que arriben als teixits diana, derivades d'una ingesta raonable d'alguna font de polifenols, són molt inferiors a les concentracions utilitzades en aquest tipus d'estudi.

Nombrosos estudis epidemiològics suggereixen que els polifenols, en particular els flavonoids (que són els majoritaris) i el resveratrol (pràcticament només present al vi), tenen un paper protector contra les malalties cardiovasculars (Geleijnse, 2002; Knekt, 2002). Algunes de les propietats per les quals als flavonoids se'ls atribueixen efectes protectors enfront a les malalties cardiovascular són per les seves característiques antioxidants, antiateroscleròtiques, antiagregants plaquetaris, antitrombogèniques i antiinflamatòries (Nijveldt, 2001).

4.2.1. Polifenols del vi i aterosclerosi

En ratolins deficitaris d'ApoE s'ha observat que després d'una suplementació dietètica de liofilitzat d'extractes de raïm fresc (150 micrograms de polifenols totals per dia durant 10 setmanes) la lesió ateroscleròtica es reduïa un 41%. En conills Watanabe amb hiperlipèmia heretable el consum d'un extracte de pell de raïm negre es va associar amb un retard del desenvolupament de l'aterosclerosi aòrtica (determinat pel contingut de colesterol dins de l'aorta abdominal) en conills mascles però no en femelles (Leifert, 2008). En un model d'aterosclerosi de hàmster, el percentatge d'aorta coberta de cèl·lules escumoses es va reduir en un 50% i 63% després de la suplementació dels animals amb 50 i 100 mg/kg de proantocianidines de llavors de raïm, respectivament (Vinson, 2002).

En dones pre- i post- menopàusiques, la ingesta d'un extracte liofilitzat de raïm va disminuir significativament les concentracions de TNF-α i IL-6, mostrant un efecte antiinflamatori per part dels polifenols del raïm. També s'ha vist en aquesta línia, que el resveratrol afecta l'expressió de citocines inflamatòries i molècules d'adhesió a través de la inhibició del NF-κB, així com a través de la inhibició del sistema de la tirosina quinasa (Zern, 2005).

4.2.2. Polifenols del vi, funció endotelial i pressió arterial

En un estudi amb 40 pacients hipertensos, s'observà una reducció de la pressió arterial sistòlica 7,2 mmHg i la diastòlica uns 6,2 mmHg després del consum de proantocianidines d'extractes de llavors de raïm, i també va modular beneficiosament altres paràmetres cardiovasculars com el flux coronari o aòrtic (Leifert, 2008). En un altre estudi dut a terme en 36 adults amb alt risc cardiovascular, després del consum diari d'extracte de llavor de raïm durant 4 setmanes, no s'observaren diferències en la PA sistòlica i diastòlica, lípids sèrics o LDL oxidades, però si que augmentà la FMD (Clifton, 2004). Aquestes observacions concorden amb Botden *et al*. (2012), que observaren que el

consum de polifenols del vi negre no afectà la pressió arterial en individus normotensos o amb hipertensió de grau 1 i postularen que els polifenols del vi negre només afecten favorablement a la pressió arterial en persones amb disfunció endotelial, com és el cas de les observacions anteriors.

En cèl·lules endotelials (Corder, 2001; Corder, 2006), es va trobar una correlació altament significativa entre el contingut del vi negre en polifenols totals i especialment en procianidines i la supressió de la síntesi d'entolina-1, un dels més potents vasoconstrictors. Per tant, sembla que els efectes vasodilatadors dels polifenols del vi negre podrien ésser atribuïbles principalment a les procianidines oligomèriques tipus B.

4.2.3. Polifenols del vi i resistència a la insulina

Hi ha estudis que han posat de manifest que altres polifenols (no només el resveratrol), presents al vi negre o a altres aliments com el te, podrien tenir un paper protector enfront a la resistència a la insulina, així com altres estudis epidemiològics posen de manifest que una dieta rica en polifenols exerceix un efecte positiu en el metabolisme de la glucosa. Els mecanismes d'acció proposats dels polifenols en el metabolisme de la glucosa són diversos, com ara la inhibició de la digestió de carbohidrats i l'absorció de glucosa a l'intestí, l'estimulació de la secreció d'insulina per part de les cèl·lules β pancreàtiques, la modulació de l'alliberament i producció de glucosa des del fetge, l'activació dels receptors d'insulina i la captació de glucosa en teixits sensibles a la insulina (Hanhineva, 2010). Si bé s'han fet molts estudis en models animals dels diversos efectes del resveratrol a diferents nivells, en humans s'ha estudiat menys, sobretot en referència al metabolisme de la glucosa. En un estudi en homes amb diabetis tipus 2, la ingesta de 10 mg de resveratrol diaris (en gelatina) millorà la resistència a la insulina, disminuí els nivells plasmàtics d'insulina i retardà l'aparició del pic de glucèmia postprandial, sense afectar la funció de les cèl·lules β (Brasnyó, 2011). No obstant, encara no s'han tret

conclusions definitives respecte el paper dels diferents aliments i els diferents polifenols en la resistència a la insulina.

4.2.4. Polifenols del vi i perfil lipídic

En un model d'aterosclerosi de hàmster, Vinson *et al*. (2002) van trobar que les proantocianidines de llavors de raïm van induir a una reducció del 25% en el colesterol i de fins al 34% dels nivells de triglicèrids plasmàtics.

En humans diversos estudis han estudiat els efectes dels antocians de les fruites vermelles (però també presents al raïm negre, i per tant, al vi negre) sobre el perfil lipídic (Erlund, 2008; Hassellund, 2012). El consum d'antocians eleva lleugerament el colesterol HDL, però sembla que no té efectes sobre el colesterol total o els triglicèrids. D'altra banda, sembla que el consum d'aliments rics en flavan-3-ols com ara el raïm, el vi, les baies, les pomes o la xocolata millora la homeòstasis lipídica, no només perquè podria reduir els nivells plasmàtics de l'ApoB, sinó també perquè podria millorar el perfil de colesterol en sèrum. En models animals l'efecte és molt clar però en humans els estudis són contradictoris, pel que és difícil treure conclusions clares sobre l'eficàcia d'aquests polifenols en la millora del perfil lipídic (Bladé, 2010).

4.2.5. Polifenols del vi i estatus oxidatiu

L'estrès oxidatiu, que es caracteritza per un augment de la producció endògena d'espècies reactives d'oxigen i peròxid d'hidrogen, és una de les causes centrals de la disfunció endotelial (Perez-Vizcaino, 2006). Per tant, sembla lògic pensar que molècules amb característiques antioxidants com els polifenols podrien exercir un efecte preventiu en la disfunció endotelial.

Els efectes antioxidants dels polifenols potser són els efectes més ben estudiats. Els efectes dels flavonoids sobre la salut humana s'atribueixen parcialment a la seva activitat antioxidant (capacitat per reduir la formació i per

eliminar radicals lliures). Aquesta propietat antioxidant es basa en dos mecanismes: la capacitat del grup funcional fenol per donar un àtom d'hidrogen d'un radical lliure i la formació d'un catió radical estable. Presència, nombre i posició relativa dels grups hidroxil fenòlics addicionals són els factors més importants alhora de determinar la capacitat antioxidant dels flavonoids. La rutina i l'epicatequina són dels flavonoids més potents alhora de neutralitzar radicals lliures (Nijveldt, 2001).

Aquests efectes antioxidants han estat ben demostrats *in vitro*, o *ex vivo,* on a curt termini, uns investigadors van observar que la ingesta de suc de raïm negre suplementat amb proantocianidines (provinents d'un extracte de llavor de raïm) disminuïa la susceptibilitat a l'oxidació de les LDL en pacients amb malaltia arterial coronària i en individus hipercolesterolèmics (Leifert, 2008). Altres autors mostren una major capacitat antioxidant del plasma després de la ingesta d'aliments rics en polifenols (Pecorari, 2010).

A l'hora d'avaluar els efectes antioxidants dels polifenols del vi *in vivo*, sembla que els polifenols del vi són capaços de contrarestar l'estrès oxidatiu postprandial i un possible efecte prooxidant de l'alcohol (Covas, 2010).

4.2.6. Polifenols del vi i agregació plaquetària

En un estudi que va incloure a 10 subjectes, l'agregació plaquetària es va reduir significativament després de beure suc de raïm negre, però no suc d'aranja o taronja durant 1 setmana i aquests efectes es van correlacionar amb el nivell de polifenols totals, ja que el suc de raïm negre, el suc de taronja i suc d'aranja, contenien 2.26, 0.75 i 0.86 EAG/L, respectivament. A més, el suc de raïm negre conté flavonols, antocianidines i proantocianidines, polifenols no contingut en els sucs d'aranja i taronja (Keevil, 2000). Un altre estudi en humans també observà una disminució de l'agregació plaquetària després d'una suplementació oral amb suc de raïm negre (Freedman, 2001). Els efectes antiagregants del resveratrol, un dels polifenols del vi negre més estudiats,

s'han observat en concentracions tan baixes com 1,2 μg/L (de vi negre diluït 1000 vegades), que són capaces d'inhibir l'agregació plaquetària en un 41,9% (Bertelli, 1995). Per tant, la reducció del nivell d'agregació de les plaquetes pot ser un dels factor que contribueix als efectes cardioprotectors dels polifenols.

4.2.7. Polifenols del vi i prevenció secundària de les MCV

El consum de 600 mg d'un extracte de polifenols de raïm negre (que contenia 4.32 mg d'epicatequina, 2.72 mg de catequina, 2.07 mg d'àcid gàl·lic, 0.9 mg de *trans*-resveratrol, 0.47 mg de rutina, 0.42 mg d'ε-viniferin, 0.28 mg d'àcid p-cumàric, 0.14 mg d'àcid ferúlic i 0.04 mg de quercetina per gram) va provocar un augment de la FMD, amb un pic màxim als 60 minuts, que va ser significativament més gran que els valors basals en 30 homes amb malaltia coronària prèvia (Lekakis, 2005). Per tant, sembla que els polifenols del raïm negre podrien millorar la funció endotelial, almenys de manera aguda, en pacients amb malaltia coronària.

4.3. Alcohol i salut cardiovascular

Encara que és indiscutible que un consum excessiu o desmesurat d'alcohol augmenta la probabilitat de mortalitat per qualsevol causa, el consum moderat d'alcohol està associat a una menor mortalitat en general i especialment per malaltia cardiovascular (**Figura 6**), sobretot en persones majors de 60 anys (Klatsky, 1992), i a una disminució dels factors de risc cardiovascular (Brien, 2011; Ronksley, 2011). A més a més, diverses línies d'evidència suggereixen que el vi negre pot conferir beneficis addicionals a altres begudes alcohòliques a nivell cardiovascular.

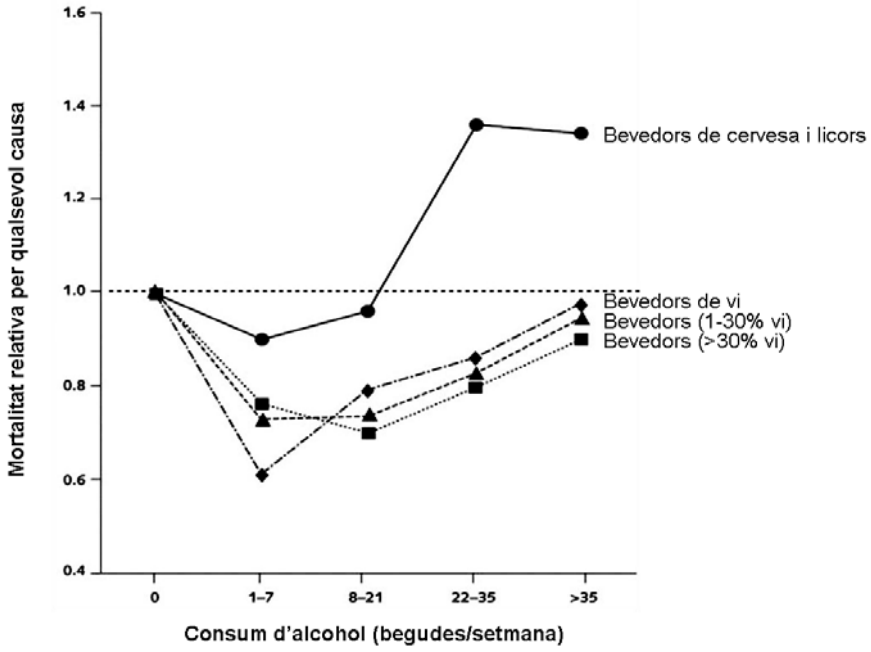

Figura 6. Risc relatiu de mort per totes les causes en relació a la ingesta total d'alcohol (Grønbaek, 2000). El risc relatiu s'ha fixat en 1.00 entre els no bevedors (<1 beguda / setmana). Les estimacions es van ajustar per edat, sexe, nivell d'educació, tabaquisme, activitat física i IMC.

En aquest context, l'any 1992 es van publicar els resultats d'un estudi ecològic que observà una correlació directe entre la incidència de malaltia coronària de diferents països i el seu consum de greix saturat, excepte França que malgrat un alt consum de greix saturat (derivats lactis) tenia una incidència de malalties cardiovasculars semblant a la dels països amb baix consum de greix, com Espanya o Itàlia. Aquest fet es coneix com la paradoxa francesa (**Figura 7**) i va atribuir aquest fenomen al seu consum de vi, i no només a l'alcohol, ja que els nivells plasmàtics de colesterol HDL van ser similars a els d'altres països amb major prevalença de MCV (Renaud, 1992).

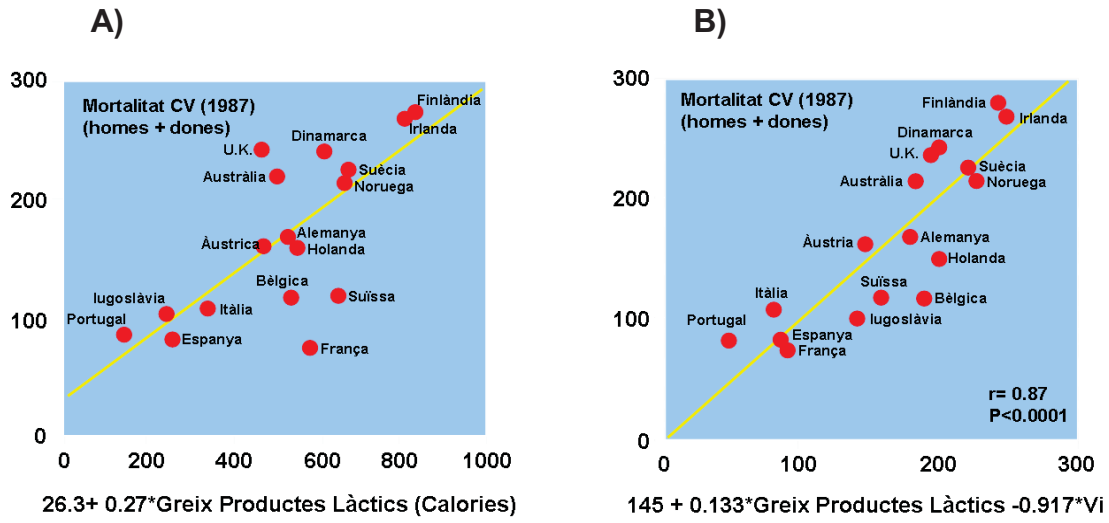

Figura 7. La paradoxa francesa: relació entre mortalitat cardiovascular (estandarditzada per edat) a diferents països d'Europa consumidors de vi en funció del consum de a) greix làctic i b) greix làctic i vi (Renaud & de Lorgeril, 1992).

Aquesta correlació va desencadenar una gran amalgama d'estudis sobre els components del vi negre (més concretament polifenols i per sobre de tots ells, el resveratrol) per tal d'explicar aquesta paradoxa i varen obrir el debat de quin tipus de beguda alcohòlica és més cardioprotectora (les fermentades -que contenen molts compostos fenòlics- o les destil·lades -que en contenen molt menys-). No obstant, encara hi ha força discussió sobre el paper cardioprotector de les diferents begudes alcohòliques i saber si aquest paper és degut al seu contingut en alcohol (etanol) o als compostos no alcohòlics, principalment polifenols que conté. Així, hi ha estudis com el *Copenhagen City Heart Study*, on 13.285 homes i dones de 30 a 70 anys d'edat van ser seguits durant aproximadament 12 anys, en el que s'observà que el risc de mortalitat per malalties cardiovasculars disminuïa de manera constant amb l'increment de la ingesta de vi negre comparat amb altres begudes alcohòliques o l'abstinència (Grønbaek, 1995). Però altres estudis (Baliunas, 2009; Koppes, 2005; Ronksley, 2011) no troben diferències entre els efectes de les diferents begudes alcohòliques pel que atribueixen els efectes als seu principal i constant component, l'etanol. En altres paraules, encara no és del tot clar a què

es deu l'efecte protector del vi negre, si a l'alcohol (etanol) o al seu alt contingut en polifenols, objectiu d'estudi de la present tesi doctoral.

4.3.1. Alcohol i aterosclerosi

En un metanàlisi força recent (Brien, 2011), l'associació entre el consum moderat d'alcohol i els nivells de PCR, IL-6 i TNF-α no va ser significativa. No obstant, separant per tipus de beguda alcohòlica, es va observar que el vi negre i la ginebra (30 g/dia durant 4 setmanes) disminuïa la concentració plasmàtica d'IL-1α i que el vi negre, però no la ginebra, reduïa la PCR en individus sans (Estruch, 2004), cosa que podria explicar per què quan només es considera l'efecte de l'alcohol, els nivells de PCR no es veuen afectats. Així mateix, en un estudi en voluntaris sans, 15 g/dia d'alcohol durant 3 setmanes en forma de vi negre no van afectar significativament la concentració plasmàtica d'IL-6 (Djurovic, 2007). Aquest fet indicaria que els efectes de les begudes alcohòliques depenen de les dosis administrades i potser també del tipus de persona estudiada (sana o afectada d'una malaltia cardiovascular).

Pel què fa a les molècules d'adhesió cel·lular relacionades amb l'aterosclerosi, les begudes alcohòliques també en modulen la seva expressió. En voluntaris sans (Estruch, 2004), el consum moderat de vi negre, però no de ginebra, disminuí significativament la secreció endotelial de VCAM-1, ICAM-1 i IL-1α, i també va disminuir l'expressió limfocitària de VLA-4 i LFA-1, així com l'expressió monocitària de Mac-1, VLA-4 i MCP-1. En un altre assaig realitzat amb voluntaris sans per Djurovic *et al.*, (2007) no es van observar canvis significatius en les concentracions plasmàtiques de VCAM-1 i ICAM-1 després de 15 g/dia d'alcohol en forma de vi negre. A més, el consum lleu i moderat d'alcohol s'ha associat *in vivo* a una menor càrrega ateroscleròtica en l'arc aòrtic proximal (Kohsaka, 2011). Amb la revisió d'aquests resultats, sembla plausible afirmar que el consum moderat d'alcohol té un efecte protector sobre l'aterosclerosi a diferents nivells, però el mecanisme d'acció és dependent de la dosi i del tipus de beguda alcohòlica, així com de l'estat de salut de l'individu.

4.3.2. Alcohol, funció endotelial i pressió arterial

Encara que és indiscutible que el consum excessiu d'alcohol està associat a la hipertensió (i a una reducció de la FMD), el consum moderat d'alcohol sembla exercir l'efecte contrari, un patró observat per la malaltia cardiovascular en general (Bau, 2007). Existeixen resultats contradictoris dels efectes del consum moderat d'alcohol sobre la funció endotelial, d'on en sorgeixen dues qüestions. En primer lloc, si els efectes aguts derivats del consum de vi negre també són aplicables a altres begudes alcohòliques i segon, si els efectes aguts descrits persisteixen més de 4 hores després del consum d'alcohol. El consum moderat d'alcohol augmenta la secreció endotelial de NO mentre que el consum crònic i excessiu d'alcohol en disminueix la biodisponibilitat (Toda, 2010). No obstant, sembla que cada tipus de beguda alcohòlica consumida juga un paper diferent, perquè Huang *et al.* (2010), observaren en voluntaris sans que el consum de 100 mL/dia de vi negre durant 3 setmanes va augmentar el NO plasmàtic, però el NO no va augmentar després de consumir quantitats equivalents d'alcohol en forma de cervesa o vodka, encara que no es van registrar canvis en la pressió arterial després del consum de cap de les begudes alcohòliques. En un altre estudi, les persones que consumien entre >1 beguda/mes i 2 begudes/dia (és a dir, un consum de baix a moderat d'alcohol) eren més propensos a tenir una FMD major que els abstemis i els que consumien >2 begudes/dia, independentment del tipus de beguda alcohòlica (Suzuki, 2009). En un assaig aleatoritzat, individus sans joves van rebre 30 g d'alcohol/dia en forma de vi negre, vi blanc, cervesa, whisky o aigua (com a control) i observaren que el vi negre exercia un efecte beneficiós sobre la funció endotelial 1-4 hores després de la ingesta; la cervesa i el vi blanc exerciren un efecte marginal sobre l'endoteli vascular 1 hora després de la ingesta, mentre que el consum de whisky no exercí cap efecte (Tousoulis, 2008). També s'ha observat en voluntaris sans que una dosi única de vi negre desalcoholitzat augmentà la FMD als 30-60 minuts postingesta en comparació amb el vi negre i amb abans de beure (Agewall, 2000). Per tant,

sembla que els efectes beneficiosos del consum moderat d'alcohol sobre la funció endotelial depenen del tipus de beguda alcohòlica consumida i no persisteixen més de 4 hores.

En quant a la pressió arterial, un metanàlisi conclou que el consum d'alcohol augmenta el risc d'hipertensió arterial de manera dosi-depenent (Taylor, 2009), sembla que a partir del llindar de 18mL d'alcohol (Okubo, 2001) i d'una manera parcialment reversible ja que s'ha observat una relació dosi-resposta entre la reducció del consum d'alcohol en els bevedors de grans quantitats d'alcohol (≥ 3 begudes/dia) i la reducció de la pressió arterial (Xin, 2001), encara que altres autors (Frisoli, 2011; Stranges, 2004) no han observat una associació consistent entre el consum de cervesa, vi o licor i el risc d'hipertensió. Per tant, encara existeix certa controvèrsia sobre els efectes del consum moderat d'alcohol i la pressió arterial, així com en els efectes de cada tipus de beguda. S'ha de tenir en compte també, a més de la quantitat d'alcohol ingerida, el patró de consum, ja que s'ha observat que el consum de begudes alcohòliques fora dels menjars augmenta el risc d'hipertensió independentment de la quantitat d'alcohol consumida (Stranges, 2004).

4.3.3. Alcohol i resistència a la insulina

L'efecte del consum moderat d'alcohol en la reducció de la incidència de la diabetis (Baliunas, 2009; Koppes, 2005; Wannamethee, 2003), un potent factor de risc de MCV (Reaven, 2003), podria ser un dels mecanismes pel quals el consum moderat d'alcohol disminueix el risc de MCV. Un metanàlisi de 20 estudis de cohorts que comprenen 477.200 individus conclou que el consum moderat d'alcohol (<60 g/dia en homes i <50 g/dia en dones) està inversament associat al risc de diabetis, d'una manera dosi-dependent, amb l'associació més forta en el consum de 22-24 g/dia d'alcohol (Baulinas, 2009). A més, en un metanàlisi de 15 estudis prospectius, el risc relatiu de desenvolupar diabetis tipus 2 va ser menor en els bevedors moderats d'alcohol que en els abstemis o els grans bevedors, independentment del tipus de beguda alcohòlica

consumida (Koppes, 2005). No obstant això, en un estudi prospectiu en dones sanes, una associació inversa entre el consum moderat d'alcohol i menor risc de diabetis va ser més evident en aquells que consumien vi o cervesa en comparació amb les dones que bevien licors (Wannamethee, 2003).

Els resultats dels pocs estudis clínics que han examinat els efectes del consum moderat d'alcohol sobre la sensibilitat a la insulina (IS), han estat inconsistents. Dos estudis observaren una millora significativa en la IS en homes sans, un després de consumir whisky 17 dies (Sierksma, 2004) i l'altre després de consumir 4 setmanes vi negre amb i sense alcohol (Beulens, 2006). Un tercer estudi va comparar els efectes del consum de vi negre i vodka durant 8 setmanes en 20 individus resistents a la insulina i observaren una modesta millora de la IS amb les dues begudes (Kim, 2009). No obstant, un quart estudi (Napoli, 2005) observà en pacients diabètics que el consum moderat durant 2 setmanes de vi negre provocà una millora del 43% en la IS. En la població femenina, el consum de vi negre a curt termini no té cap efecte en la IS en dones de mitjana edat amb sobrepès (Cordain, 2000) o en dones menopàusiques (Naissides, 2006). No obstant, en un altre assaig fet amb dones menopàusiques, el consum de 30 g/dia d'alcohol es va associar amb una millora de 7,2% en la IS en comparació amb consums de 0 o 15 g/dia (Davies, 2002).

En resum, els efectes del consum de begudes alcohòliques en la sensibilitat a la insulina són encara controvertits i sembla que hi ha diferències d'aquests efectes entre sexes.

4.3.4. Alcohol i perfil lipídic

L'efecte més ben descrit del consum moderat d'alcohol és l'augment del colesterol HDL i es creia que era el principal efecte protector cardiovascular de la ingesta moderada d'alcohol. Sembla inqüestionable que el consum moderat d'alcohol (sigui quina sigui la beguda alcohòlica consumida) eleva de manera

dosi-dependent les concentracions plasmàtiques d'HDL (Brien, 2011; Park, 2012; Tognon, 2012). Actualment però, hi ha l'opinió que l'augment dels nivells d'HDL no és un dels mecanismes més importants pels quals l'etanol exerceix la seva funció cardioprotectora (Magnus, 2011). Els efectes del consum moderat d'alcohol sobre els triglicèrids, les LDL, les VLDL, les Apo i la lp(a) encara estan en debat. En 2014 homes hipertensos, un major consum d'alcohol es va associar amb una disminució del risc de baixos nivells d'HDL i un augment en forma de J del risc d'hipertrigliceridèmia, corresponent els nivells més baixos de triglicèrids als consumidors moderats d'alcohol (Park, 2012). Un recent metanàlisi (Brien, 2011) conclou que el consum moderat d'alcohol augmenta significativament l'ApoA-I i no influeix significativament en els nivells de colesterol total, LDL, triglicèrids o lp(a), igual que en un estudi transversal en 2900 persones sueques (Tognon, 2012) el consum d'etanol va ser inversament relacionat amb el ratio ApoB/ApoA-I i el ratio colesterol/HDL, és a dir, a un augment de l'ApoA-I i el colesterol HDL, atribuïble principalment al consum de vi en les dones i de cervesa i vi en els homes. En un estudi en voluntaris sans, el consum moderat d'alcohol disminuí la concentració plasmàtica d'ApoB mentre que el vi negre (però no la ginebra) augmentà l'ApoA-I i ApoA-II (Avellone, 2006; Estruch, 2011).

En referència a la lp(a), pocs estudis s'han dut a terme i els resultats no són concloents. Donada la naturalesa d'aquesta molècula, és un tema que requereix més investigació. Encara que en un estudi amb vi negre durant 4 setmanes en individus sans (Avellone, 2006) no s'observaren canvis en la lp(a) després de la intervenció, en un altre assaig clínic també realitzat en homes sans, el vi negre, però no la ginebra, es va associar a una reducció de la concentració plasmàtica de lp(a) (Estruch, 2011), d'acord a un altre estudi, on la lp(a) disminuí després de 10 dies de consum moderat de vi negre però no de vi blanc (Sharpe, 1995).

4.3.5. Alcohol i estatus oxidatiu

En un assaig clínic en què es varen administrar 30 g d'alcohol/dia a voluntaris sans durant 4 setmanes en forma de vi negre o ginebra, la concentració plasmàtica de malondialdehid (MDA) i l'activitat de la superòxid dismutasa van disminuir, mentre que el *lag time* va augmentar amb el consum de vi negre, però no amb el de ginebra (Estruch, 2011). No obstant això, en el mateix estudi es va observar una disminució en l'oxidació de les LDL després tant del vi negre com de la ginebra, i la quantitat de diens conjugats formats al plasma només va disminuir significativament després del període de ginebra. Per tant, en aquest estudi s'arribà a la conclusió que tant l'alcohol com els polifenols del vi negre podrien contribuir a millorar l'estat d'oxidació vascular. En un estudi en voluntaris sans en els quals es va administrar etanol (40 g/dia) als dos àpats principals durant 30 dies en forma de cervesa, vi o alcohol, l'etanol va disminuir els nivells de paràmetres antioxidants i augmentà els paràmetres de lipoperoxidació. No obstant, alguns d'aquests canvis semblaven atenuar-se quan l'etanol es consumia en forma de cervesa o vi (Addolorato, 2008), arribant a la conclusió que l'alcohol és prooxidant. En un altre estudi, el consum diari de 400 mL de vi negre es va comparar amb l'abstèmia en voluntaris sans i el consum de vi negre va augmentar la capacitat antioxidant total del plasma i va disminuir els nivells de glutatió i MDA (Micallef, 2007). També s'ha observat que el consum de 375 mL de vi negre al dia durant 2 setmanes en voluntaris sans redueix les concentracions màximes de diens conjugats i substàncies reactives a l'àcid tiobarbitúric de les LDL (Tsang, 2005). A nivell postprandial també s'ha observat una reducció de l'estrès oxidatiu després del consum de vi negre (Covas, 2003), mentre que altres autors han observat que el vi negre no sembla influir en la peroxidació lipídica (Blackhurst, 2006). Per tant, mentre el consum desmesurat d'alcohol indueix a estrès oxidatiu, encara no està clar quin és el paper del consum moderat d'alcohol o de cada tipus de beguda alcohòlica, ja que en un estudi transversal, el consum moderat d'alcohol, independentment de la beguda alcohòlica consumida, es va

associar a una major concentració plasmàtica de partícules LDL oxidades (Schroder, 2006).

4.3.6. Alcohol i agregació plaquetària

Com a constant patró bilateral dels efectes del consum d'alcohol en la malaltia cardiovascular, existeix una associació consistent entre el consum excessiu d'alcohol i una menor capacitat fibrinolítica, un estat procoagulant i una major viscositat de la sang, però d'altra banda, el consum moderat d'alcohol també està associat consistentment amb una disminució de l'estat pro-coagulant (mitjançant la reducció de diversos factors de coagulació) i de la viscositat sanguínia, així com a una major capacitat fibrinolítica. En individus sans, la ratio inhibidor de l'activador del plasminogen-1/activador del plasminogen (PAI-1/tPA) no es va veure afectada després del consum agut de vi negre, mentre que es va incrementar després de la ingesta de cervesa o alcohol. Aquests resultats suggereixen que altres substàncies diferents de l'alcohol, com els polifenols continguts en el vi negre, poden disminuir l'efecte del consum agut d'alcohol en l'activitat fibrinolítica general (Tousoulis, 2008). En un altre assaig clínic aleatoritzat, realitzat també en individus sans, els nivells plasmàtics de fibrinogen varen disminuir després del consum de vi negre i ginebra mentre que el temps de protrombina només va augmentar després de la ingesta de vi (Estruch, 2011). En un estudi de casos i control en pacients amb trombosi venosa (Pomp, 2008), en comparació amb els abstemis el consum d'alcohol es va associar amb una reducció del risc de segona trombosi venosa i amb una disminució dels nivells de fibrinogen, sobretot en els que consumien 2-4 gots per dia, on es va veure un major efecte beneficiós. Per tant, sembla que l'efecte anticoagulant de consum moderat d'alcohol s'exerceix tant en individus sans com en pacients amb un perfil pro- coagulant.

4.3.7. Alcohol i prevenció secundària de les MCV

Com s'ha comentat en els sub-apartats anteriors, el consum moderat d'alcohol confereix efectes cardioprotectors en individus sans i en persones amb alt risc cardiovascular. A partir d'aquesta afirmació sorgeix una pregunta: són aquests efectes beneficiosos aplicables al pacients amb un episodi cardiovascular previ? Sempre tenint en compte les situacions individuals, on l'alcohol no interfereixi en la polimedicació, situació en que s'hauria d'evitar absolutament el consum d'alcohol, alguns estudis assenyalen els efectes beneficiosos del consum moderat d'alcohol en aquest tipus de població. En un assaig clínic en pacients hospitalitzats després d'un esdeveniment cardiovascular, el consum moderat de vi negre tingué alguns avantatges sobre els diferents paràmetres sanguinis lipídics i de l'estat oxidatiu (disminució de colesterol total i LDL) després d'accidents isquèmics coronaris, així com un augment de la fluïdesa de la membrana eritrocitària paral·lel a l'augment de l'estat antioxidant (Rifler, 2012). En un estudi en pacients amb MCV demostrada angiogràficament, el consum agut tant de vi blanc com negre acompanyat d'un àpat va provocar un augment de la FMD al cap de 360 minuts de l'ingesta, independentment del contingut en polifenols del vi (Whelan, 2004). En un estudi prospectiu (Pai, 2012), un consum moderat d'alcohol a llarg termini es va associar a una disminució de la mortalitat per qualsevol causa i CV entre homes que havien sobreviscut un infart de miocardi. Addicionalment, un metanàlisi sobre els efectes del consum d'alcohol en pacients amb un episodi cardiovascular documentat va trobar una associació significativa entre el consum entre baix i moderat d'alcohol (5-25 g/dia) i una menor incidència de mortalitat cardiovascular i de mortalitat per qualsevol causa (Costanzo, 2010).

4.3.8. Alcohol i diferències entre sexes

A l'hora d'assessorar la població general sobre el consum moderat d'alcohol, s'han de tenir en compte les diferències en el metabolisme de l'alcohol relacionades amb el sexe. El consens general és que el consum

moderat d'alcohol és de 30 g/dia d'alcohol (dues begudes) en els homes i de 15-20 g/dia d'alcohol (una beguda) en el cas de les dones. Aquestes diferències s'han posat de manifest en diferents assaigs clínics i metanàlisis, on els efectes protectors observats són majors en homes que en dones (Di Castelnuovo, 2002; Taylor, 2009), excepte en el cas del risc de trombosi venosa, on les dones tenen més beneficis derivats del consum moderat d'alcohol (Pomp, 2008). No obstant, altres estudis han observat efectes beneficiosos similars en el risc d'infart de miocardi derivats del consum moderat d'alcohol en ambdós sexes (Mukamal, 2005).

A més de les diferències dels efectes entre sexes, també s'han de tenir en compte les diferències ètniques en el metabolisme de l'alcohol i, per tant, en els efectes beneficiosos derivats d'un consum moderat d'alcohol (Bau, 2007).

En conclusió, la majoria dels estudis epidemiològics que correlacionen el consum moderat d'alcohol amb efectes cardioprotectors no tenen en compte el tipus de beguda alcohòlica consumida (és a dir, amb i sense polifenols). Per tant, per tal d'avaluar els diferents efectes sobre el sistema cardiovascular derivats del consum de diferents tipus de begudes alcohòliques, són necessaris més assaigs clínics aleatoritzats ben dissenyats, sempre en el context d'un consum moderat d'alcohol. La present tesi doctoral té com a objectiu avaluar els efectes protectors enfront a les malalties cardiovasculars de les diferents fraccions del vi negre (alcohòlica i polifenòlica), per tal d'establir els efectes diferencials i/o sinèrgics de les diferents fraccions del vi negre en persones d'alt risc cardiovascular.

HIPÒTESI

HIPÒTESI GENERAL

El consum moderat d'alcohol s'ha associat a una disminució del risc cardiovascular. A més, el consum d'aliments rics en polifenols (com les fruites i verdures, el te, el cacau i el vi, especialment negre) també està associat a una disminució del risc cardiovascular.

El consum moderat de vi negre (30 g d'alcohol/dia), degut al seu contingut en alcohol i a l'alt contingut en polifenols, disminuirà els factors de risc cardiovascular en una població d'alt risc cardiovascular, ja que els seus components (alcohol i polifenols) exerciran un efecte sinèrgic.

HIPOTESIS CONCRETES

El consum moderat d'alcohol, independentment del tipus de beguda alcohòlica, tindrà un efecte antiinflamatori i disminuirà l'expressió de molècules d'adhesió leucocitàries en homes amb un alt risc cardiovascular.

El consum moderat d'alcohol, independentment del tipus de beguda alcohòlica, elevarà el colesterol HDL en homes amb un alt risc cardiovascular.

El consum de polifenols del vi negre tindrà un efecte modulador sobre els marcadors sistèmics i cel·lulars d'inflamació i adhesió en homes amb un alt risc cardiovascular.

El consum de polifenols del vi negre millorarà la sensibilitat a la insulina.

El consum de polifenols del vi negre tindrà un efecte hipotensor en homes amb un alt risc cardiovascular.

OBJECTIUS

OBJECTIU GENERAL

Conèixer el balanç benefici/risc del consum de 30 grams d'alcohol al dia en forma d'etanol (ginebra), vi negre (beguda alcohòlica amb alt contingut en polifenols) o la quantitat equivalent de vi desalcoholitzat (beguda sense alcohol amb alt contingut en polifenols), per a considerar separadament els efectes dels polifenols i l'alcohol sobre el sistema cardiovascular en una sèrie d'homes amb alt risc cardiovascular.

OBJECTIUS CONCRETS

Analitzar l'efecte del consum moderat d'alcohol durant quatre setmanes en forma de ginebra (30 g d'alcohol/dia), vi negre (30 g d'alcohol/dia) o la quantitat equivalent de vi desalcoholitzat en una sèrie amplia d'homes amb alt risc cardiovascular sobre els següents paràmetres:

1) Molècules relacionades amb l'aparició i progressió de l'aterosclerosi:

1.1. Expressió de biomarcadors endotelials solubles d'inflamació i adhesió que participen en la interacció leucòcit-endoteli relacionats amb l'aparició i progressió de l'aterosclerosi.
1.2. Expressió de molècules d'adhesió en la superfície de limfòcits T circulants.
1.3.Expressió de molècules d'adhesió en la superfície de monòcits i macròfags circulants.

2) Metabolisme glucídic i perfil lipídic:

2.1. Glucosa, insulina i índex HOMA.
2.2. Adipocines (leptina i adiponectina).
2.3. Perfil lipídic: Triglicèrids, colesterol i apoproteïnes AI, AII, B, CI i CIII.
2.4. Homocisteïna, vitamina B_{12} i àcid fòlic.

3) Pressió arterial i òxid nítric:

3.1. Pressió arterial sistòlica i diastòlica.

3.2. Freqüència cardíaca.

3.3. Concentració plasmàtica d'òxid nítric.

RESULTATS

RESULTAT 1

Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial.

Am J Clin Nutr. 2012;95(2):326-34.

Erratum in: Am J Clin Nutr. 2012 Jun;95(6):1506.

Chiva-Blanch G, Urpi-Sarda M, Llorach R, Rotches-Ribalta M, Guillén M, Casas R, Arranz S, Valderas-Martinez P, Portoles O, Corella D, Tinahones F, Lamuela-Raventos RM, Andres-Lacueva C, Estruch R.

INTRODUCCIÓ

Molts estudis epidemiològics han estudiat els efectes cardioprotectors del consum moderat d'alcohol, però pocs estudis clínics s'han centrat en els efectes dels polifenols del vi negre independents de l'alcohol en el sistema cardiovascular.

OBJECTIU

Avaluar els efectes de l'alcohol i els polifenols del vi negre en l'expressió de biomarcadors inflamatoris relacionats amb l'aparició i progressió de l'aterosclerosi en homes que presenten un elevat risc de patir malalties cardiovasculars.

DISSENY DE L'ESTUDI

Seixanta set homes amb alt risc cardiovascular van ser inclosos en un estudi clínic d'intervenció dietètica aleatoritzat i creuat. Després d'un període de 15 dies, en els que se'ls demanà que no consumissin alcohol, tots els voluntaris van rebre vi negre (30 g alcohol/d), la quantitat equivalent de vi desalcoholitzat o ginebra (30 g alcohol/d) durant quatre setmanes cada intervenció. Abans i després de cada període es van analitzar set molècules d'adhesió leucocitàries i 18 biomarcadors inflamatoris sèrics.

RESULTATS

El consum moderat d'alcohol (intervencions amb vi negre i ginebra) va incrementar la IL-10 i va disminuir la MDC. Els polifenols del vi negre (intervencions amb vi negre i vi negre desalcoholitzat) disminuïren les concentracions sèriques d'ICAM-1, E-Selectina i IL-6, així com inhibiren l'expressió del LFA-1 als limfòcits T i la de Mac-1, SLe^x i CCR2 a la superfície dels monòcits. Tant l'etanol com els polifenols del vi van disminuir les concentracions circulants de CD40a, CD40L, IL-16, MCP-1 i VCAM-1. Ni l'alcohol ni els polifenols continguts al vi negre van afectar les concentracions plasmàtiques de CRP, IL-1α, IL-18, MCP-2, MCP-3, MIP-1α, MPIF-1 i TNF-α, ni l'expressió limfocitària de Mac-1, VLA-4 i CD40, ni tampoc l'expressió monocitària de LFA-1, VLA-4, CD40 i CD36.

CONCLUSIONS

Els resultats observats suggereixen que els polifenols del vi negre modulen l'expressió de molècules d'adhesió leucocitàries, mentre que tant l'alcohol com els polifenols del vi negre modulen els mediadors inflamatoris solubles en homes d'alt risc cardiovascular.

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Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial[1–3]

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ABSTRACT

Background: Few clinical studies have focused on the alcohol-independent cardiovascular effects of the phenolic compounds of red wine (RW).

Objective: We aimed to evaluate the effects of ethanol and phenolic compounds of RW on the expression of inflammatory biomarkers related to atherosclerosis in subjects at high risk of cardiovascular disease.

Design: Sixty-seven high-risk, male volunteers were included in a randomized, crossover consumption trial. After a washout period, all subjects received RW (30 g alcohol/d), the equivalent amount of dealcoholized red wine (DRW), or gin (30 g alcohol/d) for 4 wk. Before and after each intervention period, 7 cellular and 18 serum inflammatory biomarkers were evaluated.

Results: Alcohol increased IL-10 and decreased macrophage-derived chemokine concentrations, whereas the phenolic compounds of RW decreased serum concentrations of intercellular adhesion molecule-1, E-selectin, and IL-6 and inhibited the expression of lymphocyte function-associated antigen 1 in T lymphocytes and macrophage-1 receptor, Sialil-Lewis X, and C-C chemokine receptor type 2 expression in monocytes. Both ethanol and phenolic compounds of RW downregulated serum concentrations of CD40 antigen, CD40 ligand, IL-16, monocyte chemotactic protein-1, and vascular cell adhesion molecule-1.

Conclusion: The results suggest that the phenolic content of RW may modulate leukocyte adhesion molecules, whereas both ethanol and polyphenols of RW may modulate soluble inflammatory mediators in high-risk patients. The trial was registered in the International Standard Randomized Controlled Trial Number Register at http://www.isrctn.org/ as ISRCTN88720134. *Am J Clin Nutr* 2012;95:326–34.

INTRODUCTION

CAD[4] is generally due to atherosclerosis and is the leading cause of morbimorbidity in developed countries. Atherogenesis is a multifactorial process that involves a combination of environmental, genetic, and metabolic components that act synergistically to induce oxidative stress and a chronic inflammatory state. Thus, atherosclerosis is considered a low-grade inflammatory disease in which the cell and endothelial expression of adhesion molecules and chemokines participate in the recruitment of circulating leukocytes to the vascular endothelium and further migration into subendothelial spaces (1), which leads to the formation of atherosclerotic lesions (2). In this process, chemokines, the secreted proteins that recruit specific cell types to inflammatory sites, have emerged as major contributors to vascular inflammation. Therefore, the study of cir-

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[4] Abbreviations used: CAD, coronary artery disease; CCR2, C-C chemokine receptor type 2; CD40a, CD40 antigen; CD40L, CD40 ligand; CRP, C-reactive protein; DRW, dealcoholized red wine; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; Mac-1, macrophage-1 receptor; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP-1α, macrophage inflammatory protein 1α; PBMC, peripheral blood mononuclear cell; RW, red wine; SLe^x, Sialil-Lewis X; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late activation antigen 4.

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culating chemokines may provide a greater understanding of the underlying pathophysiology of this disease (3).

Several epidemiologic studies have reported beneficial effects of moderate alcohol consumption on cardiovascular disease (4, 5). Other studies that differentiated the actions of different alcoholic beverages have observed that moderate consumption of RW has greater effects on lowering risk of CAD than do other beverages (6–9). RW contains alcohol and nonalcoholic compounds, mainly polyphenols such as anthocyanosides, catechins, proanthocyanidins, stilbenes, and other phenolic compounds (6). The cardiovascular effects of RW phenolic compounds have been well studied in vitro (10–12) and in animal models (13–16), but few human clinical trials have focused on the alcohol-independent effects of phenolic compounds of RW (17–20). Therefore, the differential cardiovascular effects of both components in RW are not well known.

In contrast, previous clinical trials in healthy men (21, 22) and women (23) have shown a reduction in circulating markers of inflammation and monocyte adhesion to endothelial cells after the daily intake of 20–30 g alcohol as RW, which potentially modulates atherosclerosis development. However, it is unknown whether these effects can be extrapolated to high–cardiovascular risk populations, and whether these effects are attributed to alcohol, polyphenols of RW, or the synergistic effect of both.

Therefore, we embarked on a randomized, crossover, controlled clinical trial to evaluate and compare the effects of moderate consumption of 30 g alcohol/d of gin (a nonpolyphenolic alcoholic beverage), RW (a high-polyphenolic alcoholic beverage), and the same amount of DRW (a high-polyphenolic nonalcoholic beverage) on the expression of soluble and leukocyte adhesion molecules as well as proinflammatory cytokines related to the early stages of atherosclerosis in subjects at high risk of CAD in whom diet and exercise were carefully monitored.

SUBJECTS AND METHODS

Subjects

A total of 73 high-risk subjects aged between 55 and 75 y were recruited for the study in the outpatient clinic of the Internal Medicine Department at Hospital Clínic of Barcelona. Subjects included in the trial were moderate alcohol consumers (1–3 drinks/d) and had diabetes or ≥3 of the following cardiovascular disease risk factors: tobacco smoking, hypertension, plasma LDL cholesterol concentrations ≥160 mg/dL, plasma HDL cholesterol concentrations ≤35 mg/dL, overweight or obesity [BMI (in kg/m^2) ≥25], and/or family history of premature CAD. Exclusion criteria included documented CAD, stroke, or peripheral vascular disease, HIV infection, alcoholic liver disease, malnutrition, and neoplastic or acute infectious diseases. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent before participation in the study.

Study design

The study was an open, randomized, crossover, and controlled clinical trial, which included 3 4-wk periods. After a run-in period of 2 wk in which subjects were asked not to consume any alcoholic beverage, they received gin (100 mL; 30 g ethanol/d), RW (272 mL; 30 g ethanol/d), and the same amount of polyphenols as RW in the form of DRW (272 mL) according to a computer-generated random-number table. None of the subjects consumed multivitamin or vitamin E supplements or antiinflammatory drugs (steroids, nonsteroidal antiinflammatory drugs, or aspirin >100 mg/d). The RW and DRW were from the Penedès appellation and elaborated with the Merlot grape variety. The phenolic composition of the 3 beverages used in the study is detailed in **Table 1**. The total phenolic content of the 3 beverages was determined by using the Folin-Ciocalteu method (24), the phenolic profile of RW and DRW was determined by using HPLC–diode-array detection as described previously (25), and resveratrol and piceid contents were determined by using HPLC–diode-array detection as described by Romero-Pérez et al (26). No significant differences were observed between phenolic compositions of RW and DRW (Table 1).

Diet and exercise monitoring

Subjects were asked to exclude alcoholic beverages 15-d before the first intervention (run-in period) and during the study. Subjects were also asked not to change their dietary pattern during the study. Natural foods rich in antioxidants, especially fruit and vegetables, were especially monitored so that individual diets had similar antioxidant contents throughout the study. Participants were not blinded to the type of drink they ingested. At the beginning of the study and after each intervention period, a medical record and Minnesota Leisure Time Physical Activity Questionnaire validated in Spain (27) were performed, and a 7-d food record questionnaire (5 weekdays and 2 weekend days), which was also validated in our population (28), was used to assess nutrient intake and to monitor adherence to the study protocol. This information was converted into dietary data by using the Food Processor Nutrition and Fitness Software (*esha* Research). Nutritional variables analyzed included total energy, total protein, carbohydrates, dietary fiber, sugars, total lipids, saturated fatty acids, MUFAs, PUFAs, cholesterol, vitamins C, A, and E, folic acid, and total polyphenols. Subjects were asked to maintain their lifestyle habits and to report any illness or abnormality presented during the study period. At the end of each study period, a clinician assessed any adverse effects from the interventions by administering a checklist of symptoms, including bloating, fullness, or indigestion, altered bowel habits, dizziness, and other symptoms that were possibly associated with the interventions.

Methods

Clinical and laboratory measures

Anthropometric measures were performed with standardized methods (29). Fasting blood and 24-h urine samples were collected at baseline (the last day of the washout period) and the day after the last day of each intervention (RW, DRW, and gin). Serum, EDTA plasma, and urine samples were stored at −80°C until assayed. Clinical investigators and laboratory technicians were blinded to the interventions. To ensure that ethanol consumption did not cause side effects, plasma aminotransferases (aspartate aminotransferase and alanine aminotransferase), γ-glutamyl transpeptidase, albumin, vitamin B-12, and serum and intraerythrocytary folic acid concentrations were measured.

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TABLE 1
Phenolic composition of beverages used in the study: RW, DRW, and gin[1]

	RW	DRW	Gin	P^2
Alcohol (%)	14.2	0.42	38	
Phenolic compounds[3]				
Total phenols (mEqGA/L)	2933.35 ± 377.31	2694.92 ± 86.79	ND	0.426
Gallic acid (mg/L)	68.48 ± 6.40	73.17 ± 7.01	ND	0.306
Protocatechuic acid (mg/L)	5.22 ± 0.62	5.85 ± 0.51	ND	0.246
Tyrosol (mg/L)	43.59 ± 4.73	47.81 ± 3.90	ND	0.298
Catechin (mg/L)	123.51 ± 11.30	126.45 ± 13.35	ND	0.786
Epicatechin (mg/L)	67.86 ± 7.74	70.57 ± 8.22	ND	0.699
*t*rans-Caftaric (mg/L)	18.62 ± 1.45	19.21 ± 1.62	ND	0.595
trans-Caffeic (mg/L)	11.50 ± 0.79	12.18 ± 0.92	ND	0.246
trans-Coutaric (mg/L)	5.21 ± 0.45	5.62 ± 0.52	ND	0.182
2-S-glutationylcaftaric (mg/L)	10.30 ± 1.00	10.76 ± 1.26	ND	0.956
Quercetin-3-glucuronide (mg/L)	11.88 ± 1.38	11.25 ± 1.42	ND	0.770
Quercetin (mg/L)	26.66 ± 0.78	23.82 ± 2.37	ND	0.161
Isorhamnetin (mg/L)	3.34 ± 0.27	2.96 ± 0.14	ND	0.114
Delphinidin-3-glucoside (mg/L)	15.25 ± 0.89	14.71 ± 1.62	ND	0.589
Petunidin-3-glucoside (mg/L)	12.29 ± 1.06	12.04 ± 1.15	ND	0.755
Peonidin-3-glucoside (mg/L)	6.78 ± 0.62	6.68 ± 0.57	ND	0.797
Malvidin-3-glucoside (mg/L)	48.83 ± 4.45	49.86 ± 4.27	ND	0.787
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.97 ± 0.96	10.41 ± 1.20	ND	0.563
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	4.15 ± 0.27	3.54 ± 0.33	ND	0.066
Total resveratrol (*trans* and *cis*) (mg/L)	5.26 ± 0.83	5.01 ± 0.86	ND	0.566
trans-Resveratrol (mg/L)	2.92 ± 0.36	2.73 ± 0.23	ND	0.352
cis-Resveratrol (mg/L)	2.79 ± 0.15	2.75 ± 0.15	ND	0.761
Total Piceid (*trans* and *cis*) (mg/L)	18.43 ± 1.57	17.98 ± 3.21	ND	0.785
trans-Piceid (mg/L)	9.41 ± 1.12	10.53 ± 0.96	ND	0.160
cis-Piceid (mg/L)	7.71 ± 0.34	7.08 ± 0.87	ND	0.226
Total stilbenes (mg/L)	23.61 ± 1.61	24.53 ± 1.49	ND	0.408

[1] DRW, dealcoholized red wine; mEqGA/L, mEq Gallic acid/L; ND, not detected; RW, red wine.
[2] *P* values are for comparison between RW and DRW polyphenols (Student's *t* test for independent samples).
[3] Mean ± SD (n = 2) (all such values).

Resveratrol conjugates derived from phase II and microbial metabolism were measured in 24-h urine samples by using the validated methodology described by Urpi-Sarda et al (30), which was adapted to this study, as a biochemical marker of RW- and DRW-intervention compliance. Ethylglucuronide was measured in 24-h urine samples by using liquid chromatography as a biomarker of alcohol intake. High-performance liquid chromatography was performed on an LC Agilent series 1200 (Agilent Technologies) coupled with a hybrid quadrupole time-of-flight mass spectrometer QSTAR Elite (Applied Biosystems/MDS Sciex).

The following serum-soluble adhesion molecules and cytokines and other regulator molecules of adhesion and inflammation processes were quantified by customized human multianalyte profiling (Human MAP; Rules Based Medicine Inc): CD40a, CD40L, CRP, E-selectin, ICAM-1, IL-1α, -10, -16, -18, and -6, MCP-1, -2, and -3, MDC, MIP-1α, myeloid progenitor inhibitory factor 1, tumor necrosis factor-α, and VCAM-1.

PBMC immunophenotyping

PBMCs were isolated from whole blood by Ficoll-Hypaque (Pharmacia) 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 by following the manufacturer's instructions. The adhesion molecules analyzed were as follows: VLA-4 (CD49-d; Cytogmos), LFA-1 (CD11a; (Bender MedSystems), Mac-1 (CD11b/CD18; Bender MedSystems), SLe^x (CD15s; Beckman Coulter), CD40 (Caltag Laboratories), CD36 (Beckman Coulter), and CCR2 (R&D Systems). Monocytes were identified and selected with the CD14 monoclonal antibody (Caltag Laboratories), and T lymphocytes were identified and selected with the CD2 monoclonal antibody (Caltag Laboratories). Cell counting (5000 events for T lymphocytes and 2000 events for monocytes) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton Dickinson) with the use of CellQuest software (version 3.3; BD Biosciences).

Statistical analysis

Statistical analysis was performed with SAS Statistical Analysis Systems software (version 9.2; SAS Institute Inc). Descriptive statistics (means ± SDs) were used for baseline characteristics of participants. Values with a skewed distribution (ICAM-1 and MCP-1) were transformed to their natural logarithm for analyses. ANCOVA with baseline value as the covariate was used to compare changes in outcome variables in response to intervention treatments. One-factor ANOVA for repeated measures was used to compare changes in outcome variables in response between intervention treatments and baseline. To exclude the presence of a carryover effect for the 3 periods, the interaction between treatment (RW, DRW, and gin) and sequence of treatment was analyzed in the repeated-measures ANCOVA analyses. Within- and between-group differences are expressed as means and 95% CIs. $P < 0.05$was considered to be significant.

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RESULTS

Baseline characteristics, intervention compliance, diet, exercise monitoring, and side effects

Of the 73 subjects included, 6 subjects withdrew before completing the 3 phases of the study because of physical illness ($n = 2$), journeys ($n = 2$), or not being able to drink DRW ($n = 2$). Therefore, 67 subjects were included in the study. Baseline characteristics of the 67 participants are shown in **Table 2**. Most participants were overweight or obese (~91%), more than one-half of the population had hypertension (~57%), more than three-quarters of subjects had a family history of cardiovascular disease (~78%), and more than one-fifth of subjects had dyslipemia (~24%), had type-2 diabetes (~22%), or were smokers (~24%). Biochemical safety analytes (serum and intraerythrocytary folic acid, vitamin B-12, albumin, aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transpeptidase) remained within the normal range throughout the study. None of the subjects reported adverse effects related to the interventions.

Protocol adherence was optimum in all subjects, and complete agreement was observed between the reports of participants and the number of empty bottles returned. All the subjects complained about the taste and texture of the DRW. As a measure of intervention compliance, a sum of total resveratrol metabolites, which is a marker of RW and DRW consumption (31), was determined in 24-h urine samples collected the last day of the run-in period and the last day of each intervention. After consumption of RW and DRW, 24-h urinary excretion of total resveratrol metabolites increased in relation to baseline amounts from 1.24 μmol (95% CI: 0.91, 1.65 μmol) to 4.69 μmol (95% CI: 3.86, 5.53 μmol) and 8.33 μmol (95% CI: 6.86, 10.19 μmol), respectively ($P < 0.001$, both). Resveratrol metabolites concentrations were statistically higher after the DRW compared with RW intervention ($P = 0.002$) and were also statistically higher after RW and DRW interventions than those obtained after gin consumption [0.76 μmol (95% CI: 0.48, 1.11 μmol); $P < 0.0001$]. After the gin intervention, the concentration of resveratrol metabolites did not change significantly compared with that at baseline ($P = 0.832$). Ethylglucuronide was used as a biomarker of alcohol consumption. Urinary ethylglucuronide concentrations increased significantly after the RW and gin periods compared with those observed at baseline, with increases of 342% (95% CI: 245%, 773%) and 256% (95% CI: 179%, 599%), respectively ($P < 0.001$, both). Moreover, concentrations after the RW and gin interventions were also higher than those obtained after the DRW intervention: 634% (95% CI: 468%, 1424%) and 491% (95% CI: 359%, 1121%), respectively ($P < 0.001$, both). No significant differences were observed between DRW and baseline periods [66% (95% CI: 64%, 75%); $P = 1.000$] and between RW and gin interventions [24% (95% CI: 24–25%); $P = 1.000$]. According to these results, compliance with the 3 interventions was excellent.

TABLE 2
Baseline characteristics of 67 subjects[1]

	Values
Age (y)	60 ± 8[2]
Current smokers [*n* (%)]	16 (23.9)
Sedentarism [*n* (%)]	40 (59.7)
Family history of premature CAD [*n* (%)]	52 (77.6)
BMI (kg/m^2)	29.6 ± 3.9
BMI ≥25 kg/m^2 [*n* (%)]	61 (91.0)
WHR	0.975 ± 0.045
Type 2 diabetes [*n* (%)]	15 (22.4)
Hypertension [*n* (%)]	38 (56.7)
Dyslipemia [*n* (%)]	16 (23.9)
Medications [*n* (%)]	
ACE inhibitors	28 (41.8)
Diuretics	5 (7.5)
Statins	22 (32.8)
Oral hypoglycemic drugs	14 (20.9)
Aspirin or antiplatelet drugs	15 (22.4)
Systolic blood pressure (mm Hg)	142 ± 18
Diastolic blood pressure (mm Hg)	81 ± 8
Heart rate (beats/min)	69 ± 10
Glucose (mg/dL)	111 ± 34
Triglycerides (mg/dL)	128 ± 60
Total cholesterol (mg/dL)	204 ± 33
LDL cholesterol (mg/dL)	133 ± 32
HDL cholesterol (mg/dL)	43 ± 7
LDL cholesterol:HDL cholesterol ratio	3.08 ± 0.10
Folic acid (serum) (ng/mL)	9.8 ± 4.0
Intraerythrocytary folic acid (ng/mL)	386 ± 98
Vitamin B-12 (pg/mL)	406 ± 163
Albumin (mg/mL)	45.4 ± 2.8
ASAT (UI/L)	25.8 ± 9.5
ALAT (UI/L)	29.2 ± 11.7
GGT (UI/L)	30.7 ± 12.6

[1] ACE, angiotensin-converting enzyme; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CAD, coronary artery disease; GGT, γ-glutamyl transpeptidase; WHR, waist-to-hip ratio.
[2] Mean ± SD (all such values).

Dietary intake data for the 3 intervention periods are shown in **Table 3**. No significant differences were observed in nutrient intake at the beginning of the study and after each intervention. No significant differences were observed in the daily intake of antioxidants or fat before and after each intervention period or in the daily average energy expended in physical activity during the period of intake of RW, DRW, and gin. In addition, no changes were reported in drug intake (Table 2) in any subjects throughout the study. No effect of the treatment from the previous time period on the response in the current time period was observed ($P > 0.05$, all), and thus, no carryover effect was observed for any variables.

Changes in circulating inflammatory markers

Changes in circulating inflammatory markers are shown in **Table 4.** Serum E-selectin concentrations were lower after the DRW intervention than after the RW and gin interventions [$P = 0.009$ and 0.016, respectively (Bonferroni post hoc test)]. Serum ICAM-1 and IL-6 concentrations were significantly lower after the RW and DRW interventions than after the gin intervention ($P = 0.014$, 0.016, 0.026, and 0.023 for ICAM-1 and IL-6, respectively). The IL-10 concentration was shown to be significantly higher and MDC was shown to be significantly lower after the RW and gin interventions than after the DRW intervention ($P = 0.046$, 0.023, 0.003, and 0.007, for IL-10 and MDC, respectively). Serum concentrations of CD40a and CD40L decreased significantly after the 3 interventions (RW, DRW, and gin) compared with the baseline situation ($P = 0.008$, 0.043, 0.001, 0.001, 0.019, and 0.015 for CD40a and CD40L, respectively) as

TABLE 3
Daily energy and dietary intakes in 67 subjects at baseline and after 3 interventions[1]

	Baseline	RW intervention	DRW intervention	Gin intervention	*P*
Energy (kcal/d)	1816 ± 457	1782 ± 325	1862 ± 320	1887 ± 336	0.109
Total protein (g/d)	90.39 ± 20.60	89.06 ± 18.35	94.29 ± 17.56	95.04 ± 18.73	0.133
Carbohydrates (g/d)	193 ± 51	201 ± 44	193 ± 38	206 ± 40	0.111
Dietary fiber (g/d)	19.67 ± 9.83	20.63 ± 8.08	19.88 ± 6.82	22.05 ± 10.01	0.254
Sugars (g/d)	62.89 ± 23.55	66.74 ± 25.59	67.33 ± 20.08	70.11 ± 20.84	0.489
Total lipids (g/d)	74.87 ± 24.91	72.72 ± 16.27	78.55 ± 20.41	79.86 ± 21.89	0.194
SFA (g/d)	18.65 ± 8.66	18.40 ± 7.06	18.90 ± 6.15	19.01 ± 5.48	0.804
MUFA (g/d)	36.85 ± 12.06	35.84 ± 8.23	37.97 ± 10.18	38.31 ± 9.60	0.522
PUFA (g/d)	10.31 ± 3.89	10.65 ± 4.21	11.94 ± 4.42	10.71 ± 3.29	0.644
Cholesterol (mg/d)	346 ± 142	355 ± 125	342 ± 103	360 ± 162	0.710
Vitamin C (mg/d)	112 ± 75	125 ± 83	121 ± 68	133 ± 91	0.534
Vitamin A (μg retinol equivalent/d)	640 ± 360	688 ± 340	729 ± 343	791 ± 481	0.345
Vitamin E (mg/d)	9.49 ± 3.88	9.65 ± 3.22	9.47 ± 3.34	10.29 ± 4.65	0.153
Folic acid (μg/d)	426 ± 198	454 ± 155	443 ± 143	498 ± 226	0.181
Total polyphenols (mg/d)	340 ± 185	318 ± 142	311 ± 146	327 ± 170	0.555

[1] All values are means ± SDs. Energy, nutrient, and total polyphenol contributions from interventions were excluded. Changes in outcome variables were determined by using repeated-measures ANCOVA with the baseline value as the covariate. No changes were observed between baseline and interventions determined by repeated-measures ANOVA ($P > 0.05$, all). DRW, dealcoholized red wine; RW, red wine.

did IL-16 ($P = 0.013$, 0.005, and 0.008 for RW, DRW, and gin, respectively), MCP-1 ($P = 0.016$, 0.009, and 0.007 for RW, DRW, and gin, respectively) and VCAM-1 ($P = 0.002$, 0.003, and 0.027 for RW, DRW, and gin, respectively) concentrations. Serum ICAM-1 and IL-6 concentrations decreased significantly after the RW and DRW interventions compared with those at baseline ($P = 0.005$, 0.003, 0.019, and 0.009 for ICAM-1 and IL-6, respectively), and IL-10 concentrations increased and MDC concentrations decreased after the RW and gin interventions compared with those at baseline ($P = 0.013$, 0.023, 0.008, and 0.013 for IL-10 and MDC, respectively). Otherwise, serum concentrations of the other molecules evaluated were shown to remain practically unaltered between baseline and after the 3 interventions.

Expression of cell adhesion molecules on leukocyte cell surfaces

Changes in the expression of cell adhesion molecules on leukocyte cell surfaces are shown in **Table 5**. LFA-1 expression on T-lymphocyte membrane was significantly higher after the gin intervention than after the RW and DRW interventions [$P =$

TABLE 4
Expression of soluble adhesion molecules and cytokines in 67 subjects at baseline and after 3 interventions[1]

	Baseline	RW intervention	DRW intervention	Gin intervention	*P*
CD40a (ng/mL)	0.865 ± 0.225	0.805 ± 0.195[2]	0.818 ± 0.201[2]	0.799 ± 0.202[2]	0.734
CD40L (ng/mL)	2.15 ± 1.16	1.62 ± 1.23[2]	1.81 ± 1.24[2]	1.79 ± 1.24[2]	0.477
CRP (μg/mL)	2.18 ± 0.31	2.17 ± 0.33	1.76 ± 0.20	2.15 ± 0.28	0.210
E-selectin (ng/mL)	9.38 ± 4.30	8.78 ± 3.25[a]	8.33 ± 3.17[b]	8.91 ± 3.93[a]	0.008
ICAM-1 (ng/mL)	91.41 ± 28.63	81.33 ± 25.98[a,2]	79.31 ± 21.46[a,2]	87.82 ± 20.39[b]	0.029
IL-1α (pg/mL)	4.46 ± 2.23	4.24 ± 2.23	4.41 ± 2.58	4.82 ± 2.77	0.188
IL-10 (pg/mL)	6.44 ± 2.63	7.68 ± 1.96[a,2]	6.69 ± 2.63[b]	7.89 ± 2.38[a,2]	0.043
IL-16 (pg/mL)	478 ± 142	450 ± 143[2]	428 ± 168[2]	436 ± 147[2]	0.582
IL-18 (pg/mL)	399 ± 123	384 ± 150	384 ± 129	383 ± 127	0.985
IL-6 (pg/mL)	3.67 ± 1.15	2.76 ± 1.06[a,2]	2.44 ± 1.29 [a,2]	3.70 ± 1.14[b]	0.039
MCP-1 (pg/mL)	488 ± 205	455 ± 201[2]	454 ± 196[2]	446 ± 219[2]	0.817
MCP-2 (pg/mL)	43.67 ± 12.57	43.67 ± 10.37	43.17 ± 12.19	43.02 ± 11.60	0.724
MCP-3 (pg/mL)	60.64 ± 20.03	60.72 ± 17.50	59.10 ± 17.69	59.10 ± 17.11	0.546
MDC (pg/mL)	450 ± 104	416 ± 115[a,2]	446 ± 120[b]	418 ± 104[a,2]	0.009
MIP-1α (pg/mL)	141 ± 30	138 ± 30	140 ± 31	142 ± 29	0.248
MPIF-1 (ng/mL)	1.41 ± 0.29	1.41 ± 0.27	1.43 ± 0.31	1.39 ± 0.31	0.274
TNF-α (pg/mL)	7.60 ± 3.79	8.09 ± 5.59	7.034 ± 3.96	7.64 ± 5.95	0.121
VCAM-1 (ng/mL)	608 ± 114	572 ± 112[2]	572 ± 113[2]	576 ± 112[2]	0.952

[1] All values are means ± SDs. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANCOVA with the baseline value as the covariate. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). CD40a, CD40 antigen; CD40L, CD40 ligand; CRP, C-reactive protein; DRW, dealcoholized red wine; ICAM-1, intercellular adhesion molecule 1; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP-1α, macrophage inflammatory protein 1α; MPIF-1, myeloid progenitor inhibitory factor 1; RW, red wine; VCAM-1, vascular cell adhesion molecule 1.

[2] Significantly different from baseline, $P < 0.05$ (repeated-measures ANOVA and Bonferroni post hoc test).

TABLE 5
Expression of adhesion molecules on the surface of T lymphocytes and monocytes in 67 subjects at baseline and after 3 interventions[1]

	Baseline	RW intervention	DRW intervention	Gin intervention	*P*
T lymphocytes					
LFA-1 (MFI)	66.03 ± 15.72	65.95 ± 17.51[a]	66.98 ± 13.61[a]	75.92 ± 12.98[b]	0.003
Mac-1 (MFI)	43.44 ± 13.79	39.73 ± 10.90	37.80 ± 14.29	40.76 ± 15.79	0.271
VLA-4 (MFI)	36.67 ± 8.99	36.96 ± 8.34	37.27 ± 7.73	37.08 ± 7.97	0.775
SLe^x (MFI)	77.30 ± 14.06	68.46 ± 13.62[2]	68.94 ± 14.21[2]	73.28 ± 14.31	0.268
CD40 (MFI)	38.44 ± 12.73	37.36 ± 13.84	38.72 ± 11.39	38.37 ± 12.12	0.585
Monocytes					
LFA-1 (MFI)	30.59 ± 8.41	31.50 ± 9.29	31.01 ± 8.90	31.66 ± 9.19	0.781
Mac-1 (MFI)	30.05 ± 12.78	26.32 ± 9.60[a,b,2]	25.46 ± 6.41[a,2]	28.83 ± 11.48[b]	0.044
VLA-4 (MFI)	23.16 ± 8.07	22.74 ± 6.22	23.03 ± 9.57	22.69 ± 6.25	0.909
SLe^x (MFI)	40.12 ± 10.95	36.90 ± 10.40[2]	36.93 ± 11.05[2]	37.39 ± 10.53	0.861
CD40 (MFI)	23.83 ± 6.94	24.22 ± 5.23	23.67 ± 5.49	23.53 ± 5.21	0.266
CD36 (MFI)	19.89 ± 7.67	20.73 ± 7.90	20.50 ± 8.23	20.79 ± 7.74	0.279
CCR2 (MFI)	169.53 ± 36.78	116.94 ± 41.04[a,2]	109.52 ± 38.81[a,2]	168.83 ± 35.10[b]	0.009

[1] All values are means ± SDs. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANCOVA with the baseline value as the covariate. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). CCR2, C-C chemokine receptor type 2; DRW, dealcoholized red wine; LFA-1, lymphocyte function-associated antigen 1 (CD11a); Mac-1, macrophage-1 receptor; (CD11b/CD18); MFI: mean fluorescence intensity; RW, red wine; SLe^x, Sialil-Lewis X, CD15s; VLA-4, very late activation antigen 4 (CD49-d).
[2] Significantly different from baseline, $P < 0.05$ (repeated-measures ANOVA and Bonferroni post hoc test).

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0.003 and 0.005, respectively (Bonferroni post hoc test)], Mac-1 monocyte expression was lower after the DRW intervention than after the gin period ($P = 0.014$), and CCR2 monocyte fluorescence intensity was also lower after the RW and DRW interventions than after the gin intervention ($P = 0.019$ and <0.0001, respectively). In addition, compared with the baseline situation, we observed that SLe^x lymphocyte and monocyte expression decreased significantly after the RW and DRW interventions ($P = 0.006$, 0.012, 0.027, and 0.037 for lymphocyte and monocyte SLe^x, respectively) as did the Mac-1 ($P = 0.018$ and 0.003, respectively) and CCR2 monocyte fluorescence intensity ($P = 0.024$ and 0.006, respectively). The expression of VLA-4, Mac-1, and CD40 on the lymphocyte surface remained practically unaltered. The expression of LFA-1, VLA-4, CD40, and CD36 on monocyte cell membrane were not altered.

DISCUSSION

Atherosclerosis is a low-grade inflammatory disease characterized by local inflammation in the vessel wall, as well as a systemic immune response (32), in which recruitment and migration of leukocytes through cell adhesion molecules into the arterial wall is a crucial step in early atherogenesis (33). We measured several chemokines and adhesion molecules implicated in the onset and progression of the atherosclerotic process after a 1-mo intervention with RW (constituted mainly by alcohol plus polyphenols), DRW (polyphenols), and gin (alcohol) in high-risk subjects. A downregulation of serum concentrations of CD40a, CD40L, IL-16, MCP-1, VCAM-1, and E-selectin was observed after the RW, DRW, and gin interventions. Therefore, these effects could be attributed to both ethanol and the phenolic compounds of RW but in a nonadditive manner. However, an additive effect of ethanol and RW polyphenols was suggested in the case of CD40L, albeit without achieving significance. RW polyphenols also exert a protective effect on atherosclerosis because the RW and DRW interventions decreased ICAM-1 and IL-6 serum concentrations and inhibited the expression of LFA-1 and SLe^x in the T-lymphocyte surface and Mac-1, SLe^x, and CCR2 expression in monocytes. Finally, ethanol by itself exerts a dual protective effect by increasing IL-10 and decreasing MDC concentrations.

To our knowledge, no previous consumption trials have compared the antiinflammatory effects of DRW with those of RW and gin in humans. However, our results agree with those obtained in experimental studies such as the study by Norata et al (34) in which MCP-1, MIP-1α and-1β, and IL-6 decreased in the arterial wall of apolipoprotein $E^{-/-}$ mice after oral administration of a mixture of polyphenols. Similarly, in the human umbilical vein endothelial cells cell line, Cullen et al (35) showed that ethanol inhibited the endothelial production of MCP-1, and Carluccio et al (36) observed that resveratrol inhibits the synthesis of VCAM. Lastly, the incubation platelets from healthy humans with extracts of grape seed and skin inhibited the release of soluble CD40L (37).

We observed increased antiinflammatory IL-10 concentrations only after the gin intervention. Similarly, Mandrekar et al (38) showed an increase of IL-10 in human monocytes after acute alcohol intake. We also showed a diminution of MDC after the RW and gin interventions. To our knowledge, this is the first time that MDC and IL-16 expression has been studied after moderate RW or ethanol consumption.

More interestingly, the polyphenols of RW after the RW and DRW interventions decreased plasma concentrations of ICAM-1 and IL-6 and inhibited the expression of LFA-1 and SLe^x in the T-lymphocyte surface and that of Mac-1, SLe^x, and CCR2 on monocytes. These results agree with in vitro or animal models that analyzed the effect of polyphenols of RW, mainly resveratrol, in the regulation of these molecules. Resveratrol reduced ICAM-1 expression in human umbilical vein endothelial cells (39) and IL-6 in vascular smooth-muscle cells (40). The preincubation of polymorphonuclear leukocytes with *trans*-resveratrol resulted in a concentration-dependent inhibition of fMLP (formyl methionyl leucyl phenylalamine)-induced Mac-1 expression (41). Norata et al also showed a decrease in CCR2 expression in the arterial

wall of apolipoprotein $E^{-/-}$ mice after oral administration of resveratrol (34), and Cullen et al (42) showed that CCR2 expression was inhibited in a time- and dose-dependent manner by resveratrol in THP-1 monocytes.

Only a few clinical trials have analyzed the changes in the cellular expression of adhesion molecules (21, 23) or in the inflammatory biomarkers related to atherosclerosis (43) after 1-mo of moderate RW consumption in healthy humans. Therefore, we wondered whether the results observed may be extrapolated to a high–cardiovascular risk population. In a previous study in which 30 g ethanol/d in the form of gin and RW was administered to healthy men, significant reductions of VCAM-1 (16.5%), ICAM-1 (9.2%), and IL-1α (20.3%) concentrations were shown after RW consumption (21). In addition, a reduction of 13.9% in VLA-4 lymphocyte expression and LFA-1 (27.1%), Mac-1 (26.7%), VLA-4 (32.5%), and MCP-1 (45.7%) monocyte expression was also observed after RW consumption. However, no significant effects were shown after the gin intervention, except for a decrease in IL-1α concentrations (22.6%). No significant effects were detected in TNF-α, transforming growth factor β1, IL-6, ICAM-1, and VCAM-1 in another human trial performed by Djurovic et al (43) that included healthy subjects (men and women) who consumed 15 g alcohol/d of RW during 3 wk. Differences in the cardiovascular effects observed between the 2 studies (21, 43) may have been attributed to the different amount of alcohol administered (15 compared with 30 g ethanol/d). In the current study, in which 30 g ethanol/d was administered, reductions of ~6%, 11%, and ~12% of VCAM-1 and ICAM-1 concentrations and Mac-1 monocyte expression, respectively, were observed after RW consumption. Nevertheless, in difference from the aforementioned studies, we also observed a significant decrease of ~6% and ~7% in E-selectin and MCP-1 concentrations, respectively, as well as an increase of 15% in LFA-1 lymphocyte expression after the gin period. Thus, the effects of moderate RW consumption observed in healthy people could not be strictly extrapolated to the high–cardiovascular risk population. The fact that more than one-half of the high-risk subjects consumed drugs with known antiinflammatory effects (mainly statins and aspirin) (44) may explain, in part, the differences observed. However, ethanol (gin) exerted some antiinflammatory effect in high-risk subjects, which were actions that were not previously observed in healthy subjects.

One of the main limitations of our study is that there were no washout periods between interventions. Washout periods between interventions would have extended the study 6 wk more, which would have made it difficult to ensure compliance and subject withdrawal more likely. However, because previous studies have shown that changes in cellular and soluble adhesion molecules were already observed after 2 wk of intervention (21, 45), and no carryover effect was observed, the absence of a washout period would probably not have changed the results.

Although the study was far too short to deal with atherosclerosis itself and morbid mortality due to cardiovascular disease, RW polyphenols may provide additional benefits because of its antiinflammatory effects, namely the decreased expression of adhesion molecules related to early stages of atherosclerosis. Thus, another limitation of our study was that our results can only lead to limited conclusions that link the findings to a reduction in atherosclerosis because the biomarkers used in our study are not commonly examined in the clinical setting (46). Moderate alcohol consumption (independent of the type of beverage) is associated with lower risk of cardiovascular disease mortality and endpoints for CAD (47). Because most alcoholic beverages contain alcohol and polyphenols (except for gin and vodka among other alcoholic beverages), it is very difficult to exclude the effects of ethanol from other compounds of alcoholic beverages. Few studies have shown an inverse association between moderate RW consumption and risk of stroke in men (48). Therefore, we cannot conclude that the changes observed were of a potentially clinical relevant magnitude, and thus, additional studies are required regarding the clinical implication of the modulation of these biomarkers (and the role of each compound of alcoholic beverages) on the beneficial effects of alcoholic beverages on the cardiovascular system.

In conclusion, to our knowledge, this is the first randomized, controlled, clinical trial that studied the effects of RW and DRW separately on the expression of adhesion molecules and inflammatory cytokines related to early stages of atherosclerosis and provided information on the separate role of ethanol and phenolic compounds of RW in low-grade inflammation in the arterial wall and endothelial dysfunction. Our results suggest that both ethanol and nonalcoholic compounds contribute to antiinflammatory effects of RW. The phenolic content of RW may modulate leukocyte adhesion molecules, whereas both ethanol and polyphenols of RW may modulate soluble inflammatory mediators in patients at high risk of cardiovascular disease. These positive changes in the inflammatory profile in high–cardiovascular risk patients could contribute to the benefits of moderate wine consumption against early stages and the progression of atherosclerosis.

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The authors' responsibilities were as follows—RE, CA-L, RML-R, DC, and MG: conceived and designed the research; GC-B, MU-S, RL, and MR-R: conducted the research; GC-B, SA, PV-M, and RE: performed statistical analysis and interpreted data; GC-B, MU-S, and RE: wrote the manuscript; RC, OP, and FT: critically revised and gave final approval of the manuscript; RE: was responsible for the final content of the manuscript and received funding; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

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Erratum

Chiva-Blanch G, Urpi-Sarda M, Llorach R, Rotches-Ribalta M, Guillén M, Casas R, Arranz S, Valderas-Martinez P, Portoles O, Corella D, et al. Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial. Am J Clin Nutr 2012;95:326–34.

An error was identified in the 24-h urinary excretion of total resveratrol metabolites, which was incorrect at baseline and after 3 interventions. The statistical analysis between interventions has now been updated. The aim of this measure was to provide intervention compliance through the calculation of the biomarker of wine consumption. This has been previously defined as the sum of 7 phase II metabolites of resveratrol specified in reference 31. These metabolites are as follows: *trans*- and *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4′-*O*-glucuronide, *trans*- and *cis*-resveratrol-4′-*O*-sulfate, and *trans*- and *cis*-resveratrol-3-*O*-sulfate.

On page 328, the first sentence of the left-hand column of the Methods section should read as follows: "Resveratrol conjugates derived from phase II metabolism were measured in 24-h urine samples by using the validated methodology described by Urpi-Sarda et al (30) quantitatively adapted due to the commercial and available standards. *trans*- and *cis*-Resveratrol-3-*O*-glucuronide (98% purity each), *cis*-resveratrol-4′-*O*-glucuronide (96% purity), and *trans*-resveratrol-3-*O*-sulfate (98% purity) were purchased from Toronto Research Chemicals Inc. *trans*- and *cis*-Resveratrol-4′-*O*-sulfate and *cis*-resveratrol-3-*O*-sulfate were quantified using the *trans*-resveratrol-3-*O*-sulfate calibration curve."

Incorrect values were given on page 329 in the right-hand column of the Results section. The second sentence of the first paragraph should read as follows: "After consumption of RW and DRW, 24-h urinary excretion of total resveratrol metabolites increased in relation to baseline amounts from 0.94 μmol (95% CI: 0.43, 1.46 μmol) to 6.04 μmol (95% CI: 4.76, 7.31 μmol) and 6.28 μmol (95% CI: 5.10, 7.46 μmol), respectively ($P < 0.001$, both). Resveratrol metabolite concentrations were not statistically different after DRW and RW interventions ($P = 1.00$) and were statistically higher after RW and DRW interventions than after gin consumption [0.51 μmol (95% CI: 0.08, 0.94 μmol); $P < 0.001$]. After the gin intervention, the concentration of resveratrol metabolites did not change significantly compared with that at baseline ($P = 1.00$)." Total resveratrol metabolites have been used only as a measure of compliance in this report and were not used in any of the calculations, did not affect the results or discussion of the article, and therefore have no implications for the main results or their interpretation.

doi: 10.3945/ajcn.112.038810.

Erratum

Vrieling A, Kampman E. The role of body mass index, physical activity, and diet in colorectal cancer recurrence and survival: a review of the literature. Am J Clin Nutr 2010;92:471–90.

The DOI was published incorrectly in the September 2010 issue of *The American Journal of Clinical Nutrition* as 10.3945/ajcn.2010.29005. The correct DOI is as follows: 10.3945/ajcn.2009.29005.

doi: 10.3945/ajcn.112.039115.

The American Journal of Clinical Nutrition

RESULTAT 2

Effects of red wine polyphenols and alcohol on glucose metabolism and the lipid profile: a randomized clinical trial.

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Gemma Chiva-Blanch, Mireia Urpi-Sarda, Emilio Ros, Palmira Valderas-Martinez, Rosa Casas, Sara Arranz, Marisa Guillén, Rosa M Lamuela-Raventós, Rafael Llorach, Cristina Andres-Lacueva and Ramon Estruch.

INTRODUCCIÓ

Nombrosos estudis epidemiològics suggereixen que el consum moderat de vi negre redueix la mortalitat cardiovascular i la incidència de diabetis. No obstant, si aquests efectes són causats per l'etanol o els components no alcohòlics del vi negre encara roman indefinit.

OBJECTIU

Comparar els efectes del consum moderat de vi negre, vi negre desalcoholitzat i ginebra en el metabolisme de la glucosa i el perfil lipídic en homes que presenten un elevat risc de patir malalties cardiovasculars.

DISSENY DE L'ESTUDI

Seixanta set homes amb alt risc cardiovascular van ser inclosos en un estudi clínic d'intervenció dietètica aleatoritzat i creuat. Després d'un període de 15 dies, en els que se'ls demanà que no consumissin alcohol, tots els voluntaris van rebre vi negre (30 g alcohol/d), la quantitat equivalent de vi desalcoholitzat o ginebra (30 g alcohol/d) durant quatre setmanes cada intervenció. Abans i després de cada període es van analitzar en dejú la glucosa, insulina, lipoproteïnes, apolipoproteïnes i adipocines plasmàtiques. Amb la glucosa i la insulina es va calcular l'índex HOMA.

RESULTATS

La concentració de glucosa en dejú es va mantenir constant al llarg de l'estudi, mentre que la concentració d'insulina plasmàtica i, en conseqüència, l'índex HOMA van disminuir després del vi negre amb i sense alcohol. El colesterol HDL, l'apolipoproteïna A-I i A-II van augmentar després de les intervencions amb vi negre i ginebra. La lipoproteïna(a) va disminuir després de la intervenció amb vi negre. La concentració plasmàtica de GH, leptina, adiponectina, colesterol total, triglicèrids, colesterol LDL, ApoB, ApoCI i ApoCIII no es van modificar després de cap de les intervencions.

CONCLUSIONS

Els resultats observats suggereixen un efecte beneficiós de la fracció no alcohòlica del vi negre (sobretot polifenols) en la resistència a la insulina, conferint al vi negre majors efectes protectors sobre la patologia cardiovascular que altres begudes alcohòliques.

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Randomized control trials

Effects of red wine polyphenols and alcohol on glucose metabolism and the lipid profile: A randomized clinical trial

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SUMMARY

Background & aims: Epidemiological data suggest that moderate red wine consumption reduces cardiovascular mortality and the incidence of diabetes. However, whether these effects are due to ethanol or to non-alcoholic components of red wine still remains unknown. The aim of the present study was to compare the effects of moderate consumption of red wine, dealcoholized red wine, and gin on glucose metabolism and the lipid profile.

Methods: Sixty-seven men at high cardiovascular risk were randomized in a crossover trial. After a run-in period, all received each of red wine (30 g alcohol/d), the equivalent amount of dealcoholized red wine, and gin (30 g alcohol/d) for 4 week periods, in a randomized order. Fasting plasma glucose and insulin, homeostasis model assessment of insulin resistance (HOMA-IR), plasma lipoproteins, apolipoproteins and adipokines were determined at baseline and after each intervention.

Results: Fasting glucose remained constant throughout the study, while mean adjusted plasma insulin and HOMA-IR decreased after red wine and dealcoholized red wine. HDL cholesterol, Apolipoprotein A-I and A-II increased after red wine and gin. Lipoprotein(a) decreased after the red wine intervention.

Conclusions: These results support a beneficial effect of the non-alcoholic fraction of red wine (mainly polyphenols) on insulin resistance, conferring greater protective effects on cardiovascular disease to red wine than other alcoholic beverages.

www.isrctn.org: ISRCTN88720134.

1. Introduction

Consistent epidemiological data suggest that moderate alcohol consumption is associated with a reduced risk for fatal and nonfatal coronary heart disease and for cardiovascular disease (CVD) mortality, independently of the type of alcoholic beverage consumed.[1] The effect of alcohol in moderation on reducing the incidence of diabetes,[2–4] a strong risk factor for CVD, may be a mediating mechanism. A meta-analysis of 20 cohort studies comprising 477,200 subjects indicated that moderate alcohol consumption (<60 g/d in men and <50 g/d in women) was inversely associated with diabetes risk. The dose–response trend showed that the strongest inverse association was observed for 22–24 g/d.[2] Furthermore, in a meta-analysis of 15 prospective studies, the relative risk of developing type-2 diabetes was lower in moderate alcohol drinkers than in abstainers or heavy drinkers, independently of the type of alcoholic beverage consumed.[3] Nevertheless, in a prospective study in healthy women, an inverse association between moderate alcohol intake and lower diabetes risk was most apparent in those who reported wine or beer drinking compared to women who reported liquor intake.[4]

A salient feature of alcohol consumption is the increase in HDL-cholesterol (HDL-C) and apolipoprotein (Apo) A-I concentrations.[5] HDL-C and ApoA-I positively affect insulin secretion and pancreatic β-cell survival, thereby enhancing insulin sensitivity (IS).[6] Since insulin resistance increases the risk of both CVD and diabetes,[7]

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moderate alcohol consumption could possibly decrease these risks by improving IS. However, clinical trials assessing the short-term effects of moderate consumption of different alcoholic beverages on IS are few and the results are contradictory, as some studies have shown a positive effect[8,9] while most have reported no benefit.[10–14]

Among alcoholic beverages, red wine (RW) is of note because it provides both alcohol and abundant polyphenolic compounds, which are thought to provide additional benefits on lowering CVD risk.[15] To determine the possible differential effects on risk markers of alcohol and polyphenols in RW, dealcoholized red wine (DRW) or grape extracts, which are rich in grape polyphenols but devoid of ethanol, may be used. Consumption of DRW in two studies had no effect on fasting concentrations of lipids and lipoproteins or IS.[12,13] In another study lyophilized grape powder (an analog of a polyphenolic extract of wine) decreased LDL-cholesterol (LDL-C) and ApoB concentrations in women.[16] Furthermore, concentrated red grape juice decreased LDL-C and ApoB and increased HDL-C and ApoA-I concentrations in healthy volunteers as well as in hemodialysis patients,[17] but IS was not assessed in these studies using grape products.[16,17] Thus, it remains unclear whether the protective effects of alcoholic beverages on the risk of CVD and diabetes are due to ethanol or to their non-alcoholic components (mainly polyphenols). Therefore, we designed a randomized clinical trial to compare the effects of moderate alcohol consumption (30 g alcohol/d) through the ingestion of gin, a non-polyphenolic alcoholic beverage, RW, a high polyphenolic alcoholic beverage, and the equivalent amount of DRW, a high-polyphenol non-alcoholic beverage, on IS, serum lipids, and other cardiometabolic markers in subjects at high risk of CVD.

2. Subjects and methods

2.1. Subjects

A total of 73 male moderate alcohol consumers aged between 55 and 75 years were recruited for the study in the outpatient clinic of the Internal Medicine Department of our institution from January 2008 to December 2010. The subjects included were at high risk for CVD because of family history of premature CVD and/or the presence of diabetes, hypertension, dyslipidemia, and overweight/obesity. Exclusion criteria included documented CVD, human immunodeficiency virus infection, chronic liver disease, malnutrition, neoplastic or acute infectious diseases, and customary use of vitamin supplements. Participants were offered free beverages but no monetary compensation. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent.

2.2. Study design and diet monitoring

The study was an open, randomized, controlled, crossover trial with three intervention periods (Fig. 1). Two weeks prior to the study the subjects were asked to maintain their usual diet and to refrain from consuming any alcoholic beverage. Baseline data were collected after this run-in period. Following this, participants were individually randomized by the dietitian in a crossover design among three treatment sequences lasting 4 weeks each, in which the test beverages were provided. Randomization was based on a computer-generated random number table, resulting in six possible diet sequences. A dietitian assigned participants to interventions and instructed them to consume gin (100 mL/day, containing 30 g of ethanol), RW (272 mL/day, containing 30 g of ethanol and 798 mg of total polyphenols), or DRW (272 mL/day, containing 1.14 g of ethanol and 733 mg of total polyphenols). No washout periods were included between the interventions. The phenolic composition of the RW and DRW used in the study is detailed in Table 1. The total phenolic content of the three beverages was determined with the Folin-Ciocalteu method, and the phenolic profile was determined by HPLC-DAD, as described previously.[18] No significant differences were observed in the phenolic content of RW and DRW, while gin contained no detectable phenolic compounds.

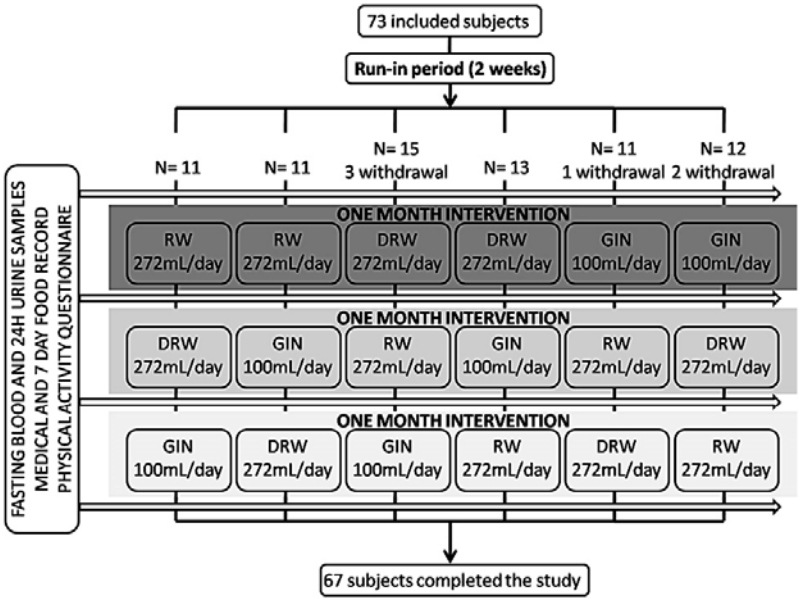

Fig. 1. Flow chart of the subjects included in the study. RW: red wine; DRW: dealcoholized red wine.

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Table 1
Phenolic composition of the beverages used in the study: RW, DRW, and gin.[a]

	RW	DRW	Gin	P^b
Alcohol (%)	14.2	0.42	38	
Phenolic compounds[c]				
Total Phenols (meqGA/L)	2933.35 ± 377.31	2694.92 ± 86.79	ND	0.426
Gallic acid (mg/L)	68.48 ± 6.40	73.17 ± 7.01	ND	0.306
Protocatechuic acid (mg/L)	5.22 ± 0.62	5.85 ± 0.51	ND	0.246
Tyrosol (mg/L)	43.59 ± 4.73	47.81 ± 3.90	ND	0.298
Catechin (mg/L)	123.51 ± 11.30	126.45 ± 13.35	ND	0.786
Epicatechin (mg/L)	67.86 ± 7.74	70.57 ± 8.22	ND	0.699
trans-Caftaric (mg/L)	18.62 ± 1.45	19.21 ± 1.62	ND	0.595
trans-Caffeic (mg/L)	11.50 ± 0.79	12.18 ± 0.92	ND	0.246
trans-Coutaric (mg/L)	5.21 ± 0.45	5.62 ± 0.52	ND	0.182
2-S-Glutationylcaftaric (mg/L)	10.30 ± 1.00	10.76 ± 1.26	ND	0.956
Quercetin-3-glucuronide (mg/L)	11.88 ± 1.38	11.25 ± 1.42	ND	0.770
Quercetin (mg/L)	26.66 ± 0.78	23.82 ± 2.37	ND	0.161
Isorhamnetin (mg/L)	3.34 ± 0.27	2.96 ± 0.14	ND	0.114
Delphinidin-3-glucoside (mg/L)	15.25 ± 0.89	14.71 ± 1.62	ND	0.589
Petunidin-3-glucoside (mg/L)	12.29 ± 1.06	12.04 ± 1.15	ND	0.755
Peonidin-3-glucoside (mg/L)	6.78 ± 0.62	6.68 ± 0.57	ND	0.797
Malvidin-3-glucoside (mg/L)	48.83 ± 4.45	49.86 ± 4.27	ND	0.787
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.97 ± 0.96	10.41 ± 1.20	ND	0.563
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	4.15 ± 0.27	3.54 ± 0.33	ND	0.066
trans-resveratrol (mg/L)	2.92 ± 0.36	2.73 ± 0.23	ND	0.352
cis-resveratrol (mg/L)	2.79 ± 0.15	2.75 ± 0.15	ND	0.761
trans-piceid (mg/L)	9.41 ± 1.12	10.53 ± 0.96	ND	0.160
cis-piceid (mg/L)	7.71 ± 0.34	7.08 ± 0.87	ND	0.226

[a] DRW, dealcoholized red wine; mEqGA/L, mEq Gallic acid/L; ND, not detected; RW, red wine.
[b] *P* values are for comparison between RW and DRW polyphenols (Student's *t* test for independent samples).
[c] Mean ± SD ($n = 2$) (all such values).

Throughout the study the participants were asked to maintain their usual dietary habits, physical activity level and medications and to abstain from alcohol-free beer or alcoholic beverages except for those provided by the investigators. Natural foods rich in antioxidants, especially fruit and vegetables, were carefully monitored in order to achieve a similar dietary antioxidant content during the interventions. Participants were not blinded to the type of drink they ingested. After the run-in period and the day after each intervention period, a medical record and the Minnesota Leisure Time Physical Activity Questionnaire, which has been validated in Spain, were administered. In addition, during the last week of the run-in period and the last week of each intervention period, subjects were asked to fill in a validated 7-d food record questionnaire of 5 week-days and 2 week-end days (Fig. 1). The food records were used to assess nutrient intake and to monitor adherence to the study protocol. Compliance with the test drinks was also assessed by measures of urinary biomarkers of both alcohol and polyphenol intake. Foods were converted into nutrients by using the Food Processor Nutrition and Fitness Software (*esha* Research, Salem, OR), adapted to local foods. At the end of the study, a clinician assessed any possible adverse effects from the interventions by administering a checklist of symptoms.

2.3. Clinical and laboratory measurements

Fasting blood and 24-h urine samples were collected at baseline and after each intervention. Serum, EDTA-plasma, and urine samples were stored with a blinded code at −80 °C until assayed. The clinical investigators and laboratory technicians were blinded to the interventions. For each subject, the parameters determined in thawed samples of whole serum or plasma, as appropriate, were as follows: blood glucose with the glucose oxidase method; cholesterol and triglycerides with enzymatic procedures; HDL cholesterol after precipitation with phosphotungstic acid and magnesium chloride; and homocysteine and vitamin B_{12} by an automated electrochemiluminiscence immunoassay system (Advia-Centaur, Siemens, Barcelona, Spain). Plasma ApoA-I, ApoA-II, ApoB, ApoC-I, ApoC-III, lipoprotein(a), growth hormone (GH), insulin, adiponectin and leptin concentrations were quantified in whole serum samples by a customized Human Multi Analyte Profiling assay (Human MAP, Rules Based Medicine Inc., Austin, TX). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by multiplying the fasting insulin concentration (mIU/L) by the fasting glucose concentration (mM) and dividing by 22.5.[19] The effects of the interventions on inflammatory biomarkers related to atherosclerosis have been published elsewhere.[18]

Ethylglucuronide, a biomarker of alcohol intake, was measured in 24-h urine samples by liquid chromatography, performed on an Agilent 1200 apparatus coupled with a hybrid quadrupole time-of-flight QSTAR Elite (Applied Biosystems/MDS Sciex). Resveratrol metabolites from phase II metabolism, as a biomarker of RW and DRW intake,[20] were also measured in 24-h urine samples using the validated methodology of Urpi-Sarda et al.[21] with slight modifications.[18]

2.4. Statistical analyses

Sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a maximum loss of 10% participants. To detect mean differences for the HOMA Index of 0.1 with a conservative SD of 0.15, 26 subjects would be needed to complete the study (α risk = 0.05, power = 0.9). However, to obtain greater differences, the sample size was more than doubled. The HOMA Index was used to determine the sample size, but changes in all endpoints were of equal interest.

Statistical analyses were performed using SAS Statistical Analysis Systems (version 9.2, SAS Institute Inc, Cary, North Carolina). Descriptive statistics [mean ± SD or *n* (%)] were used to describe the baseline characteristics of the participants and the outcome variables. Variables with a skewed distribution [GH, Insulin and lipoprotein(a)] were transformed to their natural logarithms for analyses and are shown as antilogarithmic values to facilitate the interpretation of the results. To compare changes in outcome variables from baseline in response to the interventions, one-factor analysis of variance (ANOVA) for repeated measures was used. To compare among-treatment changes in outcome variables, analysis of covariance (ANCOVA) for repeated measures with the previous intervention value (or the baseline value in the first intervention) as the covariate was used. To exclude the presence of a carryover effect for the three periods, the interaction between the type of treatment (RW, DRW and G) and the period sequence (1st, 2nd or 3rd) was analyzed in the repeated measures ANCOVA analyses. The Bonferroni post hoc test for multiple comparisons was used in both the ANOVA and ANCOVA analyses. For urinary biomarkers, results are expressed as means and 95% CIs for the among-intervention differences in the ANOVA analyses. *P* was considered significant when <0.05.

3. Results

3.1. Characteristics of study subjects

Of the 73 subjects included, six withdrew before completing the study. The reasons for withdrawal were intercurrent illness ($n = 2$), need to travel ($n = 2$), and refusal to drink DRW after tasting it

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($n = 2$). Therefore, 67 subjects completed the study. The baseline characteristics of the completers (Table 2) shows that they had a sizeable burden of cardiovascular risk factors, with a high prevalence of family history of early-onset CVD, hypertension, overweight or obesity (with a maximum BMI of 40.5 kg/m^2) and ≈25% prevalence of smoking, dyslipidemia, and diabetes. None of the diabetics was treated with insulin. The baseline characteristics of non-completers were similar to those of completers (independent samples Mann–Whitney U test; $P > 0.170$, all).

3.2. Measures of compliance and dietary control

There were no individual deviations from the interventions according to the participants' dietary reports. Urinary ethylglucuronide concentrations increased significantly after the RW and gin periods compared to baseline and DRW (Table 3). In addition, after consumption of RW and DRW, the 24-h urinary excretion of total resveratrol metabolites was higher than after the gin intervention and the baseline values (Table 3). According to these findings, compliance with the three interventions was excellent.

No significant differences in energy and nutrient intake (Table 4) or energy expenditure in physical activity were observed before and after each intervention according to food records and physical activity questionnaires. No individual changes in drug intake were reported and no adverse effects were observed. No carryover effect was observed.

3.3. Effects on glucose metabolism and adipokines

Changes in glucose metabolism are shown in Table 5. Fasting glucose concentrations did not change in any intervention, while the mean adjusted insulin values decreased significantly after the RW and DRW interventions compared to both baseline (21% and 20%, respectively) and the gin period (15% and 13%, respectively). Therefore, HOMA-IR decreased 30% and 22% from baseline after the RW and DRW interventions, respectively, as well as in comparison to gin (22% and 14%, respectively). On exclusion of the 15 participants with diabetes the results remained practically unchanged (adjusted HOMA-IR 1.47 ± 0.07, 1.08 ± 0.06, 1.13 ± 0.06, and 1.32 ± 0.07 at baseline and after RW, DRW, and gin, respectively). No significant changes were observed in GH, leptin and adiponectin from baseline or among the three interventions.

Table 2
Baseline characteristics of the 67 study subjects.

	Mean ± sd
Age (years)	60 ± 8
Family history of premature CAD (n, (%))	52 (77.6)
Current smokers (n, (%))	16 (23.9)
Hypertension (n, (%))	38 (56.7)
Dyslipidemia (n, (%))	16 (23.9)
Type-2 diabetes (n, (%))	15 (22.4)
Sedentariness (n, (%))	40 (59.7)
Body mass index (BMI) (kg/m^2)	29.6 ± 3.9
Overweight or obese (BMI>25 kg/m^2) (n, (%))	61 (91.0)
Waist-to-hip ratio	0.97 ± 0.05
Systolic blood pressure (mm Hg)	142 ± 18
Diastolic blood pressure (mm Hg)	81 ± 8
Heart rate (beats/min)	69 ± 10
Drug treatment	
Statins (n, (%))	22 (32.8)
ACE Inhibitors (n, (%))	28 (41.8)
Diuretics (n, (%))	5 (7.5)
Oral hypoglycemic drugs (n, (%))	14 (20.9)
Sulfonylurea (n, (%))	2 (3.0)
Biguanide (n, (%))	8 (11.9)
Combination of both (n, (%))	4 (6.0)
Antiplatelet agents (n, (%))	15 (22.4)

Data ($n = 67$) are expressed as mean ± SD or n (%) when indicated.
CAD: Coronary Artery Disease.

Table 3
Changes in urinary resveratrol metabolites and ethylglucuronide after the three interventions in the 67 study subjects.

	Mean percentage of increase from baseline (95% Confidence Interval)			
	Red wine	Dealcoholized red wine	Gin	P
Resveratrol metabolites	549% (401%, 1011%)[a]	565% (411%, 1089%)[a]	−46% (−36%, −82%)[b]	<0.0001
Ethylglucuronide	342% (245%, 773%)[a]	−66% (64%, 75%)[b]	256% (179%, 599%)[a]	<0.0001

Results are expressed as mean percentage of increase compared to baseline (95% CI) ($n = 67$). Changes in outcome variables in response to the intervention treatment were analyzed by repeated-measures ANOVA. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).

3.4. Effects on lipoproteins and apolipoproteins

Changes in the lipid profile are also detailed in Table 5. The mean adjusted LDL-C concentrations decreased 4.5% from baseline after the RW intervention. HDL-C concentrations increased from baseline after the RW and gin periods and in comparison to the DRW intervention (7% and 5%, respectively, for both). Therefore, the LDL/HDL ratio decreased by 8% and 5% after the RW and gin interventions compared to the DRW period and after the RW intervention compared to baseline (7%). The mean adjusted lipoprotein(a) concentration decreased by 12% after RW compared to DRW and gin. ApoA-I and ApoA-II concentrations increased in parallel after RW and gin compared to DRW (12% for ApoA-I both, and 12% and 8% for ApoA-II), but only ApoA-II increased from baseline after RW and gin (9% and 5%, respectively). The mean adjusted ApoB concentration decreased by 5% after DRW compared to baseline. No significant changes were observed in total cholesterol, triglycerides, ApoC-I, and ApoC-III from baseline and among the three interventions.

3.5. Changes in other cardiovascular risk factors and B_{12} vitamin

As shown in Table 5, no significant changes were observed for homocysteine, and vitamin B_{12}. The mean adjusted serum folic acid concentrations were lower compared to baseline after RW (−12%) and DRW (−11%) and versus the gin period (−18% and −17%, respectively).

4. Discussion

The main findings of our study are that RW rich in polyphenols with or without alcohol (RW and DRW interventions) but not gin, an alcoholic beverage devoid of polyphenols, improved glucose metabolism, as measured by HOMA-IR, and that RW but not DRW or gin decreased lipoprotein(a) in men at high cardiovascular risk.

The results of the few prior clinical studies examining the effects of moderate alcohol consumption on IS have been inconsistent.[8 14] Two studies reported no significant improvement in IS after 17 days of whisky in 23 healthy men[11] or 4 weeks of RW or DRW in 17 healthy men.[12] A third study compared RW and vodka for 8 weeks in 20 insulin-resistant individuals and found little improvement of IS with either beverage.[14] In contrast, a fourth study[9] reported a 43% improvement in IS after 2 weeks of RW in 9 diabetic men. In the

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Table 4
Daily energy and dietary intakes in the 67 subjects studied at baseline and after the three interventions.[a]

	Mean ± SD				P^c
	Baseline[b]	RW intervention[c]	DRW intervention[c]	G intervention[c]	
Energy (kcal/d)	1816 ± 457	1782 ± 325	1862 ± 320	1887 ± 336	0.109
Total protein (g/d)	90.39 ± 20.60	89.06 ± 18.35	94.29 ± 17.56	95.04 ± 18.73	0.133
Carbohydrates (g/d)	193 ± 51	201 ± 44	193 ± 38	206 ± 40	0.111
Dietary fiber (g/d)	19.67 ± 9.83	20.63 ± 8.08	19.88 ± 6.82	22.05 ± 10.01	0.254
Soluble fiber (g/d)	5.68 ± 0.53	5.45 ± 0.31	5.94 ± 0.40	5.99 ± 0.41	0.533
Sugars (g/d)	62.89 ± 23.55	66.74 ± 25.59	67.33 ± 20.08	70.11 ± 20.84	0.489
Total lipids (g/d)	74.87 ± 24.91	72.72 ± 16.27	78.55 ± 20.41	79.86 ± 21.89	0.194
SFA (g/d)	18.65 ± 8.66	18.40 ± 7.06	18.90 ± 6.15	19.01 ± 5.48	0.804
MUFA (g/d)	36.85 ± 12.06	35.84 ± 8.23	37.97 ± 10.18	38.31 ± 9.60	0.522
PUFA (g/d)	10.31 ± 3.89	10.65 ± 4.21	11.94 ± 4.42	10.71 ± 3.29	0.644
Trans fatty acids (g/d)	1.35 ± 0.94	1.28 ± 0.66	1.07 ± 0.59	1.17 ± 0.62	0.171
Cholesterol (mg/d)	346 ± 142	355 ± 125	342 ± 103	360 ± 162	0.710
Vitamin C (mg/d)	112 ± 75	125 ± 83	121 ± 68	133 ± 91	0.534
Vitamin A (μgRE/d)	640 ± 360	688 ± 340	729 ± 343	791 ± 481	0.345
Vitamin E (mg/d)	9.49 ± 3.88	9.65 ± 3.22	9.47 ± 3.34	10.29 ± 4.65	0.153
Folic acid (μg/d)	426 ± 198	454 ± 155	443 ± 143	498 ± 226	0.181
Magnesium (mg/d)	333 ± 107	345 ± 92	339 ± 95	342 ± 106	0.768
Manganese (mg/d)	2.78 ± 1.38	2.66 ± 1.09	2.72 ± 1.26	2.82 ± 1.18	0.683
Potassium (mg/d)	3071 ± 988	3119 ± 673	3089 ± 850	3161 ± 955	0.815
Selenium (μg/d)	136 ± 38	139 ± 37	136 ± 38	136 ± 33	0.822
Zinc (mg/d)	10.42 ± 2.60	10.48 ± 2.42	9.56 ± 2.40	10.40 ± 2.21	0.136
Total polyphenols (mg/d)	340 ± 185	318 ± 142	311 ± 146	327 ± 170	0.555

[a] Excluding the energy, nutrient and total polyphenol contributions from the interventions. Results are expressed as mean ± SD ($n = 67$).
[b] Observed mean.
[c] Adjusted mean and P value of the ANCOVA analysis. Changes in outcome variables in response to the interventions were analyzed by repeated-measures ANCOVA with the value of the previous intervention as covariate. No changes were observed between the baseline and the interventions determined by repeated-measures ANOVA ($P > 0.05$, all).

female population, short-term consumption of RW in middle-aged overweight women[10] or of RW and DRW in postmenopausal women[13] had no benefit on IS. In another trial in 51 postmenopausal women,[8] consumption of 30 g/d alcohol (ethanol in orange juice) was associated with a 7.2% improvement in IS compared with 0 g/d, while 15 g/d had no effect. In our study both RW and DRW improved IS which, together with prior findings, suggests that both ethanol and polyphenols are responsible for this beneficial effect. Nonetheless, further well-controlled clinical trials are required to prove this contention.

Table 5
Changes in glucose control, lipid profile and other cardiovascular risk factors after the three interventions in the 67 study subjects.

	Mean ± SD				P^c
	Baseline[a]	Red wine[b]	Dealcoholized red wine[b]	Gin[b]	
Glucose control and adipokines					
Glucose [mmol/L (mg/dL)]	6.16 ± 1.89 (111 ± 34)	6.10 ± 1.94 (110 ± 35)	6.05 ± 1.89 (109 ± 34)	6.16 ± 1.83 (111 ± 33)	0.25
Insulin [pmol/L (μIU/mL)]	35 ± 2.01 (5.15 ± 0.29)	28 ± 1.39 (4.04 ± 0.20)^{A,d}	29 ± 1.46 (4.13 ± 0.21)^{A,d}	33 ± 1.46 (4.74 ± 0.21)^{B}	0.035
HOMA-Insulin resistance	1.52 ± 0.06	1.07 ± 0.06^{A,d}	1.18 ± 0.06^{A,d}	1.38 ± 0.06^{B}	0.008
Growth hormone (μg/L or ng/mL)	0.54 ± 0.10	0.56 ± 0.10	0.66 ± 0.11	0.49 ± 0.10	0.63
Leptin (μg/L or ng/mL)	11.31 ± 1.09	11.28 ± 0.97	10.35 ± 1.03	10.90 ± 0.95	0.21
Adiponectin [μg/L (μg/mL)]	3270 ± 90 (3.27 ± 0.09)	3220 ± 90 (3.22 ± 0.09)	3210 ± 80 (3.21 ± 0.08)	3310 ± 90 (3.31 ± 0.09)	0.46
Lipids, lipoproteins and apolipoproteins					
Total cholesterol [mmol/L (mg/dL)]	5.28 ± 0.85 (204 ± 33)	5.23 ± 0.88 (202 ± 34)	5.08 ± 0.83 (196 ± 32)	5.15 ± 0.91 (199 ± 35)	0.16
Triglycerides [g/L (mg/dL)]	1.28 ± 0.60 (128 ± 60)	1.31 ± 0.60 (131 ± 60)	1.25 ± 0.58 (125 ± 58)	1.24 ± 0.61 (124 ± 61)	0.28
LDL-cholesterol [mmol/L (mg/dL)]	3.44 ± 0.83 (133 ± 32)	3.29 ± 0.72 (127 ± 28)^{d}	3.37 ± 0.65 (130 ± 25)	3.31 ± 0.72 (128 ± 28)	0.64
HDL-cholesterol [mmol/L (mg/dL)]	1.11 ± 0.18 (43 ± 7)	1.19 ± 0.23 (46 ± 9)^{A,d}	1.11 ± 0.26 (43 ± 10)^{B}	1.16 ± 0.26 (45 ± 10)^{A,d}	0.002
LDL/HDL ratio	3.08 ± 0.10	2.86 ± 0.09^{A,d}	3.10 ± 0.09^{B}	2.94 ± 0.10^{A}	0.001
Lipoprotein(a) [μmol/L (mg/dL)]	1.94 ± 0.38 (54.4 ± 10.6)	1.79 ± 0.42 (50.2 ± 11.9)^{A}	2.04 ± 0.41 (57.2 ± 11.4)^{B}	2.05 ± 0.41 (57.4 ± 11.4)^{B}	0.012
Apolipoprotein A-I [g/L (mg/dL)]	7.54 ± 0.18 (754 ± 18)	8.02 ± 0.17 (802 ± 17)^{A}	7.13 ± 0.17 (713 ± 17)^{B}	8.03 ± 0.16 (803 ± 16)^{A}	0.009
Apolipoprotein A-II(μg/L or ng/mL)	318 ± 9	347 ± 9^{A,d}	309 ± 9^{B}	334 ± 10^{A,d}	0.013
Apolipoprotein B [g/L (mg/dL)]	1.10 ± 0.02 (110 ± 2)	1.08 ± 0.01 (108 ± 1)	1.05 ± 0.01 (105 ± 1)^{d}	1.06 ± 0.01 (106 ± 1)	0.86
Apolipoprotein C-I (g/L or ng/mL)	254 ± 6	258 ± 5	245 ± 5	247 ± 5	0.45
Apolipoprotein C-III [g/L (μg/mL)]	0.155 ± 0.006 (155 ± 6)	0.159 ± 0.006 (159 ± 6)	0.149 ± 0.007 (149 ± 7)	0.152 ± 0.006 (152 ± 6)	0.56
Other cardiovascular risk factors					
Homocysteine [μmol/L (mg/L)]	12.1 ± 0.2 (1.64 ± 0.03)	12.6 ± 0.2 (1.70 ± 0.03)	12.4 ± 0.2 (1.68 ± 0.03)	12.3 ± 0.2 (1.66 ± 0.03)	0.56
Vitamin B_{12} [pmol/L (pg/mL)]	300 ± 120 (406 ± 163)	281 ± 127 (381 ± 172)	284 ± 117 (385 ± 159)	290 ± 138 (393 ± 187)	0.31
Folic acid [nmol/L (ng/mL)]	22 ± 9 (9.8 ± 4)	18 ± 9 (8.0 ± 4)^{A,d}	18 ± 9 (8.1 ± 4)^{A,d}	21 ± 9 (9.1 ± 4)^{B}	0.001

Results are expressed as mean ± SD ($n = 67$).
[a] Observed mean.
[b] Adjusted mean by the ANCOVA analyses.
[c] P value of the ANCOVA analysis. To compare the effects of the different interventions, changes in outcome variables in response to the interventions were analyzed by repeated-measures ANCOVA with the value of the previous intervention as covariate. Values in a row with different superscript capital letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). To compare differences between each intervention and the baseline value a repeated-measures ANOVA and the Bonferroni post hoc test for multiple comparisons was performed.
[d] Significantly different from baseline ($P < 0.05$).

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The effects of moderate alcohol consumption on plasma lipoprotein(a), an independent risk factor for CVD and atherosclerosis, with a strong genetic basis that is relatively refractory to both lifestyle and drug intervention,[22] are controversial. A recent meta-analysis concluded that moderate alcohol consumption had no effect on lipoprotein(a).[5] A similar conclusion was reached in another study using RW for 4 weeks in healthy subjects.[23] However, in a clinical trial performed in healthy men, RW but not gin was associated with reduced lipoprotein(a)[24] and in another study lipoprotein(a) decreased after 10 days of RW but not white wine.[25] Furthermore, social alcohol consumption was associated with lower plasma lipoprotein(a) in a cross-sectional study of middle aged Finnish men.[26] In our study mean adjusted lipoprotein(a) was reduced by 12% after RW (ethanol plus polyphenols) but not after the DRW or gin interventions. This reduction led to a lipoprotein(a) concentration that coincides with the margin between very high and high risk of cardiovascular disease (1.785 μmol/L; 50 mg/L), limiting the clinical relevance of the observed results. Nonetheless, given the paucity of effective therapy for elevated lipoprotein(a), the potential lowering efficacy of ethanol and/or polyphenols deserves further research.

In the present study, LDL-C and ApoB were slightly, albeit significantly, reduced from baseline after RW and DRW, respectively. However, post-treatment LDL-C and ApoB values were similar across the three interventions, although the oxidized LDL fraction may differ between the interventions, as red wine polyphenols decrease postpandrial lipid oxidation.[27] As expected,[5] moderate consumption of RW and G, but not DRW, increased plasma HDL-C and ApoA-I and ApoA-II concentrations and decreased the LDL/HDL ratio. This increase in Apo-II concurs with data from a prior study of RW in healthy men and women.[23] Therefore, the beneficial effects of moderate RW intake on lipid and lipoprotein metabolism are dependent on its alcohol component. Nevertheless, regarding glucose metabolism, we observed that the increased IS (in the interventions with polyphenols) does not correlate with the increase in HDL-C and ApoA-I concentrations (in the interventions with alcohol), as proposed by von Eckardstein et al.[6]

Regarding the effects of alcohol on plasma homocysteine, Gibson et al.[28] and Marcucci et al.[29] found that RW increased its plasma concentrations. No differences in homocysteine were observed after the three interventions in our study. Nevertheless, after RW and DRW consumption, we observed a significant decrease in serum folic acid, albeit within the physiological range. This can be explained by the fact that some RW polyphenols inhibit intestinal folate uptake.[30]

This study has some limitations. First, consumption of alcohol was not blinded, which is difficult to achieve given the known physiologic effects and distinct taste of alcoholic beverages. Second, our study sample was made up of older men at high cardiovascular risk, thus the results may not be extrapolated to other populations. Third, gin may contain some bioactive aromatic and other substances derived from the aging process. We measured the total polyphenols in gin and these were under the lower limit of detection (Table 1). Given the low bioavailability of these compounds it seems reasonable to assume that gin contains only ethanol and no other interfering substances. In addition, DRW contains 0.42% of alcohol (Table 1). This means that individuals ingested approximately 1 g ethanol/day during that intervention. This limitation could be avoided by administering grape or red fruit juice but another limitation would be added. DRW has the same phenolic composition as red wine, whereas the juices do not have exactly the same composition and phenolic profile as red wine, adding more confounding variables to the analyses and impeding comparison of the effects of red wine polyphenols in an alcoholic and a non-alcoholic matrix. Lastly, our study duration of 4 weeks may not represent the potential beneficial effects of long-term moderate alcohol consumption.

In conclusion, while ethanol itself exerts a protective effect on the lipid profile, the non-alcoholic fraction of RW (mainly polyphenols) has a beneficial effect on insulin resistance and RW appears to decrease lipoprotein(a) plasma concentrations. These findings suggest that RW has greater protective effects than other alcoholic beverages on cardiovascular risk.

Conflict of interest

Dr. Ramon Estruch and Rosa M Lamuela-Raventos are members of the FIVIN (Foundation for the study of wine and nutrition), and in the past they received grants from this foundation and the Spanish Foundation of Beer and Health. Nevertheless, these foundations had no involvement in the study design, the collection, analysis and interpretation of data, the writing of the manuscript or the decision to submit the manuscript for publication. The other authors declare no conflict of interests.

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Statement of authorship

RE, CA-L, RML-R and MG were involved in the conception and design of the research; GC-B, MU-S and RL in the conduction of the research; GC-B, ER, SA, PV-M and RE in the statistical analysis and interpretation of data; GC-B, MU-S, ER and RE wrote the paper and RC, MG, RML-R and CA-L, critically revised and finally approved the manuscript.

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RESULTAT 3

Dealcoholized Red Wine Decreases Systolic and Diastolic Blood Pressure and Increases Plasma Nitric Oxide.

Circ Res. 2012;111:1065-1068.

Gemma Chiva-Blanch, Mireia Urpi-Sarda, Emilio Ros, Sara Arranz, Palmira Valderas-Martínez, Rosa Casas, Emilio Sacanella, Rafael Llorach, Rosa M Lamuela-Raventos, Cristina Andres-Lacueva and Ramon Estruch.

INTRODUCCIÓ

Alguns estudis experimentals han observat un potencial efecte reductor de la pressió arterial per part dels polifenols del vi negre. No obstant, els efectes del consum moderat d'alcohol i polifenols en la pressió arterial als humans encara no està dilucidat.

OBJECTIU

Avaluar els efectes de les fraccions del vi (alcohòlica i no alcohòlica) en la pressió arterial i l'òxid nítric plasmàtic en homes que presenten un elevat risc de patir malalties cardiovasculars.

DISSENY DE L'ESTUDI

Seixanta set homes amb alt risc cardiovascular van ser inclosos en un estudi clínic d'intervenció dietètica aleatoritzat i creuat. Després d'un període de 15 dies, en els que se'ls demanà que no consumissin alcohol, tots els voluntaris van rebre vi negre (30 g alcohol/d), la quantitat equivalent de vi desalcoholitzat o ginebra (30 g alcohol/d) durant quatre setmanes cada intervenció. Abans i després de cada període es van analitzar la pressió arterial, l'òxid nítric plasmàtic i els paràmetres antropomètrics.

RESULTATS

La pressió sistòlica i diastòlica van disminuir significativament després de la intervenció amb vi negre desalcoholitzat i aquests canvis es correlacionaren amb un augment de la concentració plasmàtica d'òxid nítric. La pressió sistòlica i diastòlica van disminuir i l'òxid nítric va augmentar, encara que no significativament, després de la intervenció amb vi negre, i també comparat amb a la ginebra encara que tampoc significativament. El consum moderat de ginebra no va tenir cap efecte en la pressió arterial ni en la concentració plasmàtica d'òxid nítric. L'índex de massa corporal, la relació cintura/cadera i la freqüència cardíaca no es van veure modificats al llarg de l'estudi.

CONCLUSIONS

El vi negre desalcoholitzat disminueix la pressió sistòlica i diastòlica a través d'un augment de l'òxid nítric plasmàtic. El consum diari de vi negre desalcoholitzat podria ser útil per la prevenció de la hipertensió baixa- moderada.

Clinical/Translational Research

Dealcoholized Red Wine Decreases Systolic and Diastolic Blood Pressure and Increases Plasma Nitric Oxide

Short Communication

Gemma Chiva-Blanch, Mireia Urpi-Sarda, Emilio Ros, Sara Arranz, Palmira Valderas-Martínez, Rosa Casas, Emilio Sacanella, Rafael Llorach, Rosa M. Lamuela-Raventos, Cristina Andres-Lacueva, Ramon Estruch

Rationale: **Experimental studies have shown a potential blood pressure (BP) lowering effect of red wine polyphenols, whereas the effects of ethanol and polyphenols on BP in humans are not yet clear.**

Objective: **The aim of the present work was to evaluate the effects of red wine fractions (alcoholic and nonalcoholic) on BP and plasma nitric oxide (NO) in subjects at high cardiovascular risk.**

Methods and Results: **Sixty-seven men at high cardiovascular risk were studied. After a 2-week run-in period, subjects were randomized into 3 treatment periods in a crossover clinical trial, with a common background diet plus red wine (30g alcohol/day), the equivalent amount of dealcoholized red wine, or gin (30g alcohol/day), lasting 4 weeks each intervention. At baseline and after each intervention, anthropometrical parameters, BP and plasma NO were measured. Systolic and diastolic BP decreased significantly after the dealcoholized red wine intervention and these changes correlated with increases in plasma NO.**

Conclusions: **Dealcoholized red wine decreases systolic and diastolic BP. Our results point out through an NO-mediated mechanism. The daily consumption of dealcoholized red wine could be useful for the prevention of low to moderate hypertension. Trial registered at controlled-trials.com: ISRCTN88720134. (*Circ Res*. 2012;111:1065-1068.)**

Key Words: red wine ■ polyphenols ■ alcohol ■ blood pressure ■ nitric oxide

Epidemiological evidence has associated moderate alcohol consumption with decreased cardiovascular risk.[1] However, red wine (RW) seems to confer greater protective effects because of its high polyphenolic content. In vitro and experimental studies have shown a potential blood pressure (BP)-lowering effect and/or enhancement of endothelial nitric oxide (NO) production by RW.[2] It is unclear whether these effects can be extrapolated to humans, because the amount of RW polyphenols used in these studies is usually higher than that achieved through moderate RW consumption. Recently, small amounts of RW, but not other alcoholic beverages, were shown to increase plasma NO concentrations.[3] Although the negative effects of heavy or binge alcohol drinking on BP are well known, the effects of moderate alcohol consumption are controversial, because some studies have observed a linear trend and others a nonlinear or J-shaped association, independently of the beverage consumed.[4–6] Therefore, the aim of the present study was to evaluate the effects of RW fractions (alcoholic and nonalcoholic) on BP and plasma NO concentration in high cardiovascular risk subjects.

Editorial, see p 959

Methods

The study was an open, randomized, crossover, controlled clinical trial comprising three 4-week periods. Detailed Methods have been published[7] and are provided in the Online Data Supplement.

Seventy-three men at high cardiovascular risk, aged between 55 and 75 years were included in the study. All subjects had diabetes mellitus or ≥3 cardiovascular disease risk factors.[7] After a 2-week run-in period wherein subjects were asked not to consume any alcoholic beverage, they were randomized using a computer-generated table into 3 treatments in a crossover design, with a common background diet plus gin (100 mL, 30 g ethanol/day), RW (272 mL, 30 g ethanol/day; total polyphenols: 798 Eq Gallic Acid/day, EGA/day), and the same amount of polyphenols as RW in the form of dealcoholized red

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Non-standard Abbreviations and Acronyms	
BP	blood pressure
DBP	diastolic blood pressure
DRW	dealcoholized red wine
NO	nitric oxide
RW	red wine
SBP	systolic blood pressure

wine (DRW) (272 mL - total phenols: 733 EGA/day), resulting in 6 possible beverage sequences lasting 4 weeks each intervention. No washout periods were included between the interventions.

After the run-in period (baseline) and the day after each intervention period (RW, DRW, and gin), BP and heart rate were measured 3 times at 5-minute intervals on the nondominant arm with an oscillometer (Omron 705 CP; Omron Matsusaka Co Ltd, Matsusaka City, Japan) after 15 minutes resting in a seated position. The mean of the second and the third measures was considered for statistical analysis.

Fasting blood samples for the NO analyses were collected at baseline and after each intervention, and stored at −80°C until assayed. For measurement of NO, the release of NO_2^- and NO_3^-, the stable breakdown products of NO in plasma, were determined by a chemiluminescence detector in an NO analyzer (Sievers Instruments, Boulder, CO).

Statistical analyses were performed using the Statistical Analysis Systems (version 9.2, SAS Institute Inc, Cary, NC). To analyze the changes within each treatment, a Student *t* test for paired samples was performed between the data obtained before and after each intervention. One-factor ANOVA for repeated measures and the Bonferroni post-hoc test were used to compare the differences of the changes in outcome variables between the interventions. See the Online Data Supplement for further details of statistical analyses.

Table 1. Baseline Characteristics of the Study Subjects

Characteristic	Mean±SD*
Age, y	60±8
Current smokers [n (%)]	16 (23.9)
Sedentarism [n (%)]	40 (59.7)
Family history of premature CHD n (%)	52 (77.6)
Type-2 diabetes [n (%)]	15 (22.4)
Hypertension [n (%)]	38 (56.7)
Dyslipemia [n (%)]	16 (23.9)
Medications [n (%)]	
ACE inhibitors	28 (41.8)
Diuretics	5 (7.5)
Statins	22 (32.8)
Oral hypoglycemic drugs	14 (20.9)
Aspirin or antiplatelet drugs	15 (22.4)
Triglycerides (mg/dL)	128±60
Total cholesterol (mg/dL)	204±33
LDL-cholesterol (mg/dL)	133±32
HDL-cholesterol (mg/dL)	43±7
LDL/HDL ratio	3.08±0.10

*Mean+SD or n (%), when indicated (n=67).
CHD indicates coronary heart disease; ACE, angiotensin-converting enzyme.

Results

The baseline characteristics of the 67 subjects who completed the study are detailed in Table 1. Reasons for exclusion of 6 participants are described in the Online Data Supplement. No significant differences in body mass index, waist-to-hip ratio, and heart rate were observed (Table 2). Systolic BP (SBP) and diastolic BP (DBP) decreased significantly after the DRW intervention (P=0.0001 and 0.017, respectively) (Figure). These changes were significantly different from those observed after the gin intervention (P=0.026 and 0.045 for SBP and DPB, respectively) (Table 2). Plasma NO concentration increased after the DRW intervention (P=0.041) and the change was also significantly different from that observed after the gin intervention (P=0.026). The changes in BP and NO after the DRW period were correlated (r=0.598; P<0.001 and r=0.362; P=0.002 for SBP and DBP, respectively; Online Figure I). The intervention with RW did not differ from the DRW and gin interventions, although SBP and DBP tended to decrease and NO tended to increase after the RW intervention compared to the gin period (P=0.069, 0.075, and 0.079 for SBP, DBP, and NO, respectively). In addition, changes in SBP correlated with changes in NO after the RW intervention (r=0.251, P=0.035). Exclusion of participants with hypertension or antihypertensive treatment did not materially change the results (Online Table I). Intervention compliance and dietary data during the three interventions are also shown in the Online Data Supplement. No carryover effect was observed for any outcome.

Discussion

After the 4-week interventions with RW, DRW, and gin in a crossover study in high cardiovascular risk subjects, we observed that DRW decreased SBP and DBP while increasing plasma NO concentration. RW tended to have similar effects to those of DRW but BP changes were nonsignificant and gin had no effect. Therefore, the BP-lowering and NO-raising effects should be attributed to the RW polyphenols and not to alcohol, which seems to counteract the effects of the nonalcoholic fraction of RW.

Botden et al observed that RW polyphenol consumption for 4 weeks did not affect the BP in subjects with high-normal BP or grade 1 hypertension[8] or in healthy young women[9] and postulated that RW polyphenols could only favorably affect BP in subjects with endothelial dysfunction.[8] Our study included subjects with high-normal BP or grade 1 hypertension, but we did not measure endothelial function. However, considering the load of cardiovascular risk factors of the study subjects, their probability of having endothelial dysfunction was very high. On the other hand, Huang et al[3] reported increased plasma NO in healthy volunteers consuming 100 mL/day of RW during 3 weeks, but not when they consumed equivalent amounts of alcohol as beer or vodka, although no BP changes were reported after any intervention.

The results of our study point out that moderate alcohol consumption does not affect BP. Okubo et al[6] observed a J-shaped association between alcohol consumption and BP changes in a normotensive population, with a threshold effect at 18 mL of daily ethanol consumption. Besides, the meta-analysis of Xin et al.[10] described a dose-response relationship between the reduction of alcohol consumption in heavy

Table 2. Changes in Anthropometric Parameters, Blood Pressure, and Plasma Concentrations of Nitric Oxide in the 67 Subjects Studied After the 3 Interventions

	Red Wine Intervention	Dealcoholized Red Wine Intervention	Gin Intervention	P*
Body mass index (kg/m²)	0.6 (–0.7, 0.2)	–0.1 (–0.4, 0.1)	–0.1 (–0.3, 0.1)	0.200
Waist-to-hip ratio	–0.006 (–0.013, 0.001)	–0.001 (–0.006, 0.005)	0.007 (–0.002, 0.015)	0.118
Systolic blood pressure (mm Hg)	–2.3 (–5.1, 0.5)†,‡	–5.8 (–8.9, 2.7)†	–0.8 (–4.1, 2.5)‡	0.028
Diastolic blood pressure (mm Hg)	–1.0 (–2.5, 0.5)†,‡	–2.3 (–4.1, 0.4)†	0.1 (–1.8, 1.9)‡	0.027
Heart rate (beats/min)	–0.2 (–1.9, 1.5)	–1.7 (–3.4, 0.1)	1.1 (–0.8, 3.0)	0.187
Nitric oxide (μmol/L)	–0.6 (–3.3, 4.3)†,‡	4.1 (0.5, 7.6)†	–1.4 (–4.1, 1.3)‡	0.022

Results expressed as mean differences (95% confidence interval) between after and before each intervention. Before each intervention is the value of the previous intervention or the baseline (run-in period) in the first intervention. *P value of the repeated-measures ANOVA from the differences between interventions.

†,‡ Values in a row with different symbols are significantly different.

alcohol drinkers (≥3 drinks/day) and the reduction of BP. We studied moderate alcohol consumers who followed a run-in period with abstention from alcohol, and 4 weeks of moderate consumption of RW or gin had little effect on BP, suggesting that moderate alcohol consumption does not affect BP, at least in high cardiovascular risk subjects. These results concur with those of Frisoli et al[4] and Stranges et al,[11] who observed no consistent association of beer, wine, or liquor

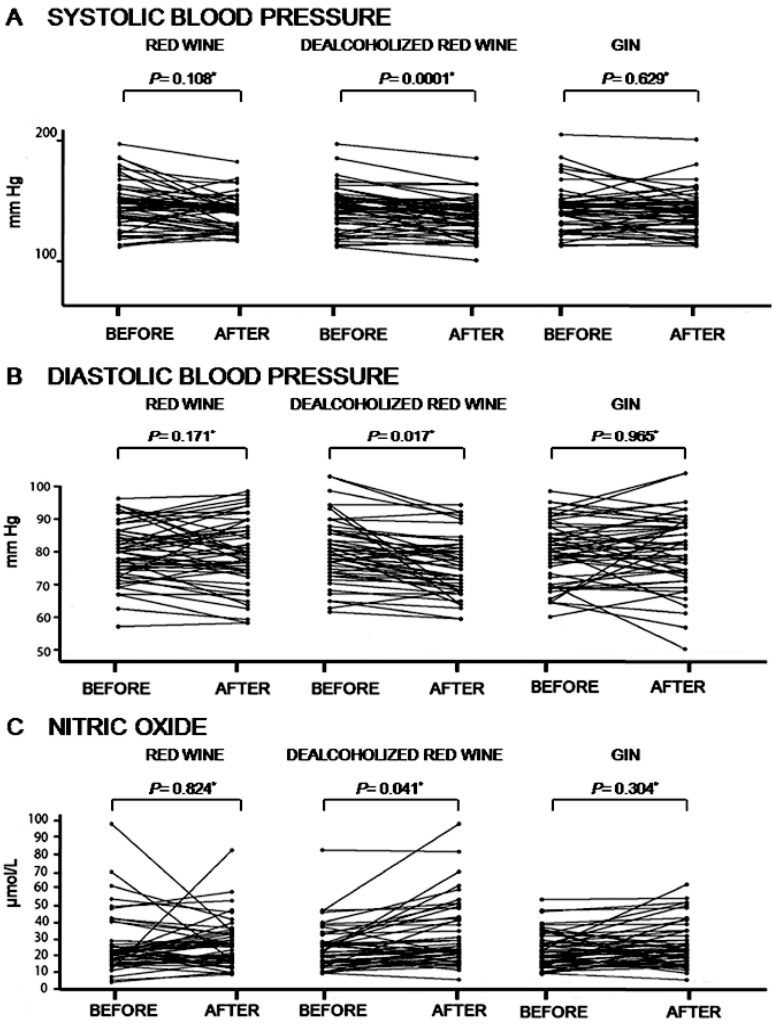

Figure. Blood pressure and plasma nitric oxide in the 67 subjects studied. *Comparisons between before and after the interventions (Student *t* test for paired samples). Before each intervention is the value of the previous intervention or the baseline in the first intervention.

consumption with the risk of hypertension. Stranges et al[11] also observed that drinking outside meals increased the risk of hypertension independently of the amount of alcohol consumed. Our study subjects were advised to consume the beverages during meals and this may explain, in part, why moderate alcohol consumption did not affect BP.

Finally, although the BP reduction after DRW consumption was modest (5.8 and 2.3 mm Hg of SBP and DBP, respectively), decreases of 4 or 2 mm Hg in SBP or DBP respectively, have been associated with a 14% and 20% reduction in coronary heart disease and stroke risk, respectively,[12] conferring clinical significance to our results, especially in the case of DRW.

Our study has limitations. A 4-week intervention may not represent the potential effects of long-term consumption. In addition, the specific substances responsible for the observed effects could not be identified and endothelial function was not measured. In conclusion, DRW decreases SBP and DBP, possibly through an NO-mediated mechanism. Therefore, the daily consumption of DRW may be useful for the prevention of low to moderate hypertension.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Hypertension is a major cardiovascular risk factor and is associated with decreased life expectancy.
- Endothelial secretion of nitric oxide (NO), a potent vasodilator, contributes to lower blood pressure.
- In experimental studies, dietary compounds such as polyphenols (contained in fruits, vegetables, and fermented alcoholic beverages such as red wine) have been shown to stimulate the secretion of endothelial NO, potentially decreasing blood pressure.
- The relationship between moderate alcohol consumption and blood pressure has not been clearly established.

What New Information Does This Article Contribute?

- Moderate red wine consumption (alcohol plus polyphenols) does not significantly affect blood pressure or NO production.
- Moderate gin consumption (alcohol without polyphenols) does not significantly affect blood pressure or NO production.
- Dealcoholized red wine consumption (red wine polyphenols without alcohol) significantly decreases systolic and diastolic blood pressure and increases plasma NO concentration.

Although an inverse relationship between moderate alcohol consumption and the incidence of hypertension has been described, the effects of the different alcoholic beverage fractions (alcoholic and nonalcoholic) on blood pressure are unclear. We observed that moderate alcohol consumption, independently of beverage type (red wine or gin) did not significantly affect blood pressure, but dealcoholized red wine decreased blood pressure and these changes were correlated with plasma NO increases. The findings provide new insights into the role of dietary components such as red wine polyphenols in cardiovascular health, particularly in blood pressure regulation. Consumption of dealcoholized red wine might be useful in preventing low- to moderate-degree hypertension.

Supplemental Material

Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide

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Supplemental Detailed Methods

Subjects

A total of 73 high-risk subjects aged between 55 and 75 years were recruited for the study in the outpatient clinic of the Internal Medicine Department at our Institution. The subjects included in the trial were moderate alcohol consumers (1-3 drinks/day) and had diabetes mellitus or ≥3 of the following cardiovascular disease risk factors: active smoking, hypertension, plasma LDL cholesterol >160 mg/dL, plasma HDL cholesterol <35 mg/dL, overweight or obesity (body mass index ≥25 kg/m^2), and/or family history of premature coronary heart disease (CHD). Exclusion criteria included documented CHD, stroke or peripheral vascular disease, human immunodeficiency virus infection, alcoholic liver disease, malnutrition and neoplastic or acute infectious diseases. None of the study subjects were consumers of multivitamin or vitamin E supplements or anti-inflammatory drugs (steroids, non-steroidal anti-inflammatory agents or aspirin at doses >100 mg/day).

After a 2-week run-in period wherein subjects were asked not to consume any alcoholic beverage, they were randomized using a computer-generated table into three treatments in a cross-over design, with a common background diet plus gin (100mL – 30g ethanol/day), RW (272mL – 30g ethanol/day; total polyphenols: 798 Eq Gallic Acid/day -EGA/day-), and the same amount of polyphenols as RW in the form of dealcoholized red wine (DRW) (272mL - total phenols: 733 EGA/day), resulting in six possible beverage sequences lasting 4 weeks each intervention. No washout periods were included between the interventions.

The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent before participation in the study. This trial was registered at controlled-trials.com as ISRCTN88720134.

Diet and exercise monitoring

Subjects were asked to exclude alcoholic beverages 2 weeks before the first intervention (run-in period) and throughout the study. They were also asked not to change their dietary habits or level of physical activity during the study. Natural foods rich in antioxidants, especially fruit and vegetables, were especially monitored so that individual diets had similar antioxidant content throughout the study. Given the characteristics of the tested beverages, participants were not blinded to the type of drink they ingested. At the beginning of the study and after each intervention period, a medical record and Minnesota Leisure Time Physical Activity Questionnaire validated in Spain[4] were administered, and a 7-d food record questionnaire (5 weekdays and 2 weekend days), also validated in our population[5] was used to assess nutrient intake and to monitor adherence to the study protocol. The dietary information was converted into nutrient data using the Food Processor Nutrition and Fitness Software (*esha* Research, Salem, OR). Subjects were asked to maintain their lifestyle habits and to report any illness or abnormality presented during the study period. At the end of each study sequence, a clinician assessed any adverse effects from the interventions by administering a checklist of symptoms, including bloating, fullness, or indigestion, altered bowel habit, dizziness and other symptoms possibly associated with consumption of the test beverages.

Composition of wines

The RW and DRW were from the Penedès appellation and elaborated with the Merlot grape variety. The total phenolic content of the three beverages was determined with the Folin-Ciocalteu method[1], the phenolic profile of RW and DRW was determined by HPLC-DAD as described previously[2] and resveratrol and piceid content was determined by HPLC-DAD as described by Romero-Perez *et al*[3]. There were no significant differences between the phenolic composition of RW and DRW (ref. 7 of the main manuscript).

Laboratory Analyses

After the run-in period (baseline) and the day after the end of each intervention period (RW, DRW and gin), BP and heart rate were measured 3 times at 5-min intervals on the nondominant arm with an oscillometer (Omron 705 CP; Omron Matsusaka Co Ltd, Matsusaka City, Japan) after 15 minutes resting in a seated position. The mean of the second and the third measures was considered for statistical analysis.

Fasting blood samples for the NO analyses and safety biochemistry determinations were collected at baseline and after each intervention, and stored at –80℃ until assayed. For measurement of NO, the rele ase of NO_2^- and NO_3^-, the stable breakdown products of NO in plasma, were determined by a chemiluminescence detector in a NO analyzer (Sievers Instruments, Inc., Boulder, CO). Plasma aminotransferases (ASAT and ALAT), gamma glutamyl transpeptidase (GGT) and albumin were measured by molecular absorption spectrometry and vitamin B12 and serum and intraerythrocytary folic acid concentrations by immunoanalyses.

Compliance assessment

Resveratrol conjugates derived from phase II metabolism were measured in 24-h urine samples from the last day of the run-in period and the last day of each intervention, using the validated methodology described by Urpi-Sarda *et al.*[6] quantitatively adapted to the commercial and available standards. trans- and cis-Resveratrol-3-O-glucuronide (98% purity each), *cis*-resveratrol-4'-O-glucuronide (96% purity) and *trans*-resveratrol-3-O-sulfate (98% purity) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). *trans*- and *cis*-Resveratrol-4'-O-sulfate and *cis*-resveratrol-3-O-sulfate were quantified using the *trans*-resveratrol-3-O-sulfate calibration curve. Ethylglucuronide was measured in 24-h urine samples as a biomarker of alcohol intake by liquid chromatography (LC) (Agilent series 1200) coupled with a hybrid quadrupole time-of-flight (TOF) QSTAR Elite (Applied Biosystems/MDS Sciex).

Statistical analyses

Statistical analyses were performed using the SAS Statistical Analysis Systems (version 9.2, SAS Institute Inc, Cary, NC). Descriptive statistics [mean ± standard deviation (SD)] were used to describe the baseline characteristics of the participants. To exclude the presence of a carryover effect for the three periods, the interaction between treatment (RW, DRW and gin) and period (1^{rst}, 2^{nd} and 3^{rd}) was analyzed by the repeated measures Analysis of Covariance (ANCOVA) with the baseline values (the values of the previous intervention or the run-in period if the first intervention) as the covariates. To analyze the changes within each treatment a Student's t test for paired samples was performed between the data obtained before and after each intervention. One-factor analysis of variance (ANOVA) for repeated measures and the Bonferroni *post-hoc* test were used to compare the differences of the changes in outcome variables between the interventions. Pearsons' correlation analysis was used to quantify relationships between changes in blood pressure and nitric oxide plasma concentrations. Within- and between-group differences are expressed as means and 95% confidence intervals (CI). *P* was considered significant when <0.05.

Supplemental Results

Baseline characteristics, intervention compliance, diet, exercise monitoring, and side effects

Of the 73 subjects included, six withdrew before completing the three phases of the study because of physical illness (n = 2), journeys (n = 2) or taste intolerance to DRW (n = 2). Therefore, 67 subjects completed the study. Most were overweight or obese (~91%), more than half the population had hypertension (~57%), more than three

quarters had a family history of cardiovascular disease (~78%), and more than one fifth had dyslipemia (~24%), type-2 diabetes (~22%) or were active smokers (~24%). Biochemical safety analytes (serum and intraerythrocytary folic acid, vitamin B12, albumin, ASAT, ALAT and GGT) remained within the normal range throughout the study. None of the subjects reported adverse effects related to the interventions.

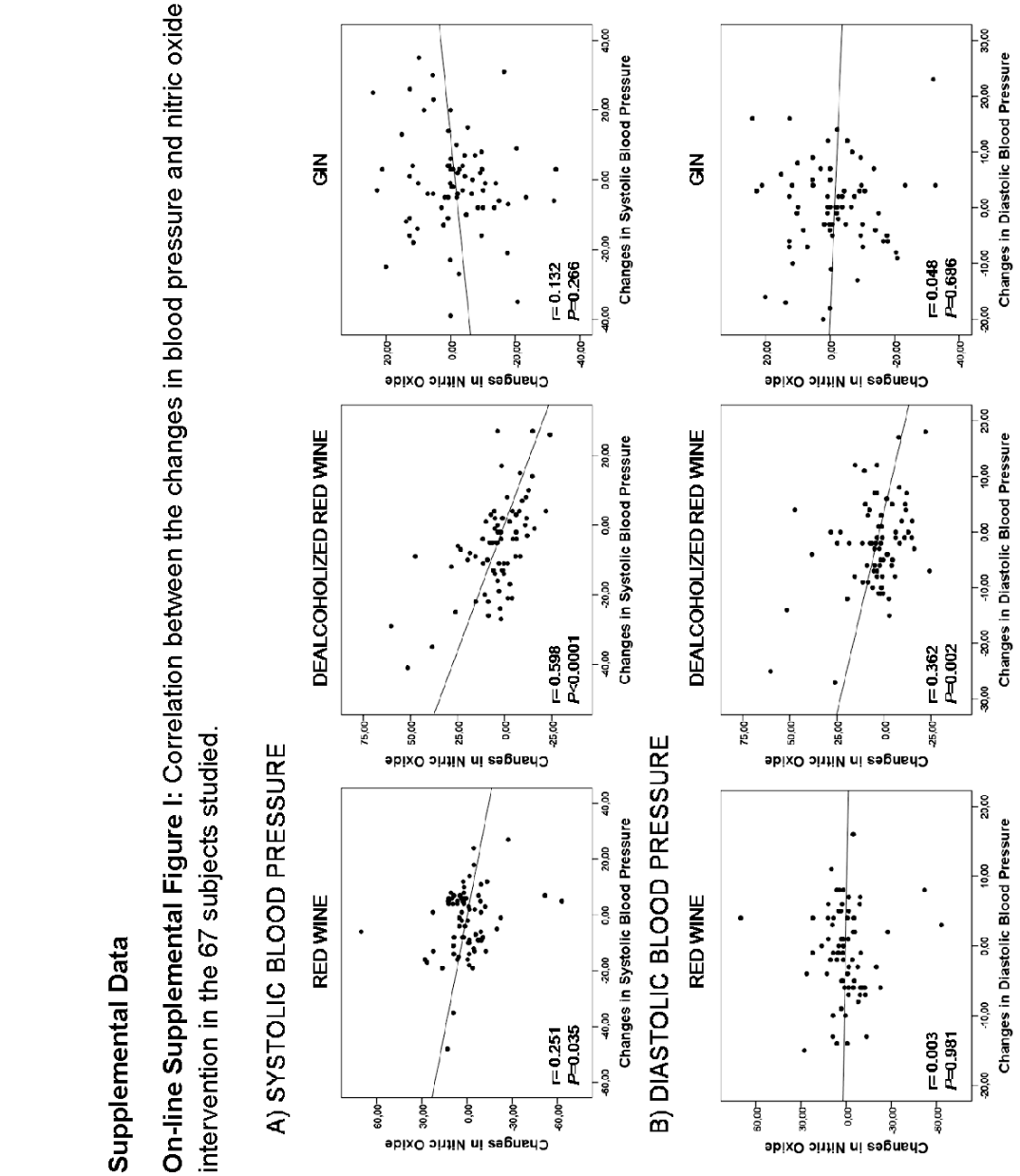
Protocol adherence was optimum in all subjects, and complete agreement was observed between the participants' reports and the number of empty bottles returned. As a measure of intervention compliance, a sum of total resveratrol metabolites -a marker of RW and DRW consumption[7]- was determined in 24-h urine samples collected the last day of the run-in period and the last day of each intervention. After consumption of RW and DRW, 24-h urinary excretion of total resveratrol metabolites increased above baseline from 0.94 µmol (95% CI: 0.43, 1.46 µmol) to 6.04 µmol (95% CI: 4.76, 7.31 µmol) and 6.28 µmol (95% CI: 5.10, 7.46 µmol), respectively ($P < 0.001$, both). Resveratrol metabolites concentrations were not statistically different after DRW and RW interventions ($P = 1.00$) and were significantly higher after RW and DRW interventions compared to gin period [0.51 µmol (95% CI: 0.08, 0.94 µmol); $P \leq 0.001$]. After the gin intervention, urinary resveratrol metabolites were similar to baseline values ($P = 1.00$). Urinary ethylglucuronide concentrations, a biomarker of alcohol consumption, increased significantly after the RW and gin periods compared to baseline values, with increases of 342% (95% CI: 245, 773%) and 256% (95% CI: 179, 599%), respectively ($P < 0.001$, both). Moreover, concentrations after the RW and gin interventions were also higher than those obtained after DRW: 634% (95% CI: 468, 1424%) and 491% (95% CI: 359, 1121), respectively ($P < 0.001$, both). No significant differences were observed between the DRW and baseline periods [66% (95% CI: 64, 75%); $P = 1.000$] and between the RW and gin interventions [24% (95% CI: 24, 25%); $P = 1.000$]. According to these results, compliance with the three interventions was excellent.

Exclusion of the participants with hypertension or under antihypertensive treatment did not materially change the results (**Online Table I**). Nevertheless, in the hypertensive subgroup, the diastolic blood pressure and the plasma nitric oxide concentrations remained practically unchanged during the study. Interestingly, we observed that baseline BMI and waist-to-hip ratios were significantly different between the hypertensive and the non-hypertensive subgroup (P=0.009 and 0.047, respectively, Student's t test for independent samples), without changes throughout the study.

Dietary intake data for the three intervention periods are shown in **Online Table II**. No significant changes from baseline in energy, nutrient, mineral, and antioxidant intake or in the daily average energy expended in physical activity were observed. Likewise, none of the study subjects reported changes in medication use throughout the study. No carryover effect was observed for any outcome, and the values before each intervention were not significantly different between them for any of the outcomes.

Supplemental Data

On-line Supplemental Figure I: Correlation between the changes in blood pressure and nitric oxide between before and after each intervention in the 67 subjects studied.



Online Supplemental Table I: Comparison of anthropometric parameters, blood pressure and plasma concentrations of nitric oxide between 38 hypertensive and 29 non-hypertensive subjects at baseline and after the 3 interventions (n=67).

		Red wine intervention			Dealcoholized red wine intervention			Gin intervention			
		Mean±SD*		Mean differences (95% CI)†	Mean± SD *		Mean differences (95% CI)†	Mean± SD *		Mean differences (95% CI)†	
		Before	After		Before	After		Before	After		P‡
Body mass index (kg/m²)	Total	29.4 ± 3.8	29.5 ± 3.9	0.6 (-0.7, 0.2)	29.3 ± 3.9	29.4 ± 4.1	-0.1 (-0.4, 0.1)	29.4 ± 3.8	29.5 ± 4.0	-0.1 (-0.3, 0.1)	0.200
	Hypertensive	30.9 ± 4.2	30.9 ± 4.3	0.2 (-0.1, 0.4)	30.9 ± 4.5	30.9 ± 4.3	-0.1 (-0.2, 0.2)	30.8 ± 4.4	30.9 ± 4.3	-0.2 (-0.5, 0.1)	0.084
	Non-hypertensive	28.4 ± 3.1	28.3 ± 3.2	-0.1 (-0.2, 0.1)	28.4 ± 3.0	28.3 ± 3.2	0.1 (-0.3, 0.4)	28.4 ± 3.2	28.2 ± 4.0	-0.1 (-0.5, 0.2)	0.876
Waist-to-hip ratio	Total	0.97 ± 0.04	0.97 ± 0.05	-0.006 (-0.013, 0.001)	0.97 ± 0.04	0.97 ± 0.05	-0.001 (-0.006, 0.005)	0.98 ±0.05	0.98 ± 0.05	0.007 (-0.002, 0.015)	0.118
	Hypertensive	0.99 ± 0.06	0.98 ± 0.05	-0.009 (-0.020, 0.003)	0.98 ± 0.05	0.98 ± 0.05	-0.001 (-0.010, 0.008)	0.98 ± 0.05	0.99 ± 0.06	0.003 (-0.010, 0.017)	0.400
	Non-hypertensive	0.97 ± 0.04	0.96 ± 0.04	-0.003 (-0.012, 0.006)	0.96 ± 0.04	0.96 ± 0.04	-0.001 (-0.008, 0.006)	0.97 ± 0.04	0.97 ± 0.05	0.011 (-0.001, 0.022)	0.202
Systolic blood pressure (mm Hg)	Total	137 ± 16	135 ± 15	-2.3 (-5.1, 0.5)[a,b]	138 ± 18	132 ± 16	-5.8 (-8.9, -2.7)[a,§]	138 ± 17	136 ± 19	-0.8 (-4.1, 2.5)[b]	0.028
	Hypertensive	139 ± 19	138 ± 15	-2.4 (-6.2, 1.5)[a,b]	143 ± 20	136 ±18	-7.7 (-12.2, -3.0)[a,§]	138 ± 19	139 ± 18	0.8 (-3.5, 5.1)[b]	0.024
	Non-hypertensive	134 ± 13	131 ± 14a	-2.1 (-6.5, 2.3)[a,b]	133 ± 16	129 ±15	-3.5 (-7.8, -0.8)[a,§]	135 ± 15	134 ± 19	-1.0 (-8.0, 2.0)[b]	0.042
Diastolic blood pressure (mm Hg)	Total	80 ± 8	79 ± 9	-1.0 (-2.5, 0.5)[a,b]	79 ± 10	77 ± 8	-2.3 (-4.1, -0.4)[a,§]	79 ± 8	79 ± 10	0.1 (-1.8, 1.9)[b]	0.027
	Hypertensive	80 ± 10	78 ± 10	-2.1 (-4.6, 0.3)	80 ± 11	77 ± 9	-2.2 (-4.8, 0.5)	78 ± 10	78 ± 9	-0.4 (-2.4, 1.7)	0.401
	Non-hypertensive	79 ± 7	79 ± 9	-0.8 (-4.2, 0.5)[a,b]	78 ± 9	77 ± 8	-2.7 (-5.6, -0.6)[a,§]	79 ± 7	80 ± 11	2.3 (-0.1, 4.9)[b]	0.015
Heart rate (beats/min)	Total	68 ± 10	68 ± 10	-0.2 (-1.9, 1.5)	67 ± 9	68 ± 9	-1.7 (-3.4, 0.1)	68 ± 10	69 ± 11	1.1 (-0.8, 3.0)	0.187
	Hypertensive	69 ± 12	68 ± 12	-1.2 (-3.7, 1.2)	67 ± 9	67 ± 10	-0.7 (-4.2, 1.0)	68 ± 11	69 ± 12	1.2 (-2.0, 4.4)	0.428
	Non-hypertensive	68 ± 8	69 ± 9	0.3 (-2.0, 2.6)	68 ± 10	69 ± 8	-1.4 (-3.9, 1.1)	68 ± 8	69 ± 11	1.1 (-1.3, 3.6)	0.499
Nitric oxide (µmol/L)	Total	27.5 ± 15.8	27.8 ± 13.0	0.6 (-3.3, 4.3)[a,b]	26.1 ± 12.1	29.6 ± 17.8	4.1 (0.5, 7.6)[a,§]	27.1 ± 11.4	25.8 ± 12.0	-1.4 (-4.1, 1.3)[b]	0.022
	Hypertensive	28.1 ± 19.1	27.4 ± 13.7	-0.8 (-8.2, 6.5)	24.8 ± 9.7	27.1 ±14.1	2.2 (-2.6, 7.1)	27.1 ± 10.7	25.8 ± 11.2	-2.0 (-6.4, 2.3)	0.464
	Non-hypertensive	26.8 ± 10.9	28.2 ± 12.5	2.1 (-0.5, 4.8)[a,b]	27.8 ± 14.1	32.0 ± 19.3	5.7 (0.2, 11.2)[a,§]	27.0 ± 12.4	25.8 ± 12.8	-1.0 (-4.6, 2.5)[b]	0.015

Results are expressed as *mean ± SD (n=38 and n=29 for the hypertensive and the non-hypertensive population) and †mean differences (95% CI) between after and before each intervention. Before each intervention is the value of the previous intervention or the baseline in the first intervention. ‡P value of the repeated-measures ANOVA from the differences between interventions. §Significant differences ($P<0.05$) between

Online Supplemental Table II: Daily energy and nutrient intakes in the 67 subjects studied at baseline and after the three interventions*.

	Red wine intervention			Dealcoholized red wine intervention			Gin intervention			
	Mean± SD†		Mean differences	Mean± SD†		Mean differences	Mean± SD†		Mean differences	
	Before	After	(95% CI)‡	Before	After	(95% CI)‡	Before	After	(95% CI)‡	P§
Energy (kcal/d)	1863 ± 256	1782 ± 325	-85 (-238, 67)	1804 ± 396	1862 ± 320	90 (-63, 244)	1896 ± 403	1887 ± 336	-15 (-183, 153)	0.359
Total protein (g/d)	92.63 ± 16.92	89.06 ± 18.35	-2.3 (-9.9, 9.5)	93.15 ± 15.78	94.29 ± 17.56	2.3 (-8.3, 8.8)	92.1 ± 2.0	95.04 ± 18.73	1.8 (-8.2, 11.8)	0.967
Carbohydrates (g/d)	205 ± 37	201 ± 44	-6.3 (-32, 30)	184 ± 40	193 ± 38	14.4 (-12.3, 41.3)	194 ± 39	206 ± 40	13.2 (-7.8, 34.2)	0.763
Dietary fiber (g/d)	22.31 ± 8.2	20.63 ± 8.08	-2.1 (-5.7, 1.5)	17.85 ± 5.14	19.88 ± 6.82	1.6 (-1.2, 4.4)	19.53 ± 9.04	22.05 ± 10.01	1.6 (-1.1, 4.4)	0.277
Sugars (g/d)	71.77 ± 20.08	66.74 ± 25.59	-9.7 (-19.6, 0.2)	61.36 ± 23.34	67.33 ± 20.08	6.5 (-4.7, 17.8)	68.88 ± 23.84	70.11 ± 20.84	2.1 (-10.3, 14.4)	0.153
Total lipids (g/d)	75.51 ± 13.99	72.72 ± 16.27	-5.7 (-13.9, 2.5)	73.48 ± 19.31	78.55 ± 20.41	9.1 (-1.3, 19.6)	73.70 ± 23.16	79.86 ± 21.89	-0.6 (-11.5, 10.3)	0.118
SFA (g/d)	19.86 ± 4.93	18.40 ± 7.06	-0.7 (-3.4, 2.0)	17.36 ± 8.33	18.90 ± 6.15	3.9 (-0.3, 7.4)	19.20 ± 6.51	19.01 ± 5.48	-1.0 (-4.9, 2.8)	0.128
MUFA (g/d)	36.99 ± 7.30	35.84 ± 8.23	-3.1 (-7.1, 0.9)	36.05 ± 8.38	37.97 ± 10.18	3.8 (-0.5, 8.2)	37.04 ± 11.27	38.31 ± 9.60	0.2 (-4.9, 5.4)	0.171
PUFA (g/d)	11.18 ± 2.93	10.65 ± 4.21	-0.8 (-2.4, 0.7)	10.87 ± 3.81	11.94 ± 4.42	0.7 (0.7, 2.0)	10.53 ± 4.83	10.71 ± 3.29	0.2 (-1.1, 1.5)	0.464
Cholesterol (mg/d)	365 ± 108	355 ± 125	-14 (-65, 36)	336 ± 136	342 ± 103	33 (-21, 86)	345 ± 124	360 ± 162	25 (-55, 61)	0.462
Vitamin C (mg/d)	118 ± 75	125 ± 83	11 (-11, 32)	124 ± 59	121 ± 68	-2.6 (-21, 16)	129 ± 87	133 ± 91	-12 (-37,11)	0.507
Vitamin A (µgRE‖/d)	605 ± 255	688 ± 340	89 (-195, 374)	696 ± 532	729 ± 343	83 (-125, 231)	725 ± 452	709 ± 481	-65 (-158,77)	0.234
Vitamin E (mg/d)	9.28 ± 2.82	9.65 ± 3.22	-0.5 (-1.5, 0.5)	9.55 ± 2.86	9.47 ± 3.34	-0.1 (-1, 0.8)	9.37 ± 2.98	10.29 ± 4.65	0.5 (-0.4, 1.3)	0.399
Folic acid (µg/d)	493 ± 166	454 ± 155	-43 (-121,35)	394 ± 138	443 ± 143	64 (-20, 148)	484 ± 186	498 ± 226	11 (-45, 67)	0.335
Calcium (mg/d)	714 ± 73	718 ± 36	61 (-24, 146)	663 ± 21	678 ± 35	39 (-47, 125)	731 ± 31	712 ± 30	-70 (-200, 605)	0.342
Magnesium (mg/d)	398 ± 74	345 ± 92	-87 (-420, 245)	316 ± 94	339 ± 95	97 (-100, 295)	368 ± 96	342 ± 106	-31 (-40.1, 34.0)	0.551
Manganese (mg/d)	2.77 ± 1.17	2.66 ± 1.09	-0.1 (-0.7, 0.5)	2.61 ± 1.06	2.72 ± 1.26	0.1 (-0.3, 0.8)	2.76 ± 1.41	2.82 ± 1.18	0.2 (-1.0, 5.4)	0.617
Potassium (mg/d)	2918 ± 718	3119 ± 673	87 (-149, 323)	2967 ± 751	3089 ± 850	49 (-186, 285)	3254 ± 790	3121 ± 955	-66 (-271, 139)	0.515
Selenium (µg/d)	146 ± 37	139 ± 37	-21 (-186, 144)	134 ± 35	136 ± 38	47 (-121, 215)	131 ± 33	136 ± 33	52 (-85, 189)	0.790
Sodium (mg/d)	2296 ± 161	2298 ± 198	-127 (-434, 180)	2137 ± 202	2205 ± 191	359 (-750, 794)	2293 ± 213	2278 ± 121	-87 (-294, 119)	0.218
Zinc (mg/d)	10.18 ± 1.59	10.48 ± 2.42	1.1 (-4.6, 5.8)	10.32 ± 2.64	9.56 ± 2.40	-2.2 (-5.7, 2.2)	10.07 ± 2.54	10.40 ± 2.21	-2.5 (-5.4, 3.4)	0.549
Total polyphenols (mg/d)	308 ± 134	318 ± 142	31 (-23, 87)	320 ± 168	311 ± 146	-33 (-89,23)	313 ± 169	327 ± 170	-32 (-77, 13)	0.146

* Excluding the energy, nutrient and total polyphenol contributions from the tested beverages. Results are expressed as †mean ± SD (n=67) and ‡mean differences (95% CI) between after and before each intervention. Before each intervention is the value of the previous intervention or the baseline in the first intervention. §P value of the repeated-measures ANOVA from the differences between interventions. No changes were observed between after and before the intervention, measured by a Student's *t* test for paired samples. ‖Retinol Equivalents.

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DISCUSSIÓ

DISCUSSIÓ

Els tres capítols de la present tesi doctoral tenen per objectiu avaluar els efectes de les dues fraccions del vi (alcohòlica i no alcohòlica) conjuntament i per separat en diferents marcadors de risc cardiovascular en homes amb un alt risc cardiovascular en un estudi creuat i aleatoritzat, que permet obtenir conclusions amb la màxima evidència científica. Per un cantó, la fracció alcohòlica del vi (intervencions amb vi negre i ginebra), a través d'un consum moderat d'alcohol, augmentà la concentració de IL-10, HDL, ApoAI i ApoAII, i disminuí la concentració de MDC, així com el *ratio* LDL/HDL. D'altra banda, la fracció no alcohòlica del vi (intervencions amb vi negre i vi negre desalcoholitzat) va disminuir la concentració d'ICAM-1, IL-6 i insulina basal, l'índex HOMA, i també va disminuir l'expressió limfocitària de LFA-1, la monocitària de Mac-1 i CCR2 així com l'expressió de SLe^x tant en limfòcits com en monòcits. No obstant, només la intervenció amb vi negre disminuí la concentració de LDL i Lp(a), i només la intervenció amb vi negre desalcoholitzat fou capaç de disminuir la concentració d'E-Selectina, de reduir la pressió sistòlica i diastòlica, així com d'augmentar la concentració de NO plasmàtic. Aquest fet, tal com varen comentar Iqbal S i Kazory (2012), condueix a pensar que, a part dels polifenols presents al vi, compostos encara no ben identificats tant al vi negre com al vi negre desalcoholitzat (com algun subproducte del procés de desalcoholització) tenen potencials efectes hipotensors.

D'aquests resultats se n'extreu una primera conclusió que és que l'alcohol i els polifenols (majoritaris en la fracció no alcohòlica) presents al vi negre no tenen efectes sinèrgics. Si bé és cert, malgrat petites excepcions, que el vi negre té els efectes additius de les dues fraccions (alcohol i polifenols), aquests efectes no són majors en el vi negre respecte a les dues fraccions per separat. No obstant, l'alcohol i els polifenols no tenen efectes antagònics en la majoria de paràmetres estudiats (excepte en la pressió arterial), pel que una primera valoració general és que el vi té més efectes cardioprotectors que

altres begudes alcohòliques que no contenen polifenols, com per exemple la ginebra.

Els resultats derivats d'aquesta tesi aporten més evidència científica en el paper de l'alcohol i els polifenols sobre el sistema cardiovascular. Si bé es creia que els beneficis cardiovasculars del consum moderat d'alcohol eren pràcticament atribuïbles a l'augment del colesterol HDL, la millora del control glicèmic i la disminució del fibrinogen (Rimm, 2007), actualment aquests factors no expliquen per si sols la disminució del risc cardiovascular en el marc d'un consum moderat d'alcohol (Magnus, 2011). Per tant, hi han més mecanismes que contribueixen a la disminució del risc cardiovascular i possiblement interactuen entre si, ja que per exemple, una disminució de la pressió arterial està associada a una disminució dels nivells circulants de IL-6 (Vázquez-Oliva, 2005). Tal com s'ha observat en diversos estudis, el vi negre sembla ésser més cardioprotector que altres begudes alcohòliques (Avellone, 2006; Estruch, 2004; Sacanella, 2007). Els resultats exposats en aquesta tesi suggereixen que els mecanismes addicionals pels quals el vi negre exerceix un efecte cardioprotector més gran que altres begudes alcohòliques són derivats de l'acció del seu major contingut en polifenols. De fet, el vi negre és una de les begudes amb un contingut més alt en polifenols, especialment resveratrol (Zamora-Ros, 2008). El resveratrol ha estat objecte d'estudi per les seves accions a molts nivells, tant antioxidant, antiinflamatòria, antiagregant plaquetari i antitrombòtica entre altres (Baur, 2006; Delmas, 2005). No obstant, altres polifenols del vi com les catequines, quercetines o els antocians també tenen accions protectores (Auger, 2010; Kurin, 2012; Loke, 2010), suggerint que els polifenols continguts al vi negre sí que actuen de manera sinèrgica.

El consum moderat de vi negre en persones amb alt risc cardiovascular exerceix un paper protector enfront a l'aparició i progressió de l'aterosclerosi. Un efecte global similar també s'ha observat en homes sans (Estruch, 2004) i en dones sanes, tant consumint vi negre (Sacanella, 2007) com cava (Vazquez-Agell, 2007). No obstant, un dels problemes de la valoració dels efectes del vi en persones amb risc vascular ve derivat dels tractaments

farmacològic que reben. Així, les estatines o l'aspirina tenen efectes antiinflamatoris (Spite, 2010) i els inhibidors de l'enzim convertidor d'angiotensina estimulen la NO sintasa en diferents graus (Comini, 2007). No obstant, en els estudis reunits en aquesta tesi doctoral, tots els participants han mantingut el mateix tractament farmacològic durant les tres intervencions, fet que fa pensar que els efectes observats són deguts a la intervenció rebuda (vi, vi desalcoholitzat o ginebra) i no a la medicació que prenen.

L'efecte observat del consum moderat de vi negre (o vi negre desalcoholitzat) sobre la pressió arterial o el metabolisme glucídic en homes amb alt risc cardiovascular no pot ésser extrapolable a persones sanes o a dones amb alt risc cardiovascular. S'han observat resultats contradictoris a altres estudis on no es van detectar canvis en la pressió arterial després del consum de polifenols del vi negre en persones amb un grau moderat d'hipertensió (Botden, 2012) o en dones sanes (Botden 2011). Per tant, són necessaris més estudis aprofundint en aquestes qüestions.

Encara que aquestes dades no han estat publicades, no s'ha observat cap efecte col·lateral perjudicial derivat del consum moderat d'alcohol ni en la funció hepàtica ni en la renal. No obstant, després del consum moderat de vi negre i vi negre desalcoholitzat hem observat una disminució de la concentració plasmàtica d'àcid fòlic. Es fa difícil explicar aquest fenomen, però sembla que alguns polifenols podrien inhibir l'absorció intestinal de folats (Lemos, 2007).

Un altre aspecte a tenir en compte alhora d'interpretar els resultats d'aquest estudi clínic d'intervenció dietètica és la significació o implicació clínica de la modulació del vi i les seves fraccions sobre els paràmetres estudiats. En el cas de la pressió arterial, la reducció de 4 o 2 mm Hg en la SBP o la DBP, respectivament, s'ha associat amb una reducció del 14% en la malaltia coronària i en un 20% en el risc d'ictus, conferint certa significació clínica als nostres resultats. En referència a les ApoAI i ApoB, també hi ha una relació clara entre l'augment i/o reducció de l'ApoAI i ApoB, respectivament, i la

disminució del risc d'infart agut de miocardi (McQueen, 2000). L'índex HOMA, almenys en diabètics tipus 2, és un predictor independent de risc cardiovascular. Una disminució de l'índex HOMA de 0.45 a 0.34 punts com passa després del consum de vi negre i desalcoholitzat, respectivament, podria estar associat a una reducció del 5 i 3% respectivament, del risc de malaltia cardiovascular (Bonora, 2002). D'altra banda, la Lp(a) està estratificada en diferents nivells de risc cardiovascular cosa que permet afirmar que la seva modulació per part del consum moderat de vi negre no és clínicament significativa. No obstant, en el cas de les molècules d'adhesió cel·lulars i els marcadors inflamatoris solubles relacionats amb l'aparició i progressió de l'aterosclerosi, és molt difícil donar una implicació clínica als resultats obtinguts ja que no existeixen ni intervals de referència en persones sanes ni en estadis pre- o ateroscleròtics. El mateix passa amb l'ApoAII, que no es correlaciona clarament amb un augment del colesterol HDL o amb un retràs de la progressió de l'aterosclerosi (Blanco-Vaca, 2002).

Complementàriament a les implicacions clíniques, els resultats observats després d'una intervenció de quatre setmanes no tenen perquè representar els potencials efectes a llarg termini. Aquests resultats tampoc es poden extrapolar a individus sans, ja que els efectes observats sobre els biomarcadors solubles d'inflamació i les molècules d'adhesió relacionades amb l'aterosclerosi en homes amb alt risc cardiovascular són diferents que els observats en homes sans (Estruch, 2004), ni tampoc es poden extrapolar a la població femenina (sana o amb risc cardiovascular).

Finalment, el disseny i anàlisi d'aquest estudi no ha permès identificar ni els compostos ni els mecanismes responsables dels efectes observats. A través dels resultats afirmats podem concloure que el consum de vi negre o vi negre desalcoholitzat té uns efectes protectors a nivell cardiovascular, però existeix una forta interrelació entre la inflamació, l'aterosclerosi, la disfunció endotelial, la hipertensió arterial i la sensibilitat a la insulina, que suggereix que alguns dels efectes observats podrien ésser secundaris a altres. A més, l'associació de biomarcadors inflamatoris amb un futur risc de complicacions

ateroscleròtiques no demostra causalitat. Encara que l'evidència experimental i clínica combinada és molt suggerent (Libby, 2011), el problema de la causalitat segueix sense ésser resolt.

Aquest estudi presenta certes limitacions. Primer de tot, el disseny de l'estudi no inclou períodes de rentat entre les intervencions. Això es deu a que incorporar períodes de rentat entre intervencions hagués allargat l'estudi 6 setmanes més i, per tant, l'acompliment de les intervencions hagués estat més dificultós i els voluntaris haguessin estat més proclius a abandonar l'estudi. Encara que el més adequat i correcte és incorporar un període de rentat als estudis creuats, el fet d'aleatoritzar les intervencions per a cada voluntari, en part, contraresta aquesta carència. A més, estadísticament no s'observà un efecte d'arrossegament de la intervenció anterior (*carryover effect*).

En segon lloc, encara que els perfils fenòlics dels vins amb i sense alcohol són pràcticament iguals i les composicions dels dos vins pràcticament només difereixen amb el contingut en alcohol, el vi desalcoholitzat conté un 0.42% d'etanol. Això implica que els voluntaris consumiren aproximadament 1 g d'alcohol al dia, quantitat però, pràcticament negligible, si es consideren diversos estudis on s'ha observat que els efectes d'un consum moderat d'alcohol es perceben a partir d'un llindar de 15-18 g d'alcohol/dia (Davies, 2002; Okubo, 2001), encara que en dos metanàlisis s'observen diferències en el risc relatiu de factors de risc i mortalitat cardiovascular a partir de menys de 2.5 g d'alcohol al dia (Brien, 2011; Ronksley, 2011). Un altre inconvenient de les intervencions de l'estudi és que la ginebra podria contenir traces d'elements bioactius que podrien emmascarar algun possible efecte sinèrgic entre l'alcohol i els polifenols, actuant ells mateixos en sinèrgia amb l'alcohol.

La tercera limitació de l'estudi és que, a pesar d'estudiar la modulació del vi negre i les seves fraccions en forces biomarcadors de risc cardiovascular, no s'han contemplat altres molècules d'adhesió expressades en neutròfils i plaquetes que també juguen un paper important en l'aparició i progressió de l'aterosclerosi (Weber, 2011), ni tampoc cap paràmetre d'oxidació que pogués

estar afectant l'estat inflamatori, la sensibilitat a la insulina, la pressió arterial o la funció endotelial (Perez-Vizcaino, 2006). A més, tampoc s'ha mesurat la funció endotelial ni la secreció endotelial d'altres vasodilatadors més enllà de l'òxid nítric o de vasoconstrictors com l'endotelina (Giles, 2012).

Resumint, el consum diari de dues copes de vi negre dins els àpats (Stranges, 2004) en homes amb alt risc cardiovascular podria ajudar a retardar l'aparició i progressió de l'aterosclerosi ja que, sobretot a causa del seu contingut en polifenols, disminueix l'expressió de molècules d'adhesió leucocitàries i marcadors inflamatoris circulants. A més també podria contribuir a la prevenció de la resistència a la insulina, ja que pel seu contingut en alcohol augmenta la concentració plasmàtica de colesterol HDL, el que podria incrementar la sensibilitat a la insulina (von Eckardstein, 2011), i pel seu contingut en polifenols disminueix l'índex HOMA. No obstant, per a la prevenció de la hipertensió arterial, el consum de dues copes de vi sense alcohol al dia podria ésser més beneficiós que el consum de vi amb alcohol, almenys en homes no hipertensos amb altres factors de risc cardiovascular.

CONCLUSIONS

CONCLUSIONS GENERALS

El consum moderat i regular de vi negre dins els àpats principals exerceix un efecte protector en l'aparició i progressió de l'aterosclerosi, així com sobre molts dels factors clàssics de risc cardiovascular en homes amb un risc cardiovascular moderat-alt.

El consum moderat i regular de vi negre en els àpats principals exerceix un major efecte protector a nivell cardiovascular que altres begudes alcohòliques gràcies al seu contingut en altres productes no alcohòlics, principalment polifenols.

L'alcohol i els compostos fenòlics continguts al vi negre no actuen sinèrgicament a l'organisme humà.

CONCLUSIONS CONCRETES

1. El consum moderat de vi negre, vi negre desalcoholitzat i ginebra (és a dir, tant l'alcohol com el contingut no alcohòlic (polifenols) del vi negre) disminueix les concentracions circulants de molècules proinflamatòries endotelials relacionades amb l'aterosclerosi en homes amb alt risc cardiovascular.

2. El consum moderat de vi negre i vi negre desalcoholitzat (és a dir, la fracció no alcohòlica del vi negre) disminueix l'expressió leucocitària de molècules d'adhesió relacionades amb la formació i progressió de la placa d'ateroma en homes amb factors de risc cardiovascular.

3. El consum moderat de vi negre i vi negre desalcoholitzat (és a dir, la fracció no alcohòlica del vi negre) durant els àpats millora la sensibilitat a la insulina en homes amb alt risc cardiovascular.

4. El consum moderat d'alcohol (però no de polifenols del vi negre) augmenta el colesterol HDL i l'ApoAI i ApoAII circulants en homes amb alt risc cardiovascular.

5. Ni el consum d'alcohol ni de polifenols del vi negre tenen un efecte en els nivells de triglicèrids, colesterol total, colesterol LDL, ApoB, ApoCI o ApoCII.

6. El consum moderat i regular de vi negre disminueix la Lp(a) plasmàtica en homes amb alt risc cardiovascular.

7. El consum de vi negre desalcoholitzat (però no de vi negre amb alcohol) disminueix la pressió arterial, possiblement a través d'augmentar la secreció endotelial d'òxid nítric en homes amb alt risc cardiovascular.

8. El consum moderat d'alcohol no modifica la pressió arterial ni la secreció endotelial d'òxid nítric d'una manera significativa, encara que sembla que contraresti una mica els efectes beneficiosos dels polifenols del vi negre.

9. L'alcohol i els polifenols continguts al vi negre no actuen sinèrgicament en la disminució dels factors de risc de malalties cardiovasculars, almenys en persones amb alt risc cardiovascular.

CONCLUSIONS FINALS

Amb els resultats de la present tesi doctoral podem concloure que en homes amb alt risc cardiovascular, el consum moderat de vi negre (dues copes al dia, uns 30 g d'alcohol diaris) té efectes beneficiosos a nivell cardiovascular. Per tant, sempre que no hi hagin contraindicacions, a un home que tingui el costum d'acompanyar els àpats amb vi negre, se li pot recomanar continuar-ho fent.

No obstant, i d'acord amb la postura de l'oficina regional per Europa de la OMS (Anderson *et al*., 2012), no hem d'oblidar que l'alcohol és una substància neurotòxica, procarcinogènica, teratògena, immunosupressora i que provoca dependència. Així com un consum moderat d'alcohol (sobretot de vi negre) pot tenir beneficis a nivell cardiovascular, un consum excessiu n'és indubtablement perjudicial. A més, tampoc podem oblidar els problemes socials, familiars i laborals derivats d'un consum excessiu d'alcohol. Per tant, sota cap premissa és recomanable aconsellar a una persona abstèmia el consum moderat d'alcohol, encara que si que se li podria recomanar el consum diari de vi negre desalcoholitzat.

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ANNEX I

ANNEX I

I.I) Presentacions a congressos durant l'etapa pre-doctoral

1. Effects of beer compounds in soluble and adhesion molecules related to atherosclerosis. Gemma Chiva-Blanch, Emma Magraner, Ximena Condines, Irene Roth, Palmira Valderas-Martínez, Antonio Sisó, Rosa M Lamuela-Raventós and Ramon Estruch. 80th European Atherosclerosis Society Congress, Milà, Italia, 25-28 de maig de 2012.
2. Efectos de los polifenoles y el alcohol del vino tinto en la presión arterial. Gemma Chiva-Blanch, Mireia Urpi-Sarda, Palmira Valderas-Martinez, Rosa Casas, Sara Arranz, Irene Roth, Anna Castillo, Rosa M Lamuela-Raventós, Rafael Llorach, Cristina Andres-Lacueva y Ramon Estruch, IX Congreso Internacional en Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya, 27-28 de març de 2012.
3. El consumo moderado de cerveza reduce el riesgo cardiovascular. Irene Roth, Gemma Chiva-Blanch, Ana Lucía Castillo, Rosa Casas, Palmira Valderas-Martínez, Sara Arranz, Alex Medina, Rosa M. Lamuela-Raventós y Ramón Estruch. IX Congreso Internacional en Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya, 27-28 de març de 2012.
4. El consumo de zumo de naranja como indicador de un patrón de alimentación más saludable en población de alto riesgo cardiovascular. Estudio clínico PREDIMED. Sara Arranz, Alex Medina-Remón, Palmira Valderas, Gemma Chiva-Blanch, Mireia Urpí-Sarda, Rosa Casas, Rosa Mª Lamuela y Ramón Estruch. IX Congreso Internacional en Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya, 27-28 de març de 2012.
5. One Year Effect of a Mediterranean-Style Dietary Pattern in Inflammatory Biomarkers Related with Atherosclerosis. Mireia Urpi-Sarda, Rosa Casas, Gemma Chiva-Blanch, Edwin Saúl Romero-Mamani, Palmira Valderas-Martínez, Jordi Salas-Salvadó, María Isabel Covas, Estefanía Toledo, Cristina Andres-Lacueva, Rafael Llorach, Ana García-Arellano, Monica Bulló, Valentina Ruiz-Gutierrez, Rosa Mª

Lamuela-Raventos, Ramon Estruch. IX Congreso Internacional en Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya, 27-28 de març de 2012.

6. ¿Es necesario estandarizar las recomendaciones de consumo de frutas y verduras que establecen las guías alimentarias? Valderas-Martínez Palmira, Arranz Sara, Chiva-Blanch Gemma, Martínez-Huélamo Miriam, Casas Rosa, Urpi-Sarda Mireia, Lamuela-Raventós Rosa Maria, Estruch Ramón. IX Congreso Internacional en Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya, 27-28 de març de 2012.

7. La ingesta de verduras crucíferas (brócoli y derivados) contribuye a la disminución de la expresión de los marcadores inflamatorios relacionados con la aterogénesis tras un año de intervención con Dieta Mediterránea. Rosa Casas, Emilio Sacanella, Ana Lucia Castillo, Irene Roth, Gemma Chiva-Blanch, Palmira Valderas-Martínez, Emilio Ros, Ramón Estruch. IX Congreso Internacional en Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya, 27-28 de març de 2012.

8. Efectos de los polifenoles del vino tinto en el metabolismo de la glucosa y el perfil lipídico. Gemma Chiva-Blanch, Mireia Urpi-Sarda, Rosa Casas, Palmira Valderas-Martinez, Sara Arranz, Rosa M Lamuela-Raventos, Cristina Andrés-Lacueva, Ramon Estruch. IV Symposium CIBERobn, Màlaga, Espanya, 10-11 de novembre de 2011.

9. Estudio PREDIMED: efecto antiinflamatorio a largo plazo de la dieta mediterránea sobre los biomarcadores relacionados con la inflamación de la pared vascular y la inestabilidad de la placa. Rosa Casas, Emilio Sacanella, Mari-Pau Mena, Gemma Chiva-Blanch, Emilio Ros, Miguel-Angel Martínez-González, Maribel Covas, Rosa Mª Lamuela-Raventos, Jordi Salas-Salvadó, Miquel Fiol, Fernando Arós, Ramon Estruch. IV Symposium CIBERobn, Màlaga, Espanya, 10-11 de novembre de 2011.

10. Changes in cellular inflammatory biomarkers related to atherosclerosis after consumption of tomato sauces. Valderas-Martínez P, Casas R, Arranz S, Chiva-Blanch G, Medina-Remón A, Urpi-Sarda M,

Andrés-Lacueva C, Lamuela-Raventós RM, Estruch R. 11th FENS European Nutrition Conference. Madrid, Espanya, 26–29 d'octubre de 2011.

11. Mediterranean diet: effect of its components in the inflammatory biomarkers related with atherosclerosis. Mireia Urpi-Sarda, Rosa Casas, Gemma Chiva-Blanch, Palmira Valderas-Martínez, Sara Arranz, Maribel Covas, Miguel Ángel Martinez, Ramon Estruch. 11th FENS European Nutrition Conference. Madrid, Espanya, 26–29 d'octubre de 2011.

12. Red wine polyphenols decrease HOMA index in high cardiovascular risk subjects. Gemma Chiva-Blanch, Mireia Urpi-Sarda, Palmira Valderas-Martínez, Rosa Casas, Sara Arranz, Rosa M Lamuela-Raventós, Cristina Andrés-Lacueva, Ramón Estruch. 11th FENS European Nutrition Conference, Madrid, Espanya, 26–29 d'octubre de 2011.

13. 11-NMR based metabolomic analysis of the effect of moderate wine consumption in subjects with cardiovascular risk factors. Rosa Vazquez-Fresno, Rafael Llorach, Francesca Alcaro, Miguel Angel Rodriguez, Maria Vinaixa, Gemma Chiva-Blanch, Ramon Estruch, Xavier Correig, Cristina Andres-Lacueva. 5th International Conference on Polyphenols and Health (ICPH), Sitges, Barcelona, Espanya, 17-20 d'octubre de 2011. Llibre d'abstracts, pàg. 203.

14. Early changes in cellular inflammatory biomarkers related to atherosclerosis after consumption of tomato products. Valderas-Martínez Palmira, Casas Rosa, Chiva-Blanch Gemma, DeLama-Santasusagna Laia, Arranz Sara, Urpí-Sarda Mireia, Torrado Xavier, Andrés-Lacueva Cristina, Lamuela-Raventós Rosa María, Estruch Ramón. 5th International Conference on Polyphenols and Health (ICPH), Sitges, Barcelona, Espanya, 17-20 d'octubre de 2011. Llibre d'abstracts, pàg. 198.

15. Effects of polyphenols and/or alcohol in glucose metabolism and expression of cytokine and adhesion molecules related with atherosclerosis. Gemma Chiva-Blanch, Mireia Urpí-Sardà, Rafa Llorach, María Rotchés-Ribalta, Palmira Valderas-Martínez, Rosa Casas, Sara

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16. Positive effects of orange juice consumption in high cardiovascular risk Mediterranean population. The PREDIMED randomized trial. Ramón Estruch, Sara Arranz, Alex Medina-Remón, Palmira Valderas, Mireia Urpi-Sarda, Gemma Chiva-Blanch, Rosa Casas y Rosa Mª Lamuela-Raventos. 5th International Conference on Polyphenols and Health (ICPH), Sitges, Barcelona, Espanya, 17-20 d'octubre de 2011. Llibre d'abstracts, pàg. 222.

17. Effects of Polyphenol-Rich Diets (Mediterranean-Style Dietary Pattern) in Inflammatory Biomarkers Related with Atherosclerosis. Mireia Urpi-Sarda, Rosa Casas, Gemma Chiva-Blanch, Palmira Valderas-Martínez, Sara Arranz, Maribel Covas, Miguel Ángel Martinez, Ramon Estruch. 5th International Conference on Polyphenols and Health (ICPH), Sitges, Barcelona, Espanya, 17-20 d'octubre de 2011. Llibre d'abstracts, pàg. 220.

18. Food Matrix effect on the resveratrol bioavailavility after acute and long-term intervention studies with moderate intake of red wine, and other resveratrol rich-products. Maria Rotchés-Ribalta, Mireia Urpi-Sarda, Elvira Escribano, Rafael Llorach, Gemma Chiva-Blanch, Luisa Perez-Garcia, Ramon Estruch, Cristina Andrés-Lacueva. 5th International Conference on Polyphenols and Health (ICPH), Sitges, Barcelona, Espanya, 17-20 d'octubre de 2011. Llibre d'abstracts, pàg. 146.

19. Antiinflammatory effects of polyphenols from the Mediterranean diet: experiences from the PREDIMED study. Ramon Estruch, Mireia Urpi-Sarda, Rosa Casas, Gemma Chiva-Blanch, Edwin Saúl Romero-Mamami, Sara Arranz, Palmira Valderas-Martínez, Rafael Llorach, Rosa Mª Lamuela-Raventos, Cristina Andres-Lacueva. 5th International Conference on Polyphenols and Health (ICPH), Sitges, Barcelona, Espanya, 17-20 d'octubre de 2011. Llibre d'abstracts, pàg. 91. Comunicació oral.

20. Cambios en los biomarcadores inflamatorios celulares relacionados con la arteriosclerosis tras el consumo de salsas tomate. Valderas-Martínez P, Casas R, Arranz S, Chiva-Blanch G, Medina-Remón A, Urpi-Sarda M, Lamuela-Raventós RM, Estruch R. XXIV Congreso Nacional de la Sociedad española de Arteriosclerosis (SEA) / XIII Congreso Internacional de la Sociedad Iberolatinoamericana de Arteriosclerosis (SILAT), Sevilla, Espanya, 25-27 de maig de 2011.

1. Inhibición de la activación de las células inmunes circulantes relacionadas con la atherogenesis tras un año de intervención con Dieta Mediterránea. Rosa Casas, Emilio Sacanella, Gemma Chiva-Blanch, Palmira Valderas-Martínez, Rosa M. Lamuela-Raventós, Emilio Ros, Ramón Estruch. XXIV Congreso Nacional de la SEA, Sevilla, Espanya, 25-27 de maig de 2011.

2. Effects of polyphenolic compounds of red wine on the expression of cellular and serum chemokines and adhesion molecules related to atherosclerosis. Chiva-Blanch G, Llorach R, Rotchés-Ribalta M, Urpí-Sardà M, Lamuela-Raventos R.M, Andres-Lacueva C, and Estruch R. 4th International Conference on Polyphenols and Health, Yorkshire, 7–10 de desembre de 2009. Llibre d'abstracts, pàg. 283.

3. Moderate consumption of red wine,but not gin,decreases erythrocyte superoxide dismutase activity. Estruch R, Sacanella E, Mota F, Chiva-Blanch G, Antunez E, Casals E, Deulofeu R, Andres-Lacueva C, Lamuela-Raventos R.M, and Urbano-Marquez A. 4th International Conference on Polyphenols and Health, Yorkshire, 7–10 de desembre de 2009. Llibre d'abstracts, pàg. 283.

4. Polyphenolic and alcohol nutritional biomarkers in an interventional clinical study on wine consumption. M Rotchés-Ribalta, R Llorach, G Chiva-Blanch, M Urpi-Sardà, M Boto-Ordoñez, M Rabassa-Bonet, RM Lamuela-Raventos, R Estruch, C Andres-Lacueva. 4th International Conference on Polyphenols and Health, Yorkshire, 7–10 de desembre de 2009. Llibre d'abstracts, pàg. 259 y 141. Pòster i comunicació oral.

5. Effects of red wine components on the expression of cellular and serum inflammatory biomarkers related to atherosclerosis. G Chiva-Blanch, N Vila, R Casas, Boto M, RM Lamuela-Raventos, C Andres-Lacueva, R Estruch. III Symposium Ciber Fisiopatología de la Obesidad y Nutrición, Isla de la Toja, Galícia, Espanya, 25-27 de novembre de 2009.

6. Effects of mediterranean diet on body weight, adiposity and related hormones. R Casas, Estruch R, Vila N, G Chiva-Blanch, M Boto, M Bullo, MP Portillo, J Salas-Salvadó. III Symposium Ciber Fisiopatología de la Obesidad y Nutrición, Isla de la Toja, Galícia, Espanya, 25-27 de novembre de 2009.

7. Metabolomic and targeted study of biomarkers in an interventional clinical study on wine consumption. M Urpi-Sarda, R Llorach, M Rotchés-Ribalta, G Chiva-Blanch, M Boto-Ordoñez, M Rabassa-Bonet, RM Lamuela-Raventos, R Estruch, C Andres-Lacueva. III Symposium Ciber Fisiopatología de la Obesidad y Nutrición, Isla de la Toja, Galícia, Espanya, 25-27 de novembre de 2009. Guanyador del premi àrea clínica.

8. A LC-MS based metabolomics strategy to explore the biological effects of polyphenols-enriched diets. Issues, quality control and guidelines. R. Llorach, M. Urpi-Sardà, O. Jauregui, N. Khan, G. Chiva, C. Andres-Lacueva. XII Jornadas de Análisis Instrumental, Barcelona, Espanya, 21-23 d'octubre de 2008. Comunicació oral.

9. High sensitive determination of resveratrol in beer by liquid chromatography tandem mass spectrometry. Gemma Chiva-Blanch, Raul Zamora-Ros, Mireia Urpí-Sarda, Alexander Medina-Remón, Ramon Estruch, Cristina Andres-Lacueva. XII Jornadas de Análisis Instrumental, Barcelona, Espanya, 21-23 d'octubre de 2008. Llibre d'abstracts pàg. 349.

10. Determination of total polyphenols and resveratrol in Beer. Gemma Chiva-Blanch, Raul Zamora-Ros, Mireia Urpí-Sarda, Alexander Medina-Remón, Ramon Estruch, Cristina Andres-Lacueva. Salamanca, Espanya, 8-11 de juliol de 2008. Llibre d'abstracts pàg. 487.

I.II) Articles i capítols en revisió de l'etapa pre-doctoral

1. Chiva-Blanch G, Arranz S, Lamuela-Raventos RM, Estruch R. Alcohol and Polyphenols on Cardiovascular Disease Risk Factors. Alcohol and Alcoholism.
2. Sara Arranz, Palmira Valderas-Martínez, Gemma Chiva-Blanch, Mireia Urpi-Sarda, Rosa Casas, Rosa M Lamuela-Raventos and Ramón Estruch. Cardioprotective effects of cocoa: Clinical evidences from randomized studies in humans. *Molecular Nutrition & Food Research.*
3. Sara Arranz, Gemma Chiva, Rosa M. Lamuela-Raventós and Ramon Estruch. Wine polyphenols in the management of cardiovascular risk factors. Book chapter in: Polyphenols in prevention and treatment of vascular and cardiac disease and cancer. Editors: Ronald R. Watson, Victor R. Preedy, Sherma Zibadi.

I.III) Presentacions a congressos i publicacions prèvies a l'etapa pre-Doctoral

I.III.I) Publicacions

4. Chiva-Blanch, G.; Giménez-Bonafe, P.; Llaudó, I.; Torras, J.; Cruzado, JM.; Petriz, J.; Castaño, E.; Franquesa, M.; Herrero-Fresneda, I.; Tortosa, A.; Rama, I.; Bestard, O.; Grinyó, JM.; Lloberas, N. Different Storing and Processing Conditions of Human Lymphocytes do not Alter P-Glycoprotein Rhodamine 123 Efflux. *Journal of Pharmacy and Pharmaceutical Sciences* 2009;12(3):357-366.

I.III.II) Presentacions a congressos

5. Actividad de extrusión de Pgp en el rechazo: uso de linfocitos congelados en estudios multicéntricos. Chiva-Blanch G., Lloberas N., Torras J., Grinyó JM y Giménez-Bonafé P. Novè Congrés de la Societat Catalana de Transplantament. Barcelona, Espanya, 23- 28 de febrer de 2007. Llibre d'abstracts, pàg. 76. Comunicació oral.

6. Opinión sobre la influencia que tienen los estudios de Nutrición humana y Dietética sobre los hábitos alimentarios de los alumnos del CESNID, UB. Puchal A, Cantos D, Chiva G, Rodríguez J y Ruiz C. Segundo Congreso de la Associación Española de Dietistas-Nutricionistas. Pamplona, Espanya, 15-16 d'octubre de 2004. Llibre d'abstracts, pàg. 39.

ANNEX II

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Determination of resveratrol and piceid in beer matrices by solid-phase extraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

Beer is one of the most commonly consumed undistilled alcoholic beverages in many countries. In recent studies, the stilbenes resveratrol and piceid have been found in some hop varieties which are used in the production of beer. Therefore, they could be transferred to beer. The aim of the present work was to validate a method to study the potential content of *trans*- and *cis*-resveratrol and piceid in 110 commercial beers from around the world. The resveratrol and piceid contents of 110 beers were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after a solid-phase extraction (SPE) using optimized and validated procedures for the beer matrix. The beer matrix effect was also studied. Stilbenes were found in quantifiable amounts in 92 beers, while concentrations below the limit of quantification (LOQ) were found in 18 beers. Resveratrol was found in the range of 1.34–77.0 μg/L in 79% of the beers analyzed, and piceid was found in the range of 1.80–27.3 μg/L in only 33% of them. The mean of total resveratrol in all the beers was 14.7 ± 20.5 μg/L. The content of resveratrol has been compared with other resveratrol containing foods. A serving of beer contains similar amounts of stilbenes as berries, less than chocolate and grape products but more than pistachios, peanuts or tomatoes. Overall, beer is one of the products with the lowest levels of total resveratrol (μg/L), and despite its high consumption it should not be considered as a representative source of resveratrol.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a phenolic *phytoalexin* with potential preventive activity in several human diseases [1–6]. The described health effects depend on the ingested amount and bioavailability of these compounds. The presence of *trans*-resveratrol, *trans*-piceid (the resveratrol glucoside) and their respective *cis*-isomers in the human diet is limited. The major sources of resveratrol include grapes and grape products such as wines and grape juice [7]. Although it has been found in other foods such as peanuts, pistachios and some berries, their total resveratrol levels are from 10 to 100-times less than those in grape products [7]. Recently, it has also been found at low levels in the skin of some kinds of tomatoes [8] and in chocolate products [9].

Beer is one of the most commonly consumed undistilled alcoholic beverages in many countries. It is a complex mixture of bioactive substances including carbohydrates, amino acids, minerals, vitamins and phenolic compounds [10]. The majority of phenolic compounds in beer are non-tannic and non-flavonoid compounds (98% of total phenolic compounds), such as phenolic acids [11,12]. Other minor phenols found in beer are flavonols, catechins, procyanidins, tannins and chalcones [13,14]. The content of polyphenols in beer is largely influenced by the genetic factors of its raw materials and therefore by the environmental conditions in which they grow, and also by technological brewing factors [13,15]. Hops are used in the brewing industry to add flavor and bitterness to beer [16]. Although it has been observed that the nature of the harvest year can have a strong influence [17–19], *trans*- and *cis*-piceid have been found in different hop cultivars and in hop pellets in concentrations ranging from 0.5 to 11.7 mg/kg, up to 2 mg/kg of *trans*-resveratrol [20], and *cis*-resveratrol has been found up to

Abbreviations: SPE, solid-phase extraction; LC–ESI-MS/MS, liquid chromatography–electrospray ionization-tandem mass spectrometry; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantification; MF, matrix factor; PE, process efficiency.

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0.3 mg/kg in hop pellets [21] and up to 1.2 mg/kg in hop cones [22]. Moreover, about 20–30% of beer polyphenols originate from hops, and 70–80% from malt [10,20], although hops are added in 100-times lesser amounts than malt [23,24]. Therefore, a low content of stilbenes from the hops could be expected to be found in the final beer product [18]. Recently, low amounts of *trans*-resveratrol and *trans*-piceid (5 and 15 μg/L, respectively) have been found in four and five regular beers, respectively [25]. Other authors, after analyzing only two beers by high performance liquid chromatography (HPLC-UV), with detection at 280 nm, found up to 200-times higher concentrations of resveratrol (ranging between 0.3 mg/L and 3 mg/L) [26], however neither the *trans*- or *cis*-forms nor the piceid contents were specified. Therefore, very sensitive, selective and validated analytical methods are necessary to strengthen scientific evidence of the presence of *trans*- and *cis*-resveratrol and piceid in beers. Added to this, when performing MS analysis of food components, a large matrix effect can be observed, which leads to a diminution in the signal intensity of the analytes and the sensitivity of the method. The matrix effect during the validation of analytical methods may be best examined by comparing the response of an analyte at any given concentration spiked into the target matrix, to the response of the same analyte present in the "neat" mobile phase [27–29].

The aim of this study was to validate an analytical method for beer matrix and to study the content of *trans*- and *cis*-resveratrol and piceid in 110 beers from around the world, including alcohol-free, lager, ale, weissbier, stout, and abbey beers, and compare them with other dietary sources of resveratrol, like red wine or grape products and other foods with stilbenes.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled avoiding exposure to light. Standards of *trans*-resveratrol (99% purity), *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity) and ethyl gallate were purchased from Sigma–Aldrich–Fluka (St. Louis, MO), *cis*-resveratrol (97% purity) from Toronto Research Chemicals Inc (Toronto, ON, Canada), and taxifolin (>90% purity) from Extrasynthèse (Genay, France). Methanol, acetone, glacial acetic acid, ethyl acetate and acetonitrile of HPLC grade were purchased from Scharlab (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from Millipore system.

2.2. Samples

A total of 110 international commercial beers were analyzed (Table 1): 52 lagers, 20 ales, 15 abbey beers, 11 weissbiers, 7 stouts and 5 alcohol-free beers. The alcohol content ranged between less than 0.05 and 14% (v/v). All beers were purchased from local commercial markets. Some of the beers selected in this study are the most widely consumed in Spain (19%), while the others are a variety of beers consumed in Europe (66%) and worldwide (15%).

2.3. Sample preparation

Prior to the analysis, all beers were sonicated for 4 min for degasification. All experiments were performed on ice, avoiding light exposure, and all reagents were maintained in an N_2 atmosphere to avoid the oxidation of phenolic compounds. All beers were analyzed immediately after being opened.

2.4. Quality parameters of the method

To obtain the maximum detectivity and sensitivity in the analysis of resveratrol in beer by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS), sample extraction was optimized, and the quality of the method and the matrix effect were evaluated. Optimization of sample extraction was carried out through the analysis of different parameters, including beer volume and pre-cleaning of samples as recommended by Jerkovic et al. [25]. Different volumes of samples (5 and 1 mL) were considered for loading onto HLB® cartridges (30 mg; 30 μm particle size and 80 Å pore size) (Waters). The pre-cleaning of beers before the solid-phase extraction (SPE) consisted of cleaning beers with toluene (1:1, v/v) followed by a double extraction with cyclohexane (1:1, v/v) as described previously by Jerkovic et al. [25].

After the optimization of the sample extraction, the method was evaluated for selectivity, detectivity, sensitivity, linearity, recovery, accuracy and precision, according to the Food and Drug Administration (FDA) acceptance criteria [30]. Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This was assessed by analyzing blank beer samples. A blank beer sample was obtained after applying the SPE analysis procedure to 1 mL of blank beer (beer number 57, Table 1). The selected blank beer for the evaluation of the method was a lager beer because it is the most widely consumed kind of beer worldwide and more common on the market. The detectivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was determined as the concentration of analytes with a signal-to-noise ratio of at least 3 and the LOQ was the lowest standard with a signal-to-noise ratio of at least 10. Sensitivity was expressed as the slope of the analytical curve. Linearity was evaluated by spiking blank beer matrix with known concentrations of analytes at 6 concentration levels (4–100 μg/L for *trans*-resveratrol and 2–50 μg/L for *cis*-resveratrol and *trans*-piceid). Recovery was calculated as the ratio of the mean peak area of the analytes spiked before extraction to the mean peak area of the analytes spiked post-extraction multiplied by 100 in a six-point calibration curve of beer matrix ($n=3$).

The precision and accuracy of the method were evaluated using three different concentrations [low (near the LOQ), medium and high] within the linear range of the calibration curve of the analytes in the beer matrix ($n=8$). Accuracy and precision were calculated as the percentage and relative standard deviation, respectively, of the ratio of the mean calculated concentration and the true value of the known added concentration in blank beer samples ($n=8$) for each concentration.

The matrix effect of the beers was also evaluated by calculating the matrix factor (MF or suppression coefficient), process efficiency (PE) [28] and the variations in the sensitivity of the method [29]. The MF was expressed as the ratio of the mean peak area of the analytes spiked after the SPE procedure in blank beer matrix to the mean peak area of the same analyte standards in aqueous matrix without SPE procedure multiplied by 100. PE was calculated as the ratio of the mean peak area of the analytes spiked before the SPE procedure to the mean peak area of the same analytes standards in aqueous matrix without SPE procedure multiplied by 100. Differences in the sensitivity of the method within the two matrices (beer and water) were expressed as the ratio of the slope of the analytical curve in beer matrix (spiked after the SPE procedure) to the slope of the analytical curve in aqueous matrix without SPE procedure multiplied by 100.

Short and long term stabilities of standards were previously evaluated by our group [31].

Table 1
Type, alcoholic degree (A.D. (%, v/v)), manufacturing country and concentrations of *trans*- and *cis*-resveratrol (μg/L) and *trans*- and *cis*-piceid (μg/L) of the 110 analyzed beers.

ID	Type	A.D. (%, v/v)	Country	*trans*-Resveratrol (μg/L)	*cis*-Resveratrol (μg/L)	*trans*-Piceid (μg/L)	*cis*-Piceid (μg/L)[a]
1	Lager	4.4	Czech Republic	n.d.	4.39	n.q.	6.48
2	Lager	5	Czech Republic	n.d.	n.q.	n.q.	n.q.
3	Lager	4.5	Mexico	n.d.	n.q.	n.d.	n.d.
4	Stout	8	Ireland	11.94	10.86	n.q.	n.d.
5	Stout	5	Holland	7.79	16.12	n.d.	n.q.
6	Stout	5.8	Germany	n.d.	4.84	n.q.	4.53
7	Lager	4.8	Brazil	n.q.	n.q.	n.q.	n.q.
8	Ale	8.4	Belgium	n.q.	n.q.	n.q.	n.q.
9	Lager	4.9	Germany	n.d.	3.45	n.q.	1.80
10	Alcohol-free	<0.05	Italy	4.09	4.74	n.q.	n.q.
11	Lager	4.7	Mexico	11.94	n.d.	n.q.	n.d.
12	Lager	4.8	Germany	n.d.	2.81	n.q.	n.q.
13	Lager	5	Holland	n.q.	n.d.	n.q.	n.q.
14	Stout	4.2	Ireland	10.56	n.d.	n.d.	n.q.
15	Alcohol-free	<0.05	Germany	5.67	n.d.	n.q.	n.q.
16	Alcohol-free	<0.05	Germany	n.d.	n.d.	n.d.	n.d.
17	Lager	<0.5	Germany	n.d.	5.28	n.q.	n.q.
18	Stout	5	Italy	30.86	7.13	n.q.	n.q.
19	Lager	5.1	Italy	n.d.	4.59	n.q.	n.q.
20	Lager	4.7	Italy	4.53	2.29	n.q.	n.q.
21	Lager	5.6	Poland	n.q.	n.q.	n.q.	n.q.
22	Lager	4.8	Germany	4.73	n.d.	n.q.	n.q.
23	Lager	4.6	Italy	7.36	2.58	n.q.	n.q.
24	Ale	4.5	Italy	32.69	17.83	n.d.	2.62
25	Ale	6.5	Italy	32.72	n.q.	n.d.	n.q.
26	Abbey beer	10.5	Belgium	23.18	n.d.	3.16	9.73
27	Lager	4.5	Mexico	n.d.	n.q.	n.q.	n.d.
28	Lager	3.5	Spain	n.q.	n.q.	n.q.	2.85
29	Lager	6.2	Spain	n.d.	1.91	n.q.	n.q.
30	Stout	4.5	England	11.61	3.43	n.q.	3.31
31	Weissbier	5.4	Germany	10.68	2.87	n.d.	n.d.
32	Weissbier	5.5	Germany	n.q.	n.q.	n.d.	n.d.
33	Abbey beer	10	Holland	n.q.	2.92	n.q.	n.q.
34	Ale	8.5	Belgium	n.q.	n.q.	n.d.	n.d.
35	Weissbier	5.3	Germany	n.d.	1.99	n.d.	n.q.
36	Weissbier	8.5	Belgium	n.d.	1.77	n.q.	n.q.
37	Ale	4.7	England	n.q.	1.72	n.q.	n.q.
38	Ale	4.5	England	12.47	n.q.	n.q.	3.51
39	Lager	5.1	Uruguay	n.d.	2.21	n.q.	n.q.
40	Lager	14	Austria	10.22	n.d.	n.d.	2.06
41	Lager	4.7	USA	8.56	n.q.	n.q.	n.q.
42	Abbey beer	8.2	Belgium	9.60	1.34	n.d.	n.q.
43	Stout	4.3	Spain	10.36	2.32	n.d.	n.q.
44	Lager	4.9	Argentina	4.42	1.82	n.d.	n.q.
45	Ale	4.6	Spain	17.78	2.68	n.d.	n.q.
46	Lager	4.8	Italy	n.d.	n.q.	n.q.	n.q.
47	Weissbier	12	France	6.25	6.07	n.q.	4.91
48	Alcohol-free	<0.05	Spain	4.60	n.q.	n.d.	3.13
49	Lager	4.5	Mexico	n.d.	n.q.	n.q.	n.q.
50	Lager	5.3	Mexico	9.66	2.56	n.d.	n.q.
51	Weissbier	5.2	Germany	13.93	n.d.	n.q.	n.q.
52	Lager	5	England	n.q.	n.d.	n.d.	n.q.
53	Lager	4	Colombia	n.d.	1.49	n.q.	n.q.
54	Ale	10	Belgium	6.12	n.q.	n.q.	n.q.
55	Abbey beer	7.3	Belgium	n.d.	3.23	n.q.	2.35
56	Lager	5	UK/Japan	n.d.	n.d.	n.q.	n.q.
57	Lager	4.8	UK/India	n.d.	n.d.	n.d.	n.q.
58	Lager	5	UK/India	n.d.	5.38	n.q.	2.56
59	Lager	5	Germany	n.q.	4.43	n.d.	n.q.
60	Weissbier	8.5	Belgium	18.00	2.70	n.q.	n.q.
61	Lager	5.2	Germany	n.d.	2.04	n.d.	n.q.
62	Weissbier	9	Belgium	14.88	5.74	3.08	24.24
63	Abbey beer	6.5	Holland	6.42	3.41	n.q.	2.15
64	Abbey beer	8	Holland	18.15	8.09	n.q.	5.38
65	Weissbier	5	Germany	n.d.	n.d.	n.d.	n.q.
66	Weissbier	8	Belgium	27.46	5.84	n.q.	n.q.
67	Abbey beer	6.6	Belgium	n.d.	n.q.	n.q.	n.d.
68	Abbey beer	5.5	Holland	n.d.	2.54	n.q.	3.07
69	Lager	4	Colombia	18.97	6.17	n.q.	n.q.
70	Abbey beer	7	Holland	n.d.	5.52	n.q.	n.q.
71	Weissbier	4.9	Belgium	38.74	8.70	n.d.	n.q.
72	Abbey beer	8.2	Belgium	10.06	5.86	n.q.	n.d.
73	Ale	8	Belgium	n.d.	n.d.	1.80	2.68
74	Ale	5.2	England	19.96	3.02	n.q.	4.83
75	Ale	5.1	Spain	33.33	8.33	n.d.	n.d.
76	Lager	3.5	Spain	42.19	5.91	n.d.	1.81

Table 1 (*Continued*)

ID	Type	A.D. (%, v/v)	Country	*trans*-Resveratrol (μg/L)	*cis*-Resveratrol (μg/L)	*trans*-Piceid (μg/L)	*cis*-Piceid (μg/L)[a]
77	Ale	7	Spain	26.21	5.86	n.d.	n.q.
78	Ale	5.4	Spain	32.47	5.66	n.d.	n.q.
79	Lager	4.7	Jamaica	6.65	n.q.	n.q.	n.q.
80	Lager	4.8	Germany	n.q.	n.q.	n.q.	1.89
81	Lager	5	Belgium/Australia	6.00	3.45	n.q.	n.q.
82	Lager	5	Holland	n.d.	n.q.	n.q.	n.q.
83	Ale	3.8	Spain	26.91	22.65	n.d.	n.q.
84	Ale	5.4	England	10.00	1.68	n.d.	3.69
85	Lager	6.4	Spain	13.82	7.40	n.d.	6.55
86	Lager	4.8	Italy	n.d.	2.00	n.d.	n.q.
87	Lager	5.2	Lithuania	n.q.	1.46	n.q.	1.80
88	Lager	4.8	Germany	n.d.	5.21	n.q.	n.q.
89	Ale	5.5	Spain	11.26	10.77	n.d.	n.q.
90	Ale	4.5	England	22.01	n.q.	n.q.	4.47
91	Lager	6.5	Spain	26.82	3.65	n.q.	1.84
92	Lager	4.9	Germany	n.q.	2.88	n.q.	n.q.
93	Alcohol-free	<0.05	Spain	6.03	1.34	n.q.	n.q.
94	Lager	5	Germany/Russia	4.63	3.68	n.q.	2.81
95	Lager	4.7	Italy	n.q.	2.61	n.q.	2.46
96	Abbey beer	6.5	Spain	n.d.	8.95	n.q.	4.77
97	Lager	7.2	Spain	n.d.	2.64	n.q.	n.q.
98	Lager	5.4	Spain	5.39	3.26	n.q.	3.77
99	Abbey beer	7	Belgium	54.52	n.q.	n.q.	4.54
100	Lager	4.5	Spain	n.d.	2.02	n.q.	n.d.
101	Lager	7.2	Spain	4.65	n.q.	n.d.	n.q.
102	Lager	4.8	Spain	n.d.	4.23	n.d.	n.q.
103	Lager	4.8	Spain	n.d.	3.04	n.d.	n.d.
104	Abbey beer	7.5	Belgium	66.74	10.31	n.d.	4.17
105	Abbey beer	9.2	Belgium	7.29	n.d.	n.q.	8.15
106	Ale	11	Belgium	13.97	3.98	2.30	6.66
107	Abbey beer	9.5	Belgium	22.61	1.37	9.31	16.88
108	Ale	8.5	Belgium	23.77	n.q.	1.99	4.47
109	Ale	10.5	Belgium	12.14	n.q.	n.q.	3.65
110	Lager	5.4	Spain	n.d.	n.q.	n.d.	1.80

n.d.: not detected; n.q.: not quantified.

[a] Expressed as *trans*-piceid equivalents.

2.5. Determination of resveratrol and piceid in beer by LC–MS/MS

Resveratrol and piceid analyses were carried out by LC–ESI-MS/MS after an SPE, based on the Urpi-Sarda et al. [31,32] method and optimized for beer samples.

2.5.1. Extraction procedure

Samples (1 mL of each presonicated beer, diluted with ultrapure water to reduce the alcohol percentage to below 5%) with the internal standard (ethyl gallate) all maintained on ice, were loaded onto a Waters Oasis® HLB® 96-well plate that had been preconditioned with 1 mL of methanol and equilibrated with 1 mL of 2 mol/L acetic acid in water. Samples were washed with 1 mL of 2 mol/L acetic acid in water and 1 mL of 2 mol/L acetic acid in water/methanol (85/15, v/v). Elution was achieved with 0.5 mL of 1 mol/L acetic acid in methanol and 2 × 0.75 mL of 1 mol/L acetic acid in ethyl acetate. The eluate was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 100 μL of initial mobile phase, with 1.64 μmol/L of taxifolin as an additional external standard. Ethyl gallate was used as the internal standard (mean recovery: 99%, CV = 10%) and taxifolin was used as an additional external standard to assess the performance of the mass spectrometer. Both compounds are absent in beers.

2.5.2. LC–MS/MS analyses

LC analyses were performed using an Agilent 1100 system equipped with a quaternary pump and a refrigerated plate autosampler (Waldbronn, Germany). An Applied Biosystems API 3000 triple quadrupole mass spectrometer, equipped with a turbo ion spray source ionizing in the negative mode, was used to obtain the mass spectrometry data.

A Phenomenex Luna C_{18} column, 50 mm × 2.0 mm i.d., 5 μm (Torrance, CA) maintained at 40 °C was used for chromatographic separation. The injection volume was 15 μL, and the flow rate was 500 μL/min. Gradient elution was carried out with 0.5 mL/L acetic acid as mobile phase A and 700 mL/L acetone, 300 mL/L acetonitrile with 0.4 mL/L acetic acid as mobile phase B. A non-linear gradient profile was applied as follows: 0–0.5 min, 10–15%B; 0.5–5 min, 15–100%B and 5–5.6 min, 100%B. The column was re-equilibrated for 6 min, to return to 10%B.

The MS and MS/MS parameters were as previously described [31]. Briefly, the following parameters were used: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 6 (arbitrary units), focusing potential −200 V, entrance potential −10 V, declustering potential −50 V, drying gas (N_2) heated to 400 °C and introduced at a flow rate of 6000 cm^3/min. The collision energy was −25 V for resveratrol, piceid and taxifolin, and −30 V for ethyl gallate.

2.5.3. Quantification of analytes

For the quantification of *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid in beer samples, the multiple reaction monitoring (MRM) mode was used with a dwell time of 300 ms, monitoring four transitions for each analysis: *trans*- and *cis*-resveratrol (227/185), *trans*- and *cis*-piceid (389/227), ethyl gallate (197/169) and taxifolin (303/285). *trans*-Resveratrol was quantified using a six-point calibration curve determined by weighted ($1/x^2$) linear regression between 4 and 100 μg/L in the beer matrix and *cis*-resveratrol and *trans*-piceid between 2 and 50 μg/L in the beer matrix ($n = 3$ for each calibration curve). Ethyl gallate was used for quantification purposes. *cis*-Piceid was expressed as *trans*-piceid equivalents as no commercial standard was available. To identify *cis*-piceid, *trans*-

Table 2
LOD, LOQ and between-day ($n=8$) precision and accuracy data obtained from the LC–MS/MS of *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid in the beer matrix after solid-phase extraction.

Analyte	LOD (μg/L)	LOQ (μg/L)	Added concentration (μg/L)	Calculated concentration[a] (mean, μg/L)	Accuracy (%)	Precision (RSD[b], %)
trans-Resveratrol	1.10	3.68	4	4.08	102	2.5
			50	48.95	98	8.5
			100	99.43	99	1.0
cis-Resveratrol	0.41	1.34	2	2.11	105	13.6
			10	10.64	106	6.1
			50	49.9	100	8.7
trans-Piceid	0.54	1.80	2	1.85	92	15.1
			10	9.86	99	8.2
			50	49.93	100	0.8

[a] Values are the mean of $n=8$.
[b] Relative standard deviation.

piceid isomerated by light exposure for 10 min on ice was used [33].

3. Results and discussion

3.1. Quality parameters of the method

Optimization of the sample extraction procedure and evaluation of the methodology were performed.

3.1.1. Sample extraction optimization

The main objective of the sample extraction optimization was to reach the highest detectivity and sensitivity while minimizing the matrix effect in LC–MS/MS. In the first step, we compared the recovery of resveratrol and piceid from the spiked beer matrix (1 μg/mL of final concentration) using different sample volumes (5 and 1 mL) with or without the pre-cleaning procedure ($n=4$). The recovery values of *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid from the pre-cleaned samples increased by 86%, 157% and 112% respectively, compared to the non-pre-cleaned ones when 5 mL were used, and this is in accordance with Jerkovic et al. [25]. However, when we compared 1 mL of a sample with and without the pre-cleaning procedure, no differences were observed for the recovery of the compounds. These results suggested a higher matrix effect when a higher volume was considered, affecting the analyte ionization and obtaining best signal-to-noise ratio for the analytes extracted with lesser volumes. When we compared different volumes of pre-cleaned samples (1 and 5 mL), the recovery values for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid after loading 1 mL were 2, 2.5 and 1.3-fold higher, respectively, than when 5 mL were considered. Again, the volume of the sample influenced the matrix effect and the resveratrol ionization. Therefore, as no differences were observed loading 1 mL of sample with and without pre-cleaning, and the recovery was higher than after loading 5 mL of pre-cleaned sample, 1 mL of beer without the pre-cleaning procedure was the selected volume used for the total resveratrol determination in the commercial beers and for the standard calibration curves.

3.1.2. Evaluation of the method

The method met the criteria of selectivity because no endogenous peaks were observed at the same retention time as the analytes in the blank beer samples. The LOD and LOQ of analytes are shown in Table 2, and sensitivity was 3.62×10^{-5}, 2.60×10^{-4} and 9.47×10^{-5} cpm L/μg for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively. The 6-point calibration concentrations ($n=3$) in blank beer matrix determined by weighted ($1/x^2$) least-square regression analysis showed correlation coefficients for all analytes > 0.99. The calibration curves were linear over the concentration range studied. Recovery was evaluated comparing a 6-point calibration curve of analytes with and without the SPE procedure in the beer matrix ($n=3$). *cis*-Resveratrol, *trans*-resveratrol and *trans*-piceid showed recovery values of 99%, 90% and 102%, respectively, after SPE in the beer matrix. The recovery for the internal standard ethyl gallate was also evaluated at the concentration used in the analysis (50 μg/L) ($n=8$). Ethyl gallate showed a recovery of 99%. The precision and accuracy of the analytes in the beer matrix after

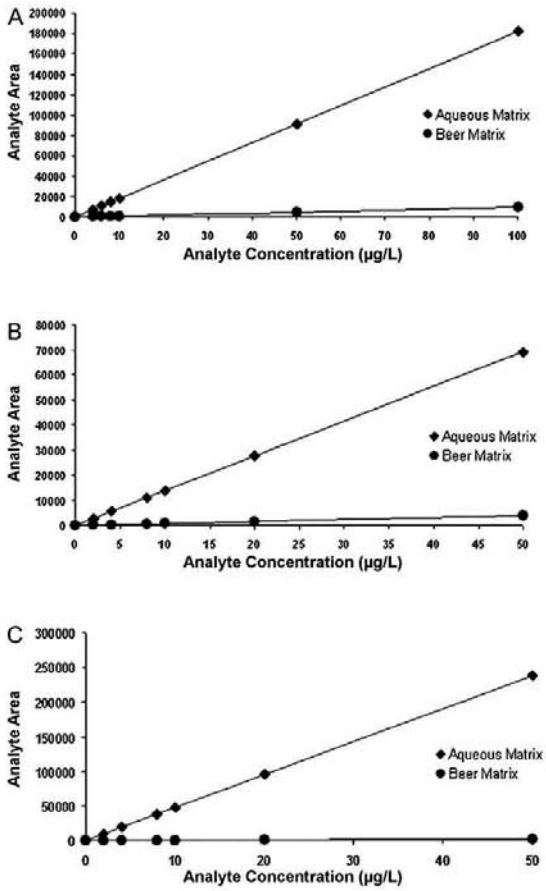

Fig. 1. Matrix effect of beer for: (A) *trans*-resveratrol; (B) *cis*-resveratrol; and (C) *trans*-piceid.

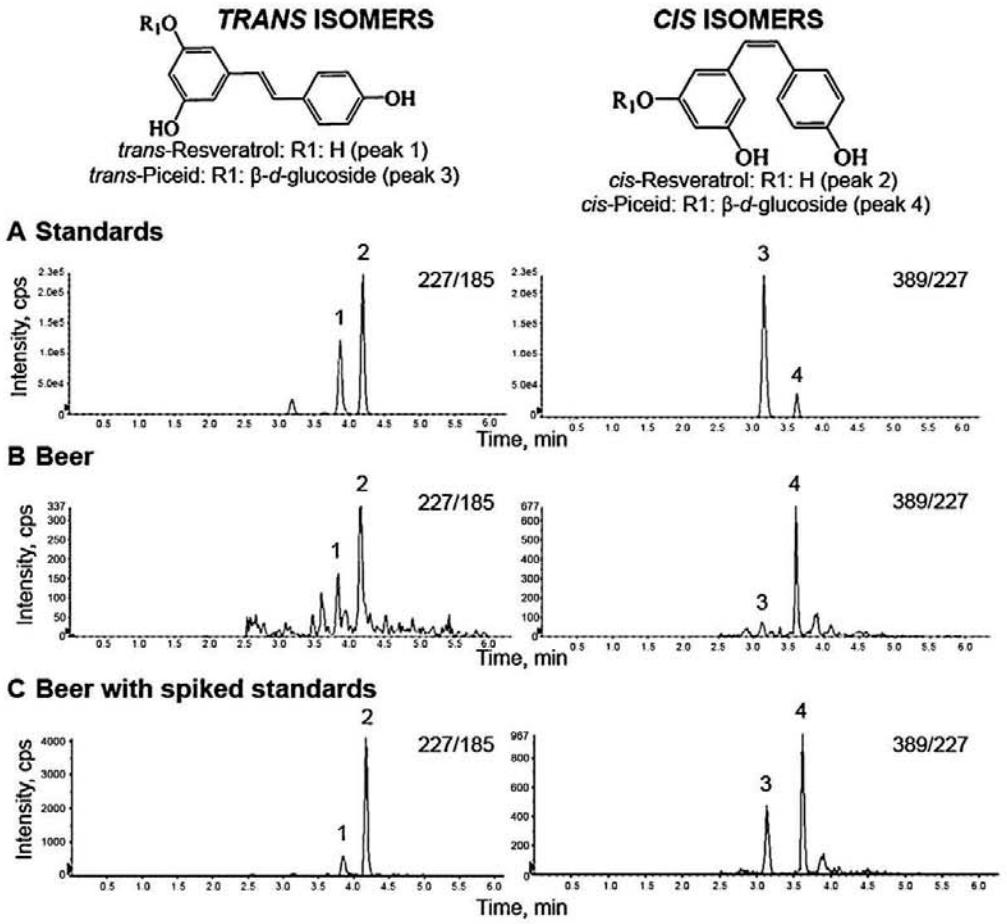

Fig. 2. Chemical structures of resveratrol and piceid in their *trans*- and *cis*-forms and multiple reaction monitoring (MRM) chromatograms for: (A) standards of *trans*-resveratrol (1), *cis*-resveratrol (2), *trans*-piceid (3) and *cis*-piceid (4); (B) beer 62 and (C) beer 62 spiked with standards.

SPE met the acceptance criteria of the FDA [30] and are shown in Table 2.

3.1.3. Evaluation of the matrix effect

To assess the strength of the matrix effect, 6-point calibration curves of analytes ($n=3$) in the beer matrix and in an aqueous matrix (pure solvent) without the SPE procedure were compared. Briefly, blank beer was prepared with the full extraction procedure and standards were added *a posteriori* to this matrix to compare the differences of peak signal intensity of the analytes due to the matrix effect to calculate the MF, and *a priori* to calculate the PE. The MF for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid were 5.2%, 5.8% and 1.1% respectively. These matrix factors highlight a great suppression of the ionization of the analytes due to the matrix effect [28,29]. The PE of the method considers the MF and the recovery. The PE was 12.6%, 28.2% and 3.25% for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively. Taking into account that the recovery is >90% for all the analytes, the low PE value is attributable to the high MF. As shown in Fig. 1, standard curves in the beer matrix showed a decrease of the sensitivity of 95%, 94% and 99% for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively, when compared to the aqueous matrix. This great loss in the peak intensity signal highlights that calibration curves in an adequate matrix, in this case the beer matrix, are needed to avoid an underestimation of the analyte concentration in beer samples. This enormous matrix effect may also explain the differences in the enhancement of the peak intensity when loading 1 or 5 mL of pre-cleaned beer samples compared to non-pre-cleaned samples. To our knowledge, this is the first time that matrix effect of beer in the resveratrol analysis by LC–MS/MS has been highlighted and calculated.

The matrix effect (MF) for the internal standard ethyl gallate was also evaluated at the concentration used in the analysis (50 μg/L) ($n=8$). Ethyl gallate in the beer matrix spiked after the SPE procedure showed a decrease of the peak intensity of 85% compared with the aqueous matrix without SPE procedure.

3.2. Determination of resveratrol and piceid in beers

The 110 commercial beers were quantified using MRM transitions of 227/185 for *trans*- and *cis*-resveratrol and 389/227 for *trans*- and *cis*-piceid in LC–MS/MS. In this study, *trans*- and *cis*-piceid showed a retention time of 3.15 and 3.62 min, respectively, and *trans*- and *cis*-resveratrol showed a retention time of 3.85 and 4.18 min, respectively (Fig. 2A). The confirmation of resveratrol and piceid in beer samples was based on their retention time and ion fragmentation in the MS/MS mode as compared with those of commercially available standards. Finally, to verify the identity of the

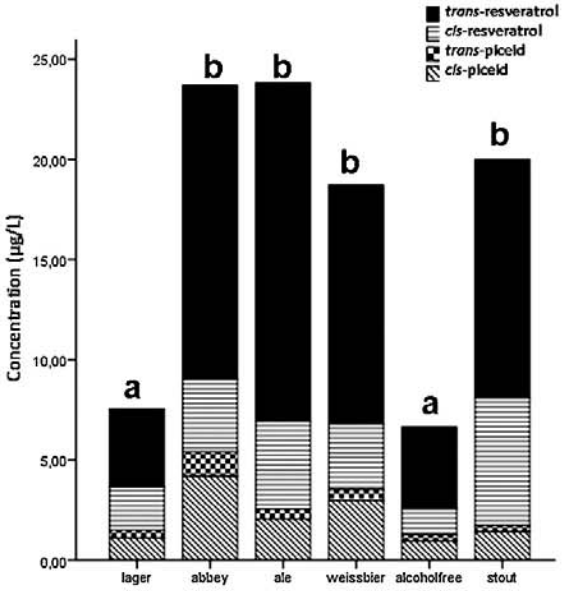

Fig. 3. Mean concentration (µg/L) of *trans*- and *cis*-resveratrol and piceid contents in different beer varieties. Bars with different letters are significantly different in total resveratrol content ($p < 0.05$).

peaks, beer samples (Fig. 2B) and spiked beer samples (Fig. 2C) were injected and compared, confirming the presence of *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid in beers.

Of the 110 analyzed beers, 79% of them contained free resveratrol (mainly in the *trans*-form), while only 33% of the beers analyzed contained piceid in quantifiable amounts. Table 1 shows that 59 beers contained *trans*-resveratrol between 3.68 and 66.74 µg/L, 69 beers contained *cis*-resveratrol in a range between 1.34 and 22.65 µg/L, 6 beers contained *trans*-piceid between 1.8 and 9.31 µg/L, and 38 beers contained *cis*-piceid between 1.80 and 24.24 µg/L. The beer with the greatest amount of stilbenes (beer number 104) contained 66.74 µg/L, 10.31 µg/L and 4.17 µg/L of *trans*- and *cis*-resveratrol and *cis*-piceid, respectively.

Jerkovic et al. [17,18,21,22] found *trans*-resveratrol (up to 1 mg/kg) and *cis*-resveratrol (up to 1.2 mg/kg) in significantly lower quantities than *cis*- and *trans*-piceid in hops (2–6 mg/kg and 4–9 mg/kg, respectively). In our analyses, resveratrol (mainly in its *trans*-form) has been found to be the most abundant stilbene in beer. This can be attributable to the fact that resveratrol (in the *trans*- and *cis*-form) can be partially regenerated by its glucoside, although piceid in beer remains more stable during the brewing process than resveratrol [26]. It could also be possible that hydrolysis of glycosides by yeast or bacterial β-glucosidase activity through beer fermentation may lead to piceid hydrolysis yielding free resveratrol [17,18]. In addition, an isomerase activity on phenols by the yeast during fermentation has been described previously by Jeandet et al. [34] and other authors [35]. As well as these factors, the amount of resveratrol and piceid extraction from hops to beer may depend on the commercial form of the hops [21,36], as well as on hop freshness [22]. These factors can explain the differences in the *trans*- and *cis*-amounts of resveratrol and piceid in hops and beers.

The mean concentration of total resveratrol distributed in *trans*- and *cis*-resveratrol and piceid in different kinds of beers is shown in Fig. 3. Abbey beers, ale, weissbier and stout beers contained significantly higher amounts of total resveratrol than lager and alcohol-free beers ($p < 0.05$, Mann–Whitney test). These differences in stilbene concentrations could be due mainly to the different hop varieties used, as well as maceration, fermentation and the hopping rate in the boiling kettle during the brewing process [18].

The mean of piceid and resveratrol in their *trans*- and *cis*-forms, as well as the total resveratrol expressed per serving and per liter or kg in beers and in other resveratrol-containing foods is shown in Table 3. On an equal volume basis, beer had ~580-fold lower levels of total resveratrol than red wine [7], ~60-fold lower levels than grape juice and ~50-fold lower levels than white wine [7]. This means that ~260 L of beer contains the equivalent amount of total resveratrol found in one glass of wine (150 mL). Nevertheless, because of their alcoholic content, they should be consumed in moderation.

In conclusion, a method to analyze resveratrol in beer matrix has been developed and evaluated and matrix effect of beer was determined. Total resveratrol was found in a range of 1.99–81.22 µg/L in 92 of the 110 commercial beers studied. *trans*-Resveratrol was the stilbene found in the highest levels and in the largest number of beers. Overall, beer contains only low levels of total resveratrol (µg/L), and despite its high consumption, it is not a representative source of dietary resveratrol.

3.3. Safety

We followed the general guidelines for working with organic solvents and acids. Universal precautions for the handling of chemicals were applied.

Table 3
Average of total resveratrol content (µg) per serving and per liter or kilogram of different food items.

Food	*trans*-Resveratrol (µg/L or µg/kg)	*cis*-Resveratrol (µg/L or µg/kg)	*trans*-Piceid (µg/L or µg/kg)	*cis*-Piceid (µg/L or µg/kg)	Total resveratrol (µg/L or kg)	Serving (g or mL)	Total resveratrol (µg/serving)	Reference
Red wine	1810	440	4950	1270	8470	150	1270	[7,9]
White wine	100	160	260	220	740	150	111	[7]
Grapes, not specified	1560	–	670	–	2230	100	223	[7]
Grape juice	100	tr	360	430	890	250	222	[7]
Peanuts, toasted	60	–	–	–	60	30	1.8	[7,9]
Pistachios, toasted	70	–	–	–	70	30	2.1	[7]
Berries, not specified	80	–	–	–	80	50	4.0	[7]
Tomatoes[a]	11	1.7	0.1	0.2	12.6	100	1.3	[8]
Chocolate, not specified[b]	692	–	2633	–	3325	10–40	88	[9]
Beer, not specified[c]	9	3.1	0.5	1.9	14.7	330	4.8	This work

tr, traces.
[a] Calculated from skin tomato values (5% of dry weight and 92% humidity) from MicroTom, Beafsteak, UglyRipe, Heirloom and PlumTom varieties [8].
[b] Chocolate included: cocoa powder, unsweetened, semi-sweet, dark and milk chocolate, and chocolate syrup [9]. Serving depends on chocolate product.
[c] Values <LOQ (traces) considered as the LOD value.

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Invited Review

Polyphenols and health: Moving beyond antioxidants

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Abstract. Diets in which plant foods, including berries, provide a relevant portion of caloric intake are associated with a reduced risk of certain degenerative diseases like cancer and atherosclerosis. As known, vegetables and fruits such as berries are rich in polyphenols, which are products of secondary metabolism. In the past few years, research on polyphenols has remarkably expanded and is constantly reporting interesting biological activities of these compounds. Due to the participation of oxidative processes in the onset and development of degenerative diseases, much attention has been paid to the antioxidant properties of polyphenols. Alas, the discovery of their low bioavailability – especially when compared to the concentrations of endogenous antioxidants – is questioning the actions of polyphenols as mere antioxidants.

In this review we critically discuss the current limitations of polyphenol research and we contend that, in addition to their putative antioxidant action, several biochemical and physiological processes might be influenced by polyphenols.

Keywords: Polyphenols, cardiovascular disease, microbiota, biomarkers

1. Introduction

The very vast majority of epidemiological studies yields unequivocal results: consumption of foods rich in plants' secondary metabolites, namely polyphenols such as flavonoids is associated with a reduced risk of atherosclerosis [1], cancer [2], and neurodegenerative diseases. In the vain attempt to come up with a unified hypothesis, several mechanisms have been proposed to biologically explain how polyphenols protect from degenerative diseases. The most publicized mechanism is that polyphenols act as antioxidants by scavenging free radicals or limiting their formation [3]. As reviewed here, this view is being challenged by recent research and more complex actions are being investigated.

In addition to the most biochemical theories, we need to take into consideration that high-polyphenol food consumption might actually be healthful "simply" because it allows limiting the intake of other potentially noxious foods such as those rich in animal protein and saturated fat. In synthesis, it might not be plants and their polyphenols that provide protective effects, but, rather, the exclusion or strong limitation in the intake of other foods, namely those rich in animal protein that would actually increase the risk of disease. Even though this is disputable, we should not overlook the fact that – in addition to polyphenols - plants contain polyunsaturated fats whose actions (once food is

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ingested) intermingle with those of polyphenols, resulting in an overall benefit on human health that is difficult to ascertain to individual components [4, 5]. Still within the realm of animal protein, their substitution with vegetable proteins, e.g. those of whole grain and soy (foods which are also rich in polyphenols) is associated with cardioprotection [6–8]. However, randomized trials with polyphenols do exist and are strongly suggestive of beneficial effects. In summary, it is difficult to disentangle and accurately estimate the contribution of individual plants' components such as polyphenols, especially within a complex diet such as the Western one. Furthermore, it is difficult to ascertain the exact mechanism of action of polyphenols; the widespread notion that these molecules act merely because they are antioxidant appear to be naïve and in contrast to the complexity of biological systems.

The question to be asked, in turn, is if really plant (including berries) polyphenols are endowed with the pharmacological activities everyone is talking about and, if so, what are the mechanisms of action responsible for such healthful activities. In this article we will briefly review the biological activities of polyphenols, challenge the notion that they act as antioxidant *in vivo*, and discuss the most advanced research in the area of polyphenols and human health.

2. The role of polyphenols in human health

2.1. Why so much emphasis on antioxidant activity?

The participation of excessive reactive oxygen species (ROS) production in the origination of several diseases is now supported by a wealth of experimental data. A paradigmatic example is the formation of oxidatively-modified human low-density lipoprotein (LDL), which renders these particles highly atherogenic. *In vivo* evidence of the formation of oxidized LDL includes their presence in human atherosclerotic plaques and in the bloodstream (where they circulate as negatively charged); further, their circulating levels have been positively correlated with the progression of carotid lesions. Moreover, many epidemiological studies have correlated a high dietary intake of antioxidants (e.g. tocopherols, carotenoids, flavonoids, and polyphenols) with a lower incidence of cardiovascular disease. It is noteworthy, though, that the implication of ROS in the above mentioned diseases has often been suggested on the basis of indirect observations, i.e. antioxidant supplementation contributed to alleviate certain diseases, almost exclusively in animal models. Furthermore, negative results of clinical trials have been published, dampening the enthusiasm and calling for a more cautious approach to antioxidant therapy [9, 10]. Intrinsic uncertainties in the selection of appropriate markers of ROS-mediated processes, related to diseases as well as in the dosage and duration of treatments, make it difficult to plan interventional studies and to evaluate the results.

A role for antioxidants in the prevention and treatment of CVD has gained support mostly because of their widespread availability and ease of supplementation.

Indeed, the human body cannot synthesize lipid- and water-soluble vitamins (including the antioxidant ones E and C) that, therefore, must be derived from food; moreover, plants' secondary metabolism (in particular the shikimate and acetate pathways) generates products that are currently grouped under the rubric of "phytochemicals", of which the most popular ones are the polyphenols [11]. These molecules (at least those with ortho-diphenolic structures) are endowed with potent *in vitro* antioxidant activities. As oxidative damage appears to be central in cardiovascular aging, antioxidants such as polyphenols may provide significant protection. Notably, the studies advocating the use of antioxidants suggest that intake should be well above the general levels of consumption, shifting the focus from dietary consumption to pharmacological treatment. If the animal results that show improved conditions following antioxidant supplementation are true, an increase of antioxidant intake in the population could reduce disease rates, improve treatment, and reduce hospital stays. Thus, it is conceivable that dietary interventions with antioxidants could provide an effective means to improve or maintain myocardial function in the elderly (who often do not consume antioxidants in adequate amounts) or in the malnourished. As mentioned, despite this strong theoretical rationale, there is currently conflicting evidence to suggest that individual antioxidant supplements actually lessen risk for CVD in the population.

When we address polyphenols, it must be underscored that their simple antioxidant activity - which is the frequent subject of several lay press proclamations – has been almost exclusively proven *in vitro*. In this regard, there is much controversy as to whether polyphenols retain their antioxidant features *in vivo*, following ingestion, due to their low

bioavailability [12–14]. While there are a number of papers showing increased plasma antioxidant capacity following the intake of polyphenol-rich food items [15], many investigators suggest that polyphenols' bioavailability is too low to allow significant contributions to the endogenous antioxidant machinery [12, 14]. In addition, human cells already contain several layers of antioxidants, some of which enzymatic in nature, e.g. superoxide dismutase and catalase. Intracellular antioxidants often reach millimolar concentrations, whereas polyphenols' circulating concentrations normally do not exceed the low micromolar range. In synthesis, the real contribution of polyphenols to the overall antioxidant activity appears to be negligible, at least from a theoretical viewpoint. Moreover, whether the observed increase in antioxidant capacity is really due to polyphenols or is it just an epiphenomenon is as yet to be ascertained [12]. In the end, even though circumstantial evidence does suggest an antioxidant role played by polyphenols *in vivo*, the jury is still out and most of the current marketing claims are unsubstantiated.

2.2. *If not [just] antioxidants then what?*

In the past few years, research on polyphenols has remarkably expanded and is unveiling several nutrition-pharma biological activities of these compounds, most of which extend beyond antioxidant activity [16, 17]. Unfortunately, the marketing departments of food and pharma industries are jumping ahead of solid scientific evidence; as a consequence, unsubstantiated claims are being made and whole foods or fortified, enriched, or enhanced foods are being created and sold as "functional foods", "nutraceuticals" or "designer foods" based on the sole antioxidant ability. In this respect, we are witnessing a "race" toward to most antioxidant extract or single compound. Notably, these claims are made on the bases on *in vitro* techniques (usually the ORAC) that, though providing suggestive information, cannot translate into proven *in vivo* actions. Much more research is needed and several myths are to be disproven.

2.3. *Is there an RDA for polyphenols?*

A frequent question that the lay public asks scientists and nutritionists is that of "how much" antioxidants and polyphenols should be eaten everyday, i.e. if we have an RDA for epicatechin, hydroxytyrosol, etc. It should be immediately underscored that even the RDA for antioxidant vitamins is not really based on their antioxidant actions, but, rather, on their multifaceted biological activities. In should be also mentioned that "polyphenol deficiencies" in diets are as yet to be described. In brief, there is no evidence-based indication of the lowest amount of polyphenols that should be consumed everyday to maintain optimal health. The same line of reasoning applies to antioxidants, for which there is no clear indication of how much is needed to contrast excessive ROS production. In brief, the discovery that several phenolic molecules exert interesting biological activities – some of which may even be classified as "pharmacological" [18] – calls for an evaluation of their intake and consequent correlation with incidence of degenerative diseases. Indeed, one of the current limitations in assessing the intake of polyphenols (either as a group or individually) is the scantiness of information on their content in foodstuff. This is due on one side to the developing technology that will allow precise measurement of their concentrations and on the other side to the relatively recent interest triggered by polyphenols. It is noteworthy that, in the area of diet and health, the predominant topic is still that of caloric intake and of macronutrient repartition, rather than that of micronutrients. Notably, composition databases are being built (e.g. www.phenol-explorer.eu) and metabolomic techniques are rapidly advancing [19]. Hence, we can foresee, in the near future, accurate correlations between intakes and disease incidence/prognosis. One example is that of a French population study that has given us recent information on total polyphenol intake. The SU.VI.MAX cohort consisted of 4942 middle aged participants who consumed a total of 337 polyphenols with at least half of the population consuming 258 polyphenols [20]. The estimation of intake was done by using the recent Phenol-Explorer database mentioned above. The authors reported that 98 polyphenols were consumed at levels of more than 1 mg/day and the mean total intake was 1193 mg/d (820 mg/d as aglycones). The maximum intake was 1.8 g/day and the highest contributors were non-alcoholic beverages (mainly coffee) and fruits.

2.4. *There are no reliable biomarkers of antioxidant activity*

When dealing with food, we face what Linus Pauling called "orthomolecular medicine" [21]. In this case we should talk about orthomolecular nutrition. Indeed, though the gap between pharmacology and nutrition is narrowing

[17], nutrition and the study of bioactive compounds face the challenge of addressing the fate of molecules that our body already contains. While drugs are almost exclusively composed of molecules that our body does not habitually contain, food provides substances whose levels are constantly modulated by their intake. These differences are underscored by two major obstacles that are as yet to be overcome. One is that of accurately measure the variations in concentrations of micronutrients that follow their ingestion. Technology is still limited, and the field of metabolomics is rapidly evolving, but it cannot – as yet – provide accurate information through the use of methods approved and shared worldwide [19]. In practice, it is quite difficult to have access to methods and techniques agreed upon by investigators. Therefore, we still cannot carry out accurate dose-response studies with polyphenols and we cannot accurately follow their absorption, distribution, metabolism, and excretion (ADME) as we routinely do with drugs (as mandated by regulatory bodies).

Another major limitation we are facing in polyphenol research is that of the scantiness of biomarkers to assess their actions *in vivo*, including the antioxidant activities that are making them famous. Some biomarkers have been shown to be influenced by polyphenols. The most notable ones are those of inflammation, which can be positively modulated by polyphenols [22] and whose consequences on cardiovascular outcome are, likely, more important than those due to antioxidant actions [23–25]. Polyphenols also influence lipid metabolism [26, 27].

As mentioned above, food components are different than drugs and their actions on human physiology are usually moderate. This translates into a difficulty to measure *in vivo* activities, because the effects of polyphenols are (and should be) of modest magnitude. One reflection is that, while medicines are habitually employed for limited timeframes and address very specific conditions, food and its macro- and micro-components are ingested throughout a lifetime, during which even modest daily effects would become noteworthy.

3. Mechanisms of action alternative to the antioxidant ones. Current fields of research

3.1. Activation of Phase II enzymes

It is now thought more likely that some phytochemicals, including polyphenols, are processed by the body as xenobiotics. They stimulate stress- related cell signalling pathways that result in increased expression of genes encoding cytoprotective genes. Nrf2 (NF-E2-related factor 2) is a transcription factor which binds to the Antioxidant Response Element (ARE) in cells and thus regulates enzymes involved in antioxidant functions or detoxification (e.g. thioredoxin reductase-1 and glutathione peroxidases). Polyphenols might increase gene transcription of Nrf2 mediated by such response elements. This provides grounds for the theory of hormesis, i.e. when mild stress triggers defence mechanisms. In the case of polyphenols it indicates how they could have an indirect antioxidant action.

One human example of these effects can be found in Visioli and colleagues [28], who reported a study in which 98 Chinese/Malay subjects ingested an olive preparation which was high in phenolics. After one hour, no difference in plasma antioxidant capacity was observed, but a significant increase in total plasma glutathione concentration was measured. The authors postulated that the observed effects of the olive phenols on glutathione levels might be governed by the antioxidant response element (ARE)-mediated increase in Phase II enzyme expression.

3.2. Novel targets of polyphenols: The microbiota

One often-overlooked – though being very actively investigated – aspect concerns the contribution of the intestinal microbiota to the actions of polyphenols [29, 30]. The interaction between the gut flora and polyphenols is two-ways: on one hand the ingested compounds modify the qualitative composition of the flora [31]. Most of the studies on polyphenol bioavailability and, indirectly, on their bioactivity, have focused on their absorption in the small intestine. Nevertheless, the role of the colonic microflora has emerged in the last years as paramount in the global bioavailability of polyphenols (and, therefore, their bioactivity) [32–37]. It has been estimated that only 5–10% of the total polyphenols that we ingest is absorbed in the small intestine. The remaining polyphenols (90–95% of total polyphenol intake), together with the deconjugated polyphenols excreted in the bile, reach the colon [35], where they are metabolized by the colonic microbiota [30] before being either reabsorbed [34] or eliminated [35]. Consequently, the compounds that reach our cells and tissues are chemically, biologically, and (in many instances)

functionally distinct from their dietary form [32, 38, 39]. As mentioned above, the interaction between polyphenols and the gut microbiota is bidirectional [40]. Hence, the microbiota modifies the chemical structure and, therefore, the bioavailability and bioactivity of polyphenols and these polyphenol metabolites are able to modulate gut microbiota.

3.3. Microbial metabolism of polyphenols

Only a few out of the many species of intestinal bacteria responsible for phenolic metabolism have been identified and there is scarce knowledge of the mechanisms involved in their activities [34]. The microbial community of the human colon comprises 10^{12} bacteria/g of colonic content, composed of hundreds of different species. Firmicutes and Bacteroidetes make up over 90% of the intestinal microbiota [37], although this proportion varies substantially among individuals [36, 41–43]. The microbiota metabolites of polyphenols are better absorbed in the intestine and their enterohepatic circulation ensures that the residence time in plasma for the metabolites is longer than that of their parent compounds (indeed they appear in the systemic circulation 6–8 h post-ingestion [43]) before being excreted in the urine [30, 44]. After microbial enzyme-catalyzed deconjugation of any polyphenol conjugates that reach the colon, there are two possible routes available: (a) absorption of the intact polyphenol through the colonic epithelium and passage into the bloodstream (as free or conjugated forms) or (b) breakdown of the original polyphenol structure into metabolites.

Concerning polyphenols, several studies have been performed on the microbial metabolism of green tea [38, 42, 45, 46] and cocoa [47–49]. They all confirm that green tea and cocoa flavanols are metabolized by the colonic microbiota into valerolactones and then phenolic acids [45, 47, 48], considered to be the main microbial metabolites derived from the biotransformation of flavan-3-ols [50]. These flavanol-derived microbial metabolites have also been identified after wine powder consumption [42], suggesting that different polyphenols are transformed by the colon microbiota into the same final metabolites. Hydroxycinnamic acid esters, quercetin [39] and polyphenols linked to rhamnose are also degraded to phenolic acids by the microbiota [41, 47]. Chlorogenic acids from coffee are poorly hydrolyzed in the stomach or small intestine. When coffee is ingested, a relatively small absorption of caffeic and ferulic acids in the small intestine and a low absorption of intact chlorogenic acids are recorded. The major absorption occurs in the colon, where dihydroferulic and dihydrocaffeic acids – products of microbial biotransformation – are the major products that have been recovered [51]. In another study, 3-hydroxyphenylpropionic and benzoic acids were the main microbial metabolites of caffeic acid and its esters, chlorogenic acid and caftaric acid. 3-Hydroxyphenylpropionic was the main metabolite of caffeic acid and its esters and accounted for 9–24% of the initial dose of the substrates [52]. Data regarding berries polyphenolic microbial metabolism have shown that protocatechuic acid is one of the quantitatively most relevant product formed from anthocyanins [51, 53, 54]. In addition, in a trial with 20 healthy volunteers who consumed thermally-processed strawberry puree, all of the volunteers produced urolithin A, but only 3 of 20 volunteers produced and excreted urolithin B from the ellagitanins. The trial confirmed that some volunteers were efficient producers of urolithins, whereas other produced much lower amounts [53]. Furthermore, punicalagins transformation of pomegranate products – in a model of colonic microbiota – yielded urolithins C and D while urolithin B was not detected [55]. As seen with urolithin B, microbial fermentation of polyphenols shows a great interindividual variability. In this regard, isoflavones from soy and prenylflavonoids from hop deserve special attention. Equol, a gut bacterial metabolite of the soy polyphenols daidzin [44] and daidzein, is more estrogenic than its precursors and posses higher antioxidant capacity than its parent molecules [39]. Notably, only ~30–40% of the population is able to convert dadidzein into equol [33]. The inability of some subjects to produce equol is a consequence of the lack of specific components of the intestinal microflora [34].

Isoxanthohumol is a prenylated flavanone from hops and beer that has interesting biological activities, at least *in vitro*. It can be metabolized *in vivo* by the colonic microbiota to yield 8-prenylnaringenin [34], which possesses greater estrogenic activity than isoflavones [56]. As in the case of equol, subjects can be classified into poor (~60%), moderate (~25%) and strong (~15%) 8-prenylnaringenin producers [37].

3.4. Modulation of gut microbiota by polyphenols

As mentioned, the polyphenol-microbiota relation is two-ways and an important aspect of polyphenol biology is their modulation of microbiota composition. The mechanisms of modulation of the gut microbiota by polyphenols

is largely unknown, but polyphenols and their metabolites, in addition to their putative beneficial effect on human physiology, appear to confer added health benefits via modulation the gut microecology [30, 46]. Metabolites released into the lumen may influence the growth of the microbiota which transformed them, affecting other neighboring microflora species as well. A review of the literature suggests that polyphenol metabolites are able to enhance (or modify) some beneficial probiotic species while inhibiting the growth of non-beneficial species. In support of this notion, polyphenolic green tea extracts has been shown to have general inhibitory effects on intestinal bacteria [46], but more specifically on Bacteroides spp., Clostridium spp. (C. perfringens and C. difficile), E. coli, and Salmonella typhimurium [30]. Cocoa dietary fiber (which contains fermentable polysaccharides and free flavanol monomers, both able to modify the gut microbiota) increased the lactobacilli and Bifidobacterium spp. [49], and a pomegranate by-product and punicalagins in human fecal culture significantly inhibited the growth of the pathogenic Escherichia coli, Pseudomonas aeruginosa, clostridia and Staphyloccocus aureus. Most bifidobacteria were generally not affected, and the growth of probiotic lactobacilli and Bifidobacterium breve and Bifidobacterium infantis was significantly enhanced [55]. In another study where 25 g of wild blueberry powder in 250 mL of water were administered to healthy volunteers during six weeks, Bifidobacterium spp. increased compared to the placebo drink [54], suggesting a prebiotic effect of blueberry polyphenols. Furthermore, in a colonic model where several polyphenols were tested (caffeic acid, catechin, chlorogenic acid, epicatechin, o-coumaric acid, p-coumaric acid, phloridzin, rutin, naringenin, daidzein, genistein, quercetin and gentamicin), except rutin, all of them affected the viability of the colonic microbiota to some extent. Naringenin and quercetin were the most active molecules. In general, S. aureus was the most sensitive to polyphenols, while S. typhimurium and E. coli were comparable in their sensitivity to the treatment. The probiotic L. rhamnosus was less sensitive to the polyphenols indicating that viability of lactobacilli may be relatively unaffected by polyphenols in the gut. Generally, flavonols, isoflavones and glycosides were found to have a low antibacterial activity and phenolic acids were intermediate, while the flavanone and flavanol tested had high antibacterial activity [31]. Added to this, some phenolic acids are able to inhibit the growth of several pathogenic and non-beneficial intestinal bacteria without significantly affecting the growth of beneficial bacteria (Lactobacillus spp. and Bifidobacterium spp.), and the dihydroxylated forms (i.e. 3,4-dihydroxyphenylacetic and 3,4-dihydroxyphenylpropionic acids) efficiently destabilize the outer membrane of Salmonella. In general, non-hydroxylated and monohydroxylated phenolic acids are more potent than dihydroxylated or disubstituted phenolic acids. With regard to the saturated side chain, the order of potency, for the same benzene ring-substitution, is benzoic > phenylacetic > phenylpropionic acid [35]. Resveratrol increased Bifidobacterium and Lactobacillus counts and abolished the expression of virulence factors of Proteus mirabilis to invade human urothelial cells. Anthocyanins from berries have also proved to inhibit the growth of pathogenic Staphylococcus spp., Salmonella spp., Helicobacter pylori and Bacillus cereus. Flavonoids may also reduce the adhesion ability of L. rhamnosus to intestinal epithelial cells [30].

In summary, the interactions between polyphenols and gut microbiota are mutual, very complex, and show largely interindividual differences. Because of the biological importance of the microbial metabolites, a deeper understanding of these relationships will improve our knowledge on the health benefits of polyphenols and the factors controlling their production and whether this can be modulated advantageously for the human health.

4. Conclusions

Polyphenols exhibit a wide variety of different biological effects whose quantification *in vivo* is currently hampered by the lack of robust biomarkers. The disappointing results of antioxidant vitamins clinical trials, based on the supplementation of antioxidant vitamins in pure form [57] suggest that the interaction between the above-mentioned dietary components concomitant with a high intake of fibers, a low caloric density, and a paucity of atherogenic foods in the diet are likely – together – to effect protection from these diseases. Indeed, some trials do show positive modulation of surrogate markers of cardiovascular disease and cancer following the administration of defined amounts of polyphenols, e.g. from cocoa, olive oil, orange, etc. to human volunteers [58–61]. These healthful activities are the results of manifold and complex actions of polyphenols that do extend beyond their mere antioxidant actions. As briefly reviewed here, anti-inflammatory activities, activation of Phase II enzymes, and modulation of the gut microbiota play roles that are – biologically – more important that the purported *in vivo* scavenging of ROS or limitation

of free radical production. Therefore, future research (and, alas, advertisement) should focus on identifying an array of actions and their modulation of robust biomarkers. All of this – obviously – applies to berries and their minor components, which play multiple roles in human physiology and should not be heralded as mere antioxidants, but, rather, as multi-functional and biologically-important compounds.

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Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine *versus* dealcoholized red wine in humans by an optimized ultra-high-pressure liquid chromatography tandem mass spectrometry method

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ABSTRACT

Resveratrol exerts a variety of biological and pharmacological activities, which are observed despite its extremely low bioavailability and rapid clearance from the circulation due to extensive sulfation and glucuronidation in the intestine and liver. In order to more accurately quantify all known resveratrol metabolites, a sensitive and optimized analytical assay was developed and validated by pure standards. Methodology improvements aimed to the chromatographic detection of disulfates and sulfoglucuronides, improving resolution of sulfates, by using a buffered solution, with recovery values of resveratrol and its metabolites, even of sulfates, of 99%. The adapted methodology was then applied to a clinical study with high cardiovascular risk subjects, after the moderate consumption of red wine (RW) or dealcoholized red wine (DRW) for 28 days. Up to 21 resveratrol metabolites, including those formed by gut and microbial metabolism, were identified in 24-h urine samples. Interestingly, after long-term consumption of RW and DRW, resveratrol metabolite concentration significantly increased in urine with no differences between the two interventions, indicating that bioavailability and biotransformation of resveratrol is not affected by the alcoholic matrix of wine. In summary, we established a sensitive analytical assay for the quantification of a wide resveratrol metabolic profile in human urine, also regarding gut microbial-derived metabolites, which may also be applied to blood and tissue samples. The resveratrol metabolic pattern might therefore act as an excellent marker for the efficacy of resveratrol in clinical and epidemiological studies for the study of the beneficial effects of grape product consumption. In this sense, having a more precise concentration value of all the resveratrol metabolites in target tissues would finally lead to a better interpretation of the obtained results.

1. Introduction

Resveratrol (RV) is a stilbene mainly consumed through grape and grape products, such as red wine [1]. Although several studies have already demonstrated the RV ability to protect against several diseases [2], it has aroused some controversia due to its low bioavailability [3], since it is rapidly metabolized after its oral absorption [4]. Indeed, free RV found in plasma samples constituted less than 2% of the total RV consumed [5], and therefore it is mainly the metabolic forms which will reach the target tissues. Thus, more studies are necessary in order to investigate the biological mechanisms for which RV or its metabolites could exert

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a biological activity. Actually, scarce information is now available regarding the possible benefits of these metabolites [6–9], which mainly focuses on the sulfate conjugates. Prior to studying the possible beneficial effects of RV metabolites, development of highly sensitive and selective techniques is necessary for their identification and quantification in biological samples. Several liquid chromatography–mass spectrometry (LC–MS) techniques have already been validated for the analysis of RV metabolites [10–16], and although it is now widely accepted that RV is mainly metabolized to glucuronide and sulfate conjugates [3], including several isomers of mono and di-conjugates [10–12,17,18], less information is now available regarding piceid metabolism [11,19], as well as those metabolites derived from gut microbial action, represented by dihydroresveratrol (DHR) and its phase II metabolites [19–21]. Standards of these metabolites are rarely available and their quantification in biological samples is made by expressing results as aglycone equivalents [10–12], which is useful as a first approach, but this is open to error, since metabolite ionization in LC-ESI–MS techniques could be different from their aglycones. Several studies attempted to avoid this known technical drawback by measuring RV metabolites indirectly after enzymatic hydrolysis [20,22,23] or with diode-array detection (DAD) [17,24]. However, other drawbacks are shown using these techniques, such as the low efficiency of enzymatic hydrolysis, as well as losing all information of the metabolic profile, and the low sensitivity for DAD. Studies on the RV metabolic profile need more sensitive methodologies only achieved by LC-ESI–MS techniques, which are being used not only for RV metabolites [10–12] but also for other polyphenols [25,26], despite their quantification drawback. Nowadays, some purified standards of RV metabolites, along with the labeled C^{13}-resveratrol are already commercially available, allowing an accurate quantification of these compounds in biological samples; so, data from clinical and epidemiological studies could be more precise. Moreover, other drawbacks in chromatography, bearing in mind the different chemical characteristics of the high number of determined and identified metabolites, need to be minimized. It is known that while aglycone, glucuronided and glucosided metabolites of RV and other polyphenols are well resolved when reverse analytical columns are used, other metabolites, such as sulfate conjugates, show poor chromatographic behavior [21]. Although in our previous methodology [10] we solved this problem with the addition of more apolar mobile phase, such as acetone, the determination of disulfate metabolites was not evaluated. Thus, analytical solutions are required in this aspect.

Therefore, the main aim of this study is to validate a methodology for the analysis of RV metabolites in urine samples, through optimization of solid-phase extraction (SPE) and ultra-performance liquid chromatography (UPLC) analysis, using the recent available standards of glucuronide and sulfate conjugates and gut microbial metabolites. This methodology was applied to a randomized, crossover, controlled clinical trial, where volunteers with cardiovascular risk factors consumed, for one month, a daily moderate dose of RW or the same dose of dealcoholized RW (DRW) in order to investigate a possible alcoholic matrix effect on the bioavailability of a detailed RV metabolic profile, which will be accurately quantified with the corresponding standards.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled with no exposure to light. Standards of *trans*-RV (99% purity) and *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and taxifolin (>90% purity) from Extrasynthese (Genay, France).

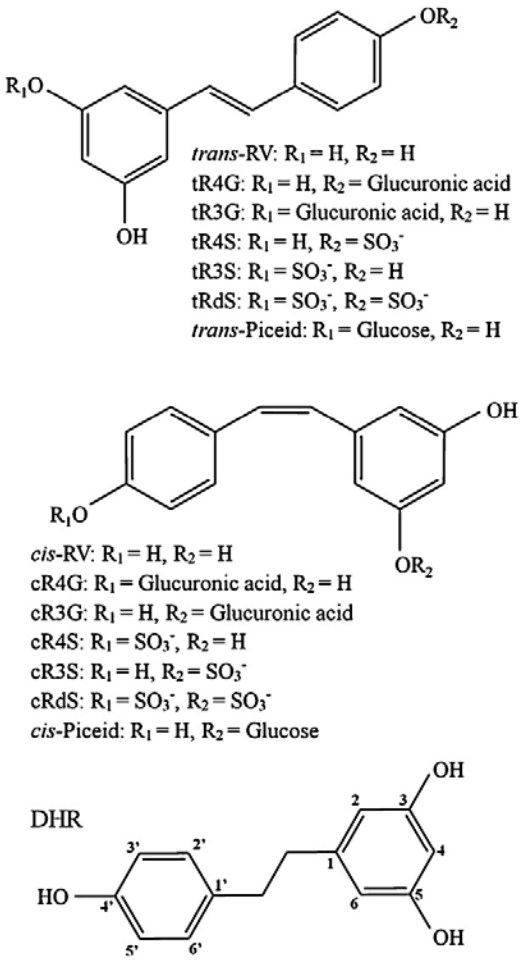

Fig. 1. Chemical structures of RV and its metabolites identified using the available commercial standards.

Standards of *cis*-RV (97% purity), *trans*- and *cis*-resveratrol-3-*O*-glucuronide (tR3G and cR3G, respectively) (98% purity each), *trans*- and *cis*-resveratrol-4′-*O*-glucuronide (tR4G and cR4G, respectively) (98% and 96% purity, respectively), *trans*-resveratrol-3-*O*-sulfate (tR3S) (98% purity) and *trans*-RV-$^{13}C_6$, to be used as internal standard, were acquired from Toronto Research Chemicals Inc. (North York, ON, Canada). *trans*-Resveratrol-4′-*O*-sulfate (tR4S) (>95% purity) and *trans*-resveratrol-3,4′-*O*-disulfate (tRdS) (>91% purity) were obtained as reported previously [27]. DHR aglycone (80% purity) was synthesized following the work by Thakkar et al. [28] (see Section 2.2), using palladium on charcoal and celite, which were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standards were prepared as 80% (v/v) methanol stock solutions.

Methanol, ethyl acetate and acetonitrile (ACN) of LC grade and ammonium acetate (>99%) were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain). LC grade solvents glacial acetic acid, acetone and ammonia (35%) were purchased from Panreac Quimica, S.A.U. (Castellar del Vallès, Spain) and the deuterated dimethyl sulfoxide (DMSO-d_6, 99.96% deuterated) from Euriso-top, SAS (Cedex, France). Ultrapure water (MilliQ) was obtained from Millipore (Bedford, MA, USA) and blank human urine from volunteers after one week of polyphenol-free diet.

Table 1
MRM transitions and optimized parameters of collision energy (CE) and declustering potential (DP).

Analyte	MRM transitions	Identified by	CE	DP
Mobile phase A: 0.05 mL/L acetic acid				
trans-RV	227/185	STD	−30	−55
cis-RV		STD		
tR4G	403/227	STD	−40	−50
tR3G		STD		
cR4G		STD		
cR3G		STD		
trans-Piceid	389/227	STD	−25	−50
cis-Piceid		Isomerized STD		
Pic-G	565/227	PIS		
DHR	229/123	STD	−25	−45
DHR-G	405/229	PIS		
Mobile phase A: ammonium acetate 10 mM				
tR4S	307/227	STD	−30	−50
tR3S		STD		
cR4S		Isomerized STD		
cR3S		Isomerized STD		
tR34dS	387/227	STD	−20	−35
cR34dS		Isomerized STD		
RV-SG	483/307	PIS	−20	−35
Pic-S	469/227	PIS	−25	−50
DHR-S	309/229	PIS	−25	−45
DHR-SG	485/309	PIS		
t-RV-$^{13}C_6$ (IS)	233/191	STD	−25	−55
Taxifolin (IS)	303/285	STD	−25	−50

2.2. Synthesis and structural identification of DHR

For DHR synthesis, 100 mg of *trans*-RV were dissolved in ethanol (120 mL) and hydrogenated at 40 psi in the presence of 10% palladium on charcoal for 24 h. The solution was filtered through celite to remove the catalyst and was evaporated to dryness. DHR structure was confirmed by nuclear magnetic resonance (^{1}H NMR) measurements using a Varian 400-MHz instrument VNMR System (Varian, Palo Alto, CA). Synthesized DHR was dissolved in deuterated DMSO (99.96% deuterated) using dinitrobenzene (1.6 mmol/L) as internal standard. The ^{1}H NMR spectrum was acquired with the following signals numbered according to Fig. 1: δ (ppm) 9.0 (broad band, 3H, —OH), 6.97 (doublet, 2H, J = 8.6 Hz, H-2′,6′), 6.63 (doublet, 2H, J = 8.6 Hz, H-3′,5′), 6.03 (doublet, 2H, J = 4.0 Hz, H-2,6), 6.00 (double doublet, 1H, J = 4.0 Hz, H-4), 2.68–2.56 (multiplet, 4H, —CH_2—CH_2—). Standard purity was calculated following the validated protocol by Malz and Jancke [29], obtaining 80% purity, for quantifying purposes. Only one peak was detected after a full-scan MS experiment (m/z 229) and no peaks of the parent compound RV were detected, since 97% purity related to the starting material was shown.

2.3. Intervention beverages

RW and DRW of the Merlot variety (Penedes appellation) were selected for its large amount of RV and piceid, in comparison to other red varieties [30]. The phenolic composition of both wines was analyzed throughout the study period [31–33] and no significant differences were obtained for any phenolic compound between RW and DRW (Supplementary Table S1).

2.4. Human experimental design

A total of 73 high-risk subjects with high cardiovascular risk, aged ≥55 years, were recruited for a randomized, crossover, controlled clinical trial [34], although in this work, we analyzed available urine from 59 included subjects to study the metabolic profile of RV. Subjects were firstly asked to follow a 15-day run-in period in which they consumed neither grape-derived products nor alcoholic beverages and, after that, they were requested to consume 272 mL of RW (30 g ethanol/day) or DRW every day for 4 weeks, following the same background diet. Twenty-four hour urine samples were collected on the last run-in period day and on the last day after each intervention period. Aliquots were immediately stored at −80 °C until analysis, after noting the corresponding total excreted volume.

This trial was registered in the Current Controlled Trials at the International Standard Randomized Controlled Trial Number Register, at controlled-trials.com, as ISRCTN88720134.

2.5. Sample extraction

RV metabolites were extracted from urine samples by SPE as previously described [10] with slight modifications. Briefly, 1 mL of urine with 100 μL of *trans*-RV-$^{13}C_6$ as internal standard (1.71 μmol/L) was loaded onto a preconditioned Waters Oasis® HLB 96-well SPE plate (30 mg) (Milford, MA, USA). After washing the plate, elution of RV metabolites was achieved with 0.5 mL of 1 mol/L acetic acid in methanol, 2 × 0.5 mL of 1 mol/L acetic acid in ethyl acetate and 0.5 mL of ammonia 5% (v/v) in methanol. The eluate was evaporated to dryness and then reconstituted with a solution of 100 μL of taxifolin (1.64 μmol/L) as secondary internal standard diluted in acidified water (0.5 mL/L acetic acid) with 10% organic mobile phase.

2.6. Chromatography optimization

The mobile phase has a significant influence on the ionization efficiency. Therefore, mobile phases were examined in order to optimize chromatographic conditions for RV metabolites, using the available pure standards (1 mg/L) in MRM mode. These mobile phases included: 0.5 mL/L acetic acid [11], and a buffered solution with ammonium acetate (10 mmol/L) at different pH (3, 4 and 5),

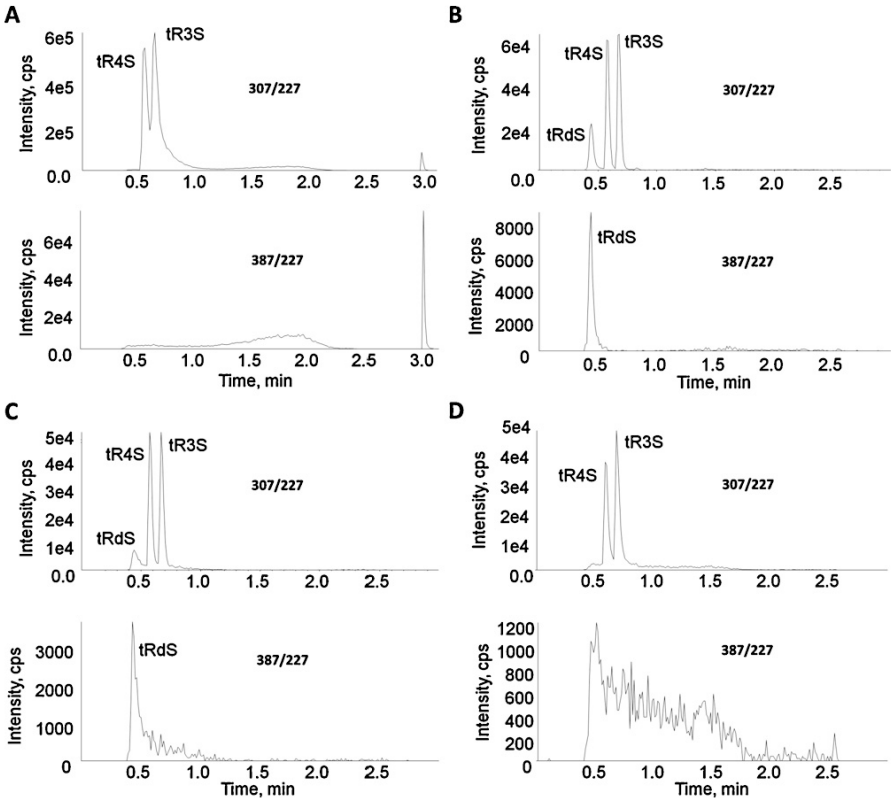

Fig. 2. Representative MRM chromatograms of tR3S (peak 5 – Fig. S1) and tR4S (peak 6 – Fig. S1) (MRM 307/227) and tRdS (peak 9 – Fig. S1) (MRM 387/227) standards, using (A) 0.5 mL/L acetic acid, (B) ammonium acetate 10 mmol/L at pH 5, (C) ammonium acetate 10 mmol/L at pH 4 and (D) ammonium acetate 10 mmol/L at pH 3.

as the aqueous solvent A and acetone:ACN (70:30, v/v) as solvent B in gradient elution (see Section 2.7). Ammonium acetate, along with formate salt, has already been used for the determination of RV-sulfates [13,27,35] as well as for other sulfate metabolites [36,37]. Acetic acid 10% (v/v) was used to adjust the mobile phase at different pH values.

2.7. UPLC–MS/MS analysis

The analysis of RV metabolites in urine samples was carried out by UPLC coupled to tandem mass spectrometry (UPLC–MS/MS) adapted from a previous validated methodology [10]. A Waters Acquity UPLC system (Milford, MA, USA), equipped with a binary solvent manager and a refrigerated autosampler plate, was used to couple an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray, ionizing in negative mode. An Acquity UPLC BEH C18 (Milford, MA, USA) analytical column (1.7 μm, 2.1 mm × 5 mm), maintained at 40 °C, was used for chromatographic separation at a flow rate of 1 mL/min, split (1:1) before MS analysis. Injection volume was 5 μL. Two different aqueous mobile phases A were used: 0.5 mL/L acetic acid and then ammonium acetate 10 mmol/L at pH 5. Mobile phase B consisted of acetone:ACN (70:30). A linear gradient profile was applied with the following proportions (v/v) of phase A [*t*(min),%A]: (0,90); (1,70); (2,0); (2.3,0); (2.31,90); (3,90). MS and MS/MS parameters were optimized in infusion experiments for each RV metabolite standard (Table 1) in the corresponding mobile phase A/B (1:1) at a flow rate of 5 μL/min in a mass spectrometer using a model 11 syringe pump (Harvard Apparatus, Holliston, MA), with the general parameters of collision cell exit potential (−15), focusing potential (−200 V), entrance potential (−10 V) and capillary voltage (−3500 V) obtained.

The availability of pure standards of RV phase II metabolites allows their identification by comparing the retention time at the corresponding MRM transitions. Other RV and piceid phase II metabolites, along with the gut microbial ones, were identified by product ion scan (PIS) (Supplementary Figs. S1 and S2) [19]. For quantitative analysis, calibration curves in blank human urine were constructed with available standards subjected to the same SPE procedure as the samples, using the MRM transitions shown in Table 1. When the metabolite standard was not available, concentrations were estimated using the most similar compound standard curve and expressed as their equivalents (Table 3). A commercial standard of cR3G was not available when urine samples were analyzed and, thus, they were quantified using a cR4G standard curve, although afterwards cR3G was validated for this method. After quantification, values under LOQ were considered as the LOD value and values under LOD were considered zero.

2.8. Assay validation

Selectivity, linearity, detection and quantification limits, recovery, accuracy and precision of the available standards were evaluated for the optimized method, according to the Food and Drug Administration (FDA) [38]. The selectivity of the method was assessed by analyzing blank human urine in order to ensure that there were no interferences at the same retention time and MRM transition for the analytes. Five calibration curves in blank human urine of all available RV metabolite standards were performed on three different analysis days for linearity evaluation at

Table 2
Limits of detection (LOD), recovery, accuracy and precision data ($n = 8$) obtained from the UPLC–MS/MS of all available standards in blank human urine for each chromatographic method.

Analyte	LOD (μg/L)	Recovery (mean ± SD, %)	Added Conc. (μg/L)	Calculed Conc. (mean, μg/L)	Accuracy (%)	Precision (RSD, %)
Mobile phase A: 0.5 mL/L acetic acid						
trans-RV	0.48 ± 0.03	105.1 ± 11.5	50.0	54.1	108.18	6.81
			500.0	506.1	101.21	9.30
			1000.0	974.4	97.44	7.68
cis-RV	0.55 ± 0.04	101.6 ± 8.4	51.8	53.5	102.86	9.31
			518.0	482.0	93.04	8.66
			1036.0	960.5	92.71	11.54
tR4G	0.76 ± 0.10	101.2 ± 5.1	50.0	54.9	109.50	0.39
			500.0	546.5	109.30	0.39
			1000.0	958.0	95.80	1.18
tR3G	1.41 ± 0.20	98.7 ± 7.2	51.2	51.4	100.76	4.04
			512.0	473.0	92.38	7.90
			1024.0	970.3	94.76	7.94
cR4G	1.75 ± 0.20	102.0 ± 6.4	51.5	50.3	96.79	8.69
			515.0	482.6	93.72	7.84
			1030.0	988.5	95.98	10.14
cR3G	1.53 ± 0.15	96.1 ± 9.1	50.5	48.6	95.28	6.22
			505.0	497.6	98.53	12.32
			1011.0	1063.6	105.20	12.12
trans-Piceid	0.42 ± 0.05	96.5 ± 5.0	50.0	47.8	95.51	8.63
			500.0	523.6	104.72	9.21
			1000.0	1002.0	100.20	10.57
DHR	0.77 ± 0.03	96.1 ± 5.4	51.6	51.3	98.67	9.13
			516.0	531.7	103.05	5.83
			1032.0	1004.0	97.29	10.32
Mobile phase B: ammonium acetate 10 mM						
tR4S	0.53 ± 0.05	97.4 ± 10.0	50.0	54.4	108.81	8.27
			500.0	523.4	104.68	5.55
			1000.0	954.7	95.47	9.05
tR3S	0.36 ± 0.04	95.6 ± 9.9	50.0	51.5	103.04	6.04
			500.0	546.1	109.23	5.19
			1000.0	981.4	98.14	9.19
tR34dS	0.62 ± 0.09	100.5 ± 6.4	53.3	49.7	93.74	10.08
			533.0	510.1	95.70	6.11
			1065.0	1043.3	97.96	10.97
trans-Piceid	0.85 ± 0.10	–	50.0	49.8	99.53	12.09
			500.0	486.2	97.23	6.06
			1000.0	919.4	91.94	11.03
DHR	1.05 ± 0.13	–	51.6	51.3	98.68	12.42
			516.0	495.0	95.93	7.48
			1032.0	904.9	87.68	9.60

eight concentration points from 1 to 1000 μg/L. Detection limit was defined as the analyte concentration that produced a signal-to-noise ratio of at least 3. The lowest concentration with accepted precision and accuracy criteria for each standard on the calibration curve was the lower limit of quantification (LLOQ). Three different concentrations (low, medium and high) of each available standard were used to evaluate their precision and accuracy ($n = 8$). The latter was obtained as the percentage of the ratio between the mean calculated concentration and the known added concentration and precision as its relative standard deviation. The same concentration levels were used to perform the extraction recovery, for which the standards were spiked to blank human urine before extraction and compared to the standards spiked to extracted blank human urine.

Validation parameters were evaluated for *trans*- and *cis*-RV, the available four glucuronide isomers, *trans*-piceid and DHR using the chromatographic method with 0.5 mL/L acetic acid as mobile phase A, while for sulfate and disulfate conjugates the validation procedure was performed using the mobile phase A consisting of ammonium acetate 10 mmol/L. Standards of *trans*-piceid and DHR were also validated for the ammonium acetate 10 mmol/L mobile phase A, since their calibration curves were used to quantify their respective sulfate and sulfoglucuronide (SG) conjugates.

2.9. Statistical analysis

As urinary RV data were skewed (Kolmogorov and Levene tests) and the natural logarithm of the variable did not normalized the data, comparisons between interventions were performed using the nonparametric Friedman test and the paired Wilcoxon test. IBM SPSS Statistics software, version 20 (Chicago, IL), was used to perform the statistical analysis. Statistical significance was defined as $P \leq 0.05$.

3. Results and discussion

3.1. Method optimization for sulfate metabolites

The availability of purified standards of RV metabolites, and specifically tRdS, has allowed some improvements of the previously validated methodology for the analysis of the RV metabolic profile

Table 3
24-h urinary excretion (mean ± SEM) of individual metabolites at baseline (BAS) and after regular consumption of dealcoholized red wine (DRW) and red wine (RW).

Metabolites	Urinary excretion (nmol/24 h)		
	Baseline	DRW	RW
RV phase II metabolites			
trans-RV	n.d.	n.d.	n.d.
cis-RV	n.d.	n.d.	n.d.
tR4G	85.82 ± 28.36*	838.11 ± 259.78	390.58 ± 145.55
tR3G	89.58 ± 24.70*	192.88 ± 29.34	192.69 ± 29.72
cR4G	53.59 ± 15.97*	487.35 ± 84.54	449.89 ± 60.64
cR3G[a]	292.35 ± 97.22*	2410.23 ± 267.05	2304.85 ± 231.88
tR4S	80.33 ± 61.95*	140.56 ± 77.24	91.08 ± 47.40
tR3S	55.83 ± 25.32*	488.62 ± 69.93	591.88 ± 93.27
cR4S[b]	180.43 ± 62.57*	1044.86 ± 152.15	931.97 ± 143.70
cR3S[c]	83.34 ± 41.40*	891.18 ± 132.27	753.16 ± 132.13
tR34dS	47.63 ± 16.78*	414.45 ± 58.92	418.60 ± 53.37
cR34dS[d]	n.d.	n.d.	n.d.
RV-SG[d]	35.35 ± 19.36*	211.14 ± 30.73	170.17 ± 22.37
Sum of the total RV phase II metabolites	1004.26 ± 252.75*	7119.38 ± 658.16	6294.88 ± 620.00
RV glucosides			
trans-Piceid	0.00 ± 0.00*	2.94 ± 0.53	2.63 ± 0.55
cis-Piceid[e]	2.26 ± 0.92*	14.71 ± 1.78	17.84 ± 2.41
Pic-G[e]	3.80 ± 1.52*	29.64 ± 2.97	31.80 ± 4.55
Pic-S 1[e]	6.91 ± 2.44*	50.46 ± 6.11	54.68 ± 7.06
Pic-S 2[e]	4.39 ± 1.56*	44.86 ± 5.08	39.42 ± 4.01
Sum of the total RV glucosides	17.36 ± 5.87*	142.60 ± 13.23	146.38 ± 13.40
Gut microbial metabolism			
DHR	1.46 ± 1.01*	18.08 ± 2.47	20.27 ± 3.56
DHR-G 1[f]	7.94 ± 3.29*	92.18 ± 8.03	73.16 ± 8.95
DHR-G 2[f]	58.21 ± 22.06*	500.81 ± 43.85	455.99 ± 42.34
DHR-S 1[f]	515.83 ± 166.71*	3286.05 ± 279.89	2799.04 ± 254.89
DHR-S 2[f]	144.81 ± 27.51*	839.04 ± 97.16	681.17 ± 106.40
DHR-SG[f]	49.75 ± 17.43*	333.12 ± 34.09	300.42 ± 34.22
Sum of the total gut microbial metabolism of RV	778.00 ± 222.87*	5069.28 ± 383.77	4330.04 ± 362.33

Changes in excretion values in response to the intervention treatment were determined by Wilcoxon non-parametric test for 2 related samples. n.d., not detected.
[a] Expressed as cR4G equivalents.
[b] Expressed as tR4S equivalents.
[c] Expressed as tR3S equivalents.
[d] Expressed as tRdS equivalents.
[e] Expressed as *trans*-piceid equivalents.
[f] Expressed as DHR equivalents.
* $P < 0.05$, comparing baseline to DRW and RW interventions.

[10,11], including also a reduction of time analysis with UPLC. The poor chromatographic behavior previously reported for RV-sulfates [21] was improved using ammonium acetate (10 mmol/L) as mobile phase. In our previous publication we improved sulfate metabolite resolution using a ternary mobile phase B with acetone [10], but disulfate metabolites were not previously considered. In fact, the use of a buffered solution in chromatography analysis allowed the identification of tRdS, since it was not observed when 0.5 mL/L acetic acid was used (Fig. 2A). Certainly, ammonium acetate improves the separation and enhances peak shapes of sulfate conjugates, avoiding adsorption and tailing previously shown [3]. MRM analysis of metabolites (Fig. 2) supports these improvements, since monosulfate conjugates provided a signal-to-noise ratio around 7-fold higher for tR4S and tR3S, respectively, with ammonium acetate at pH 5 compared with 0.5 mL/L acetic acid (Fig. 2A and B). Regarding pH values, mobile phase at pH = 5 provided signal-to-noise ratios from 9- to 20-fold higher for tRdS and around 7-fold higher for tR4S and tR3S, compared to mobile phases at pH 4 and 3. Moreover, the higher the pH value, the better the resolution of sulfate peak shapes shown (Fig. 2). However, the signal-to-noise ratios for aglycones (*trans*- and *cis*-RV and DHR), as well as their glucoside (*trans*-piceid) and glucuronide conjugates, was up to 4-fold higher with 0.5 mL/L acetic acid mobile phase, compared to the buffered solution. Bearing in mind these differences in chromatography resolution, both aqueous mobile phases were used for sample analysis, since low chromatographic times were achieved with UPLC. Regarding the benefits of ammonia solution for sulfate detection, we added a final stage of the elution process in the SPE with 5% ammonium hydroxide in methanol, obtaining recoveries from 96% to 105% for all studied standards (Table 2).

3.2. Quality parameters of the method

Selectivity of the method was evaluated in blank human urine and no interference peaks were observed at the same retention time of the metabolites. The five calibration curves were determined by weighted ($1/x^2$) least-square regression analysis, and linearity over the concentration range was studied by obtaining correlation coefficients for all analytes higher than 0.994, with accuracy values ranging from 85 to 115%. Detection limit for each standard ranged from 0.36 to 1.75 μg/L (Table 2), and the lower limit of quantification (LLOQ) was 5 μg/L, which evinced the great sensitivity achieved by the analytical method. Results for accuracy and precision, displayed in Table 2, met the acceptance criteria of the FDA [38]. Thus, results obtained with this new adapted methodology will be repetitive and exact at low, medium and high concentrations, since accuracy values were from 88 to 111%, with mean variations in precision up to 12%. Mean recovery values for all

the compounds were 99% (Table 2), with 95% for the RV isotope (*trans*-RV-$^{13}C_6$), which was used as internal standard.

3.3. Identification of metabolite profiling of RV

Up to 15 RV and derivates were unequivocally identified using the available commercial standards, including also *cis*-isomers of RV sulfates and piceid, which were identified by isomerization of the *trans*-isomer standard solutions after 30 min in the sunlight [11] (Table 1). Although *cis*-resveratrol-3,4′-*O*-disulfate (cRdS) was also identified after isomerization of the *trans*-standard, it was not found in any sample after RW or DRW consumption. Also, neither *trans*- nor *cis*-RV were found in urine samples, as was previously reported [1,17], due to their extensive metabolism [39].

The presence of other metabolites, whose standard were not yet available, was also studied through MS/MS experiments. We identified up to 9 RV metabolites using the PIS (Supplementary Figs. S1 and S2): RV-SG, piceid-glucuronide (Pic-G), two piceid-sulfates (Pic-S), two DHR-glucuronides (DHR-G), two DHR-sulfates (DHR-S) and a DHR-SG. Other metabolites such as RV-diglucuronides (MRM 579/403), DHR-diglucuronides (MRM 581/229) and DHR-disulfates (MRM 389/229) were also sought, although any peak could be confirmed in PIS experiments.

Piceid and its phase II metabolites were also previously detected in human samples [11,19] and in rats [15,40,41], indicating that RV glucoside might be absorbed in its intact form, conjugated and finally excreted in urine. Gut microbial metabolism of RV was observed with the presence of DHR and its glucuronide and sulfate conjugates, as already published in some previous works in humans [19–21], in rats [14,15,42] and in pigs [43] after RV or RW administration. However, it should be borne in mind that this is the first work linking all the resveratrol derivates and their metabolites in a unique metabolic profile of up to 21 compounds, considering resveratrol and piceid phase II metabolites and also those obtained by microbial action.

3.4. Quantitative expression of RV metabolites

With the recent commercial availability of RV metabolites, an accurate quantification of these compounds in biological samples could be achieved. In this work, we have been able to quantify 6 RV phase II metabolites with the corresponding standard: tR4G, tR3G, cR4G, tR4S, tR3S and tRdS. phase II metabolites of RV have already been studied, with glucuronide and sulfate forms being the most reported ones [3,39], although many studies measured them indirectly as free aglycone after enzymatic hydrolysis [20–23], or directly expressing results as aglycone equivalents [10–12]. In this work, the effect of quantifying a RV metabolite with its corresponding standard or as aglycone equivalents has been evaluated in the urine samples of this study (Fig. 3). Statistical differences between both quantification methods were obtained for the evaluated metabolites, with higher excretion values using aglycone equivalents, except for tR4G (Fig. 3). However, while only 0.7–1.2-fold higher values were achieved for glucuronide conjugates, amounts of sulfate metabolites were more than 8-fold higher when expressed as aglycone equivalents. Thus, sulfate conjugates seem to be greatly overestimated compared to glucuronides, when quantified as RV equivalents.

Despite all these reported differences, a wide RV metabolic profile of up to 21 compounds was considered, with 8 of those accurately quantified as the real metabolite and 13 expressed as equivalents of the most similar compound (Table 3), since commercial standards are not yet available. It would now be very interesting to have these standards available, in order to finally achieve a more accurate concentration of the whole metabolic profile of RV and its relation to the beneficial effects of grape product consumption. To

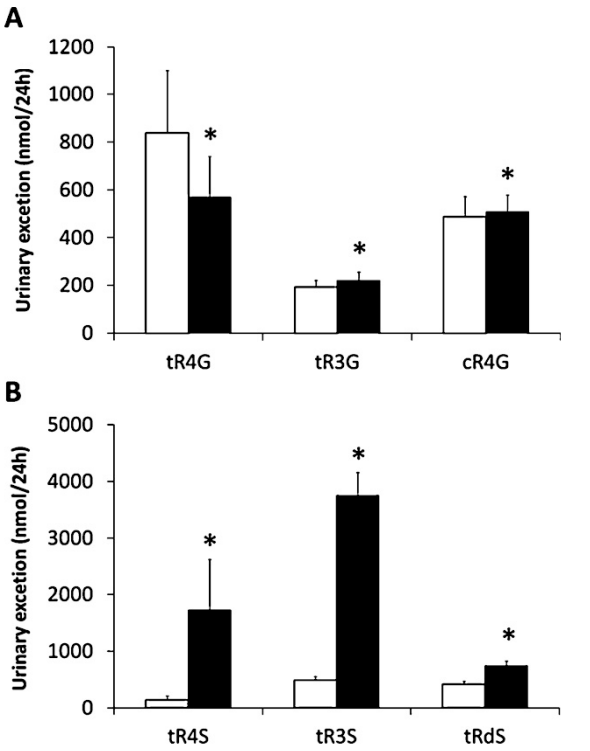

Fig. 3. Mean excreted amounts (and SEM) of RV glucuronides (A) and RV sulfates (B) accurately quantified with their corresponding standard (white boxes) or as RV equivalents (black boxes) after the consumption of DRW. *$P < 0.05$ when comparing both quantification expressions (Wilcoxon test).

our knowledge, few studies have been performed on the possible biological activity of RV metabolites [6–9] and, although the *in vitro* activity of these metabolites has been shown to be less effective than that observed for RV [6,8,9], it is thought that, *in vivo*, enzymes could release the aglycone [7], which exerts its biological activity.

3.5. Effect of alcohol on the bioavailability of RV

Statistical analysis of 24-h urinary excretion of all the RV metabolites showed significant differences between baseline period and both interventions ($P < 0.001$, Friedman test). In fact, these differences in the excreted amounts of all the metabolites studied were significantly higher after a regular consumption of RW and DRW compared with values obtained after the run-in period (Table 3) ($P < 0.05$). Increments on excreted RV metabolites after RW consumption were previously observed [10,44,45] and, indeed, Zamora-Ros et al. described urinary glucuronide and sulfate conjugates of RV as biomarkers of moderate wine intake [44,45].

Interestingly, when the 24-h excretion values of volunteers after 4 weeks of moderate RW and DRW consumption were compared, no differences were obtained for any individual metabolite or for the sum of RV phase II metabolites, RV glucosides and those of gut microbial metabolites (Table 3) ($P > 0.05$). Therefore, excretion of RV metabolites, and thus RV bioavailability, would not be influenced by the alcoholic matrix of wine. Previous reports showed that alcohol from RW could improve polyphenol absorption by increasing their solubility [46], as was reported for quercetin in *in vitro* and *in vivo* studies [22,47]. However, different conclusions are reached in the few *in vivo* human studies focused on the bioavailability of RV related to alcohol matrix effects. In one of the first RV bioavailability studies in humans [22], pure *trans*-RV was

dissolved in three different matrices considering absence/presence of alcohol – white wine, grape juice and vegetable juice – resulting in an equivalent absorption of this polyphenol either in aqueous or alcoholic matrices. These results are in line with those obtained by Ortuño et al. [20], who compared pharmacokinetics of RV after wine or grape juice administration, obtaining similar values for *trans*-RV when samples were hydrolyzed. Moreover, another study concluded that the combined intake of 2000 mg of *trans*-RV with 500 mg of quercetin and 5% of alcohol (100 mL) did not improve RV absorption [48]. The same results were obtained for other polyphenols, such as catechin, malvidin-3-glucoside, caffeic acid and production of 4-O-methylgallic acid when interventions with RW and DRW were compared [49–51]. It is important to note that the above-mentioned studies on RV bioavailability only looked for RV aglycone, as itself or after enzymatic hydrolysis, with their results being in line of those obtained in this study. Thus, the utility of this method to give a global value for the resveratrol metabolism, largely used in clinical and epidemiological studies [34,45,52], might not be discarded, since similar results were obtained not only for the individual compounds but also for the total sum. However, the importance of the study of the large metabolic profile must be highlighted, since different concentration values of RV metabolites could reach target tissues, and thus they could have different capacities to exert their possible biological activity.

4. Conclusions

Although several studies have already demonstrated the RV ability to protect against several diseases, its low bioavailability makes them quite controversial. Thus it is important to have a sensitive and accurate methodology for the resveratrol analysis in biological samples, providing a complete profiling of the metabolism, including those formed by gut and microbial metabolism, in order to elucidate their possible biological activities. In this work, the analysis of RV metabolite profiling has been optimized, specifically for disulfate and sulfate metabolites, improving their chromatographic resolution and their extraction, as well, by using ammonium solutions. The improved methodology using combined mobile phases to consider the different chemical characteristics and analytical behavior of all the compounds constituting the metabolic profile has been validated for 11 RV derivates in a wide concentration range, with the recent availability of pure standards, which also allows an accurate quantification of biological samples. Furthermore, the study of the metabolic profile of RV in humans has been extended to a total of 21 metabolites in order to get the most comprehensive metabolic profile of resveratrol described in the literature with the inclusion of tentative identification of piceid and gut microbial-derived metabolites, which have aroused a great interest in this field, due to the microbiota effect on polyphenols activity. The analysis of all these metabolites has been applied to a clinical study where RV metabolites were excreted in higher proportions after long-term consumption of RW and DRW, with no differences observed between the interventions. Thus, it seems that RV bioavailability would not be influenced by the alcoholic matrix of wine. Having a more precise concentration value of all the RV metabolites in target tissues would finally lead to a better interpretation of the obtained results from clinical and epidemiological studies for the study of the beneficial effects of grape product consumption, since it has been demonstrated that the resveratrol metabolic pattern might act as an excellent marker for the efficacy of resveratrol in such studies. However, not only a global sum of total metabolites could be provided, but also the individual excretion of all the metabolic profile and, thus, it would be achieved higher possibilities to know which compounds would possibly have higher biological activity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.09.093.

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Review

Wine, Beer, Alcohol and Polyphenols on Cardiovascular Disease and Cancer

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Abstract: Since ancient times, people have attributed a variety of health benefits to moderate consumption of fermented beverages such as wine and beer, often without any scientific basis. There is evidence that excessive or binge alcohol consumption is associated with increased morbidity and mortality, as well as with work related and traffic accidents. On the contrary, at the moment, several epidemiological studies have suggested that moderate consumption of alcohol reduces overall mortality, mainly from coronary diseases. However, there are discrepancies regarding the specific effects of different types of beverages (wine, beer and spirits) on the cardiovascular system and cancer, and also whether the possible protective effects of alcoholic beverages are due to their alcoholic content (ethanol) or to their non-alcoholic components (mainly polyphenols). Epidemiological and clinical studies have pointed out that regular and moderate wine consumption (one to two glasses a day) is associated with decreased incidence of cardiovascular disease (CVD), hypertension, diabetes, and certain types of cancer, including colon, basal cell, ovarian, and prostate carcinoma. Moderate beer consumption has also been associated with these effects, but to a lesser degree, probably because of beer's lower phenolic content. These health benefits have mainly been attributed to an increase in antioxidant capacity, changes

in lipid profiles, and the anti-inflammatory effects produced by these alcoholic beverages. This review summarizes the main protective effects on the cardiovascular system and cancer resulting from moderate wine and beer intake due mainly to their common components, alcohol and polyphenols.

Keywords: wine; beer; alcohol; polyphenols; cardiovascular disease; cancer

1. Introduction

Since ancient times wine has been closely associated with diet, particularly in Mediterranean countries [1], and for many years, moderate and regular consumption of wine has been associated with health benefits, with no scientific basis. However, over the last two decades, several studies around the world have demonstrated that intake of alcoholic beverages produces positive effects on antioxidant capacity, lipid profile and the coagulation system [2], that may explain the reduction in the risk of cardiovascular disease (CVD) [3,4], overall mortality [5] and other diseases observed in moderate drinkers. By contrast, alcohol abuse or binge drinking has undoubtedly been related to a large number of medical, social and work related problems (negative effects), including the development of alcohol dependence syndrome, several chronic diseases (liver cirrhosis, cardiomyopathy, encephalopathies, polyneuropathy, dementia) and accidents which eventually lead to death [6–8].

Several cohort studies have pointed out that light-to-moderate alcohol consumers have an increased survival compared to abstainers [9]. Current evidence also suggests the protective effects of moderate drinking on cardiovascular events including coronary heart disease (CHD) [10], ischemic stroke [11], peripheral arteriopathy and congestive heart failure [12]. Positive effects have also been reported for moderate alcohol consumption on cellular aging damage, cognitive function and dementia. These effects have been observed in a variety of patients, including diabetics, hypertensive subjects and those with previous CHD.

Beneficial effects of moderate alcohol intake against atherosclerosis have been attributed to its antioxidant and anti-inflammatory effects, as well as to its actions on vascular function. In this framework, part of these effects may be attributed to polyphenols mainly contained in wine and beer, as these compounds exhibit antioxidant [13], anticarcenogenic [14], anti-inflammatory [15], hypotensive [16] or even anticoagulant properties [17].

Since the French paradox was described two decades ago [18], several studies have focused their attention on the components of red wine (mainly polyphenols and especially resveratrol) in order to explain the inverse association observed between moderate wine consumption and the incidence of CVD, as well as the different effects of the various types of alcoholic beverages (with or without polyphenols), thereby opening the debate of which type of alcoholic beverage is more cardioprotective than others.

Although the chemical constituents of grapes and wine vary, similar beneficial effects have been observed in different varieties of red wine, with white wine seeming to benefit the cardiovascular system to a lesser extent than red wine. The greater health benefits of red wine may be related to its

higher polyphenolic content because of the distinctive production processes between red and white wine.

The mechanisms responsible for the healthy effects of wine are extremely complex due to the many different pathways involved. Both alcohol and polyphenolic compounds have been extensively studied, despite the continued controversy as to which component is the most active [19]. The underlying mechanisms to explain these protective effects against CHD include an increase in high-density lipoprotein (HDL) cholesterol, a decrease in platelet aggregation, a reduction in the levels of fibrinogen and an increase in insulin sensitivity, which have been attributed to the ethanol content in wine. Other studies have provided evidence that wine exhibits beneficial properties which are independent of the presence of alcohol, and should be attributed to their polyphenolic content [20,21].

Similarly, the compounds found in beer have different biological activities demonstrated in enzymatic assays or cell cultures such as antioxidant [22], anticarcinogenic [23–25], anti-inflammatory [26], estrogenic [27] and even antiviral properties [28]. Different profiles of *in vitro* biological activity have been described for these compounds which, combined together, could have a synergistic effect.

Therefore, the objective of this review is to summarize the main protective effects of moderate wine and beer consumption on CVD and cancer by way of their bioactive compounds.

2. Polyphenolic Compounds in Wine and Beer

Red wine polyphenols are a complex mixture of flavonoids (such as anthocyanins and flavan-3-ols) and nonflavonoids (such as resveratrol, cinnamates and gallic acid). Flavan-3-ols are the most abundant, with polymeric procyanidins (condensed tannins) composing up to 50% of the total phenolic constituents [29]. These compounds act as potent antioxidants as they reduce low-density lipoprotein (LDL) cholesterol oxidation, modulate cell signaling pathways, and reduce platelet aggregation. Red wine contains more polyphenols than white wine (around 10-fold) because during the wine making process, red wine, unlike white wine, is macerated for weeks with the skin which is one of the parts of the grape with the highest concentrations of phenolic compounds [30]. The concentrations in red wine range from around 1.2 to 3.0 g/L (Table 1).

Both flavonoids and nonflavonoid phenolic compounds have been implicated in the protective effects of wine on the cardiovascular system. Nevertheless, the stilbene resveratrol has been one of the most extensively studied nonflavonoids as a critical constituent that contributes to the health benefits of red wine. In experimental studies, resveratrol exhibited both cardioprotective and chemopreventive effects, inhibiting LDL oxidation and platelet aggregation in animal studies. It also inhibits the growth of some tumor types and exhibits anti-inflammatory, antibacterial, antifungal, antiviral, neuroprotective, antiproliferative and anti-angiogenic activities [31,32]. However, the beneficial effects of moderate wine consumption may be attributed to the overall mix of all of its components and not to a specific action of one, such as resveratrol. Indeed, progress can be achieved in the field of the cardiovascular health effects of polyphenols when the one-dimensional antioxidant view of polyphenols is replaced by a view considering their multifaceted bioactivity, as polyphenols are versatile bioactives rather than mere antioxidants.

Table 1. Polyphenolic compounds in red wine.

Phenolic compounds	(mg/L) *
Anthocyanins	
Cyanidin 3-*O*-(6′-acetyl-glucoside)	0.8
Cyanidin 3-*O*-glucoside	2.1
Delphinidin 3-*O*-(6′-acetyl-glucoside)	4.2
Delphinidin 3-*O*-(6′-*p*-coumaroyl-glucoside)	1.8
Delphinidin 3-*O*-glucoside	10.6
Malvidin 3-*O*-(6′-acetyl-glucoside)	35.2
Malvidin 3-*O*-(6′-caffeoyl-glucoside)	1.8
Malvidin 3-*O*-(6′-*p*-coumaroyl-glucoside)	19.5
Malvidin 3-O-glucoside	99.7
Peonidin 3-O-(6″-acetyl-glucoside)	4.7
Peonidin 3-*O*-(6′-*p*-coumaroyl-glucoside)	5.2
Peonidin 3-*O*-glucoside	8.2
Petunidin 3-*O*-(6′-acetyl-glucoside)	5.7
Petunidin 3-*O*-(6′-*p*-coumaroyl-glucoside)	3.9
Petunidin 3-*O*-glucoside	14.0
Pigment A	0.7
Pinotin A	2.2
Vitisin A	3.1
Dihydroflavonols	
Dihydromyricetin 3-*O*-rhamnoside	44.7
Dihydroquercetin 3-*O*-rhamnoside	9.7
Flavanols	
(+)-Catechin	68.1
(+)-Gallocatechin	0.8
(−)-Epicatechin	37.8
(−)-Epicatechin 3-*O*-gallate	7.7
(−)-Epigallocatechin	0.6
Procyanidin dimer B1	41.4
Procyanidin dimer B2	49.7
Procyanidin dimer B3	94.7
Procyanidin dimer B4	72.9
Procyanidin dimer B7	2.7
Procyanidin trimer C1	25.6
Procyanidin trimer T2	67.1
Prodelphinidin dimer B3	1.1
Flavanones	
Hesperetin	0.5
Naringenin	0.5
Naringin	7.5
Flavonols	
Isorhamnetin	3.3
Isorhamnetin 3-*O*-glucoside	2.6
Kaempferol	2.3
Kaempferol 3-*O*-glucoside	7.9
Myricetin	8.3
Quercetin	8.3
Quercetin 3-*O*-arabinoside	4.9
Quercetin 3-O-glucoside	11.4
Quercetin 3-O-rhamnoside	11.5
Quercetin 3-*O*-rutinoside	8.1
Hydroxybenzoic acids	
2,3-Dihydroxybenzoic acid	0.8
2-Hydroxybenzoic acid	0.4
4-Hydroxybenzoic acid	5.5
Gallic acid	35.9
Gallic acid ethyl ester	15.3
Gentisic acid	4.6
Protocatechuic acid	1.7
Syringic acid	2.7
Vanillic acid	3.2
Hydroxycinnamic acids	
2,5-di-*S*-Glutathionyl caftaric acid	28.6
Caffeic acid	18.8
Caffeoyl tartaric acid	33.5
Ferulic acid	0.8
o-Coumaric acid	0.3
p-Coumaric acid	5.5
p-Coumaroyl tartaric acid	11.8
Sinapic acid	0.7
Hydroxyphenylacetic acids	
4-Hydroxyphenylacetic acid	1.6
Stilbenes	
d-Viniferin	6.4
e-Viniferin	1.5
Pallidol	2.0
Piceatannol	5.8
Piceatannol 3-*O*-glucoside	9.5
Resveratrol	2.7
Resveratrol 3-*O*-glucoside	6.2
Hydroxybenzaldehydes	
Protocatechuic aldehyde	0.5
Syringaldehyde	6.6
Tyrosols	
Hydroxytyrosol	5.3
Tyrosol	31.2

* Mean value of bibliographic data from Phenol-Explorer Database, Version 1.5.7 (INRA in collaboration with the Wishart Research Group) [33].

Table 2. Polyphenolic compounds in beer.

Phenolic compounds	(mg/L) *	Phenolic compounds	(mg/L) *
Simple Phenols		**Flavanones**	
Vinil-4-fenol	≤0.15	Isoxanthohumol	0.04–3.44
Vinil-4-guayacol	≤0.55	8-Prenilnaringenin	0.001–0.24
Etil-4-fenol	≤0.01	6-Prenilnaringenin	0.001–0.56
Isoeugenol	≤0.04	6-Geranilnaringenin	≤0.074
Tyrosol	≤40	Taxifolin	≤1.0
Propil-4-siringol	≤0.2	**Flavanols**	
2,3-Dihydroxy-guaiacyl propan-1-one	≤0.034	(+)-Catechin	≤5.4
Phenolic acids		(−)-Epicatechin	≤3.3
4-Hydroxyfenilacétic	≤0.65	Catechin gallate	5–20
Homovanillic	0.05	Epicatechin gallate	5–20
Alquilphenols		Procyanidin B3	≤3.1
3-Metilcatecol	≤0.03	Prodelphynidina B3	≤3.3
4-Etilcatecol	≤0.01	Prodelphynidina B9	≤3.9
4-Metilcatecol	≤0.02	Procyanidin C2	0.3
Vinil-4-fenol	≤0.15	**Flavonols**	
Benzoic acid derivatives		Kanpherol	16.4
3,5-Dihydroxybenzoic	0.3	Kanpherol-3-rhamnoside	≤1.0
2,6-Dihydroxybenzoic	0.9	Quercetin	≤10
2-Hydroxybenzoic	≤2.0	3,7-Dimetilquercetin	0.003
3-Hydroxybenzoic	≤0.3	Miricetin	0.007
4-Hydroxybenzoic	≤9.6	Quercetin 3-*O*-Arabinoside	0.006
Protocatecuic	≤0.3	Quercetina 3-*O*-Rutinoside	0.90
Vanillic	≤3.6	Quercitrin	≤2.3
Gallic	≤0.2	Isoquercitrin	≤1.0
Siríngico	≤0.5	Rutin	≤1.8
o-Vanillin	≤1.6	**Isoflavones**	
Siringic aldehyde	≤0.7	Daidzein	≤0.005
Cinnamic acids		Genistein	≤0.01
p-coumaric	≤1.2	Formononetin	≤0.004
m-Coumaric	≤0.2	Biochanin A	≤0.015
o-Coumaric	≤1.5	**Flavones**	
5-Caffeoilquinic	≤0.8	Apigenin	0.042
Caffeic	≤0.3	**α-acids (humulones)**	1.7
Ferulic	≤6.5	***Iso*-α-acids (*iso*-humulones)**	0.6–100
Sinapic	≤0.7	**Other polyphenols**	
Chalcones		Catechol	0.1
Xanthohumol	0.002–1.2	Pirogalol	0.3

* Mean value of bibliographic data published by Estruch *et al.* 2011 [34].

Beer is one of the most consumed alcoholic beverages around the world, being rich in nutrients such as carbohydrates, amino acids, minerals, vitamins and other compounds such as polyphenols. Hop (*Humulus lupulus* L.) is one of the raw materials of beer which serves as an important source of phenolic compounds. Dried hop cones contain about 14.4% of polyphenols, mainly phenolic acids, prenylated chalcones, flavonoids, catechins and pro-antocianidins [35]. Around 30% of polyphenols from beer comes from hops and 70%–80% originates from malt [36]. Moreover, hops provide a resin containing monoacyl phloroglucinols which become bitter acids during the development process of beer, such as α-acids (humulones) and iso-α acids. Table 2 details the bioactive compounds found in beer, mainly polyphenols. The structural classes of polyphenols in beer include simple phenols, benzoic acid derivatives and cinnamic acid, coumarins, catechins, di- and tri-oligomeric proanthocyanidins, prenylated chalcones and α- and iso-α acids derived from hops.

Different profiles of *in vitro* biological activities have been described for these compounds which, in combination, exert a synergistic effect. However, to extrapolate these results and evaluate the *in vivo* physiological effects of beer consumption it is necessary to study their bioavailability in the body. The compounds found in beer have different biological activities demonstrated *in vitro* as antioxidant, anticarcinogenic, anti-inflammatory, estrogenic and antiviral. However, further studies in humans are needed to determine whether the plasma concentrations of these compounds, derived from moderate consumption of beer, have the same bioactivity observed *in vitro*.

3. Effects of Alcohol and Polyphenols on the Cardiovascular System

Although several epidemiological studies have shown a protective effect of moderate alcohol consumption on the incidence of cardiovascular events, the exact mechanisms involved are still not well known. Up to now, lower risk of myocardial infarction in both sexes has been related to the effects of moderate alcohol consumption on lipoproteins (HDL), coagulation (fibrinogen) and sensitivity to insulin (haemoglobin A1c) [11]. The modulation of circulating cholesterol is the best established protective factor of alcohol intake [37]. Except in people with liver impairment, alcohol ingestion raises serum HDL-cholesterol levels by incompletely understood pathways [38]. Inverse relations of HDL-cholesterol and CHD risk operate substantially via removal of lipid deposits in large blood vessels. HDL also binds with cholesterol in the tissues and may aid in preventing LDL cholesterol oxidation. The net effect is a reduction in the plaque building in walls of large blood vessels, such as coronary arteries. One study suggested that the major HDL subfractions, HDL_2 and HDL_3, were involved. While HDL_3 may be strongly related to alcohol intake, both HDL fractions are related to lower CHD risk [39]. Nevertheless, a recent meta-analysis has questioned this hypothesis suggesting that HDL is not one of the most important intermediate variables in the possible causal pathway between moderate alcohol intake and CHD [40].

Since CHD is also considered a disease related to oxidative stress, several studies have analyzed the effects of different alcoholic beverages on oxidant status. In a cross-sectional study, alcohol consumption showed a direct relationship with the plasma concentration of *in vivo* oxidized low density lipoproteins (LDL) [41], while another study observed that the levels of the DNA oxidation marker 8-oxo-deoxyguanosine decreased with the amount of alcohol consumed [42]. The final effect probably depends on the total amount of alcohol consumed. Thus, in interventional studies a change in

daily beer alcohol consumption from moderate-heavy to light intake lowered plasma F2-isoprostanes in healthy non-smoking men [43]. Ethanol metabolism can produce free radicals and reduce the levels of glutathione, the major cellular protection against oxidative stress. However, in addition to alcohol, wine and beer contain polyphenols which could confer beneficial health properties compared to other classes of alcoholic beverages [44,45]. In a recent study comparing the antioxidant effects of a polyphenol-rich alcoholic beverage (red wine) with those of a polyphenol-free alcoholic beverage (gin), it was observed that compared to gin, red wine reduced plasma superoxide dismutase activity and malondialdehyde levels, as well as increased lag phase time of LDL oxidation analysis, suggesting that red wine has greater antioxidant effects, probably due to its high polyphenolic content [46].

Triglycerides may play an independent role in the risk of CHD. A subset of heavy drinkers has a substantial increase in triglyceride levels, but this is infrequently seen in light to moderate drinkers [47]. Alcohol (ethanol) inhibits several promoters of clotting, including platelet stickiness and fibrinogen levels [38]. In addition, moderate alcohol consumption also affects the fibrinolytic system. In fact, it increases plasminogen activator inhibitor activity and reduces plasminogen activator activity in the postprandial period (five hours after eating), a fact that may explain the reduction in the early morning cardiovascular events observed in moderate drinkers who consume alcohol with dinner [48].

A recent meta-analysis of prospective observational studies has observed that moderate alcohol consumption lowers the risk of type 2 diabetes [49]. In addition, randomized clinical trials have also demonstrated that moderate alcohol intake has beneficial effects on insulin concentrations and insulin sensitivity in non-diabetic patients [50], suggesting that moderate alcohol consumption decreases the risk of CVD and type 2 diabetes by improving insulin sensitivity. On the other hand, the results of some experimental studies have suggested that moderate consumption of wine could also protect endothelial cells from injury produced by hyperhomocysteinemia [51], although further studies are needed on this issue.

The previously mentioned effects of ethanol, mainly those on lipoproteins and clotting factors seem to account for 50% of the beneficial effect of alcohol consumption in the prevention of atherosclerosis. However, to explain the totality of the antiatherosclerotic effects of alcohol it is necessary to resort to additional mechanisms.

Other studies have shown that alcohol and individual polyphenols modulate EC fibrinolytic protein (t-PA, u-PA, PAI-1, u-PAR and Annexin-II) expression at the cellular, molecular and gene levels to sustain increased fibrinolytic activity [52].

In summary, the mechanisms by which alcoholic beverages exert their actions involve lipid regulation and systemic anti-inflammatory effects. The alcohol component (ethanol) increases HDL-cholesterol levels, inhibits platelet aggregation, and reduces systemic inflammation. However, red wine provides additional benefits to those of other alcoholic beverages probably due to its higher polyphenolic content, by decreasing blood pressure, inhibiting the oxidation of LDL particles and other favorable effects on the cellular redox state, improving endothelial function, inhibiting platelet aggregation, reducing inflammation and cell adhesion and activating proteins that prevent cell death. These effects are weaker in the case of white wine or beer probably due to the lower concentration of polyphenols compared to red wine.

4. The Effects of Wine and Beer on the Cardiovascular System

4.1. Epidemiological Studies

In previous studies evaluating whether different alcoholic beverages protect against CVD, a J-shaped relationship was found for increasing wine consumption and vascular risk [53]. A recent meta-analysis including a parallel and separate evaluation of wine and beer consumption indicated a similar protective effect for beer and wine against cardiovascular risk [54]. On the contrary, no statistically significant association with vascular events was apparent for the intake of spirits, the type of alcoholic drink with the highest alcohol concentration and the lowest polyphenolic concentration, suggesting that the polyphenolic constituents found in wine or beer could be (mainly) responsible for the beneficial effect of alcoholic beverages on vascular events [55–60].

A great number of epidemiologic studies have evaluated the effects of the three main types of alcoholic drinks (wine, beer and distilled drinks) on the cardiovascular system. Some studies such as the Copenhagen City Heart Study [53] or that carried out in the east of France [61] found a high significant relationship between low or moderate wine consumption and a lower mortality by CVD, whereas in many other great epidemiologic studies, especially those based on the registries of the Nurse's Health Study [62] or the Health Professionals Follow-up Study [11], no differences were found between the protective effects of moderate consumption of the different types of drink consumed. In the meta-analysis by Di Castelnuovo *et al*., 2010 [59], the authors analyzed the effects of the consumption of wine and beer on cardiovascular risk separately, concluding that the relative risk of associated vascular disease with wine consumption was of 0.68 (confidence interval 95% 0.59 to 0.77) compared with non-drinkers.

However, in other prospective studies, it has been observed that the moderate consumption of alcoholic drinks with a high alcoholic grade (liquors and distillates) also has a cardioprotector effect [63]. This fact explains that part of the beneficial effects of alcoholic beverages is largely due to ethanol, and not to the other specific components of each type of drink. Thus, the question as to whether the beneficial effects of alcoholic beverages depend on the alcoholic or non-alcoholic components of these beverages remains open and may not be answered on the basis of the results of epidemiological studies alone (see later).

Finally, other confounding factors should be taken into account in the study of the relationship between alcohol consumption and health. As the incidence of coronary disease is low in men and women under 40 and 50 years of age, respectively, a recent study concluded that young adults who consume alcohol moderately also presented a significant reduction of the risk of coronary disease compared with abstainers, although the protective effects are lower than those observed in middle-aged adults or older adults [64].

Other studies have pointed out that the lower mortality and the reduced risk of ischemic cardiopathy observed in moderate drinkers could be due to other factors such as the maintenance of an uncommon lifestyle (Mediterranean diet) or due to genetic factors that also play an important role in the protection of moderate alcohol consumption against CVD [2]. These epidemiological studies have controlled both the classical vascular risk factors and other confounding factors, such as diet, exercise and certain demographic and psychosocial characteristics, leaving little doubt as to the validity of results from an epidemiological point of view.

However, to obtain the best scientific evidence, the conclusions obtained from the results of meta-analyses or systematic reviews should only be based on randomized clinical trials. It would, therefore, be necessary to analyze a number of randomised studies with long-term interventions in which hard end-points (cardiovascular mortality, non-fatal acute myocardial infarction and non-fatal stroke) have been evaluated. In the absence of these studies, the findings of epidemiological studies cannot be considered definitive, especially after striking differences have been observed between the results of epidemiological and intervention studies, assessing, for example, the effectiveness of antioxidant vitamins on ischemic CHD [65].

For more than one decade it has been known that atherosclerosis is not only due to simple lipid accumulation in the arterial wall of certain zones of the vascular system, but rather, this is accompanied by a chronic inflammatory reaction of low intensity that contributes to the formation of atheroma plaques [66]. The biochemical and cellular mechanisms that lead to the beginning and progression of atherosclerosis have been widely studied [67]. This complex process involves the participation of very diverse cells (endothelial cells, smooth muscular cells, monocytes, lymphocytes and platelets), adhesion molecules (selectins, integrins and those pertaining to the super family of immunoglobulins), cytokines (interleukin (IL)-6, monocyte chemotactic peptide-1) and enzymes (metalloproteases). The first stage of atherosclerosis consists of the adhesion of monocytes and lymphocytes to the endothelium facilitated by adhesion molecules. Later, these cells migrate to the sub-endothelial space where they accumulate lipids and produce cytokines, growth factors and hydrolytic enzymes, which also induce a migration and proliferation of smooth muscle cells in this sub-endothelial space. The perpetuation of this process gives rise to the formation of atheroma plaque that becomes symptomatic when fissure (unstable plaque) is produced and induces the generation of thrombus on ulcerated plaque which, if not quickly degraded (fibrinolysis), may significantly occlude the vascular lumen and give rise to clinical events such as acute myocardial infarction or ischemic stroke [68]. Therefore, in order to know the role of alcohol consumption on the initiation and progression of atherosclerosis, the effect of alcohol on all the factors participating in the different stages of this process should be analyzed.

4.2. Clinical Trials

As stated before, although there is general consensus concerning the lower risk for ischemic disease in moderate drinkers, there are discrepancies as to whether this cardioprotective effect is due to the ethanol in alcoholic beverages or to their non-alcoholic content, mainly polyphenolic compounds contained in some alcoholic beverages, especially wine or beer. Part of these issues may be solved only by the performance of well-designed randomized clinical trials analyzing the *in vivo* effects of wine and/or beer (in comparison with other alcoholic beverages). Randomized cross-over clinical trials will allow monitoring the diet of the subjects included and will eliminate the effects of antioxidants and other healthy compounds from the food, mainly fruits and vegetables. Up to now, the results of the different clinical trials have been contradictory.

Thus, de Rijke *et al.* [69] examined the effect of the non-alcoholic components of red wine by reducing their alcohol content. The de-alcoholized red wine did not affect the susceptibility of LDL to copper-mediated oxidation. The results of this study did not show a beneficial effect of red wine

consumption on LDL oxidation. By contrast, the study performed by Fuhrman, Lavy and Aviram [70] concluded that the consumption of red, but not white wine, by healthy volunteers reduced the propensity of LDL to undergo lipid peroxidation. However, some methodological problems related to the sample size, the sex of the subjects and dose or type of alcoholic beverage may be raised on analysis of these studies. In order to solve these issues, recent clinical trials have compared the effects of red wine, an alcoholic beverage with high polyphenol content, with gin, an alcoholic beverage without polyphenols. In this setting, Estruch *et al*. 2011 [46] showed that ethanol itself may exert significant beneficial effects on the cardiovascular system, mainly by increasing HDL-cholesterol and decreasing oxidation of LDL. However, red wine provided additional benefits due to its higher antioxidant effects, by decreasing serum malondialdehyde, blood superoxide dismutase activity and oxidized LDL plasma levels.

On the other hand, since atherosclerosis is considered an inflammatory disease, other studies have analyzed the effects of moderate consumption of wine and gin on inflammatory biomarkers [60,71]. Randomized clinical trials including wine and other alcoholic beverages without polyphenols showed anti-inflammatory effects by reducing plasma fibrinogen and IL-1α levels. Again, however, wine showed additional effects by decreasing highly sensitive C-reactive protein, as well as monocyte and endothelial adhesion molecules [60,72]. Related to the latter, new findings from a randomized, crossover trial developed with 67 high-risk male volunteers consuming red wine, the equivalent amount of de-alcoholized red wine, or gin, revealed that alcohol increased IL-10 and decreased macrophage-derived chemokine concentrations, whereas the phenolic compounds of red wine decreased serum concentrations of intercellular adhesion molecule-1, E-selectin, and IL-6 and inhibited the expression of lymphocyte function-associated antigen 1 in T-lymphocytes and macrophage-1 receptor, Sialyl-Lewis X, and C-C chemokine receptor type 2 expression in monocytes. Both ethanol and phenolic compounds of red wine downregulated serum concentrations of CD40 antigen, CD40 ligand, IL-16, monocyte chemotactic protein-1, and vascular cell adhesion molecule-1 [73]. Other studies performed in women observed that daily doses of 15–20 g of alcohol as red wine were sufficient to elicit anti-inflammatory effects similar to those observed in men who consumed higher doses of wine [74].

Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as a pure compound or whole beverage. Subjects who showed higher absorption of polyphenols showed lower serum concentrations of inflammatory biomarkers than their counterparts [75]. Table 3 summarizes data from several relevant reviews about levels of polyphenolic metabolites in plasma and urine after wine or beer consumption. The results clearly show wide variability in the bioavailability of the different polyphenols. The polyphenols that are most well absorbed in humans are isoflavones and gallic acid, followed by catechins, flavanones and quercetin glucosides, with different kinetics. The least well-absorbed polyphenols are proanthocyanidins, the galloylated catechins and the anthocyanins. Data for other polyphenols are still too limited. Moreover, metabolism by the gut microbiota probably plays a major role in the biological activity of many polyphenols as in the case of prenylflavonoids of beer.

Table 3. Main polyphenolic metabolites described in literature from wine and beer.

Polyphenol group	Polyphenol metabolite	Source	Dose per day	No. subjects	Urine		Plasma		Ref.
					Urinary excretion	Tmax (h)	Mean Concentration	Tmax (h)	
Flavanols	Catechin	Red wine	35 mg (120 mL)	9		3.1	0.091 µmol/L	1.5	[76]
		Red wine	35 mg (120 mL)	9		3.2	0.077 µmol/L	1.44	[77]
		Red wine	35 mg (120 mL)	9	3%–10% of intake				[78]
	(+)-Catechin	Red wine	120 mL	9				1.6	[76]
	3′-*O*-Methylcatechin	Red wine	120 mL	9				1.2	[76]
Anthocyanins	Total anthocyanins	Red wine	218 mg (300 mL)	6	1.5%–5.1% of intake/12 h	6			[79]
	Malvidin 3-glc	Red wine	68 mg (500 mL)	6	0.016% of intake/6 h	<3	0.0014 µmol/L	0.83	[80]
Hydroxycinnamic acids	Caffeic acid	Red wine	0.9–1.8–2.7 mg (100, 200, 300 mL)	5			0.007–0.027 µmol/L	1	[81]
		Red wine	1.8 mg (200 mL)	10			0.037–0.060 µmol/L	0.5–1	[82]
		Red wine	55 µg/kg bw	12			0.084 µmol/L	2	[83]
		Red wine	5 mL/kg bw	12					[83]
	Ferulic acid	Beer	500 mL	10			0.05–0.07 µmol/L	1	[83]
		Beer	500 mL	10			0.11 µmol/L	1	[83]
	4-Hydroxyphenylacetic acid	Beer	500 mL	10		8	1.4–1.17 µmol/L	0.5–1	[84]
	Vanillic acid	Beer	500 mL	10			0.11 µmol/L	1	[84]
	p-Coumaric acid	Beer	500 mL	10			0.05–0.07 µmol/L	1	[84]
Hydroxybenzoic acids	Gallic acid	Red wine	4 mg (300 mL)	2			0.22 GA + 1.1 4-MeGA + 0.25 3-MeGA µmol/L		[85]
	Methylgallic acid	Red wine	47 µg/kg bw	12			0.18 4-MeGA µmol/L	2	[83]
	4-*O*-Methylgallic acid	Red wine	5 mL/kg bw	12					[83]
Stilbenes	*Cis*-resveratrol 3-sulfate	Red wine	250 mL	5	221.2 nmol/g creatinine	4			[86]
	Trans-resveratrol 3-*O*-glucuronide	Red wine	250 mL	5	179.2 nmol/g creatinine	4			[86]
	Trans-resveratrol 4′-*O*-glucuronide	Red wine	250 mL	5	59.6 nmol/g creatinine	4			[86]
	Cis-resveratrol 4′ sulfate	Red wine	250 mL	5	9294.2 nmol/g creatinine	4			[86]
	Trans-Resveratrol 3-sulfate	Red wine	250 mL	5	74.7 nmol/g creatinine	4			[86]
	Trans-Resveratrol 4′-sulfate	Red wine	250 mL	5	2.4nmol/g creatinine	4			[86]
	Cis-resveratrol 3-*O*-glucuronide	Red wine	250 mL	5	893.5 nmol/g creatinine	4			[86]
	Cis-resveratrol 4′-*O*-glucuronide	Red wine	250 mL	5	355.8 nmol/g creatinine	4			[86]

Tmax, time when Cmax is achieved; Cmax: maximal concentration; GA, gallic acid; MeGA, methylgallic acid; bw, body weight.

All these data provide strong biological plausibility to the epidemiological observations that moderate wine drinking reduces cardiovascular events. However, it should be taken into account that, although moderate wine intake reduces the risk of CHD, even low alcohol consumption in women may increase the risk of certain cancers, especially breast cancer [87].

In conclusion, moderate consumption of wine exerts a protective effect on biomarkers related to the progression and development of atherosclerosis due to its alcoholic (ethanol) and non-alcoholic (polyphenols) content. Women and those subjects with high polyphenol absorption are more sensitive to the healthy effects of wine.

Beer, despite its lower antioxidant and anti-inflammatory activity, also exerts a protective role against CVD. However, most studies addressing this issue are based on *in vitro* assays and animal studies, which have shown that the compounds derived from benzoic and cinnamic acids, catechins, procyanidins, humulones [23] and prenil-chalcones [88] are the major contributors to the antioxidant capacity of beer. It has also been observed that xanthohumol inhibited the oxidation of LDL *in vitro* induced by Cu^{2+} [89], as well as lipid peroxidation of liver microsomes in rats [90].

The anti-inflammatory mechanisms of bioactive compounds of beer are mainly due to the inhibition of inducible nitric oxide synthase (iNOS) and inhibition of the activity of cyclooxygenase 1 (COX-1) [23,26]. The main anti-inflammatory effect mediated by inhibition of iNOS induction seems to be due to xanthohumol. Moreover, xanthohumol and humulone produce an anti-inflammatory effect through inhibition of endogenous synthesis of prostaglandin E2 via COX-2 inducible by TNFα [23,26].

5. Role of Wine and Beer in Cancer Prevention

The consumption of alcohol has been identified as one of the top-10 risks contributing to the worldwide burden of disease. The International Agency for Research on Cancer (IARC) has classified ethanol as carcinogenic to humans [91]. Thus, the carcinogenic effects of alcoholic beverages are essentially due to their ethanol content and increase with the amount of alcohol drunk. Although the interpretation of data obtained in epidemiological studies is difficult due to confounding factors such as smoking, diet, hormone-replacement therapy and family history, the risks are essentially due to the ethanol content of alcoholic beverages. However, there is evidence that moderate wine consumption may decrease the risk of several cancers, including colon, basal cell carcinoma, ovarian and prostate cancer [92,93].

Consumption of approximately 1 glass of wine daily was associated with a decreased risk of developing Barrett's esophagus, a precursor to esophageal adenocarcinoma, when compared to heavy drinkers or non-drinkers [94]. A meta-analysis found that modest wine consumption had an inverse association for developing lung cancer, for both average wine consumption of less than one drink per day (RR, 0.77; 95% CI, 0.59–1.00) and one drink or greater per day (RR, 0.78; 95% CI, 0.60–1.02) [95]. A study of female non-Hodgkin's lymphoma patients found a significantly better five-year overall survival (75% *vs.* 69%) and five-year disease free survival (70% *vs.* 67%) in occasional wine drinkers *vs.* abstainers [96]. Compared to non-drinkers, women who drank wine for at least 25 years previously were 33% less likely to die and 26% less likely to experience a relapse or develop a secondary cancer over the five-year period following diagnosis.

A large percentage of the literature on the cancer-preventing effects of wine are focused on one compound in particular: resveratrol. Many comprehensive reviews have been written on this subject [31,97,98]. Wine may inhibit carcinogenesis by acting as an antioxidant, anti-inflammatory agent, antimutagen, antimetastatic, anti-angiogenic, antidifferentiation, antiproliferative, and proapoptotic agent. It modulates signal transduction, immune response, transcription factors, growth factors, cytokines, caspases, interleukins, prostaglandin synthesis and cell cycle-regulating proteins.

However, although there are few doubts as to the beneficial effects of moderate drinking on the cardiovascular system, only a limited number of reports concerning alcohol and cancer have provided results in favor of a reduced risk of death by cancer. According to the data of the Million Women study [87] even light to moderate levels of alcohol consumption were predictive of an increased risk of several common cancers, mainly those of the breast. It has been suggested that alcohol intake increases circulating levels of estrogen and the production of reactive oxygen species during alcohol metabolism, inducing DNA damage that results in breast cancer [99,100]. A recent study has reported that alcohol intake, the genes involved in alcohol metabolism and their interaction, increase the risk of breast cancer in post-menopausal women [101]. In relation to the type of beverage (beer, wine or spirits), a prospective cohort study has reported that total alcohol intake of more than 27 drinks per week increases breast cancer risk in premenopausal women independently of the type of alcoholic beverage consumed [102]. However, a low to moderate alcohol intake was not associated with increased mortality after breast cancer in a cohort of middle-aged women previously diagnosed with breast cancer [103]. Additionally, the drinking pattern seems important in terms of alcohol-breast cancer association as some authors have suggested that low and regular wine consumption does not increase breast cancer risk [104].

Several components of wine (mainly resveratrol) have shown anticarcinogenic properties in experimental studies [105]. Those have been supported by its ability to inhibit proliferation of a wide variety of human tumor cells *in vitro* as well as implanted tumors, usually in mice [97,106]. However, it should be emphasized that these results are measurements of resveratrol responses on human cancer cells in culture, or taken as conclusions from epidemiological studies, rather than the results of clinical trials with cancer patients.

In the case of beer, xanthohumol is the best studied anticarcinogenic. It acts by inhibiting the metabolic activation of procarcinogenics, detoxifying enzyme inducers of carcinogens [25] and inhibiting tumor growth in early stages through inhibition of angiogenesis and inflammatory signals. Other compounds in beer with anticarcinogenic capacity are 8-prenilnaringenin, isoxanthohumol (having 10–20-fold lower concentrations than the effective doses in humans in beer) and other prenilflavonoids, as well as the flavanones, humulones and proantocianidins [24]. Considering that the bioavailability of the phenolic compounds of beer is very low, their anticarcinogenic effects are somewhat controversial as many epidemiological studies have shown [107,108]. However, polyphenols from beer can reach low but effective concentrations in the colon, acting as local anti-carcinogenic agents. The iso-α-acids (humulones) represent one of the most abundant groups of polyphenols in beer and also possess antitumor activity. It has also been described that cohumola, *n*-humulone and adhumulona activate receptors of α peroxisome proliferation (PPAR α), having potential activity in preventing cancer [23].

Several epidemiological studies have investigated the potential role of beer as a cause of cancer due to the detection of volatile nitrosamines in beer, although amounts have been reported to be lower in more recent decades because of changes in the beer-making process [109–111]. Since most epidemiological studies refer to long-term alcohol consumption, these studies cover, in part, periods of time before changes were made in the malting process. On the other hand, beer consumption (10 g alcohol/day) was reported to significantly decrease the risk of prostate cancer in comparison with non-drinking of beer in a Canadian study including 1253 subjects [112,113].

Malt-derived beer components require further investigation. Studies with melanoidins, *i.e.*, polymeric and colored final products of the Maillard reaction which are formed non-enzymatically during the roasting of malt, indicate peroxyl radical scavenging potential [114,115]. Melanoidin fractions with a relatively high molecular weight (10→200 kDa) also weakly induced NADPHcytochrome C reductase and size-dependently modulated GST activities in the Caco-2 colon cancer cell line [116]. The information available illustrates that beer is an extremely complex mixture of bioactive substances. Therefore, a thorough exploration of the chemopreventive activities of isolated prominent beer components seems to make eminent sense. Nevertheless, future studies should also focus on defined combinations to explore whether the mixture can be more efficacious than the single components.

Thus, the issue of alcohol and cancer is wide open and new studies are needed. Since self-reported data on alcohol consumption in epidemiological studies may not be reliable, especially in women, clinical studies on the effects of alcohol on health should be based on specific and accurate biomarkers of alcohol or wine/beer consumption such as ethanol (ethylglucoronide or ethylene glycol) or polyphenolic metabolites, respectively, in urine.

6. Conclusions

Sufficient evidence supports a significant inverse association between regular and moderate wine consumption and vascular risk, particularly red wine, and a similar relationship is reported for beer consumption, while lower protection is described for the consumption of any spirituous beverage.

Clinical and epidemiological studies indicate that it is mainly red wine which may protect against CVD, atherosclerosis, hypertension, certain types of cancer, type 2 diabetes, neurological disorders and metabolic syndrome.

There is evidence that certain polyphenols, such as resveratrol, anthocyanins, flavonols and catechins in wine provide an abundance of health benefits. Furthermore, rather than polyphenols themselves, their metabolites might be the real key players in cardiovascular and cancer protection. In beer, xanthohumol and its metabolites isoxanthohumol and phytoestrogen 8-prenylnaringenin also provide healthy properties such as anticarcinogenic, anti-invasive, anti-angiogenic, anti-inflammatory and antioxidant effects. The complexity increases when considering that each subject may metabolize the beverage differently, making it impossible to establish one specific constituent as being critical from a health standpoint.

It must be emphasized that the benefits associated with red wine and beer are dependent upon regular and moderate consumption. Although general recommendations are one drink (150 mL of wine or 10 g of alcohol) daily for women and two drinks (300 mL of wine or 20 g of alcohol) daily for men, individual ideals may vary based on age, gender, genetics, body type and drug/supplement use. These

different recommended daily doses of alcohol between genders are explained by the fact that women are more sensitive to the effects of alcohol on the body. In addition, any healthy effects of wine and beer are greater in combination with a healthy diet. The health benefits associated with the Mediterranean diet, which combines moderate wine and beer consumption with a diet rich in fruits, vegetables and whole grains, suggests that polyphenols have synergistic effects with compounds found in other groups of foods.

Although alcohol consumption is a two-sided coin, moderate alcohol consumption especially of wine has demonstrated the provision of a protective role for the cardiovascular system and in some types of cancer. Most medical professionals as well as the American Heart Association agree that heavy drinkers or alcohol abstainers should not be encouraged to drink wine for health reasons. Wine consumption should not replace a healthy lifestyle. However, light-to-moderate wine drinkers, without medical complications, may be assured that their wine consumption is a healthy habit.

Nevertheless, more randomized clinical trials focused on elucidating the mechanisms of the action of alcohol and polyphenols are needed.

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Conflict of Interest

The authors declare no conflict of interest.

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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, $\mu g/L$							
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, $\mu g/L$							
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, $\mu g/L$							
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) (*n* = 101)	2 (−24.5–62.7) (*n* = 112)	3 (≥62.7) (*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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Online Supporting Material

Supplemental Table 1. Baseline characteristics of participants randomly assigned to the 3 intervention groups [1].

	Total (*n*=516)	MD-VOO (*n*=178)	MD-Nuts (*n*=175)	LFD (*n*=163)
Age, *y*	67 ± 6	66 ± 6	67 ± 6	67 ± 6
Men, *n (%)*	254 (49.2)	83 (46.6)	89 (50.9)	82 (50.3)
Family history of CHD, *n (%)*	87 (16.9)	28 (15.7)	24 (13.7)	35 (21.5)
Current smokers, *n (%)*	96 (18.6)	36 (20.2)	30 (17.1)	30 (18.4)
BMI, *kg/m²*	29.4 ± 3.4	29.3 ± 3.4	29.2 ± 3.5	29.7 ± 3.2
Overweight or obese (BMI ≥ 25 kg/m²), *n (%)*	469 (90.9)	161 (90.4)	157 (89.7)	151 (92.6)
Height, *m*	1.6 ± 0.09	1.6 ± 0.09	1.6 ± 0.09	1.6 ± 0.08
Weight, *kg*	75.9 ± 11.1	76.0 ± 11.9	75.4 ± 11.0	76.5 ± 10.4
Waist circumference, *cm*	98.3 ± 10.2	98.1 ± 11.0	97.4 ± 9.9	99.7 ± 9.5
Systolic blood pressure, *mmHg*	151 ± 18.8	151 ± 19.1	154 ± 19.4	149 ± 17.5
Diastolic blood pressure, *mmHg*	85.7 ± 9.8	85.1 ± 9.2	86.8 ± 11.0	85.0 ± 9.1
Cardiac frequency, *beats/min*	70.5 ± 11.8	69.6 ± 11.5	70.4 ± 11.8	71.4 ± 12.2
Physical activity, *MET·min/d*	307 ± 252	268 ± 245	305 ± 254	293 ± 268
Type 2 diabetes mellitus, *n (%)*	262 (50.8)	93 (52.2)	82 (46.9)	87 (53.4)
Hypertension, *n (%)*	405 (78.5)	140 (78.7)	141 (80.6)	124 (76.1)
Dyslipidemia, *n (%)*	324 (62.8)	113 (63.5)	108 (61.7)	103 (63.2)
Medication, *n (%)*				
ACE inhibitors	213 (41.3)	68 (38.2)	72 (41.4)	73 (44.8)
Diuretics	119 (23.1)	50 (28.1)	37 (21.3)	32 (19.6)
Other antihypertensive agents	137 (26.6)	40 (22.5)	56 (32.2)	41 (25.2)
Statins	161 (31.2)	50 (28.1)	58 (33.3)	53 (32.5)
Other lipid-lowering agents	19 (3.7)	9 (5.1)	5 (2.9)	5 (3.1)
Insulin	24 (4.7)	7 (3.9)	10 (5.7)	7 (4.3)
Oral hypoglycemic drugs	139 (26.9)	51 (28.7)	37 (21.1)	51 (31.3)
Aspirin or other antiplatelet drugs	78 (15.1)	27 (15.2)	24 (13.8)	27 (16.6)
Occupation, *n (%)*				
Unskilled	119 (23.1)	41 (23.0)	40 (22.9)	39 (23.9)
Skilled, manual	191 (37.0)	69 (38.8)	67 (38.3)	57 (35.0)
Skilled, non manual	114 (22.1)	41 (23.0)	37 (21.1)	37 (22.7)
Directive and professional	88 (17.1)	27 (15.2)	32 (18.3)	29 (17.8)
Education level, *n (%)*				
Primary school	386 (74.8)	127 (71.3)	137 (78.3)	122 (74.8)
First-degree high school	81 (15.7)	31 (17.4)	26 (14.9)	24 (14.7)
High school or university	42 (8.1)	15 (8.4)	11 (6.3)	16 (9.8)

[1] Values are mean ± SD or *n* (%). ACE, angiotensin converted enzyme; CHD, coronary heart disease; LFD, low fat diet; MD, Mediterranean diet; VOO, virgin olive oil.

Online Supporting Material

Supplemental Table 2. Consumption of selected food items and the 14-point MD score at baseline and after 1 y and the differences between the two time points in the 3 intervention groups [1]

		MD-VOO (*n*=178)	MD-Nuts (*n*=175)	LFD (*n*=163)	Repeated-measures ANOVA[2]	*P* value for differences[3]		
					Time x treatment	MD-VOO vs MD-Nuts	MD-VOO vs LFD	MD-Nuts vs LFD
VOO, *g/d*								
	Baseline	23.5 ± 26.1	21.9 ± 26.1	16.9 ± 21.9				
	1 y	56.3 ± 16.2*[a]	28.6 ± 28.3 *[b]	19.6 ± 21.8 [c]	< 0.001	< 0.001	< 0.001	0.47
	Dif (1y-B)	32.9 (29.2 – 36.6)	6.6 (3.0 – 10.2)	2.8 (-1.1 – 6.7)				
Refined olive oil, *g/d*								
	Baseline	22.6 ± 22.8	21.8 ± 22.2	21.8 ± 20.3		< 0.001	< 0.001	0.045
	1 y	1.8 ± 9.8 *[c]	24.2 ± 26.0 *[a]	18.1 ± 20.0 *[b]	< 0.001			
	Dif (1y-B)	-20.8 (-24.4 - -17.2)	2.6 (-0.9 – 6.0)	-3.8 (-7.5 – -0.1)				
Total nuts, *g/d*								
	Baseline	9.1 ± 12.2	13.4 ± 14.3	10.8 ± 13.3				
	1 y	13.4 ± 13.7 *[b]	35.0 ± 16.4 *[a]	9.1 ± 12.1 [c]	< 0.001	< 0.001	0.002	< 0.001
	Dif (1y-B)	4.4 (1.9 – 6.9)	21.8 (19.4 – 24.2)	-2.0 (-4.5 – 0.6)				
Vegetables, *g/d*								
	Baseline	337 ± 178	316 ± 147	309 ± 153				
	1 y	366 ± 139 *[a]	343 ± 132 *[a]	306 ± 156 [b]	0.09	1.00	0.15	0.17
	Dif (1y-B)	29 (5 – 53)	28 (5 – 51)	-5 (-30 – 20)				
Legumes, *g/d*								
	Baseline	19.5 ± 11.5	18.7 ± 7.8	18.6 ± 10.6				
	1 y	24.7 ± 10.4 *[a]	23.2 ± 7.9 *[a]	19.1 ± 10.5 [b]	0.001	1.00	0.002	0.008
	Dif (1y-B)	5.3 (3.3 – 7.2)	4.6 (2.7 – 6.4)	0.4 (-1.6 – 2.4)				
Fruits, *g/d*								
	Baseline	412 ± 230	421 ± 198	374 ± 216				
	1 y	461 ± 214 *[a]	486 ± 176 *[a]	404 ± 208 [b]	0.11	0.70	1.00	0.11
	Dif (1y-B)	50 (17 – 82)	77 (45 – 109)	28 (-7 – 62)				
Cereals, *g/d*								
	Baseline	251 ± 118 [ab]	240 ± 88 [b]	257 ± 118 [a]				
	1 y	228 ± 94 *	243 ± 89	242 ± 104	0.049	0.06	1.00	0.21
	Dif (1y-B)	-23 (-40 - -6)	5 (-11 – 21)	-17 (-34 – 0.3)				
Fish and seafood, *g/d*								
	Baseline	98 ± 44	107 ± 39	104 ± 45				
	1 y	107 ± 43*	106 ± 46	96 ± 44*	0.002	0.15	0.001	0.26
	Dif (1y-B)	9 (3 – 16)	-0.03 (-6 – 6)	-8 (-15 - -1)				
Meat and meat products, *g/d*								
	Baseline	138 ± 63	144 ± 52	141 ± 51				
	1 y	128 ± 49 *	130 ± 47 *	131 ± 48 *	0.83	1.00	1.00	1.00
	Dif (1y-B)	-10 (-19 - -2)	-14 (-22 - -6)	-11 (-19 – 2)				
Pastries, cakes or sweets, *g/d*								
	Baseline	23.3 ± 27.7	24.3 ± 29.7	24.9 ± 39.2				
	1 y	19.0 ± 26.1	19.2 ± 25.8 *	19.8 ± 30.0 *	0.88	1.00	1.00	1.00
	Dif (1y-B)	-4.2 (-8.5 – 0.1)	-4.6 (-8.8 - -0.4)	-5.8 (-10.2 - -1.3)				
Low-fat dairy products, *g/d*								
	Baseline	269 ± 218	289 ± 244	278 ± 248				
	1 y	333 ± 228 *	289 ± 241	299 ± 249	0.012	0.011	0.14	1.00
	Dif (1y-B)	64 (34 – 94)	1 (-29 – 30)	20 (-12 – 51)				
Whole dairy products, *g/d*								
	Baseline	107 ± 150	111 ± 173	119 ± 174				
	1 y	84 ± 138	94 ± 157	88 ± 133	0.65	0.023	0.048	1.00
	Dif (1y-B)	-23 (-45 - -1)	-17 (-39 – 4)	-32 (-55 - -9)				
Alcohol, *g/d*								
	Baseline	10.3 ± 14.5	14.5 ± 21.6	10.9 ± 16.0				
	1 y	9.9 ± 13.6	12.6 ± 18.5	10.1 ± 15.5	0.46	0.66	1.00	1.00
	Dif (1y-B)	-0.4 (-2.1 – 1.4)	-1.9 (-3.6 - -0.2)	-0.9 (-2.7 – 0.9)				
Wine, *mL/d*								
	Baseline	76.7 ± 119	111 ± 171	76.9 ± 121				
	1 y	81.4 ± 123	106 ± 159	76.6 ± 128	0.67	1.00	1.00	1.00
	Dif (1y-B)	5 (-10 – 20)	-5 (-19 – 10)	-0.5 (-16 – 15)				
MD score								
	Baseline	9.0 ± 1.9	9.1 ± 1.7	8.6 ± 1.8				
	1 y	11.1 ± 1.7 *[a]	11.4 ± 1.5 *[a]	9.0 ± 1.7 *[b]	< 0.001	1.00	< 0.001	< 0.001
	Dif (1y-B)	2.1 (1.8 – 2.4)	2.2 (2.0 – 2.5)	0.4 (0.1 – 0.6)				

[1] Values are means ± SD. 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; LFD, low fat diet; MD, Mediterranean diet; 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 energy intake.

Online Supporting Material

Supplemental Table 3. Energy and nutrient intakes at baseline and after 1 y and the differences between the two time points in the 3 intervention groups [1]

		MD-VOO (n=178)	MD-Nuts (n=175)	LFD (n=163)	Repeated-measures ANOVA[2]	P value for differences[3]		
					Time x treatment	MD-VOO vs MD-Nuts	MD-VOO vs LFD	MD-Nuts vs LFD
Energy, *kJ/d*								
	Baseline	9994 ± 2340	10140 ± 2370	9776 ± 2801				
	1 y	10140 ± 2261 [a]	10605 ± 2119 *,[a]	9161 ± 2303 *,[b]	< 0.001	0.13	< 0.001	< 0.001
	Dif (1y-B)	151 (-120 – 421)	542 (279 – 805)	-708 (-990 - -426)				
Protein, *g/d*								
	Baseline	94 ± 23	96 ± 19	95 ± 24				
	1 y	95 ± 20 [a,b]	98 ± 18 [a]	90 ± 21 *,[b]	< 0.001	1.00	0.002	0.001
	Dif (1y-B)	2 (-1 - 4)	2 (-0.6 – 5)	-6 (-9 - -3)				
Carbohydrate, *g/d*								
	Baseline	250 ± 82 [a]	243 ± 76 [b]	244 ± 92 [a]				
	1 y	242 ± 71	249 ± 73	234 ± 71*	0.008	0.07	1.00	0.009
	Dif (1y-B)	-8 (-18 – 3)	9 (-1 – 19)	-13 (-24 - -3)				
Total fat, *g/d*								
	Baseline	104 ± 27	106 ± 24	100 ± 32				
	1 y	111 ± 26 *,[a]	117 ± 23 *,[a]	91 ± 27 *,[b]	< 0.001	0.34	< 0.001	< 0.001
	Dif (1y-B)	7 (3 – 11)	11 (8 – 15)	-10 (-13 - -6)				
SFA, *g/d*								
	Baseline	25 ± 8	27 ± 9	26 ± 10				
	1 y	26 ± 8 [a]	26 ± 7 [a]	23 ± 8 *,[b]	0.003	1.00	0.004	0.028
	Dif (1y-B)	-0.3 (-1.4 – 0.8)	-1 (-2 – 0.2)	-3 (-4 - -2)				
MUFA, *g/d*								
	Baseline	53 ± 14	52 ± 12	49 ± 16				
	1 y	59 ± 13 *,[a]	60 ± 13 *,[a]	45 ± 14 *,[b]	< 0.001	1.00	< 0.001	< 0.001
	Dif (1y-B)	6 (4 – 8)	7 (5 – 9)	-4 (-6 - -2)				
PUFA, *g/d*								
	Baseline	16 ± 6 [a]	17 ± 6 [b]	17 ± 8 [ab]				
	1 y	17 ± 5 *,[b]	22 ± 6 *,[a]	15 ± 6 *,[c]	< 0.001	< 0.001	< 0.001	< 0.001
	Dif (1y-B)	2 (0.7 – 3)	5 (4 – 6)	-2 (-3 – -1)				
αLA, *g/d*								
	Baseline	1.4 ± 0.7	1.6 ± 0.7	1.5 ± 0.7				
	1 y	1.6 ± 0.7 *,[b]	2.2 ± 0.6 *,[a]	1.3 ± 0.6 *,[c]	< 0.001	< 0.001	< 0.001	< 0.001
	Dif (1y-B)	0.2 (0.1-0.3)	0.6 (0.4 – 0.7)	-0.2 (0.3 - -0.03)				
Marine (n-3) fatty acids, *g/d*								
	Baseline	0.80 ± 0.45	0.81 ± 0.41	0.83 ± 0.43				
	1 y	0.91 ± 0.44 *,[a]	0.88 ± 0.46 *,[ab]	0.77 ± 0.46 [b]	0.004	1.00	0.005	0.023
	Dif (1y-B)	0.1 (0.03 – 0.2)	0.08 (0.01 – 0.2)	-0.06 (-0.1 – 0.01)				
Cholesterol, *mg/d*								
	Baseline	378 ± 165	386 ± 110	382 ± 130				
	1 y	360 ± 106	353 ± 113 *	355 ± 136 *	0.61	1.00	1.00	1.00
	Dif (1y-B)	-18 (-38 – 2)	-31 (-50 - -12)	-29 (-49 - -8)				

[1] Values are means ± SD. 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). αLA, α-linolenic acid; Dif, differences between 1 y and baseline; LFD, low fat diet; MD, Mediterranean diet; 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 energy intake.

Online Supporting Material

Supplemental Table 4. Concentrations of circulating inflammatory molecules at baseline and after 1 y and the differences between the two time points in all participants by tertile of change in consumption of selected foods and nutrients[1]

		Δ FOODS and NUTRIENTS TERTILES			Repeated-measures ANOVA[2]	*P* value for difference [3]		
		mean (95% CI)						
		Δ VOO consumption tertiles			Time x treatment	1 vs 2	1 vs 3	2 vs 3
		1 (≤ -0.3 g/d)	2 (-0.3 to 24 g/d)	3 (≥ 24 g/d)				
		(*n*=144)	(*n*=146)	(*n*=147)				
ICAM-1, *μg/L*	Baseline	259 (246-173)	274 (260-289)	268 (255-282)				
	1 y	273 (261-286)	267 (255- 280)	267 (255-279)	0.09	1.00	0.34	1.00
	Dif (1y-B)	14 (-1 – 28)	-7 (-23 – 8)	-1 (-14 – 10)				
		(*n*=105)	(*n*=120)	(*n*=109)				
IL6, *ng/L*	Baseline	0.92 (0.77-1.09)	1.00 (0.85-1.17)	0.90 (0.76-1.06)				
	1 y	0.77 (0.62-1.09)	0.80 (0.66-0.97)	0.69 (0.56-0.84)	0.85	1.00	1.00	1.00
	Dif (1y-B)	-0.15 (-0.33 – 0.03)	-0.20 (-0.45 – 0.05)	-0.21 (-0.40 – -0.01)				
		(*n*=128)	(*n*=127)	(*n*=130)				
TNFR80, *μg/L*	Baseline	6.2 (5.8-6.6)	6.5 (6.1-6.7)	6.5 (6.1-6.9)				
	1 y	6.4 (6.0-6.8)	6.4 (6.0-6.8)	6.1 (5.7-6.4)	0.14	1.00	0.14	0.85
	Dif (1y-B)	0.2 (-0.3 – 0.6)	-0.1 (-0.8 – 0.6)	-0.4 (-0.9 – 0.01)				
		Δ Nuts consumption tertiles						
		1 (≤ -3.7 g/d)	2 (-3.7 to 9.8 g/d)	3 (≥ 9.8 g/d)				
		(*n*=148)	(*n*=147)	(*n*=142)				
ICAM-1, *μg/L*	Baseline	266 (253-280)	266 (252-280)	269 (256-284)				
	1 y	273 (261-286)	266 (254-279)	268 (255-281)	0.64	1.00	1.00	1.00
	Dif (1y-B)	7 (-8 – 21)	0 (-16 – 15)	-1 (-27 – 25)				
		(*n*=99)	(*n*=120)	(*n*=115)				
IL6, *ng/L*	Baseline	0.85 (0.71-1.01)	1.06 (0.91-1.24)	0.91 (0.77-1.07)				
	1 y	0.76 (0.62-0.94)	0.84 (0.69-1.02)	0.66 (0.54-0.81)	0.34	0.63	0.33	1.00
	Dif (1y-B)	-0.09 (-0.32 – 0.13)	-0.22 (-0.44 – -0.02)	-0.25 (-0.49 – 0.002)				
		(*n*=123)	(*n*=137)	(*n*=125)				
TNFR80, *μg/L*	Baseline	6.5 (9.1-6.9)	6.1 (5.8-6.5)	6.6 (6.1-7.0)				
	1 y	6.3 (5.9-6.8)	6.2 (5.8-6.6)	6.3 (5.9-6.7)	0.47	1.00	1.00	0.69
	Dif (1y-B)	-0.2 (-1.0 – 0.4)	0.1 (-0.3 – 0.5)	-0.3 (-1.0 – 0.3)				
		Δ Fish and seafood products consumption tertiles						
		1 (≤ -14.4 g/d)	2 (-14.4 to 14.3 g/d)	3 (≥ 14.3 g/d)				
		(*n*=144)	(*n*=142)	(*n*=151)				
ICAM-1, *μg/L*	Baseline	272 (258-287)	271 (258-286)	259 (246-272)				
	1 y	277 (264-290)	261 (249-273)	269 (258-282)	0.07	0.32	1.00	0.07
	Dif (1y-B)	5 (-10 – 19)	-10 (-25 – 4)	10 (-5 – 23)				
		(*n*=124)	(*n*=120)	(*n*=90)				
IL6, *ng/L*	Baseline	0.93 (0.80-1.09)	0.90 (0.77-1.06)	1.00 (0.83-1.21)				
	1 y	0.83 (0.69-1.01)	0.67 (0.55-0.81)	0.76 (0.61-0.96)	0.33	0.16	0.62	1.00
	Dif (1y-B)	-0.10 (-0.29 – 0.10)	-0.23 (-0.48 – 0.03)	-0.24 (-0.46 – -0.002)				
		(*n*=127)	(*n*=127)	(*n*=130)				
TNFR60, *μg/L*	Baseline	1.5 (1.3-1.7)	1.5 (1.3-1.6)	1.6 (1.4-1.8)				
	1 y	1.6 (1.4-1.8)	1.4 (1.2-1.5)	1.5 (1.4-1.7)	0.21	0.32	0.60	1.00
	Dif (1y-B)	0.1 (-0.1 – 0.2)	-0.1 (-0.3 – 0.02)	-0.1 (-0.2 – 0.1)				
		Δ MUFA consumption tertiles						
		1 (≤ 22.5 g/d)	2 (22.5 to 38.1 g/d)	3 (≥ 38.1 g/d)				
		(*n*=147)	(*n*=145)	(*n*=145)				
ICAM-1, *μg/L*	Baseline	268 (252-285)	268 (254-282)	266 (249-283)				
	1 y	268 (253-284)	272 (260-285)	267 (252-282)	0.89	1.00	1.00	1.00
	Dif (1y-B)	0 (-13 – 12)	4 (-13 – 16)	1 (-17 – 19)				
		(*n*=108)	(*n*=109)	(*n*=117)				

IL6, *ng/L*	Baseline	0.92 (0.75-1.13)	0.97 (0.82-1.15)	0.94 (0.77-1.14)				
	1 y	0.74 (0.57-0.94)	0.76 (0.62-0.93)	0.76 (0.60-0.97)	0.96	1.00	1.00	1.00
	Dif (1y-B)	-0.18 (-0.41 – 0.07)	-0.21 (-0.41 – 0.02)	-0.18 (-0.48 – 0.10)				
		(*n*=114)	(*n*=136)	(*n*=135)				
TNFR80, *µg/L*	Baseline	6.7 (6.1-7.2)	6.3 (5.9-6.7)	6.3 (5.8-6.8)				
	1 y	6.5 (6.0-7.0)	6.2 (5.9-6.9)	6.1 (5.7-6.6)	0.90	1.00	1.00	1.00
	Dif (1y-B)	-0.2 (-0.9 – 0.4)	-0.1 (-0.8 – 0.5)	-0.2 (-1.0 – 0.5)				
		Δ Vegetables consumption tertiles						
		1 (≤ -24.5 g/d)	2 (-24.5 to 62.7 g/d)	3 (≥ 62.7 g/d)				
		(*n*=153)	(*n*=143)	(*n*=141)				
ICAM-1, *µg/L*	Baseline	263 (250-276)	275 (261-290)	264 (250-278)				
	1 y	273 (260-285)	270 (258-283)	264 (252-277)	0.28	0.35	0.90	1.00
	Dif (1y-B)	10 (-5 – 23)	-5 (-20 – 9)	0 (-15 – 14)				
		(*n*=101)	(*n*=112)	(*n*=121)				
IL6, *ng/L*	Baseline	0.86 (0.72-1.02)	0.85 (0.73-1.00)	1.11 (0.95-1.30)				
	1 y	0.65 (0.53-0.80)	0.67 (0.55-0.82)	0.95 (0.78-1.14)	0.68	1.00	1.00	1.00
	Dif (1y-B)	-0.21 (-0.46 – 0.03)	-0.18 (-0.37 – 0.04)	-0.16 (-0.36 – 0.03)				
		(*n*=122)	(*n*=125)	(*n*=138)				
TNFR80, *µg/L*	Baseline	6.5 (6.1-6.9)	6.4 (6.0-6.9)	6.3 (5.9-6.7)				
	1 y	6.4 (6.0-6.9)	6.4 (6.0-6.8)	6.0 (5.7-6.4)	0.79	1.00	1.00	1.00
	Dif (1y-B)	-0.1 (-0.8 – 0.4)	0 (-0.7 – 0.6)	-0.3 (-1.0 – 0.2)				
		Δ MD score tertiles						
		1 (≤ 0.9)	2 (0.9 to 2.4)	3 (≥ 2.4)				
		(*n*=148)	(*n*=143)	(*n*=146)				
ICAM-1, *µg/L*	Baseline	269 (255-283)	268 (254-283)	266 (252-280)				
	1 y	275 (263-289)	269 (257-282)	263 (251-275)	0.89	1.00	1.00	1.00
	Dif (1y-B)	6 (-11 – 23)	1 (-15 – 14)	-3 (-21 – 14)				
		(*n*=114)	(*n*=105)	(*n*=115)				
IL6, *ng/L*	Baseline	1.00 (0.85-1.17)	1.00 (0.84-1.18)	0.84 (0.71-0.99)				
	1 y	0.84 (0.69-1.01)	0.74 (0.60-0.91)	0.68 (0.56-0.83)	0.60	1.00	1.00	1.00
	Dif (1y-B)	-0.16 (-0.36 – 0.05)	-0.26 (-0.48 –-0.05)	-0.16 (-0.36 – 0.04)				
		(*n*=130)	(*n*=125)	(*n*=129)				
TNFR60, *µg/L*	Baseline	1.5 (1.4-1.8)	1.6 (1.4-1.7)	1.4 (1.3-1.6)				
	1 y	1.7 (1.5-1.8)	1.5 (1.3-1.7)	1.3 (1.2-1.6)	0.11	0.36	0.12	1.00
	Dif (1y-B)	0.2 (-0.003 – 0.4)	-0.1 (-0.3 – 0.01)	-0.1 (-0.3 – 0.004)				

[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; ICAM-1: intercellular adhesion molecule-1; LFD: low-fat-diet; 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, gender, energy intake, BMI, smoking status, physical activity, research center and drugs (aspirin and statins).

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Review

Virgin olive oil and nuts as key foods of the Mediterranean diet effects on inflammatory biomarkers related to atherosclerosis

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ABSTRACT

Previous epidemiological and feeding studies have observed that adherence to Mediterranean diet (Med-Diet) is associated with reduced cardiovascular risk. However, the molecular mechanisms involved are not fully understood. Since atherosclerosis is nowadays considered a low-grade inflammatory disease, recent studies have explored the anti-inflammatory effects of a Med-Diet intervention on serum and cellular biomarkers related to atherosclerosis. In two sub-studies of the PREDIMED (PREvencion con DIeta MEDiterranea) trial, we analyzed the effects at 3 months of two Med-Diet interventions supplemented with either virgin olive oil (VOO) or nuts compared with a control low-fat diet (LFD). Both Med-Diets showed an anti-inflammatory effect reducing serum C-reactive protein, interleukin-6 (IL6) and endothelial and monocytary adhesion molecules and chemokines ($P<0.05$; all), whereas these parameters increased after the LFD intervention ($P<0.05$; all). In another substudy, we evaluated the long-term (1 year) effects of these interventions on vascular risk factors in 516 high-risk subjects, as well as the effect of different Med-Diet components in the reduction of these biomarkers. At 1 year, the Med-Diet groups had significant decreases in the plasma concentrations of IL6, tumor necrosis factor receptor (TNFR) 60 and TNFR80 ($P<0.05$), while intercellular adhesion molecule 1 (ICAM-1), TNFR60 and TNFR80 concentrations increased in the LFD group ($P<0.002$). In addition, those allocated in the highest tertile of VOO and vegetables consumption had a significant diminution of plasma TNFR60 concentration compared with those in tertile 1 ($P<0.02$). In conclusion, Med-Diet exerts an anti-inflammatory effect on cardiovascular system since it down-regulates cellular and circulating inflammatory biomarkers related to atherogenesis in subjects at high cardiovascular risk.

Contents

Abbreviations: BMI, body mass index; CHD, coronary heart disease; CRP, C-reactive protein; HDL, high density lipoprotein; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL6, interleukin-6; iNOS, inducible nitric oxide synthase; LDL, low density lipoprotein; LFD, low fat diet; LPS, lipopolysaccharide; Med-Diet, Mediterranean diet; MMP-9, matrix metalloproteinase-9; MUFA, monounsaturated fatty acids; PGE, prostaglandin E; PUFA, polyunsaturated fatty acids; TNFR, tumor necrosis factor receptor; VCAM-1, vascular adhesion molecule 1; VOO, virgin olive oil.

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1. Introduction

Inflammation is essential in the development of atherosclerosis, the main cause of coronary heart disease (CHD), since appears to play a key role from the inception to the final lesions of this disease [1]. Proinflammatory stimuli activate inflammation inducing the secretion of inflammatory cytokines and generating endothelial adhesion molecules and other chemoattractants. Cytokine production and up-regulation of adhesion molecules on endothelial cells and leukocytes facilitates the recruitment of inflammatory cells from the circulation, their adhesion to endothelium and finally migration to subendothelial space. Subsequent ongoing inflammation is also crucial in the development of instability and rupture of atheroma plaques and the subsequent appearance of ischemic events in advanced stages of the disease [1,2].

CHD is the main cause of death worldwide [3] and is higher in industrialized countries. Western countries, including the US, currently continue to exhibit unacceptably high absolute rates of cardiovascular morbidity and mortality. However, surprisingly, as compared to Northern European countries or the US, there is a low incidence of CHD in countries of Southern Europe, such as France, Spain, Greece and Italy [4,5]. The traditional Mediterranean food pattern has been the factor most frequently involved to explain this health advantage. The exact mechanism of this prevention is not fully understood, but could be caused by the functional compounds of main foods characteristics of Mediterranean diet (Med-Diet). These functional compounds could be phytochemicals such as polyphenols [6,7] or fatty acids [8,9].

In this review, we discuss the effects of the Med-Diet, its components and their functional compounds on inflammatory biomarkers related with atherosclerosis.

2. The PREDIMED study

The PREvencion con Dieta MEDiterranea (PREDIMED) study is a large, parallel-group, multicenter, randomized, controlled, 5-year clinical trial that aims to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org) [10,11]. This trial was registered in the Current Controlled Trials at London, International Standard Randomized Controlled Trial Number, at www.controlled-trials.com, as ISRCTN35739639. Almost 7500 high-risk participants have been recruited for the study. Eligible participants had no documented CHD who either had type 2 diabetes or had at least 3 of the following risk factors: smoking, hypertension (blood pressure ≥140/90 mmHg or treatment with antihypertensive drugs), LDL-cholesterol concentration ≥160 mg/dL (or treatment with hypolipidemic drugs), HDL-cholesterol concentration ≤40 mg/dL, 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. Participants were randomly assigned to 3 intervention groups: 2 Med-Diet groups, one supplemented with virgin olive oil (Med-diet with VOO) and the other supplemented with mixed nuts (Med-Diet with Nuts), and a low-fat diet (LFD) group whose participants received recommendations to reduce all types of fat according to the American Heart Association guidelines [12]. Participants in both Med-Diet groups were recommended to follow a Med-diet pattern through the use of olive oil for cooking and dressings, increase of intake of vegetables (≥2 servings/d), fresh fruit (≥3 servings/d), legumes, nuts and fish or seafood (≥3 servings/wk), reduce consumption of red meats, processed meats or meat products, and a moderate alcohol intake, usually in the form of red wine consumed with meals [10,11,13]. Participants assigned to the Med-Diet with VOO were provided with VOO (1 L/wk) and those assigned to the Med-Diet with Nuts were provided with mixed nuts (30 g/d, as 15 g walnuts, 7.5 g almonds, and 7.5 g hazelnuts). Otherwise, participants in the LFD group only received small non-food gifts. At baseline and at follow-ups, participants completed a validated 14-item questionnaire assessing adherence to the Med-Diet [14], a validated 137-item validated food frequency questionnaire [15], a validated version of the Minnesota Leisure Time Physical Activity Questionnaire for men [16] and women [17] and a 47-item questionnaire about education, lifestyle, history of illnesses, and medication use. The main outcome of the PREDIMED is an aggregate of cardiovascular events (cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke).

3. Anti-inflammatory effects of the Mediterranean diet

In 2006, we analyzed the 3-month effects of these 2 Med-Diet and a LFD interventions on 4 soluble adhesion molecules [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), interleukine-6 (IL6) and C-reactive protein (CRP)] in a pilot study with the first 772 participants recruited in the PREDIMED trial [10]. The plasma concentrations of IL6, VCAM-1 and ICAM-1 significantly decreased after 3 months in the Med-Diet intervention groups while plasma concentrations of CRP only decreased in the Med-Diet with VOO ($P < 0.05$; all). Otherwise, plasma concentrations of VCAM-1 and ICAM-1 increased after 3 months in the LFD group ($P < 0.05$; both) [10].

The anti-inflammatory effects of the Med-Diet have also been tested through the analysis of circulating inflammatory biomarkers and on immune cell activation biomarkers, all related to atherogenesis, in another sub-study of the PREDIMED trial [18,19]. We analyzed the changes in peripheral blood mononuclear cells expression of cell surface inflammatory mediators (adhesion molecules and proinflammatory ligand CD40 expression on T lymphocytes and monocytes) after 3 months of the 3 PREDIMED interventions in 106 participants at high cardiovascular risk (Fig. 1). Both Med-Diet with VOO and Nuts down-regulate the following cellular inflammatory biomarkers related to atherogenesis: CD49d molecule in peripheral T-lymphocytes and CD11b, CD49d (crucial adhesion molecule for leukocyte homing) and CD40 (pro-inflammatory ligand) in monocytes [18]. The study of these mediators had not been previously investigated and we concluded that Med-Diet supplemented with VOO or Nuts modifies the process of firm adhesion of circulating monocytes and lymphocytes T to endothelial cells during inflammation, a crucial step linked to the appearance and development of atherosclerosis [1]. In this sub-study, we also analyzed the effects before and after 3 months of intervention on plasma concentrations of ICAM-1, VCAM-1, IL6, CRP, E-selectin and P-selectin in these 106 participants (Table 1). After the intervention period, ICAM-1 decreased in both Med-Diet groups, whereas VCAM-1, IL6 and CRP only decreased in Med-Diet group supplemented with VOO ($P < 0.05$). Interestingly, plasma concentrations of ICAM-1, VCAM-1 and IL6 increased in the LFD group. After 3 months, plasma concentrations of ICAM-1 and IL6 decreased in both Med-Diet groups and increased in the LFD group. These changes were different between Med-Diet groups and LFD group ($P < 0.02$). Plasma concentrations of VCAM-1 and CRP only decreased in the Med-Diet group with VOO and these changes were significantly different of the LFD group. No differences were observed for E- and P-selectin. According to these data, LFD may exert an inflammatory effect and, therefore, it seems not to be as healthy as it was supposed.

In a third sub-study of the PREDIMED trial, [20] we analyzed the 1-year effects of the 2 Med-Diet supplemented with VOO or Nuts and a control LFD on 4 molecules related to systemic

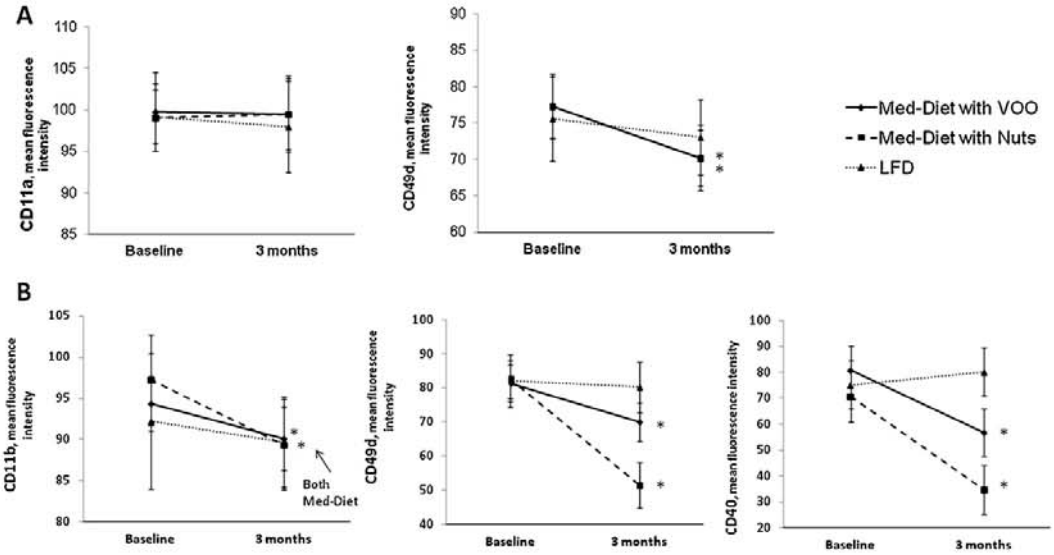

Fig. 1. Changes in adhesion molecules and proinflammatory ligand CD40 expression on T-lymphocytes (A) and monocytes (B). Values are mean ± SD; *different from baseline, $P < 0.05$ (Bonferroni post hoc test).

Data from Estruch [18] and modified from Mena et al. [19].

inflammation (TNFR60, TNFR80, ICAM-1 and IL6) (Fig. 2). The plasma concentrations of IL6, TNFR60 and TNFR80 decreased after 1 year in the Med-Diet intervention groups ($P < 0.05$; all). Otherwise, plasma concentrations of ICAM-1, TNFR60 and TNFR80 increased after 1 year in the LFD group ($P < 0.05$; all). Again, at 1-year plasma concentrations of ICAM-1, IL6, TNFR60 and TNFR80 were greater in the LFD group than in the Med-Diet groups ($P < 0.05$; all) (Fig. 2). To our knowledge, this is the first study in which TNFR concentrations were affected due to changes in the diet. Few studies have analyzed the effect of an intervention with foods with healthy compounds on TNFα receptors. Although the TNFα has an ambivalent role in relation to CHD [21], the activation of TNFR60 could induce expression of adhesion molecules and activates NF-κB, and TNFR80 play a role in T cell proliferation [22].

Several studies in humans have supported evidences that consumption of a Med-Diet pattern and its main components such as VOO and/or nuts provide beneficial anti-inflammatory effects [18,23], which are close related to the prevention of atherosclerosis, the main cause of coronary artery disease [1]. A recent study has demonstrated the association between adherence to

Table 1
Concentrations of circulating inflammatory molecules at baseline and after 3 months of intervention with Med-Diet supplemented with virgin olive oil (Med-Diet with VOO), Med-Diet supplemented with nuts (Med-Diet with Nuts) or LFD in patients at high risk for cardiovascular disease.

	Med-diet with VOO ($n = 35$)	Med-diet with nuts ($n = 35$)	LFD ($n = 36$)	Repeated-measures ANOVA[a]	*P* value for differences[b]		
				Time × treatment	MD-VOO vs MD-nuts	MD-VOO vs LFD	MD-nuts vs LFD
ICAM-1, μg/L							
Baseline	290 ± 104	270 ± 113	239 ± 103				
3 months	212 ± 93b[c]	208 ± 85b[c]	315 ± 148a[c]	<0.001	1.00	<0.001	<0.001
Change	−78 ± 122	−63 ± 118	76 ± 85				
VCAM-1, μg/L							
Baseline	1033 ± 311	962 ± 363	1023 ± 298				
3 months	857 ± 252b[c]	883 ± 329b	1147 ± 318a[c]	0.003	0.85	0.002	0.08
Change	−176 ± 275	−79 ± 440	124 ± 325				
IL6, ng/L							
Baseline	6.8 ± 4.6	6.8 ± 6.0	5.9 ± 5.3				
3 months	5.7 ± 3.7[c]	5.9 ± 5.5	7.3 ± 5.8[c]	0.002	1.00	0.004	0.019
Change	−1.2 ± 2.5	−0.9 ± 0.9	1.4 ± 2.7				
CRP, μg/L							
Baseline	4.0 ± 4.9	2.2 ± 1.9	2.8 ± 2.7				
3 months	2.5 ± 2.4[c]	2.5 ± 3.2	3.9 ± 5.7	0.024	0.20	0.025	1.00
Change	−1.5 ± 4.9	0.3 ± 2.5	1.1 ± 4.1				

Modified from Mena et al. [19].

Values are means ± SD. Means in a row with online letter (a,b) without a common letter differ, $P < 0.05$ (Bonferroni post hoc test).

[a] Data were analyzed by repeated-measures 2-factor ANOVA ($P < 0.05$).

[b] Data were analyzed by ANOVA ($P < 0.05$).

[c] Different from baseline, $P < 0.05$ (Bonferroni post hoc test).

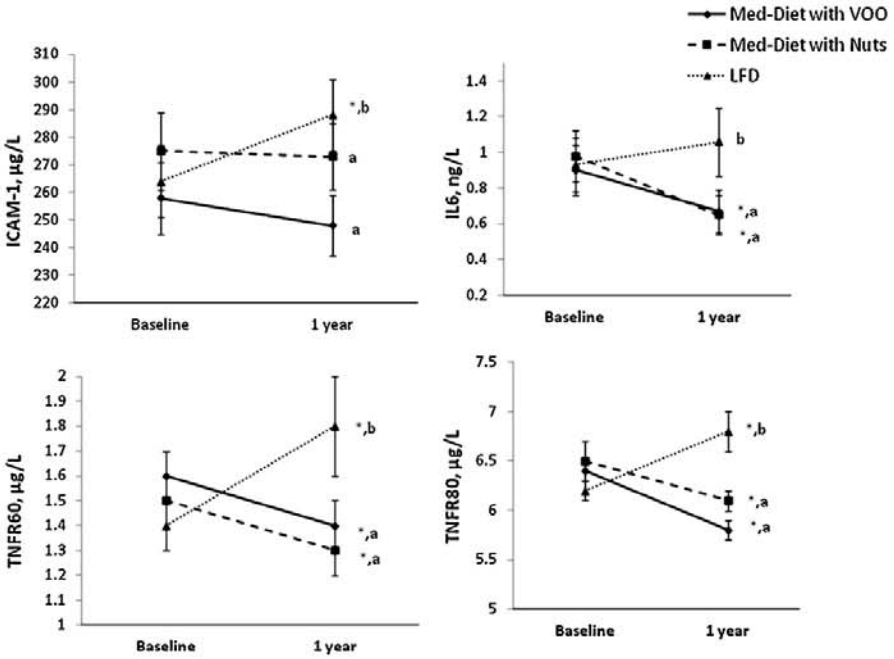

Fig. 2. Concentrations of circulating inflammatory molecules at baseline and after 1 year of intervention with Med-Diet supplemented with VOO, Med-Diet supplemented with nuts or LFD in patients at high risk for cardiovascular disease. Values are geometric means (95% CI). Data were analyzed by repeated-measures 2-factor ANOVA ($P<0.05$). Changes between 1 year and baseline in response to the intervention treatment were analyzed by ANCOVA ($P<0.05$). Repeated measures and ANCOVA were adjusted for age, gender, BMI, smoking status, physical activity and drugs (aspirin and statins). *Different from baseline, $P<0.05$ (Bonferroni post hoc test). Changes in response to the intervention without a common letter differ ($P<0.05$).
Modified from Urpi-Sarda et al. [20].

the Med-Diet and systemic inflammation independent of sharing genetic and environmental factors [24]. Thus, diet is, indeed, one modified factor that could modulate inflammation [25,26].

In 2004, the participants of a population from the Attica area of Greece who were more adhered to the traditional Med-Diet had lower plasma concentrations of CRP, IL6, homocysteine and fibrinogen, as well as a lower white blood cell count and a borderline decrease of TNFα [27]. In addition, a group of patients with metabolic syndrome who followed a Med-Diet pattern showed reduced serum concentrations of CRP, IL6, IL7 and IL18, decreased insulin resistance and improved endothelial function [28]. In the Nurses' Health Study I cohort, different dietary patterns were related with markers of inflammation and endothelial dysfunction (CRP, IL6, ICAM-1 and VCAM-1). A prudent pattern, similar than a Med-Diet pattern, was inversely associated with plasma CRP and E-selectin concentrations, whereas a Western pattern, with higher intake of red meat, sweets, fries and refined grains, was positively associated with CRP, IL6, E-selectin, ICAM-1 and VCAM-1 concentrations [29]. A recent updated meta-analysis has observed that higher adherence to the traditional Med-Diet provides protection against major chronic degenerative diseases (cardiovascular, cancer and neurodegenerative diseases) [30]. Until now, the PREDIMED study is the unique randomized trial that has evaluated the protective effect of a Med-Diet pattern vs a LFD in patients at high risk for cardiovascular disease on cardiovascular disease events and the possible mechanisms involved in this protection. In this study, we observed that those participants who reported a higher adherence to the traditional Med-Diet also showed a significant reduction in classical and novel risk factors, with a significant diminution of cellular and serum inflammatory parameters, compared to those participants with a low adherence top Med-Diet [10,19,20].

The Med-Diet is identified as the traditional dietary pattern found in olive-growing areas of Crete, Greece, and Southern Italy in the late 1950s and early 1960s. Its major characteristics are: (a) a high consumption of whole grains, legumes, nuts, vegetables, and fruits; (b) a relatively high-fat consumption (up to 40% of total energy intake), mostly from monounsaturated fatty acids (MUFA, up to 20% of energy) mainly provided by olive oil, the principal source of culinary and dressing fat; (c) moderate to high fish consumption; (d) poultry and dairy products (usually as yogurt or cheese) consumed in moderate to small amounts; (e) low consumption of red meats, processed meats, and meat products; and (f) moderate alcohol intake, usually in the form of red wine consumed with meals. VOO is one of the main components of the Med-Diet due to its almost exclusive production and consumption in the Mediterranean area. Nuts are also traditionally associated to the Med-Diet and mainly include walnuts (*Juglans regia* L.), almonds (*Prunus dulcis* (Mill.) D.A.Webb) and hazelnuts (*Corylus avellana* L.). Both foods are rich in polyphenols and healthy fatty acids, i.e. MUFA in VOO and polyunsaturated fatty acids (PUFA) in mixed nuts. Most of the beneficial effects of their regular consumption in a Med-Diet pattern may be attributed to these components.

Several studies have demonstrated the anti-inflammatory effects of the consumption of diets rich in these foods. Twenty-eight stable CHD patients receiving 50 mL of VOO and refined olive oil sequentially administered over two periods of 3 weeks showed a significant decrease in plasma IL6 and CRP concentrations after VOO intervention with respect to refined olive oil intervention [31]. The anti-inflammatory effect of VOO and cod liver oil has also been demonstrated after an intervention study where healthy humans received 50 mL of VOO and cod liver oil and their ICAM-1 and TNFα plasma concentrations decreased after 3 h of both interventions [8].

In healthy Tehran women, it was observed that the intake of olive oil also decreased plasma concentrations of TNFα, ICAM-1 and CRP [32]. Three month intervention with a traditional Med-Diet supplemented with VOO decreased the gene expression related with inflammation [INF-gamma, Rho GTPase-activating protein 15, and IL-7 receptor] and oxidative stress [adrenergic beta(2)-receptor] in peripheral blood mononuclear cells [33]. In line with this study, Camargo et al. [34] also reported that the intake of a breakfast enriched in VOO with high content in polyphenols, decreased the expression of NF-κB and COX-2, genes involved in inflammation. Diets supplemented with olive oil or walnuts, rich in MUFA and PUFA, respectively, had a larger diminution in the RNA expression of TNFα messenger than a butter breakfast diet [35]. When compare two Med-Diets in hypercholesterolemic subjects, a regular Med-Diet and the same diet replacing MUFA by walnuts, walnut diet improved endothelium-dependent vasodilation and decreased VCAM-1 concentrations while endothelium-independent vasodilation and concentrations of ICAM-1, CRP, homocysteine, and oxidation biomarkers did not differ between both Med-Diets [26]. In another controlled intervention study where patients with metabolic syndrome were supplemented with mixed nuts (including walnuts, almonds and hazelnuts) during 12 weeks, only the inflammatory biomarker IL6 decreased after the nuts intervention compared with the control diet [36]. In this line, a cross-sectional Multi-Ethnic Study of Atherosclerosis with 6080 participants from USA showed that regular nut and seed consumption was associated with lower levels of inflammatory markers (CRP and IL6) [37].

There are consistent epidemiological evidences to support a cardioprotective effect of nut consumption. In a large Californian cohort, the Adventists Health Study, the frequency of nut intake was inversely associated with CHD incidence [38]. More recently, the results of 3 additional observational studies, the Iowa Women's Health Study, the Nurses' Health Study, and the Physician's Health Study, have confirmed that frequent nut consumption is associated with a lower risk for incident CHD [39–41]. However, the results of the Iowa cohort did not reach statistical significance, and the Physician's Health Study only found protection for sudden cardiac death, but not for non-sudden coronary death or myocardial infarction.

Part of these protective effects has been attributed to their actions on lipid profile. Several small feeding trials (<50 subjects) have shown consistent decreases in total and LDL cholesterol with diets enriched with a variety of nuts in comparison with other healthy diets. Almonds and walnuts have been the nuts most frequently investigated in this regard [42,43]. The hypocholesterolemic effect is achieved with intakes of 1.5–3 servings per day. Effects on HDL cholesterol have been inconsistent. When evaluated, the ratio total/HDL cholesterol was found to decrease [44]. However, more recently, an anti-inflammatory effect of nut intake has been reported. Thus, a walnut diet has been reported to improve endothelium-dependent vasodilation and to reduce VCAM-1 levels in hypercholesterolemic subjects [26]. These results suggest mechanistic explanations for the observed CHD risk reduction associated with nut intake. Some studies have also demonstrated the effects of nuts when they are used in manufactured functional foods. Canales et al. [45] compared the effects of the consumption a functional food created with walnuts (walnut-enriched meat) versus a low-fat meat on adhesion molecules and leukotrienes (LTB4) in patients at increased cardiovascular risk. They observed that patients that consumed walnut-enriched meat had higher levels of paraoxonase activity, lower levels of ICAM-1, VCAM-1 and leukotriene B4 and lower ratios of paraoxonase-1/HDLc and paraoxonase-1/Apo A1 than those with low-fat meat consumption.

In conclusion, follow a Med-Diet pattern together with some of its main components such as VOO and nuts generally reduce the cardiovascular risk factors and down-regulate cellular inflammatory pathways related to atherosclerosis, thus recommendations to follow-up a healthy Med-Diet pattern is adequate in all stages of the disease. The results suggest that learning healthy diets and obtain dietary habits are important keys in the prevention of cardiovascular diseases in any period of life.

4. Relationship between Mediterranean diet key foods and its functional compounds and inflammation

In another sub-study of the PREDIMED trial, we analyzed the relationship between the 1-year change in consumption of 13 foods and 4 plasma inflammatory biomarkers in 516 participants at high cardiovascular risk [20]. We observed a clear relationship between the consumption of VOO and vegetables and the receptor 60 of TNF. Concretely, we observed that participants who increased more than 24 g/d their consumption of VOO after 1 year decreased their plasma concentration of TNFR60 from 1.8 μg/L (baseline levels) to 1.5 μg/L (1-year levels) ($P<0.05$) [20]. In line with this result, participants who increased more than 62.7 g/d their consumption of vegetables after 1 year decreased their plasma concentration of TNFR60 from 1.7 μg/L to 1.5 μg/L ($P<0.05$). We also observed that participants who increased their adherence to the Med-Diet in more than 2.4 points had decreases in plasma concentration of TNFR80 from 6.5 μg/L to 5.9 μg/L ($P<0.05$) [20].

The healthy effects on the prevention of inflammation derived of VOO consumption have been described above. In this section, we analyze the health effects of functional components of VOO and nuts.

The beneficial effects of VOO could be due to their components such as phenolic compounds, α-tocopherol, carotenoids and to the high unsaturated/saturated fatty acid ratio with oleic acid (MUFA) as its main fatty acid [46,47]. The polyphenols in VOO have been described as the main components that attributed the anti-inflammatory properties after VOO consumption and thus contribute to the lower incidence of CHD [48,49]. Up to 36 polyphenols from different groups have been identified and described in VOO [7]: (1) *secoiridoids*: the group with higher quantity of polyphenols in VOO (from 0.8 to 522.2 mg/kg) includes oleuropein aglycone (3,4-DHPEA-EA), oleuropein-aglycone di-aldehyde (3,4-DHPEA-EDA), ligstroside aglycon and deacetoxy-ligstroside aglycone (oleocanthal); (2) *flavonoids*, mainly dihydroflavonols such as taxifolin (up to 129.4 mg/kg) and flavones (apigenin and luteolin); (3) *phenolic alcohols*, mainly tyrosol and hydroxytyrosol (from 0.5 to 14.4 mg/kg); (4) lignans, mainly (+)-1-acetoxypinoresinol and (+)-pinoresinol (from 0.2 to 36.2 mg/kg); (5) *phenolic acids-benzoic acid derivatives*, up to 1.8 mg/kg include protocatechuic, gentisic, and gallic acids, between others; (6) *cinnamic acid derivatives*, up to 0.4 mg/kg, include caffeic, *o*-coumaric and *p*-coumaric acids; (7) *hydroxy-isocromans* include 1-phenyl-6,7-dihydroxy-isochroman and 1-(3′-methoxy-4′-hydroxy) phenyl-6,7-dihydroxy-isochroman. The content of polyphenols in VOO could vary by variety of olive fruits, region of production, agricultural techniques, fruit maturity at harvest and processing methods during extraction [7]. The cardioprotective role and the anti-inflammatory effects attributed to polyphenols of VOO depend on their bioavailability. Several studies have demonstrated the human bioavailability of olive oil polyphenols after VOO consumption through determination of tyrosol and hydroxytyrosol conjugates in plasma and its derived metabolites such as homovanillic acid and vanillin conjugates [50], as well as analyzing urinary excretion of tyrosol and hydroxytyrosol [51]. Recently, a exploratory analysis of human urine using a time-of-flight analyzer has identified more than 60 metabolites of olive oil polyphenols, including mainly those derived from secoiridoids, phenolic alcohols (mainly hydroxytyrosol) and flavonoids, after a single intake of 50 mL of VOO in healthy volunteers [52].

The anti-inflammatory and anti-atherogenic properties of individual VOO polyphenols have been observed in several experimental studies [53–55]. An in vitro study has observed that oleuropein inhibited vascular smooth muscle cell proliferation through a cell cycle block between the G1 and the S phases, which may be regulated by ERK1/2 [56], increased in a dose-dependent manner the nitrite production and the inducible nitric oxide synthase (iNOS) expression in mouse macrophages challenged with lipopolysaccharide (LPS) [54], and inhibited lipoxygenase activity and production of leukotriene B4 [57]. It has also been reported that oleuropein exerts a cardioprotective effect against acute adriamycin cardiotoxicity [58] and exhibit anti-ischemic and hypolipidemic activities [59]. Pretreatment of ischemic hearts with oleuropein significantly reduced the prompt release of oxidized glutathione and prevent membrane lipid peroxidation [60]. Oleuropein and an olive oil extract also prevented the stimulation of matrix metalloproteinase 9 (MMP-9) expression and secretion in TNFα-treated THP-1 cells in a monocyte cell line [61]. The incubations of nutritionally concentrations of oleuropein and hydroxytyrosol with human umbilical vein endothelial cells (HUVEC) for 30 min, followed by co-incubation with bacterial LPS or cytokines to trigger adhesion molecule expression, showed that these polyphenols reduced monocytoid cell adhesion to stimulated endothelium, as well as VCAM-1 mRNA and protein [53]. The effect of a phenolic extract from VOO and separate pure polyphenols from VOO (oleuropein aglycone, hydroxytyrosol and homovanillyl alcohol) were evaluated on cell surface and mRNA expression in HUVEC of 3 crucial adhesion molecules (ICAM-1, VCAM-1 and E-selectin). Oleuropein aglycone and hydroxytyrosol were the responsible compounds for the reduced expression of ICAM-1 and VCAM-1 obtained by VOO phenolic extract. Otherwise, homovanillyl alcohol decreased the expression of the 3 molecules, but the effect on mRNA expression was weaker [62]. Oleocanthal possesses a relatively similar chemical structure than ibuprofen and acts inhibiting the same cyclooxygenase enzymes in the prostaglandin-biosynthesis pathway than this well-known anti-inflammatory drug [63]. In vitro hydroxytyrosol has been reported to attenuate the TNF-α, iNOS, and COX-2 in LPS-induced human monocytic (THP-1) cells [64]. Hydroxytyrosol inhibited the production of nitric oxide and prostaglandin E (PGE) and both hydroxytyrosol and an olive aqueous extract decreased secretion of cytokines (IL1α, IL1β, IL6, TNFα and IL12) and chemokines (CXCL10/IP10 and CCL2/MCP-1), reduced the expression of genes of iNOS, IL1α, CXCL10/IP-10, MIP-1 β, matrix MMP-9, and inhibited PGE synthase in murine macrophages (RAW264.7 cells) [65]. The hydroxy-isocroman 1-phenyl-6,7-dihydroxy-isochroman inhibited in a dose-dependent manner the production of prostanoid and TNF-α in LPS-primed human monocytes in vitro [66].

Otherwise, only few studies have demonstrated the healthy properties of olive oil fatty acids (mainly oleic acid) linked to Med-Diet on inflammatory biomarkers [67]. Oleic acid was able to reduce the inflammatory effects of saturated fatty acids in human aortic endothelial cells by reducing the incorporation of stearic acid into phospholipids and by the reduction of NF-κB activation [67]. In other studies, it reverses the in vitro inhibitory effect of the inflammatory cytokine TNFα on insulin production in the rat pancreatic β cell line INS-1 [9] and decreased membrane expression of VCAM-1 and NF-κB activation in endothelial cells [68].

In summary, to follow a Med-Diet pattern together with the intake of some of its main components (i.e. VOO and nuts), which contained functional compounds (polyphenols) generally reduce the cardiovascular risk factors and down-regulate cellular inflammatory pathways related to atherosclerosis; thus, it seems adequate to recommend a Med-Diet pattern and intake of polyphenol-rich foods are adequate in patients in all stages of atherosclerosis disease. Learning healthy diets and upgrade adherence to the traditional Med-Diet are important keys in the prevention of cardiovascular diseases in all periods of life.

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Moderate consumption of red wine, but not gin, decreases erythrocyte superoxide dismutase activity: A randomised cross-over trial☆

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KEYWORDS
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Abstract *Background and Aims:* Several studies have shown that moderate alcohol consumption reduces the risk of coronary heart disease, a disease related to oxidative stress. However, the effects of different alcoholic beverages on antioxidant status are not fully known. Our aim was therefore to compare the effects of a moderate intake of an alcoholic beverage with high polyphenol content (red wine) and another without polyphenol content (gin) on plasma antioxidant vitamins, lipid profile and oxidability of low-density lipoprotein (LDL) particles.
Methods and results: Forty healthy men (mean age, 38 years) were included in a randomised cross-over trial. After a 15-day washout period, subjects received 30 g/ethanol/d as either wine or gin for 28 days. Diet and exercise were monitored. Before and after each intervention, we measured serum vitamins, malondialdehyde (MDA), superoxide dismutase (SOD) and

Abbreviations: CHD, Coronary heart disease; Mg, Magnesium; MDA, Malondialdehyde; oxLDL, Oxidized low-density lipoproteins; Se, Selenium; SOD, Superoxide dismutase.

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glutathione peroxidase activities, lipid profile, oxidized LDL and LDL resistance to *ex-vivo* oxidative stress. Compared to gin intervention, wine intake reduced plasma SOD activity [−8.1 U/gHb (95% confidence interval, CI, −138 to −25; $P = 0.009$)] and MDA levels [−11.9 nmol/L (CI, −21.4 to−2.5; $P = 0.020$)]. Lag phase time of LDL oxidation analysis also increased 11.0 min (CI, 1.2−20.8; $P = 0.032$) after wine, compared to gin, whereas no differences were observed between the two interventions in oxidation rate of LDL particles. Peroxide concentration in LDL particles also decreased after wine [−0.18 nmol/mL (CI, −0.3 to−0.08;$P = 0.020$)], as did plasma oxidized LDL concentrations [−11.0 U/L (CI,−17.3 to −6.1; $P = 0.009$)].

Conclusion: Compared to gin, red wine intake has greater antioxidant effects, probably due to its high polyphenolic content.

Introduction

Several studies have pointed out that moderate alcohol consumption reduces the incidence of coronary heart disease (CHD) [1−3]. Although beer [4] and spirits [5] may have protective effects on the cardiovascular system, wine intake seems to have additional benefits [1], due to its specific constituents, mainly polyphenols [6,7]. Thus, the role of the different alcoholic beverages in cardiovascular protection remains open.

On the other hand, studies on the mechanisms underlying the effects of alcohol on the cardiovascular system have been focused on lipid metabolism and the haemostatic profile [8−10]. However, other mechanisms have been proposed. Current evidence indicates that oxidative damage promotes pathophysiological changes occurring in oxidative stress-associated diseases, such as CHD [11]. Oxidized low-density lipoproteins (oxLDL) may play a major role in atherosclerosis [12]. However, few randomised controlled studies have assessed the efficacy of red wine on *in vivo* LDL oxidation [8,9].

We embarked, therefore, upon a randomised cross-over trial to evaluate the effects on plasma antioxidant vitamins, antioxidant status, lipid profile and oxidizability of LDL particles, of moderate intake of an alcoholic beverage with high polyphenolic content (red wine) compared to an alcoholic beverage without polyphenolic content (gin).

Methods

Study population

Healthy adult men aged 30−50 years were eligible if they reported an average daily ethanol intake between 10 and 40 g and had no cardiovascular risk factors or were receiving any medication or multivitamin or vitamin E supplements. All volunteers were healthcare staff of the Hospital Clinic of Barcelona.

Study design

An open, prospective, randomised, cross-over, clinical trial was performed. During the first 15 days, no intervention was undertaken except diet monitoring (*first washout period*). During the next 28 days, the subjects underwent the *first intervention*, according to the group they had been assigned to. Following the first intervention, the *second* 15-day *washout period* was performed. Finally, the last 28 days, they underwent the *second intervention* (Fig. 1). All volunteers received 30 g of ethanol/day either as red wine (two glasses of 160 mL/day during dinner) or gin (100 mL/day during dinner). The subjects were randomly divided into two groups using a computer-generated random-number table. Half received wine as the first intervention and gin as the second intervention, while the other half received first the gin and lastly the red wine.

Diet and exercise monitoring, clinical examination, and laboratory analyses, including plasma oxidant assessment, serum lipid levels and low-density lipoprotein (LDL) susceptibility to oxidation were performed. Other studies on the anti-inflammatory effects of red wine and gin have been published [13].

The protocol was compiled according to the Helsinki Declaration and approved by the Hospital Clinic Institutional Review Board.

Diet and exercise monitoring

Both groups followed an iso-caloric diet, being provided with detailed dietary information. Strict control of antioxidant products in the diet, especially fruit and vegetables, was performed. The diet and exercise performed were monitored before and after each intervention period. Diet monitoring was performed using a validated 3-day recall questionnaire of food consumption [14], that was converted into nutritional data using the Professional Diet Balancer software (Cardinal Health Systems, Inc., Edina, MN). Exercise monitoring was performed using the Minnesota Leisure Time Physical Activity questionnaire [15]. Weekly phone contacts ensured adherence to the protocol. Intervention compliance was assessed at the end of each intervention period by counting the empty bottles and measuring plasma polyphenolic concentrations.

Red wine and gin

The wine used was obtained from Merlot grapes. The alcoholic strength was 12.5° and contained 3381.06 ± 89.68 mEq Gallic Acid/L of polyphenolic compounds. It also contained gallic acid <66 mg/L, protocatechuic acid <10 mg/L, tyrosol <39 mg/L, catechin <157 mg/L, (-)epicatechin-gallate <4 mg/L, (-)epicatechin <0.09 mg/L, *trans*-caftaric <20 mg/L, *trans*-caffeic <13 mg/L, *trans*-coutaric <7 mg/L, 2-S-

Figure 1 Design of the clinical trial. An open, prospective, randomised, cross-over, clinical trial was performed. During the first 15 days of the study, the subjects included did not undergo any intervention except monitoring of the diet (*first washout period*). During the next month (28 days), they underwent the *first intervention*, according to the group assigned. At the end of the first intervention, 15 days followed without any intervention, except monitoring of the diet (*second washout period*). Finally, during the last 28 days, they underwent the *second intervention* with diet monitoring. The subjects included ($n = 40$) were randomly divided into two groups using a computer-generated random-number table. Half received wine as the first intervention and gin as the second intervention, and the remaining received gin as the first intervention and red wine as the second intervention.

glutationylcaftaric <11 mg/L, quercetin-3-glucuronide <14 mg/L, quercetin <28 mg/L, isorhamnetin <4 mg/L, cyanidin 3-glucoside <0.15 mg/L, delphinidin 3-glucoside <0.15 mg/L, petunidin-3-glucoside <14 mg/L, peonidin-3-glucoside <10 mg/L, malvidin-3-glucoside <0.15 mg/L, malvidin-(6-acetyl)-3-glucoside <13 mg/L, malvidin-(6-coumaroyl)-3-glucoside <5 mg/L, malvidin-(6-coumaroyl)-3-glucoside <5 mg/L, *trans*-resveratrol <2 mg/L, *cis*-resveratrol <3 mg/L, *trans*-piceid <10 mg/L, *cis*-piceid <4 mg/L, anthocyanins 624 mg/L and tannins 2.93 g/L. The phenolic content of several distilled alcoholic beverages was analysed and the alcoholic beverage selected was gin since the amount of phenols was not detectable.

Clinical and nutritional measurements

At baseline, a medical record, including ethanol intake, smoking and dietary habits, was obtained using a structured questionnaire. Blood pressure was measured using a semi-automatic oscillometric device (Lohmeier B606, München, Germany). Height, body weight, tricipital skin thickness fold and arm perimeter were also measured. The body mass index and lean body mass were calculated, as were the muscular and fatty area of the arm. Protein nutritional status was assessed using laboratory analysis.

Other blood parameters were evaluated, including haematocrit, haemoglobin, transferrin, glucose, creatinine, electrolytes, uric acid, and serum aspartate and alanine aminotransferases. Red cell and leukocyte counts and serum protein electrophoresis were determined. Platelet count, prothrombin time, and serum fibrinogen were measured. Serum levels of folic acid and vitamins B_1, B_6, and B_{12} were determined.

Lipid analyses, oxidant assessment and polyphenol determinations

Blood samples were obtained after overnight fasting, and serum and EDTA plasma were collected. Serum lipoprotein and antioxidant analysis included total cholesterol (C), triglycerides, high-density lipoprotein (HDL)-C, LDL-C, very low density lipoprotein-cholesterol (VLDL-C), apolipoprotein A-I, apolipoprotein B, lipoprotein(a), β-carotenes and vitamins A, C and E. In addition, erythrocyte superoxide dismutase (SOD) [16] and glutathione peroxidase activities [17] were determined, as a measure of antioxidant activity of hydrosoluble systems. Plasma malondialdehyde (MDA) concentration was determined by HPLC with a photodiode-array detector (HPLC-DAD) [18], as a marker of lipid peroxidation status.

LDL were separated from plasma by discontinuous density-gradient ultracentrifugation and dialysed against phosphate buffered saline (PBS) at 4 °C for 3 days (without EDTA). Fifty microgram of LDL protein were incubated in the presence of 5 μmol/L of copper sulphate at 37 °C and the following parameters were measured: (i) α-tocopherol content of LDL particles; (ii) MDA content; and (iii) LDL oxidation kinetic parameters (lag time, oxidation rate, maximal formation of conjugated dienes) [19]. Oxidized LDL was determined by ELISA using the monoclonal 4E6 antibody (Mercodia AB, Uppsala, Sweden).

For intervention compliance, plasma concentrations of catechin and its metabolites were determined before and after each intervention by HPLC-DAD, monitored at a wavelength range of 190—700 nm[20].

All serum measurements were performed in duplicate. All parameters were analysed before and after each intervention period (wine and gin intake).

Statistical analysis

Standard statistical methods from the SPSS Statistical Analysis System 15.0 (SPSS, Chicago, IL, USA) were used. The paired two-tailed *t*-test was used to compare changes in outcome variables in response to each intervention period and carryover effects for the two-period cross-over design. To exclude the presence of a carryover effect for the two periods, comparison of the outcome variables observed before the wine and gin periods was performed. We also compared the differences in the parameters obtained from the group starting with wine with those obtained from those starting with gin. The paired two-tailed *t*-test was also used to compare differences in the effects of each intervention. $P < 0.05$ was considered significant.

Results

Subject characteristics

Of the 66 eligible subjects, 24 were excluded before randomisation for the reasons shown in Fig. 2. Forty-two

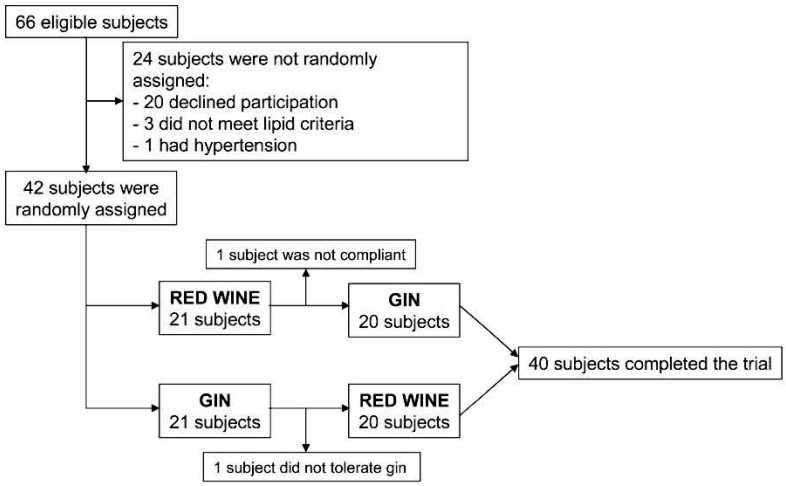

Figure 2 Flow chart of the subjects included in the study. Of the 66 eligible subjects, 24 were excluded from the study before randomisation. Forty-two healthy men who did not fulfil any of the exclusion criteria were included in the study and were randomly assigned to one of the two interventions. Two subjects withdrew before completing the two phases of the study.

healthy men (mean age 37.6 ± 7.4 years, range 30–50) were included in the study and randomly assigned to one of the two interventions. Their reported daily intake of ethanol was 23.4 ± 15.1 g over a period of 18.8 ± 8.5 years. Eleven (26%) had smoked one pack of cigarettes a day since the second decade of their lives and additional 11 subjects were ex-smokers. Two subjects withdrew before completing the two phases of the study. The baseline characteristics of these subjects were similar to the mean values of the overall group. Subsequent data refer only to the 40 subjects who completed both phases of the study.

Diet, exercise, nutritional status and polyphenol determinations

No significant differences were observed between nutrient intake before and after each intervention or in the daily average energy expended in physical activity by the subjects during wine and gin intake periods.

Protocol adherence was optimum in the 40 subjects who completed both phases and complete agreement was observed between the reports of the participants and the number of empty bottles. In addition, the nutrient content of the self-reported diets was close to that of the planned diets. No subject was non-compliant and only two reported a one-day violation of onion eating 15 and 21 days before assessment.

As another measure of intervention compliance, epicathechin-gallate (ECG)—a marker of polyphenol constituents—was determined in plasma drawn after overnight fasting, around 10 h after the last wine or gin ingestion in 29 cases. Plasma ECG levels increased from 0.178 ± 0.043 mg/L to 0.479 ± 0.399 mg/L after wine ($P = 0.001$), but did not change after gin intake (0.173 ± 0.093 to 0.188 ± 0.077 mg/L).

Body weight, blood pressure and clotting parameters

Body weight and anthropometric parameters remained unchanged throughout the study. Moreover, no significant changes were observed in any of the laboratory nutritional parameters evaluated.

Mean systolic blood pressure decreased significantly from 121 ± 16 mm Hg to 118 ± 15 mm Hg ($P = 0.023$) after gin whereas it did not change after wine intake (119 ± 16 vs. 120 ± 16 mm Hg). However, the differences in the effects of both interventions did not achieve statistical significance ($P = 0.100$). No changes were observed in diastolic blood pressure or heart rate after gin or wine intake.

Plasma fibrinogen levels decreased by 8% after gin (2.67 ± 0.71 to 2.45 ± 0.56 g/dL; $P = 0.048$) and by 10% after wine (2.59 ± 0.62 to 2.34 ± 0.59; $P = 0.001$). Prothrombin time increased by 3% after wine ($P = 0.003$), but did not change after gin intake. No differences were observed in platelet count before or after either intervention.

Serum lipids and lipoproteins

Table 1 shows the changes in serum lipids. No carryover effect was seen between the two periods. The two interventions did not differ with respect to their effects on levels of total cholesterol, triglycerides, VLDL-C and LDL-C. However, plasma apolipoprotein B-LDL concentration decreased by 3% after gin and increased by 2% after wine intake. Consequently, the differences between the effects of both interventions reached statistical significance ($P = 0.02$). On the other hand, HDL-C increased by 7% after both gin and wine intake ($P < 0.001$; both). Apolipoprotein A1 increased after both interventions, but only achieved

Table 1 Serum lipid and lipoprotein levels before and after each intervention ($n = 40$).

	Spirit		Red wine		Mean (95% CI) Differences
	Before (A)	After (B)	Before(C)	After(D)	
Cholesterol (mg/dL)	198 ± 36	204 ± 36	201 ± 38	205 ± 37	−1.1 (−10.4 to 8.3)
Triglycerides (mg/dL)	106 ± 46	120 ± 76	104 ± 40	109 ± 50	−7.4 (−30.4 to 15.6)
cVLDL (mg/dL)	16.4 ± 10.7	21.7 ± 14.8	16.8 ± 10.2	17.8 ± 11.7	−3.3 (−8.3 to 1.7)
cLDL (mg/dL)	123 ± 33	121 ± 33	123 ± 32	125 ± 34	4.9 (−1.3 to 11.1)
Apo B − LDL (mg/dL)	96.4 ± 23.4	93.6 ± 26.3	96.6 ± 26.8	98.5 ± 26.0$^{\infty}$	14.4 (2.5 to 26.3)
cHDL (mg/dL)	54.6 ± 13.6	58.5 ± 15.7*	53.9 ± 14.0	57.9 ± 14.1*	−0.44 (−3.3 to 2.4)
Apo AI − HDL (mg/dL)	145 ± 26	150 ± 26	146 ± 29	155 ± 28$^{\#\infty}$	10.4 (1.7 to 19.1)
Cholesterol/HDL ratio	3.82 ± 1.05	3.69 ± 1.07	3.91 ± 1.07	3.72 ± 1.12$^{\#}$	−0.02 (−0.3 to 0.2)
LDL/HDL ratio	2.37 ± 0.88	2.17 ± 0.78*	2.42 ± 0.89	2.29 ± 0.93$^{\#}$	−0.25 (−0.06 to 0.5)
Lipoprotein (a) (mg/dL)	17.2 ± 17.1	16.4 ± 15.5	16.6 ± 16.1	13.6 ± 13.0$^{\#}$	−2.2 (−8.2 to 3.9)

95% CI = 95% confidence intervals of the differences between the effects of red wine versus spirit. *$P = 0.001$ and $^{\#}P \leq 0.02$, compared to before intervention. $^{\infty}P \leq 0.020$, in the comparison of the effects of both interventions.

statistical significance after wine ($P = 0.012$). Again, the differences between the effects of the two interventions reached statistical significance ($P = 0.021$) (Table 1). The mean ratio total cholesterol to HDL-C only decreased by 3% after gin ($P = 0.111$), but by 5% after wine ($P = 0.015$). The mean ratio of LDL-C to HDL-C decreased by 8% after gin ($P = 0.001$) and by 5% after wine ($P = 0.015$), although these differences did not achieve statistical significance. Lipoprotein(a) levels decreased by 22% (4.4 mg/dL; $P = 0.016$) after wine and by 5% (0.7 mg/dL; $P = 0.100$) after gin.

Oxidant status and LDL oxidation

After wine, plasma MDA levels decreased by 9% (−4.1 nmol/L; $P = 0.020$), with no changes after gin (Table 2). Therefore, the differences between the effects of the two interventions on MDA levels were highly significant in favour of wine ($P = 0.016$). SOD activity decreased by 7% ($P = 0.041$) after wine, but increased by 3% ($P = 0.387$) after gin, presenting statistically significant differences ($P = 0.007$). No significant changes were observed in glutathione peroxidase activity after both interventions.

Plasma vitamins E and B1 increased by 8% ($P < 0.020$; both) after wine, but did not significantly change after gin. The differences between wine and gin plasma vitamin B1 levels achieved statistical significance ($P = 0.009$). No differences were observed in the plasma concentrations of β-carotenes and vitamins A, B_{12} and C after both interventions. Serum folate concentration decreased by 11% and 9% after gin and red wine intake, respectively, but only the effect of gin on this nutrient was statistically significant ($P = 0.011$).

Table 2 shows the results of lipid oxidation analysis. Lag phase time increased by 21% (9.1 min; $P = 0.001$) after wine and by 9% (4.4 min; $P = 0.200$) after gin, with these differences achieving statistical significance ($P = 0.03$). These changes were accompanied by a decrease of 23% and 16% in the LDL oxidation rate after gin and wine, respectively ($P < 0.02$; both) and a decrease of 5% after wine ($P = 0.151$) and 9% after gin ($P = 0.020$) in the amount of conjugated dienes formed.

According to the changes in plasma ECG concentration after wine, the subjects were divided into two groups, 18 cases with high absorption of polyphenols (difference between ECG concentration before and after wine period ≥0.1 mg/L) and 11 with low absorption (difference <0.1 mg/L). Interestingly, the subjects with high ECG absorption showed a significantly longer lag phase time of LDL oxidation than those with low absorption (45 ± 49 min vs. 40 ± 39 min, respectively; $P = 0.045$). In addition, a weak but significant correlation was observed between the lag phase time of LDL oxidation and the increase in ECG concentration after wine ($r = 0.401$; $P = 0.034$).

MDA concentration in LDL particles decreased by 29% (0.07 nmol/mL; $P = 0.02$) after wine, increasing by 9% (0.02 nmol/L; $P = 0.317$) after gin, with the differences in the effects of both interventions on LDL peroxidation being significant ($P = 0.014$). However, no significant changes were observed in the vitamin E content of LDL particles after both periods. Finally, circulating oxLDL decreased by 13% ($P = 0.024$) after wine and increased by 7% ($P = 0.066$) after gin, with these differences between the two interventions also achieving statistical significance ($P < 0.01$).

Discussion

In this trial in 40 healthy men, we observed that, compared to gin, consumption of 30 g of ethanol a day as red wine reduced SOD activity and MDA levels. Lag phase time of LDL oxidation was significantly longer after wine than after gin, and peroxide concentration in LDL particles decreased after wine as did plasma oxidized LDL concentration. These data indicate that red wine, an alcoholic beverage with high polyphenolic content, has higher additional antioxidant effects than gin, a beverage with no polyphenolic content.

The cardioprotective effect of moderate consumption of alcoholic beverages has been documented in several epidemiological studies [1–3,21,22]. However, even in prospective cohort studies assessment of alcohol consumed and control of important confounding factors, such as diet and exercise are difficult. Indeed, some

Table 2 Serum malondialdehyde, antioxidant parameters, and results related to low-density lipoprotein (LDL) oxidation analysis ($n = 40$).

	Spirit		Red wine		Mean (95%CI) Differences
	Before	After	Before	After	
Blood analysis					
Malondialdehyde (nmol/L)	49.7 ± 17.4	48.5 ± 16.1	45.2 ± 12.5	41.1 ± 16^∞	−11.9 (−21.4 to−2.5)
Glutation peroxidase (U/gHb)	2.25 ± 0.63	2.30 ± 0.54	2.36 ± 0.55	2.42 ± 0.77	−0.32 (−0.70 to 0.03)
Superoxide dismutase (U/gHb)	1111 ± 161	1147 ± 142	1146 ± 211	1070 ± 109^&#	−81 (−138− −25)
β- carotene (mg/dL)	0.29 ± 0.18	0.28 ± 0.18	0.27 ± 0.12	0.27 ± 0.13	0.02 (−0.03 to 0.07)
Vitamin C (mg/dL)	49.4 ± 15	46.7 ± 12.7	48.7 ± 16.7	46.7 ± 13.8	2.2 (−4.5 to 9)
Vitamin E (mg/dL)	29.5 ± 5.9	30.6 ± 6.6	27.2 ± 5.1	29.4 ± 6.3^+	1.9 (−0.9−4.7)
Vitamin B1 (mg/dL)	54.4 ± 10.4	54.6 ± 9.4	55.75 ± 10.5	58.4 ± 10.1^#	5.5 (1.5−9.5)
Folate (mg/L)	5.9 ± 2.15	5.26 ± 1.7^+	6.4 ± 2.3	5.8 ± 2.4	0.19 (−0.9 to 1.3)
LDL Analysis					
Lag phase time (min)	42.3 ± 9.9	46.7 ± 11.4	35 ± 11.8	44.1 ± 8*$	11.0 (1.2 to 20.8)
Ox. rate (μmol CD/g LDL/min)	35.0 ± 11.4	26.9 ± 9.7^+	34.3 ± 11.9	29.3 ± 8.3^	3.4 (−5.6 to 12.4)
Cmax (μmol CD/g LDL)	960 ± 184	877 ± 185^	928 ± 153	880 ± 153	−26.0 (−156 to 104)
LDL peroxides (nmol/ml)	0.23 ± 0.23	0.25 ± 0.21	0.24 ± 0.20	0.17 ± 0.16^∞	−0.18(−0.3 to−0.01)
LDL vit E (μmol/mmol chol)	0.032 ± 0.016	0.043 ± 0056	0.037 ± 0.036	0.044 ± 0.069	−0.001(−0.01to 0.01)
Oxidized LDL (U/L)	55.3 ± 23.43	59.31 ± 19.55	60.26 ± 19.18	52.61 ± 26.5^#	−11.7(−17.3 to−6.1)

95%CI = 95% confidence intervals of the differences between the effects of red wine versus spirit. Ox. rate: Oxidation rate. Cmax: Total amount of conjugated dienes. LDL vit. E = Concentration of Vitamin E in LDL particles.*$P = 0.001$, &$P = 0.041$, +$P = 0.011$ and ^$P \leq 0.020$ compared to before intervention; #$P = 0.009$, ∞$P < 0.020$ and $$P = 0.032$, in the comparison of the effects of both interventions.

researchers have suggested that the lower mortality of moderate drinkers may be partly due to the consumption of a healthier diet [8]. All fruit and vegetables contain high amounts of antioxidant compounds, possibly explaining the lower rates in CHD mortality observed in Mediterranean countries [23]. This issue may only be solved by the performance of well-designed clinical trials. However, even the design of the current study presented difficulties to ensure compliance which was partially offset by detailed dietary instructions and weekly diet recalls. In fact, the dietary data obtained demonstrated that compliance was very good, and plasma ECG determinations confirmed this impression.

Oxidative modification of LDL is possibly obligatory in the pathogenesis of atherosclerosis. Oxidation of LDL lipids leads to changes in the conformation of the lipoproteins that facilitate their uptake by macrophages in the arterial wall, thereby promoting the atherosclerotic process [24]. However, the clinical relevance of lipoprotein oxidation remains under debate. Despite results of epidemiological studies showing an inverse association between intake of dietary antioxidants and cardiovascular risk, clinical trials with antioxidant vitamin supplements have shown no benefit [25], thereby questioning the role of the oxidant/antioxidant imbalance in atherogenesis. The antioxidants present in fruits and vegetables in a food matrix might be more beneficial than large doses of a single antioxidant given for a finite period of time. In fact, the latter might deplete endogenous antioxidant pools, thus turning an antioxidant effect into a pro-oxidant one *in vivo*, as shown for vitamin E [26].

The decrease in plasma MDA concentration observed in the current trial suggests that the whole process of lipid peroxidation is diminished by regular wine drinking. In addition, we observed a significant reduction in SOD activity after wine. Previous studies have reported an increase in SOD activity after the intake of antioxidant-rich foods [27]. However, our results differ and a decrease in SOD activity was observed after red wine intake. In fact, high ethanol consumption seems to increase SOD activity, a fact attributed to the pro-oxidant effects of ethanol [28], whereas wine consumption provides antioxidant benefits in humans [29]. The reduction observed in SOD activity may indicate that the body saves energy and diminishes enzyme activities when not necessary.

Consumption of wine also reduces the propensity of LDL to undergo peroxidation [9,30]. In the current study, the oxidation rate of LDL particles decreased after both interventions. Thus, ethanol itself may have some effect on the oxidation of LDL particles. However, the antioxidant capacity of red wine (ethanol plus polyphenols) seems to be significantly higher than that of gin (ethanol) and this cannot be related to changes in vitamin E, since no difference was observed in its content in LDL particles before or after wine intake. The weak but significant correlation observed between changes in EPG concentration and the lag phase time of LDL oxidation suggests that part of the antioxidant effect of red wine may be attributable to its polyphenolic content. On the other hand, there is accumulating evidence showing that circulating oxLDL levels may have an added value versus classical lipid risk factors [31].

Lipoprotein(a), a LDL particle containing protein(a), is another independent risk factor for developing CHD [32]. Although inheritance plays an important role in the plasma concentrations of lipoprotein(a), there is evidence that dietary factors may modify their levels [32]. The results of a population-based study [33] and a clinical trial [34] have

indicated that moderate alcohol consumption lowers plasma lipoprotein(a) concentration. Our data confirm these previous results and provide another possible mechanism for the beneficial effect of alcoholic beverages on CHD.

Finally, a link between the "oxidation" and "inflammatory" hypothesis of atherosclerosis has been suggested since oxidized LDL particles may induce the expression of endothelial adhesion molecules. Our results confirm the link between these two theories since wine triggers a significant reduction in the expression of monocyte adhesion molecules [13,35].

In summary, the results of the current study confirm that ethanol itself may exert significant beneficial effects on the cardiovascular system, mainly by increasing HDL-C and decreasing oxidation of LDL. However, red wine provides additional benefits due to its higher antioxidant effects, by decreasing MDA, SOD and oxidized LDL plasma levels. Our data provide strong biological plausibility to the epidemiological observations that moderate wine drinking reduces cardiovascular events. However, it should be taken into account that, although moderate wine intake reduces the risk of CHD, even low alcohol consumption in women may increase the risk of certain cancers, especially breast cancer [36].

Conflict of interest

The authors have no conflict of interest.

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Research Article

^{1}H-NMR-based metabolomic analysis of the effect of moderate wine consumption on subjects with cardiovascular risk factors

Moderate wine consumption is associated with health-promoting activities. An H-NMR-based metabolomic approach was used to identify urinary metabolomic differences of moderate wine intake in the setting of a prospective, randomized, crossover, and controlled trial. Sixty-one male volunteers with high cardiovascular risk factors followed three dietary interventions (28 days): dealcoholized red wine (RWD) (272mL/day, polyphenol control), alcoholized red wine (RWA) (272mL/day) and gin (GIN) (100mL/day, alcohol control). After each period, 24-h urine samples were collected and analyzed by ^{1}H-NMR. According to the results of a one-way ANOVA, significant markers were grouped in four categories: alcohol-related markers (ethanol); gin-related markers; wine-related markers; and gut microbiota markers (hippurate and 4-hydroxphenylacetic acid). Wine metabolites were classified into two groups; first, metabolites of food metabolome: tartrate (RWA and RWD), ethanol, and mannitol (RWA); and second, biomarkers that relates to endogenous modifications after wine consumption, comprising branched-chain amino acid (BCAA) metabolite (3-methyl-oxovalerate). Additionally, a possible interaction between alcohol and gut-related biomarkers has been identified. To our knowledge, this is the first time that this approach has been applied in a nutritional intervention with red wine. The results show the capacity of this approach to obtain a comprehensive metabolome picture including food metabolome and endogenous biomarkers of moderate wine intake.

Keywords:
Biomarkers / Metabolomics / Nuclear Magnetic Resonance / Urinary profile / Wine intake
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1 Introduction

Cardiovascular disease (CVD) is one of the main causes of mortality in western countries. Diet and lifestyle are recognized as the major modifiable risk factors in CVD [1]. The low incidence of coronary heart disease (CHD) in Mediterranean countries has been partly ascribed to the dietary habits of their inhabitants [2, 3]. As one of the main components of Mediterranean diet, wine and its constituents, especially polyphenols, may provide additional health-promoting benefits [4–6]. Results of several clinical and epidemiological studies have showed the protective effect of a moderate wine consumption against CVD [7], particularly with regards to oxidative stress [8, 9], inflammation, and vascular function [10–12], the main causes behind the development of CVD [13]. However, there are discrepancies on the effects of the different types of alcoholic drinks (wine, beer, and liquors) on the cardiovascular system and whether the possible protective mechanisms of alcoholic beverages are due to their alcoholic component (ethanol), their nonalcoholic constituents, mainly polyphenols, or both [14, 15]. Therefore, new biomarkers of wine and alcohol consumption are needed in order to increase the knowledge on the effects of these beverages on the cardiovascular system and to clarify their protective mechanisms of CVD.

Foodomics is considered a new discipline that studies food and nutrition fields through advanced omics technology application: genomic, transcriptomic, proteomic, and /or metabolomic [16] using sensitive methods (NMR,

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Abbreviations: δ, chemical shift; **BCAA**, brached-chain amino acids; **CHD**, coronary heart disease; **CVD**, cardiovascular disease; **d**, doublet; **m**, multiplet; **q**, quadruplet; **RWA**, red wine alcoholized; **RWD**, dealcoholized red wine; **s**, singlet; **t**, triplet

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Colour Online: See the article online to view Figs. 1 and 3 in colour.

GC-MS, LC-MS, CE) to detect and quantify changes in dietary patterns [17]. ^{1}H-NMR-based metabolomics is a very robust technique for metabolomic applications enabling the simultaneous detection of a wide range of structurally different metabolites [18], and it can facilitate the discovery of new candidates for biomarkers of disease risk [19–21]. Similarly, NMR-based metabolomics can provide information, in combination with dietary intake, about the development of different diseases, as published by Vinaixa et al., who assessed the effect of dietary cholesterol on the progressive development of fatty liver disease [22]. As far as red wine polyphenol intake is concerned, van Dorsten et al. used a dry mix of red wine and red grape juice extracts to assess the metabolic fate of red wine polyphenols in humans by GC-MS and NMR metabolomics [23]. Despite this use of extracts from red wine, as far we know, no reports on metabolomic studies using wine (as a beverage) in a nutritional intervention with either animals or humans are available.

As a result, the aim of the present study is to apply, for the first time to our knowledge, an ^{1}H-NMR-based metabolomic strategy in order to investigate the effect of wine intake on the human metabolome in a urinary profile, identifying the most relevant markers of consumption and the endogenous effect of this beverage on human volunteers.

2 Material and methods

2.1 Subjects and study design

A total of 61 high-risk subjects aged ≥55 years without documented CHD (CHD: ischemic heart disease—angina/recent or past myocardial infarction/previous or cerebral vascular accident, peripheral vascular disease) were recruited for the study. The subjects included had diabetes mellitus or more than three of the following CHD risk factors: tobacco smoking, hypertension, hypercholesterolemia, plasma LDL cholesterol ≥160 mg/dL, plasma HDL cholesterol <40 mg/dL, obesity (BMI (in kg/m^2) ≥30), and/or a family history of premature CHD (first-line male relatives <55 years or females <65 years). Participants had to voluntarily give signed informed consent. Subjects with a previous history of CVD, any severe chronic disease, alcoholism, or other toxic abuse were excluded. The study was a prospective, randomized, crossover, and controlled trial. As a result of random computer-generated selection, participants were asked to take either, 272mL/day of red wine (hereafter, RWA) (about 30g ethanol/day), 272mL/day of dealcoholized red wine (hereafter, RWD, polyphenols control), or 100mL/day of gin (hereafter, GIN, alcohol control), every day for 4 weeks (28 days). Twenty-four-hour urine samples were collected on the last day of each period as well as the basal time. In order to evaluate the metabolic profiles, urine samples were analyzed by ^{1}H-NMR after each intake. All participants received all three interventions. Subjects had forbidden other alcoholic beverages 15 days before the first intervention and during the study, also asked not to change their dietary pattern during the study. A 7-day food record questionnaire was validated in our population [24] monitoring nutrient intake at the beginning of the study and after each intervention, also a medical record and Minnesota Leisure Time Physical Activity Questionnaire validated in Spain [25] were performed. The wine used for the interventions (RWA and RWD) was elaborated with Merlot grape variety. The daily dose of alcohol was the same in both the RWA and GIN periods and the polyphenol composition of the wine is detailed in Table 1 [26]. The total phenolic content of the three beverages was determined by the Folin–Ciocalteu method [27], the phenolic profile of RWA and RWD and resveratrol and piceid contents were measured by using HPLC-diode-array detection [28], [29].

2.2 Sample preparation

The urine samples were thawed, vortexed, and centrifuged at 13 200 rpm for 5 min. The supernatant (600 μL) from each urine sample was mixed with an internal standard solution (120 μL, consisting of 0.1% TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN_3, bacteriostatic agent), and 1.5 M KH_2PO_4 in 99% deuterium water (D_2O)). The optimized pH of the buffer was set at 7.0, with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical shifts of the NMR resonances. This mixture was transferred to a 5-mm NMR tube.

2.3 ^{1}H-NMR data acquisition and processing

The ^{1}H-NMR spectra were acquired on a Varian-Inova-500 MHz NMR Spectrometer with presaturation of the water resonance using a NOESYPRESAT pulse sequence. During the acquisition, the internal temperature was kept constant at 298 K. An exponential window function was applied to the free induction decay (FID) with a line-broadening factor of 0.3 Hz prior to the Fourier transformation. For each sample, FIDs were collected into 32 K data points (128 scans) with a spectral width of 14 ppm, an acquisition time of 2 s, relaxation delay of 5 s, and a mixing time of 100 ms. NMR spectra were phased, baseline corrected, and calibrated (TSP, 0.0 ppm) using TopSpin software (version 3.0, Bruker, BioSpin, Germany). After baseline correction, two different approaches were subsequently used to summarize spectral data. On the one hand, original spectral data were bucketed in equal-size domains of 0.005 ppm [30–32] using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Toronto, Canada). On the other hand, intensities of each 1H-NMR region conveniently identified in the urine 1D-NMR spectra were integrated for each sample entering the study (profiling integration) using the AMIX 3.8 software package (Bruker). In both cases, the spectral region between 4.68 and 5.08 ppm was excluded from the dataset to avoid spectral interference from residual water.

Table 1. Phenolic composition of beverages used in the study

	RWA	RWD	GIN
Alcohol (%)	14.2	0.42	38
Total phenols (mEq GA/L)	2933.35 ± 377.31	2694.92 ± 86.79	ND
Gallic acid (mg/L)	68.48 ± 6.40	73.17 ± 7.01	ND
Protocatechuic acid (mg/L)	5.22 ± 0.62	5.85 ± 0.51	ND
Tyrosol (mg/L)	43.59 ± 4.73	47.81 ± 3.90	ND
Catechin (mg/L)	123.51 ± 11.30	126.45 ± 13.35	ND
Epicatechin (mg/L)	67.86 ± 7.74	70.57 ± 8.22	ND
trans-Caftaric (mg/L)	18.62 ± 1.45	19.21 ± 1.62	ND
trans-Caffeic (mg/L)	11.50 ± 0.79	12.18 ± 0.92	ND
trans-Coutaric (mg/L)	5.21 ± 0.45	5.62 ± 0.52	ND
2-S-glutationylcaftaric (mg/L)	10.30 ± 1.00	10.76 ± 1.26	ND
Quercetin-3-glucuronide (mg/L)	11.88 ± 1.38	11.25 ± 1.42	ND
Quercetin (mg/L)	26.66 ± 0.78	23.82 ± 2.37	ND
Isorhamnetin (mg/L)	3.34 ± 0.27	2.96 ± 0.14	ND
Delphinidin-3-glucoside (mg/L)	15.25 ± 0.89	14.71 ± 1.62	ND
Petunidin-3-glucoside (mg/L)	12.29 ± 1.06	12.04 ± 1.15	ND
Peonidin-3-glucoside (mg/L)	6.78 ± 0.62	6.68 ± 0.57	ND
Malvidin-3-glucoside (mg/L)	48.83 ± 4.45	49.86 ± 4.27	ND
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.97 ± 0.96	10.41 ± 1.20	ND
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	4.15 ± 0.27	3.54 ± 0.33	ND
trans-Resveratrol (mg/L)	2.92 ± 0.36	2.73 ± 0.23	ND
cis-Resveratrol (mg/L)	2.79 ± 0.15	2.75 ± 0.15	ND
trans-Piceid (mg/L)	9.41 ± 1.12	10.53 ± 0.96	ND
cis-Piceid (mg/L)	7.71 ± 0.34	7.08 ± 0.87	ND

RWA, RWD, and GIN (nonsignificant differences were found for any of the phenolic compounds between RWA and RWD (student's *t*-test for independent samples). Mean ± SD.

2.4 Statistical analysis

Datasets derived from the two above-mentioned integrations were submitted to MetaboAnalyst, a web-based platform for comprehensive analysis of metabolomic data [33]. The two different matrices were row-wise normalized (rows were samples) by the sum of the intensities of the spectra [34] and column-wise normalized (columns were metabolites) using Pareto scaling [21] prior to being analyzed by ANOVA test. Fisher's LSD test for multiple comparisons was applied as a post hoc test; a $p < 0.05$ was considered to indicate statistical significance. In addition, we performed a correlation test (Pearson's correlation) [33] for verification when two or more signals came from the same metabolite. Correlation values close to 1 indicate a strong correlation, while values lower than 0.5 indicate a weak correlation, and values very close to 0 indicate no correlation.

2.5 Metabolite identification

Metabolite identification was performed according to Chenomx NMR Suite 7.0 profiler (Chenomx Edmonton, Canada) by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (HMDB; http://www.hmdb.ca), the Biological Magnetic Resonance Data Bank (BMRB, http://www.bmrb.wisc.edu), and the Madison Metabolomics Consortium Database (MMCD, www.mmcd.nmrfam.wisc.edu), along with the existing NMR-based metabolomics literature [35–37].

3 Results and discussions

The total phenolic content of the three beverages used in this study, the phenolic profile of RWA and RWD, and resveratrol and piceid content are presented in Table 1. ^{1}H- NMR resonance assignments with chemical shifts and multiplicity of the signals elucidated in ^{1}H- NMR spectra of urine of the participants are shown in Table 2. Results performed by multivariate techniques were not consistent and difficult to interpret so we choose ANOVA test to analyze selected NMR signals to determine statistical differences between interventions of the individuals [38, 39]. ANOVA test is effective determining whether a given set of NMR signals contains interesting information. The significant bins returned by the ANOVA test ($p < 0.05$) for both techniques are shown in Table 3. Results showed that significant bins from the two above-mentioned integrations were in consonance. The box plots with the Fisher's LSD test for multiple comparisons are shown in Fig. 1. Additionally, in order to present an overview of the whole representative spectrum, the significant metabolites of each spectrum in basal and the three dietary interventions are shown (Fig. 2). Identified markers were grouped in

Table 2. ^{1}H-NMR resonance assignments with chemical shifts for signals identified in samples of human urine

Code	Metabolite	δ (H^1 shift) ppm	Multiplicity
1	Acetate	1.93	s
2	Acetoacetate	2.27	s
3	Acetone	2.24	s
4	cis-Aconitate	3.11; 5.75	s; s
5	Acetylcarnitine	2.15	s
6	Alanine	1.49	d
7	Betaine	3.27	s
8	Carnitine	3.23	s
9	Citrate	2.68 + 2.55	d + d
10	Creatine	3.94, 3.04	s; s
11	Creatinine	3.06; 4.06	s; s
12	Dimethylamine (DMA)	2.72	s
13	Ethanol	1.19; 3.69	t; q
14	Formate	8.46	s
15	Fucose	5.20	d
16	Glycine	3.57	s
17	Glycylproline	3.94	s
18	Glucose	3.50; 4.66; 5.25	m; d; d
19	Hippurate	3.98; 7.57; 7.64; 7.84	d; tt; t; dd
20	Histidine	7.08	s
21	2-Hydroxyisobutyrate	1.36	s
22	3-Hydroxyisovalerate	1.26	s
23	3-Hydroxymandelate	6.82	s
24	4-Hydroxyphenylacetate	6.87	d
25	Indole-3-acetate	7.51	d
26	Lactate	1.33	d
27	Leucine	0.96	t
28	Lysine	1.73	m
29	Malonate	3.12	s
30	Mannitol	3.69; 3.77; 3.80; 3.87	dd; m; d; dd
31	n-Methylhistidine	7.09	s
32	1-Methylnicotinamide	4.47	s
33	Methylsuccinate	1.07	d
34	3-Methyl-2-oxovalerate	1.10	d
35	N-N Dimethylglycine (DMG)	2.93	s
36	Tartrate	4.35	s
37	N-Phenylacetylglycine (PAG)	7.35; 7.43	m; m
38	Succinate	2.41	s
39	Taurine	3.43; 3.26	t; t
40	Trigonelline	4.43; 8.08; 8.85; 9.13	s; m; m; s
41	Trimethylamine (TMA)	2.89	s
42	Trimethylamine-N-oxide (TMAO)	3.27	s
43	Threonine	1.33	d
44	Tyrosine	6.90	d
45	Urea	5.75–5.90	m
46	Valine	0.98; 1.05	d; d

s: singlet; d: doublet; dd: double doublet; t: triplet; tt: double triplet; q: quadruplet; m: multiplet.

four categories, namely: alcohol-related markers; gin-related markers; wine-related markers; gut microbiota markers.

3.1 Alcohol-related markers

A significant presence of ethanol (δ1.19 (t), and δ3.69 (q)) was detected in the urine of participants after RWA and GIN consumption, which suggests that this presence may correspond to the ethanol from the beverage (both gin and red wine). In fact, ethanol has been found in wine by NMR [40]. With regard to human samples, ethanol has been identified in the urine of healthy Caucasian volunteers who, after 2 days of abstinence from alcoholic beverages, consumed sake and rice wine [41]. The presence of ethanol in urine was recently used as an indicator of noncompliance of dietary protocol

Table 3. Metabolites detected after the three treatments

Compound	^{1}H-NMR Chemical shift (ppm)		After RWA intake	After RWD intake	After GIN intake	*p*-value		Metabolites information
	0.005 ppm integration	Profiling integration				0.005 ppm integration	Profiling integration	
Tartrate	4.35	4.35	↑	↑	–	4.81×10^{-9}	1.0147×10^{-7}	Wine compound
Mannitol	3.77	3.69–3.87	↑	–	–	3.68×10^{-2}	3.43×10^{-2}	Wine alcoholized compound
	3.87					4.69×10^{-2}		
Ethanol	1.19	1.19	↑	–	↑	1.43×10^{-4}	3.0324×10^{-4}	Alcohol from beverage
	3.69					8.60×10^{-4}		
3-Methyl-2-oxovalerate	1.10	1.10	↑	↑	–	1.63×10^{-5}	3.48×10^{-2}	Valine, leucine, and isoleucine degradation
4-Hydroxyphenylacetate	6.87	6.87	↑	↑	–	1.89×10^{-2}	4.31×10^{-2}	Phenylalanine and tyrosine metabolism Gut microbiota
Hippurate	7.84	7.84	–	↑	–	6.56×10^{-4}	1.26×10^{-2}	Phenylalanine metabolism
	3.98	3.98				2.59×10^{-2}	1.49×10^{-2}	Gut microbiota
	7.64	7.64				2.58×10^{-3}	3.05×10^{-2}	
	7.57	7.57				1.04×10^{-2}	3.29×10^{-2}	

-: no changes.
p-values of each metabolite ($p < 0.05$; ANOVA).

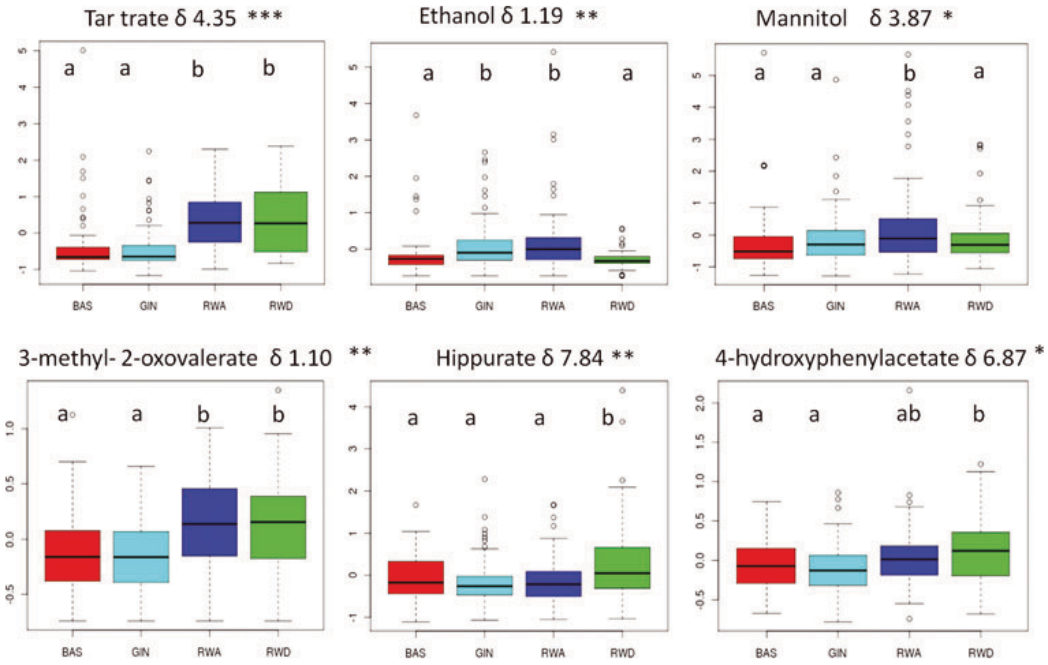

Figure 1. Box plots of the intensities of significant metabolites. ***$p < 0.00001$; **$p < 0.005$; *$p < 0.05$. Different letters indicate significant differences between interventions. BAS (basal time); RWA (red wine), RWD (dealcoholized red wine), and GIN (Gin).

in a metabolomic experiment in which alcohol intake was forbidden [42]. In our case, we view the presence of ethanol in the interventions that has the alcoholic component in the dietary protocol (RWA and GIN), concluding that the identified ethanol could be considered as a biomarker of the overall compliance of dietary interventions.

3.2 Gin-related markers

Two correlating signals (82.13 (s) and 5.69 (s), Fig. 3A) were excreted in statistically higher concentrations after GIN intake. These two signals are also present in some volunteers in basal time and after a RWA or RWD period, without a

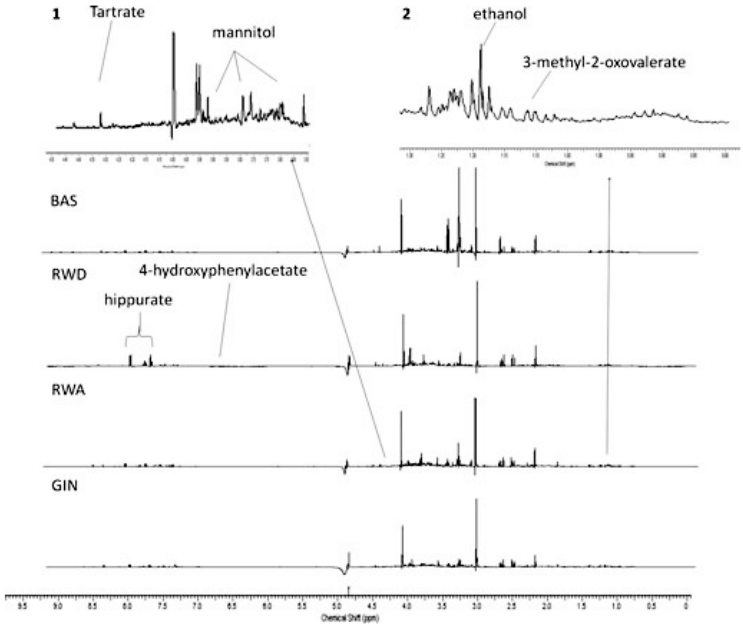

Figure 2. Representative 500-MHz ^{1}H-NMR region of spectra in basal time (BAS), RWD (dealcoholized red wine); RWA (red wine), GIN (gin). The figure exhibits the significant metabolites in ANOVA test. Zoomed regions are performed. Spectral area zoomed between 3.55 and 4.50 ppm (1); 0.80–1.30 ppm interval area (2).

clear pattern in terms of volunteers and interventions followed. This behavior indicates either possible gin compound or an endogenous compound because not all gin volunteers presented these peaks in their urinary metabolome. Querying to the NMR database failed to confirm the assignation of these signals. However, the δ2.13 peak could suggest the presence of acetylcholine or acetylcarnitine, but this hypothesis could not be confirmed by comparing the sample with a urine sample spiked with commercial standards, so it has been rejected. Therefore, we have not been able to identify these two correlated signals and they have been termed as "unknown 1."

3.3 Wine-related markers

This group comprises those markers related to the intake of red wine (RWA and/or RWD). With regards to these two classes of wine (RWA and RWD) intake, a chemical shift at 4.35 ppm (s), corresponding to tartrate, was detected. The box plot in Fig. 1 shows the significant differences between the wine, basal, and gin periods, with no differences between wines. Tartaric acid is the major acid in grapes and so it is also present in wine [40, 43]. Lord et al. tested the frequent ingestion of grape juice (28 mL) in a human population producing urinary tartrate concentrations >300 g/mg creatinine [44]. Dietary sources strongly influence the concentration of urinary tartrate, and its production by intestinal yeast or bacteria is insignificant because the majority of tartaric acid is destroyed by microbial action [45, 46], indicating that the urinary tartrate in our samples was provided by the wine composition. In addition, Yamashita et al. demonstrated that tartaric acid has the beneficial effect of enhancing the bioavailability of wine polyphenols [47].

In this context, an endogenous product of the degradation of branched-chain amino acids (BCAA) termed

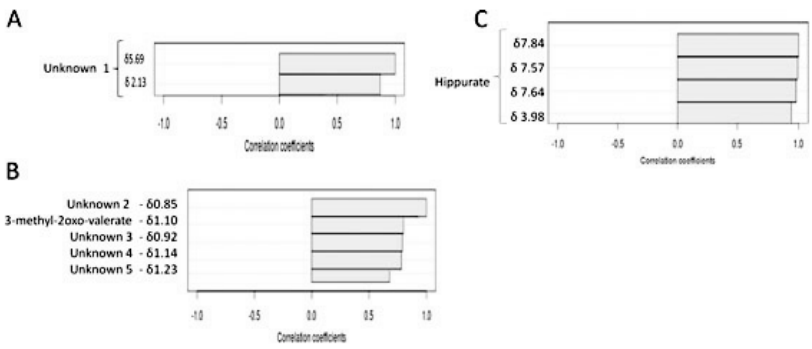

Figure 3. Correlation plot from integration bucketing corresponding to signals of unknown 1 (A); Aliphatic region metabolites, 0.84–0.86: unknown 2; 1.09–1.11: methyl-2-oxovalerate; 0.90–0.92: unknown 3; 1.13–1.15: unknown 4; 1.23–1.24: unknown 5 (B); and Hippurate (C). Correlation coefficient >0.5, for all the figures. The compounds are represented as horizontal bars. Light pink color indicates positive correlations.

3-methyl-2-oxovalerate (δ1.10, d) was identified in urines after the intake of RWA and RWD (Fig. 1, Table 2). This identification was confirmed by spiking urine samples with the commercial standard. According to the results obtained, Connor et al. found an increase in urinary levels of the BCAA valine and its metabolite 3-methyl-2-oxovalerate in diabetic mice [48]. Moreover, it was discovered that cardiovascular subjects such as diabetic [48, 49] and obese subjects [50, 51] have elevated plasma levels of BCAAs [52], because of the reduction in BCAA catabolism. The first step in the catabolism of BCAAs is a reversible step and is carried out in the brain and several nonhepatic tissues by branched-chain aminotransferase (BCAT) to convert BCAAs into branched-chain alpha ketoacids (BCKAs) such as 3-methyl-2-oxovalerate. Our results show an increase in 3-methyl-2-oxovalerate in urine as a result of the first step of BCAA catabolism, suggesting a possible upregulation of this pathway carried out by BCAT enzyme after RWA and RWD intake.

In the aliphatic region comprising 0.85–1.24 ppm, where 3-methyl-2-oxovalerate is also present, there were statistical differences between wine intake (RWA and RWD) and basal or gin treatment. An increase in several signals in this region (see nomenclature in Fig. 3B: unknown 2: δ0.85 (s), unknown 3: δ0.92 (s), unknown 4: δ1.15 (d); unknown 5: δ1.24 (d+d)) was detected after wine consumption, with a good correlation between them and with 3-methyl-2-oxovalerate (Fig. 3B). The identification of these unassigned signals (overall the 0.80–1.00 ppm region) was hampered by their low signal intensities, a broadband signal area, and the absence of other resonance patterns.

With regard to RWA intake, mannitol (δ3.78, dd) showed a significant increase in the sugar region. Mannitol is a polyol produced by heterofermentative lactic acid bacteria from the reduction of fructose [53] and is present in wine. Liu et al. tested mannitol concentration in different wines and fruit juices, finding higher values in Cabernet Sauvignon, a red wine from the same family as Merlot (the wine used in this study) [54], than in sweet white, medium-dry white, or Pinot noir wine. The presence of this polyol in urine samples after RWA intake could be explained by the fact that it is largely eliminated from the body before any metabolism can take place [55], and the absence after RWD intake suggests a possible elimination of this compound during the process of dealcoholization.

3.4 Gut microbiota markers

In samples from the RWD intake period, the intensity signals of hippurate (δ3.98 (d), δ7.57 (tt), δ 7.64 (t), δ7.84 (dd), Fig. 3C) increased in comparison with GIN and basal time. An interesting significant difference was observed between RWA and RWD (Fig. 1 and Table 3). Hippurate is a metabolite belonging to the phenylalanine and tyrosine degradation pathways and it has also been related to polyphenol microbiota catabolism. There have been several studies in rats and humans showing an increase in hippurate excretion after wine polyphenol consumption [56, 57]. Another gut microbiota metabolite, 4-hydroxyphenylacetate (δ6.87), showed an effect of wine intake, with no significant differences between RWD and RWA; however, it did show significantly higher levels after RWD intake compared to the GIN and basal period. This metabolite has been identified in urine and faeces [32, 56] after consumption of polyphenolic compounds in wine [57, 58], it could explain no differences in these results between wines (RWD and RWA) in the current results. In addition, this metabolite is also related to tyrosine metabolism. An interesting result was that no significant differences between the alcohol-containing diets (RWA and GIN) were detected (Fig. 1, Table 3).

The two markers related to gut microbiota showed a similar behavior (Fig. 1); however, slight differences were observed. The similarities were found in relation to the influence of alcohol intake where for both metabolites, RWA and GIN showed no significant difference. This fact suggests a possible impact of ethanol on the metabolic pathways related to the production of both compounds, probably modifying the relationship between microbiota and human metabolism. A recent study conducted by Gao et al. [59] compared urine from Wistar and Spraguel-Dawley rats after ethanol administration. The authors demonstrated a different excretion pattern in some metabolites affected by alcohol intake. Concretely, hippurate showed less excretion after ethanol administration, and in another hand, excretion of 4-hydroxyphenylacetate increased with alcohol administration. With regard to differences in the present results, while hippurate content was significantly different when comparing RWA and RWD, the amount of 4-hydroxyphenylacetic acid did not differ significantly between wine diets (RWA and RWD). This behavior could be explained because 4-hydroxyphenylacetate is a compound increased with wine consumption, counteracting with the effect of alcohol administration. As a result, no differences between wines (RWD and RWA) (Fig. 1, Table 2) and between alcohol-containing beverages (RWA and GIN) were observed. Hippurate could arise from different sources, such as diet (mainly polyphenols), oxidative stress, and intestinal microbiota [20]. In contrast, in the case of 4-hydroxyphenylacetic acid, this behavior (no differences between wine diets) suggests that an important part of this compound should be associated with wine intake from the intervention.

4 Concluding remarks

The results of the current work show the capability of an NMR-based metabolomic approach to detect significant changes in metabolites after moderate wine consumption. Wine-related biomarkers may be classified into two groups. The first group comprises those metabolites coming from the metabolism of food components (food metabolome), where mannitol is related to an RWA diet, and tartrate is a biomarker of wine intake (RWA and RWD). These results support the notion that this compound (tartrate) could be considered to be a possible marker of wine intake. The second group

comprises those markers related to endogenous modifications after wine consumption: BCAA metabolites and other signals in the same spectral area. In spite of this, more about BCAAs and wine intake needs to be known in order to understand the connection between the catabolic pathway of BCAA and moderate wine intake.

Despite these biomarkers, ethanol was a robust biomarker of alcohol consumption as it was related to GIN and RWA diets. In fact, combining tartrate and ethanol, we observed a global compliance of dietary intervention, important factor in metabolomics studies that other authors also has been investigated [60]. Finally, the gut microbiota metabolites, 4-hydroxyphenylacetate, and hippurate, showed a particular effect in combination with alcohol, providing new insights into the assimilation of polyphenol metabolites.

One of the main limitations of our study is that there we have no washout periods between interventions. Washout periods between interventions would have extended the study 6 weeks more, which would have made it difficult to ensure compliance and any increase in study length may increase participant dropout rate [61]. Previous studies carried out at the Hospital Clinic of Barcelona confirmed that changes in cellular and endothelial adhesion molecules due to the intervention were already observed after 15 days of treatment [62,63], and no carryover effect was observed, the absence of a washout period would probably not have changed the results. Therefore, the results observed at the end of the 4-week period could be attributed to the intervention and should only be compared with those observed at the end of the other interventions.

In conclusion, to our knowledge, this is the first time that this approach has been applied in a nutritional intervention with red wine. The results showed that H-NMR-based metabolomics is a powerful strategy for obtaining biomarkers in nutritional intervention studies. In fact, the results obtained generate new perspectives on understanding the relationship between moderate wine intake and human health.

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The authors have declared no conflict of interest.

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Cocoa consumption reduces NF-κB activation in peripheral blood mononuclear cells in humans

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Matrix effect

Abstract *Background and aims:* Epidemiological studies have demonstrated an association between high-polyphenol intake and reduced incidence of atherosclerosis. The healthy effects of cocoa-polyphenols may be due to their antioxidant and anti-inflammatory actions, although the exact mechanisms are unknown and depend on the matrix in which cocoa-polyphenols are delivered. Nuclear factor κB (NF-κB) is a key molecule in the pathophysiology of atherosclerosis involved in the regulation of adhesion molecules(AM) and cytokine expression and its activation is the first step in triggering the inflammatory process. The aim of this study was to evaluate the effect of acute cocoa consumption in different matrices related to the bioavailability of cocoa-polyphenols in NF-κB activation and the expression of AM.
Methods and results: Eighteen healthy volunteers randomly received 3 interventions: 40g of cocoa powder with milk (CM), with water (CW), and only milk (M). NF-κB activation in leukocytes and AM (sICAM, sVCAM, E-selectin) were measured before and 6h after each intervention. Consumption of CW significantly decreased NF-κB activation compared to baseline and to CM ($P < 0.05$, both), did not change after CM intervention, and significantly increased after M intervention ($P = 0.014$). sICAM-1 concentrations significantly decreased after 6h of CW and CM interventions ($P \leq 0.026$; both) and E-selectin only decreased after CW intervention ($P = 0.028$). No significant changes were observed in sVCAM-1 concentrations.

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Conclusions: The anti-inflammatory effect of cocoa intake may depend on the bioavailability of bioactive compounds and may be mediated at least in part by the modulation of NF-κB activation and downstream molecules reinforcing the link between cocoa intake and health.

Introduction

Several studies have shown that consumption of cocoa, a polyphenol-rich food [1], is related to a better health status because of its cardio preventive, anticarcinogen and neuro-preventive effects [2,3]. However, the exact mechanism by which cocoa intake produces these effects on health is not fully understood. Cardiovascular protection by flavonoid-rich diets has mainly been attributed to non-inflammatory mechanisms [4], such as vasodilatation [5], reduction in plasma cholesterol concentrations [6], insulin resistance [7] and blood pressure [8], as well as modulation of platelet function [9]. Some studies have also suggested anti-inflammatory mechanisms of polyphenol-rich diets through inhibition of proinflammatory molecules such as CRP, IL-6 and TNF-α [10]. A human clinical trial recently showed that regular cocoa consumption significantly decreased the expression of VLA-4 (very late antigen-4), CD40 and CD36 on monocyte surface and also decreased serum concentrations of endothelium-derived adhesion molecules such as P-selectin and ICAM-1 (intercellular adhesion molecule-1) [11]. In addition, *in vitro* studies have suggested that cocoa procyanidins and phenolic metabolites can also modify intracellular transduction pathways and thereby modulate the synthesis of inflammatory cytokines such as IL-1β and IL-2 [12] or even IL-6 [13].

The nuclear factor κB (NF-κB) has a central role in the development of inflammatory response because it is a crucial and regulating factor for adhesion molecules and cytokine expression [14]. In the last years NF-κB has been implicated in the pathophysiology of disorders such as atherosclerosis and cancer which have a significant impact on human health. This factor exists as an inactive form in the cytoplasm of the cells [15]. After stimulation, NF-κB dissociates from its inhibitory protein and translocates to the nucleus where it modulates the transcription of the inflammatory cytokines and adhesion molecules [15].

Thus, hypothetically, modulation of NF-κB activation could be a target to reduce inflammatory response and in this way decrease the injury to the cells which may be one of the first events in the development of certain disorders such as atherosclerosis, cancer or degenerative diseases [14]. In fact, some studies have demonstrated that acute intake of polyphenol-rich foods such as olive oil [16] and red wine [17] induce a reduction in NF-κB activation. However, no studies have evaluated the acute effect of cocoa on this pathway.

To date, several studies have evaluated the effect of cocoa intake on inflammatory markers (VCAM, ICAM), albeit with conflicting results [18–20]. This may be due to the different amount of polyphenols in cocoa products and to the effect of the different matrices in which the polyphenols are delivered [21]. In this sense, it is known that milk may reduce the bioavailability of cocoa-polyphenols [22,23] but the extent of the clinical effects is unknown.

Spain is the largest consumer of cocoa powder [reports by ACNielsen, Euromonitor International, and Caobisco Association of the Chocolate Biscuit and Confectionery Industries of the European Union (EU)] representing ~28% of the total cocoa consumption in this country [24], and cocoa powder is the main source of flavonoids in the young population, being mainly consumed with milk (Family-Food-Panel, Spain 2005–2006, Taylor Nelson Sofres).

Therefore, the aim of this study was to evaluate the effects of a single dose of cocoa consumed with water or milk on NF-κB activation in peripheral blood mononuclear cells (PBMC) and on the expression of downstream inflammatory molecules such as intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in healthy subjects.

Methods

Subjects and study design

Eighteen healthy volunteers (9 men and 9 women, aged 19–49 years) were included in this clinical trial. None reported any of the following exclusion criteria: diabetes mellitus, tobacco smoking, hypertension, LDL-c levels > 160 mg/dL, HDL-c levels <35 mg/dL, coronary heart disease (CHD), family history of premature CHD, cerebrovascular diseases, peripheral vascular diseases, human immunodeficiency virus infection, alcoholic liver disease, malnutrition, neoplastic or acute infection diseases. In addition, none were receiving any medication or vitamin supplements. Before inclusion in the trial, all the participants provided informed consent for the different procedures and none received any economic compensation. The Institutional Review Board of the Hospital Clinic approved the study protocol. This trial was registered in the Current Controlled Trials at London, International Standard Randomized Controlled Trial Number, at controlled-trials.com as ISRCTN75176807.

This study was an open, prospective, randomized, crossover, clinical trial. The participants followed a cocoa washout period of 7 days before each intervention and were instructed to abstain from alcoholic beverages and any polyphenol-rich foods for 48 h before and during the intervention days. Prior to the study a list of the allowed and forbidden foods was given to all the participants to ensure that the polyphenol free diet was strictly followed. Volunteers fasted overnight for at least 12 h before beginning the assessment period. All subjects performed the three interventions in a random order: i) 40g of cocoa powder with 250 mL of whole milk (CM), ii) 40 g of cocoa powder with 250 mL of water (CW), and iii) 250 mL of whole milk (M) as a control. The three interventions were performed during 3 consecutive weeks at 8:00 a.m. During the

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days of the study the volunteers remained in the experimental clinical ward to ensure that they only consumed the diet prescribed in this study.

Cocoa powder composition

Table 1 details the cocoa powder phenolic content used in this trial [23]. The intervention beverages were always prepared following a standardized protocol to assess that caloric intake and macronutrient composition were the same. Sugar was added to M and CW preparations in ensure that the caloric content was the same as that of CM. The CM and CW macronutrient composition (in 250 mL) were 30.75 and 58.40 g for carbohydrates, 10.91 and 2.16 g for fat, 13.54 and 5.64 g for proteins and for energy 275.35 and 276.60 kcal, respectively, as described previously [23,25]. The M macronutrient composition (in 250 mL) was 42.75 g for carbohydrates, 7.75 g for fat, 18.11g for proteins and 271.37 Kcal.

Clinical and laboratory measurements

Blood samples were obtained from all the volunteers before cocoa consumption (0 h or baseline) and 6 h after each intervention. The samples were coded with random numbers and processed immediately to perform lipid profile and immunological assays of PBMC. Blood lipid analysis, cholesterol and triglycerides were measured using enzymatic procedures and HDL cholesterol was quantified after precipitation with phosphotungstic acid and magnesium chloride. Samples were analyzed in duplicate. Serum was obtained after blood centrifugation and was immediately frozen at −80 °C until analysis.

In addition, phase II metabolites of epicatechin (ie, epicatechin–glucuronide and three epicatechin sulfates) [25] were measured in 2 h plasma and 0–6 h urine fraction samples and microbial metabolites of epicatechin (i.e. phenolic acids) [23] were measured in 0–6 h urine fraction samples by liquid chromatography tandem mass spectrometry

Table 1 Composition of the soluble cocoa powder (per 40 g) used in the study.

Nutrient	Mean value (40 g)
Carbohydrates	23 g
Fiber	6.4 g
Protein	5.6 g
Fat	2.2 g
Flavanols[a]	
(−)-Epicatechin	28.2 mg
(+)-Catechin	8.4 mg
Procyanidin B_2	25.5 mg
Flavonols[a]	
isoquercitrin	1.35 mg
quercetin	0.23 mg
quercetin-3-glucuronide	0.17 mg
quercetin-3-arabinoside	1.45 mg

[a] Measured by HPLC following the methodology of Andres-Lacueva et al., 2008 [24].

evaluating the bioavailability of cocoa-polyphenols and as biochemical markers of compliance [23,25].

Total protein isolation of PBMC

PBMC were isolated from fresh blood samples by Ficoll-Hypaque (Pharmacia, Uppsala, Sw) density gradient [26]. Total protein from PBMC was isolated using the Tripure™ isolation reagent (Roche Molecular Biochemicals) following the manufacturer's instructions. The quantification of total protein concentration from PBMC samples was carried out using the bicinchoninic acid protein assay (Pierce, Rockland, IL).

Determination of NF-κB activation by Western blot

An equal amount of proteins (20 μg) was loaded and separated into 10% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (Invitrogen, CA, USA). The blots were blocked with 5% non-fat dry milk 1 h and incubated overnight at 4 °C with a monoclonal antibody against p65 phosphorylated (P-p65) on serine 536 (Cell Signaling Technology Inc, Beverly, MA). To verify equal protein loading, the blots were reincubated with a monoclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody concentrations were used as indicated by the manufacturer's instructions. Levels of P-p65 and β-actin expression were visualized by treating the blots with a quimioluminiscent detection kit (Pierce, Rockland, IL) that enhanced the signal. The intensity of the signal was quantified with the Image-Gauge Software. Protein expression of NF-κB was assessed with the P-p65/β-actin ratio (P-p65 amount was normalized by actin content) in arbitrary units.

Determination of adhesion molecules by ELISA

Serum concentrations of soluble endothelial adhesion molecules sICAM-1, sVCAM-1 and sE-selectin were measured in duplicate by ELISA using commercial immunoassays for the quantitative detection of soluble human molecules (Bender MedSystems, Vienna, Au). Intra- and inter-assay variation coefficients of the methodology ranged from 3.1% to 5.4% for sICAM-1 and sVCAM-1 and from 5.2% to 7.6% for sE-selectin.

Statistical analysis

Statistical analyses were performed using the SPSS Statistical Analysis System (version 15.0; SPSS Inc, Chicago, IL). Continuous variables were expressed as mean ± SEM. One-factor analysis of variance for repeated measures with the LSD post-hoc test was used to compare changes in outcome variables in response to the intervention treatments. The level of significance was set at $P < 0.05$. To exclude the presence of a carryover effect for the three interventions, comparison of the outcome variables observed before the 3 intervention periods was performed. Within- and between group differences are expressed as means and 95% confidence intervals (CI).

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Results

Participant characteristics and bioavailability of cocoa-polyphenols

All the 18 participants (mean age 26 ± 7 years) completed the three interventions and none reported any adverse effect. The baseline lipid characteristics of the participants are detailed in Table 2 and no changes were observed between the three baseline periods. Nutrient intake was similar in all the study phases. There were no changes in body weight or lipid profile (data not shown).

Consumption of CW and CM resulted in urinary excretions of 0.64 and 0.55 nmol/mg creatinine, respectively, of cocoa phase II metabolites (Σ epicatechin–glucuronide and epicatechin sulfates) [25] representing an increased trend of 17% of excretion when cocoa was taken with water. The mean plasma levels of epicatehin-glucuronide 2 h after CW were higher, albeit not significantly, than those observed after CM (330 ± 156 nmol/L and 274 ± 138 nmol/L, respectively; $P = 0.07$). However, on separating the subjects by gender, the differences between the mean plasma levels of epicatechin–glucuronide 2 h after CW and CM achieved statistical significance in men ($P = 0.04$) [27]. Moreover, consumption of CW and CM resulted in the urinary excretion of 220.32 and 111.58 nmol/mg creatinine, respectively, of microbial metabolites (Σ 3,4-dihydroxyphenylacetic, protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, caffeic and ferulic acids) derived from cocoa-polyphenols representing a significant increment of 97.5% ($P < 0.001$) after CW when compared with CM [23]. On analyzing phase II and microbial metabolites together, consumption of CW and CM resulted in urinary excretions of 220.96 and 112.13 nmol/mg creatinine, respectively, with a significant increment of 97.1% after CW intake ($P < 0.001$). Before the consumption of the test meals and after the washout periods, concentrations of phase II epicatechin metabolites in plasma and urine were under the detection limits of the technique [25,27]. Moreover, no significant differences were observed in urinary excretion of microbial metabolites before the interventions and after the washout periods [23].

NF-κB activation

Data on NF-κB activation before and after the three interventions are shown in Fig. 1. Changes in NF-kB activation measurements differed between the three interventions when analyzed by one-factor analysis of variance for repeated measures ($P = 0.039$). The effects of the interventions on NF-κB activation after 6 h were significantly greater in favor of cocoa (CM and CW) compared to the M intervention ($P = 0.028$ and $P = 0.002$, respectively) and were, moreover, also significantly greater in the CW compared to the CM intervention ($P = 0.033$). Compared to baseline levels, NF-κB activation was significantly decreased after the CW intervention (−28.8% [95%CI: −157.9% to−25.9%; $P = 0.042$]), being significantly increased after the M intervention (24.7% [95%CI: 11.4%–37.9%; $P = 0.014$]). No differences were observed after the CM intervention [2.6%, 95%CI: −29.4%–34.7%, $P = 0.759$].

Table 2 Baseline lipid characteristics of the 18 participants in the study.

Lipid baseline characteristics	Mean ± SEM
Cholesterol (mg/dL)	187.37 ± 24.53
Triglicerides (mg/dL)	60.44 ± 14.80
cHDL (mg/dL)	52.41 ± 9.63
cLDL (mg/dL)	121.85 ± 24.05
LDL/HDL ratio	2.43 ± 0.66
Apo A - HDL (mg/dL)	128.81 ± 15.33
Apo B - LDL (mg/dL)	82.00 ± 12.50

Concentration of soluble adhesion molecules

Significant differences in ICAM-1 ($P = 0.006$) and E-selectin ($P = 0.048$) levels were also observed after analysis. The sICAM-1 concentrations significantly decreased 6 h after the CW and CM interventions when compared to baseline values [−12.18% (95% CI, −2.14% to −22.21%) and −8.19% (95% CI, −1.08% to −15.31%) respectively; $P \leq 0.026$ both] (Fig. 2). No significant differences were observed after the M intervention, although it did show a trend to increase. On comparison between treatments no differences were observed between the baseline periods, despite significant differences after 6 h of treatment. Specifically, sICAM-1 concentrations were significantly lower after the CW compared to the CM intervention ($P = 0.048$). In the case of sE-selectin concentrations, a significant diminution was only observed 6 h after the CW intervention when

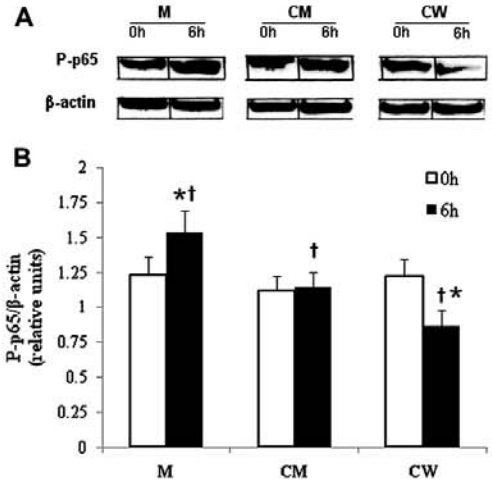

Figure 1 NF-κB activation in the 18 volunteers at 0 h and 6 h after milk (M), cocoa with milk (CM) or cocoa with water (CW) intake. A) Representative example of Western blot of P-p65 protein expression (β-actin was used to verify equal protein loading) B) Densitometric quantification of P-p65 levels. Values are mean ± SEM. Bars with an * are significantly different ($P < 0.05$, LSD test) in the same intervention period (baseline vs. intervention). Bars with a † are significantly different ($P < 0.05$, LSD test) between 6 h periods.

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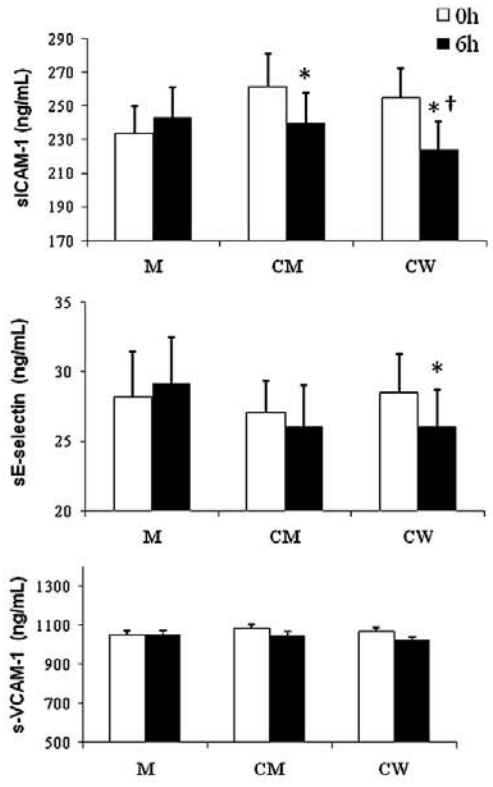

Figure 2 Serum intercellular adhesion molecule-1(sICAM-1), sE-selectin and vascular cellular adhesion molecule-1 (sVCAM-1) concentrations in 18 volunteers at baseline and 6 h after M, CM and CW intake. Values are mean ± SEM. Bars with an * are significantly different ($P < 0.05$, LSD test) in the same intervention period (baseline vs. intervention). Bars with a † are significantly different ($P < 0.05$, LSD test) compared to 6 h CM.

compared to baseline [−8.47% (95% CI, −1.01% to −15.93%), $P = 0.028$]. Otherwise, no changes were observed in serum sVCAM-1 levels in any case.

Discussion

In this clinical trial, acute consumption of 40 g of cocoa powder mixed with water significantly reduced phosphorylation of p65, the transcriptional active subunit of NF-κB, thereby significantly reducing the activation of NF-κB. On the other hand, the consumption of whole milk by volunteers resulted in a significant increment in NF-κB activation, whereas no changes in NF-κB activation were observed on consumption of 40g of cocoa powder mixed with milk. To our knowledge, there are no studies in human volunteers on the effect of cocoa powder on NF-κB activation.

NF-κB is a transcriptional modulator of genes involved in inflammation and has a crucial role in atherosclerosis and other inflammatory diseases. Nowadays, it is considered a major therapeutic target [28]. NF-κB activation is mediated by the stimulation of tumor necrosis factor alpha or interleukin-1β [29], although it may also be activated by some diet factors such as high-fat diets [30]. This activation leads to the transcription of mRNAs for ICAM-1, VCAM-1 and E-selectin resulting in high levels of ICAM-1, VCAM-1 and E-selectin proteins in the activated endothelial cells. These proteins are adhesion molecules involved in leukocyte-endothelial cell interaction [31]. Previous *in vitro* studies have shown that epicatechin, catechin and procyanidins isolated from cocoa already inhibit NF-κB activation [32].

The different effects of CM, CW and M on NF-κB activation are remarkable, and may be explained by the different fat and polyphenol content of each intervention. In fact, it has been reported that high-fat diets such as whole milk consumption, increase NF-κB activation [30]. However, this effect may be prevented by the simultaneous consumption of polyphenols [17]. The differences between consumption of CM and CW, both with 40 g of cocoa powder, could be attributable to the different bioavailability of polyphenolic compounds present in cocoa powder when taken with milk or water.

The effect of milk on the bioavailability of cocoa-polyphenols has previously been widely studied. Recent studies have shown that milk has minor or no effects on the plasma pharmacokinetics of phase II metabolites of epicatechin [22,25]. However, although greatly controversial, milk might diminish the urinary excretion of some phase II metabolites of epicatechin [22]. Cocoa-polyphenols that are not absorbed (mainly procyanidins) could reach the colon where they are degraded to phenolic acids by the intestinal microbiota and are absorbed in the organism [23]. The effect of milk on the excretion of microbial phenolic acids after acute ingestion of cocoa powder has been studied and showed that milk significantly diminishes the urinary excretion of some phenolic acids related to the metabolism of cocoa-polyphenols such as caffeic, ferulic, 3,4-dihydroxyphenylacetic, protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric acids [23]. Therefore, in general terms, metabolites derived from cocoa powder consumption seem to have greater bioavailability after CW than after CM intake. This fact could be directly related to the activation of NF-κB, since p65 phosphorylation levels after CM were significantly lower when compared with M and significantly higher when compared with CW. Moon et al. (2009) have recently shown that caffeic acid, a natural phenolic compound, reduces vascular inflammation inhibiting NF-κB activation in human umbilical vein endothelial cells [33]. Similarly, our results show that cocoa consumption, even in a single dose, might exert anti-inflammatory effects by modulating the NF-κB pathway and this could be attributable to the overall phenolic content of cocoa powder.

The activation of NF-κB may be the key step in increasing the levels of ICAM-1, VCAM-1 and E-selectin proteins in activated endothelial cells as previously reported [31]. Nevertheless, our results show that this activation does not have a direct impact on these three cytokines since only sICAM-1 concentrations decreased after the CM and CW interventions compared to baseline. Furthermore, sICAM-1 levels were significantly lower after the CW intervention when compared to the CM intervention which was also related to the bioavailability of cocoa-polyphenols. sE-Selectin concentrations only decreased after the CW

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intervention, whereas sVCAM concentrations were not modified after either intervention. These results are in accordance with the results of Kurlandsky et al., who showed a decrease in ICAM-1 concentrations without changes in VCAM-1 concentrations after dark chocolate consumption in healthy women [19]. In volunteers with cardiovascular risk factors, Monagas et al. [11] compared the effects of long-term consumption (1-month) of cocoa powder with skimmed milk (CSM) with those produced by skimmed milk (SM). Similar to our results they found a significant decrease in sICAM-1 concentrations after 1-month intake of CSM, and no differences were observed in sE-Selectin and sVCAM-1 concentrations in high cardiovascular risk subjects. However, contrary to our results, long-term studies in non-healthy subjects and with consumption of chocolate showed no changes in ICAM-1, VCAM-1 and selectin levels [18,34] or only a diminution in VCAM-1 levels [20]. The different results that we observed *in vivo* between NF-kB activation and the concentrations of the three cytokines may have two plausible explanations: the short time period between NF-kB activation and the measurement of adhesion molecules such as sVCAM-1, and also the direct effect of polyphenol metabolites of cocoa in the more bioavailable cytokines such as ICAM-1. However, further studies should be carried out in this field to determine the precise mechanism of how cocoa intake modulates NF-KB activation.

The main limitations of the current study are that only healthy subjects with low cardiovascular risk were included and we only evaluated the acute effects of cocoa intake on the mechanisms related to activation of inflammatory pathways. However, since the spectrum of the atherosclerotic lesions is a continuum, and high-risk patients show a higher inflammatory response in arterial wall [35], the protective effects of cocoa may be even greater in this type of subjects. Therefore, further studies are required to determine the time course regulation of NF-κB transcription and NF-κB activation by cocoa and other polyphenol-enriched foods. These new studies should include time course analysis of gene and protein expression profiling, which would provide information on the protective effect of cocoa during and after the postprandial phase.

In conclusion, cocoa consumption could confer beneficial anti-inflammatory effects mediated by inhibition of the NF-κB-dependent transcription pathway or by direct interaction with certain cytokines and the food matrix could play a crucial role in the modulation of this effect.

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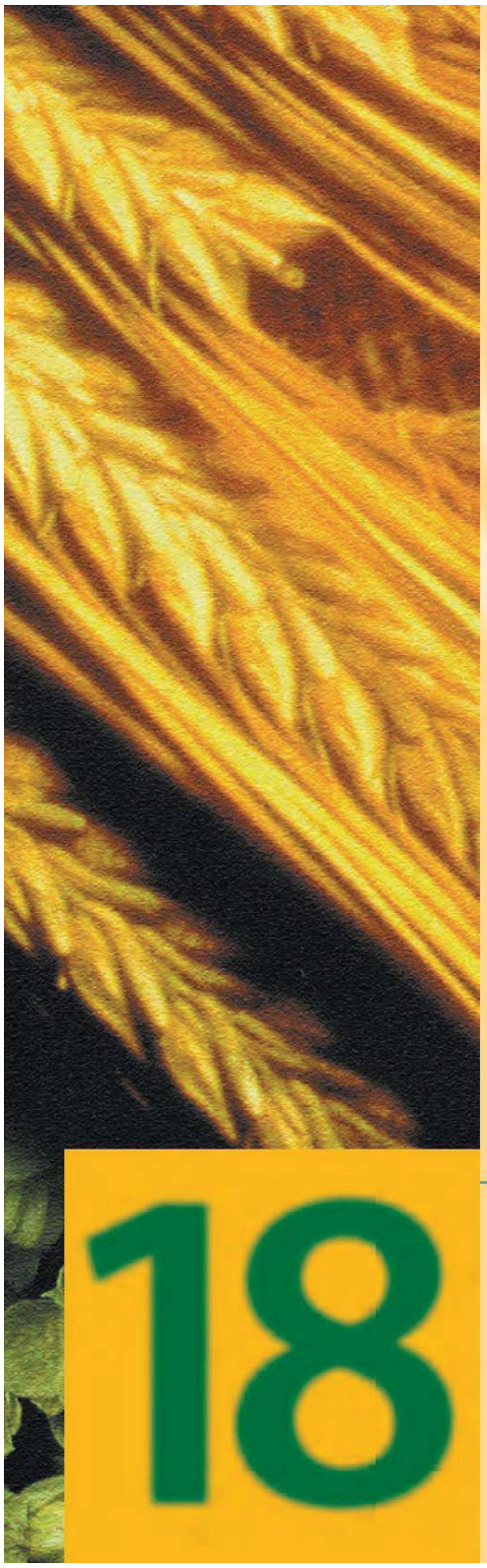

Cerveza, dieta mediterránea y enfermedad cardiovascular

Julio 2010

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CERVEZA y SALUD

Cerveza, dieta mediterránea y enfermedad cardiovascular

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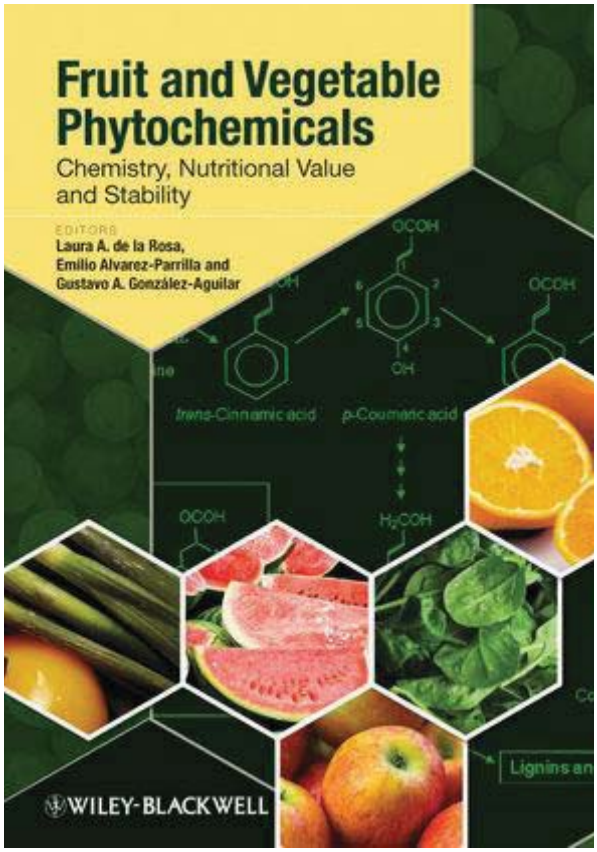

CAPÍTOL DE LLIBRE 1

Andrés-Lacueva, C.; Medina-Remón, A.; Llorach, R.; Urpi-Sarda, M.; Khan, N.; Chiva-Blanch, G.; Zamora-Ros, R.; Rotchés-Ribalta, M.; Lamuela-Raventós, R.M.

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2 Phenolic Compounds: Chemistry and Occurrence in Fruits and Vegetables

Cristina Andrés-Lacueva*, Alex Medina-Remon, Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva-Blanch, Raul Zamora-Ros, Maria Rotches and Rosa M. Lamuela-Raventós

Chemistry and Classification of Polyphenols

Polyphenols are the most abundant antioxidants in human diets. They are secondary metabolites of plants. These compounds are designed with an aromatic ring carrying one or more hydroxyl moieties. Several classes can be considered according to the number of phenol rings and to the structural elements that bind these rings.

In this context, two main groups of polyphenols, termed flavonoids and nonflavonoids, have been traditionally adopted. As seen in Figure 2.1, the flavonoids group comprises the compounds with a C6-C3-C6 structure: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones, and proanthocyanidins. The nonflavonoids group is classified according to the number of carbons that they have (Figure 2.2) and comprises the following subgroups: simple phenols, benzoic acids, hydrolyzable tannins, acetophenones and phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans, and secoiridoids.

Flavonoids

Flavonoids have a skeleton of diphenylpropanes, two benzene rings (A and B) connected by a three-carbon chain forming a closed pyran ring with the benzene A ring (see Figure 2.1).

Flavonoids in plants usually occur glycosylated mainly with glucose or rhamnose, but they can also be linked with galactose, arabinose, xylose, glucuronic acid, or other sugars. The number of glycosyl moieties usually varies from one to three; nevertheless, flavonoids have been identified with four and also five moieties (Vallejo and others 2004).

Flavonols and *flavones* have a double bond between C2 and C3 in the flavonoid structure and an oxygen atom at the C4 position. Furthermore, flavonols also have a hydroxyl group at the C3 position. *Dihydroflavonols* have the same structure as flavonols without the double bond between C2 and C3.

Flavanones are represented by the saturated three-carbon chain and an oxygen atom in the C4 position.

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Chapter

FLAVONOIDS FROM FOOD AND ITS IMPLICATION IN HUMAN HEALTH

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ABSTRACT

Flavonoids comprise a large group of phenolic compounds synthesized by plants as secondary metabolites, with a common diphenylpropane skeleton (C6-C3-C6). Over 9,000 structurally distinct flavonoids have been identified in nature, although less than 200 were found in a sample French population. Flavonoids can be classified according to their chemical structure into eight subclasses: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols or flavanols, anthocyanidins, isoflavones and proanthocyanidins.

Flavonoids occur ubiquitously in plants and plant products. They are generally found in glycosylated forms, mainly with glucose or rhamnose moieties, but other sugars may also be involved. Flavonoids in foods contribute to their flavor, providing color, astringency and bitterness. Flavonols are the most frequent flavonoids in foods, with

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CAPÍTOL DE LLIBRE 2

Montse Rabassa, Raul Zamora-Ros, Sara Tulipani, Maria Boto, Gemma Chiva-Blanch, Mar Garcia-Aloy, Mireia Urpi-Sarda, Rafael Llorach, Rosa Vázquez, Maria Rotches-Ribalta, Cristina Andres-Lacueva.

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ANNEX III

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Red Wine and Cardiovascular Health

Huige Li and Ulrich Förstermann

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Editorial

Red Wine and Cardiovascular Health

Huige Li, Ulrich Förstermann

There is strong epidemiological evidence that light-to-moderate consumption of alcoholic drinks, but neither zero nor more than moderate intake, reduces mortality from all causes and also diminishes cardiovascular risk.[1] The lowest risk for coronary heart disease mortality is seen with 1 to 2 drinks (12.5–25 g alcohol) per day.

Article, see p 1065

Hypertension is a major risk factor for cardiovascular disease, and there is a clear inverse relationship between light-to-moderate alcohol intake and blood pressure. Thus, the reduced cardiovascular risk associated with moderate consumption of alcoholic drinks may be due, in part, to a reduction in blood pressure. The greatest blood pressure benefit seems to be obtained with 1 drink per day for women and with 2 drinks per day for men.[2]

In most published studies, red wine has been used as the alcoholic test beverage. Red wine produces concentration-dependent vasodilator effects in subcutaneous small resistance arteries obtained from patients with essential hypertension.[3] In addition, red wine and dealcoholized red wine increase flow-mediated vasodilation of the brachial artery in healthy subjects.[4,5] Intake of red wine also counteracts endothelial dysfunction produced by high-fat diet in human volunteers,[6] and red wine increases flow-mediated vasodilation and decreases blood pressure in adult cigarette smokers.[7,8]

Alcohol Versus Polyphenols in Red Wine

With red wine, both the alcoholic and the polyphenolic components seem to contribute to its beneficial effects.[9] The alcoholic component is known to increase high-density lipoprotein cholesterol and to decrease fibrinogen concentrations.[10] The polyphenols present in red wine have been shown to provide beneficial effects by inhibiting prooxidant enzyme systems and by stimulating antioxidant enzyme systems in the vasculature (Figure). In addition, wine polyphenols inhibit platelet aggregation, attenuate vascular inflammation, and improve endothelial function.[9]

In the current issue of *Circulation Research,* Chiva-Blanch et al[11] demonstrate in a small prospective clinical study that a reasonable daily dose of dealcoholized red wine (275 mL/day) decreases systolic and diastolic blood pressures. This finding is in agreement with previous findings showing that the vasodilator effects of red wine may be mediated, at least in part, by polyphenolic compounds.[4] Also other nonalcoholic, polyphenol-rich beverages such as grape juice produce vasodilation (increase flow-mediated vasodilation) in healthy individuals.[12]

Cardiovascular risk factors are associated with oxidative stress in the vasculature because of an increased production and/or impaired inactivation of reactive oxygen species.[13,14] Oxidative stress, particularly the increased vascular production of superoxide, rapidly oxidizes and inactivates bioactive NO. In addition, there is evidence that persisting oxidative stress can render endothelial NO synthase (eNOS) dysfunctional such that it no longer produces NO but superoxide.[13,14] This is likely to represent the pathophysiological situations of the subjects participating in the current study by Chiva-Blanch et al.[11] All these individuals had several cardiovascular risk factors.

Red Wine Polyphenols Reduce Oxidative Stress and Increase Bioactive NO in the Vasculature

When the effects of 3 alcoholic beverages, red wine, beer, and vodka, were compared in a recent study,[15] only red wine provided protection against vascular oxidative stress. Indeed, red wine polyphenols seem to shield the vasculature by reducing reactive oxygen species (eg, by decreasing the expression of p22phox) and by inhibiting endothelin-1 expression.[16–18] At the same time, red wine polyphenols have been shown to increase bioactive NO in the vasculature. Also other protective factors, such as endothelium-derived hyperpolarizing factor, are stimulated.[16] In the current study by Chiva-Blanch et al,[11] the decrease in blood pressure by dealcoholized red wine was associated with elevated plasma levels of nitrite and nitrate, the oxidation products of NO. Thus, the study supports the notion that, also in humans in vivo, polyphenolic compounds from red wine can improve the functionality and activity of eNOS and can increase vascular levels of NO. Although a causal relationship between this effect and the observed decrease in blood pressure seems plausible, it is not being demonstrated in the study by Chiva-Blanch et al.[11]

Resveratrol and More

Red wine is a complex mixture containing numerous phenolic compounds. This raises the question of the chemical entities actually responsible for the beneficial effects. There is evidence that particularly the phytoalexin resveratrol can provide health benefits.[19] Experimentally, many positive effects of red wine can be mimicked with (usually relatively high doses of) resveratrol.[20] For instance, both red wine[21] and resveratrol[22] have been shown to enhance eNOS expression in cultured human endothelial cells. However, the effect of red wine cannot be explained by resveratrol alone; it is largely mediated by

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

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resveratrol (≈70%), but several other polyphenols and phenolic acids contribute.[23] Also, both red wine and resveratrol enhance eNOS enzymatic activity. Whereas the effect of resveratrol on eNOS phosphorylation (and activation) mainly involves estrogen receptor–mediated activation of extracellular signal-regulated kinases 1 and 2,[24] the phosphorylation (and activation) of eNOS by phenolic extracts from red wine also involves the phosphatidylinositol 3-kinases/Akt pathway.[25] Resveratrol has a low bioavailability.[19] Therefore, many in vivo effects of red wine are likely to be attributable to resveratrol metabolites in concert with several other polyphenolic compounds (and possibly their metabolites).

Red Wine and Unresolved Questions

The beneficial effects of red wine can be enhanced by a healthy diet. Indeed, a synergistic effect of red wine and green olive oil (both are components of the Mediterranean diet) on flow-mediated vasodilation has been demonstrated.[26] The average moderate wine drinker is more likely to exercise, to be health conscious, and to be of a higher educational and socioeconomic status.[9]

Nevertheless, there is growing evidence, corroborated by the current study of Chiva-Blanch et al,[11] that chemical constituents present in red wine confer health benefits beyond alcohol and are independent of potential confounding factors. However, numerous issues need to be resolved to clearly assess the preventive or therapeutic potential of red wine constituents. Specific chemical entities responsible for the beneficial effects have to be identified. In this context, one has to realize that there are probably major differences between different red wines, depending on the grape and the growing area.[21] Pharmacological mechanisms leading to increases in vascular NO and other beneficial effects have to be elucidated, and it has to be clarified whether the decrease in blood pressure is causally related to the increased NO levels or what other mechanisms may be involved.

Finally, the question whether white wine offers a similar benefit as red remains unresolved. White wine, which is usually fermented without skin and seeds, is missing many of the polyphenols (eg, resveratrol, catechin, quercetin) mainly found in these parts of the grape. There are a few studies[27] demonstrating superior effects of red versus white wine on certain cardiovascular parameters. However, epidemiological evidence demonstrating a specific benefit of red over white wine is poor,[27] and conclusive studies comparing the 2 are missing. In the long run, a prospective, randomized study would be needed to prove or drop the red-better-than-white hypothesis.

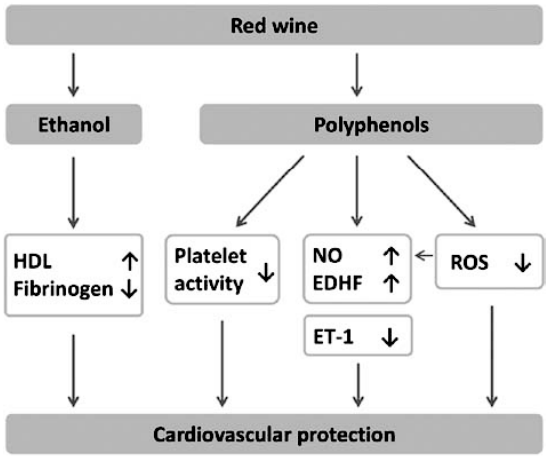

Figure. Factors potentially contributing to the vasoprotective effects of red wine. The alcoholic component of red wine increases high-density lipoprotein (HDL) cholesterol level and decreases fibrinogen concentrations. Polyphenols present in red wine independently inhibit platelet aggregation, enhance bioactive NO, stimulate the formation of endothelium-derived hyperpolarizing factor (EDHF), reduce reactive oxygen species (ROS) generation, and inhibit endothelin-1 (ET-1) expression.

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None.

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KEY WORDS: wine ■ polyphenols ■ alcohol drinking ■ blood pressure

Letter to the Editor

Letters to the Editor will be published, if suitable, as space permits. They should not exceed 1000 words (typed double-spaced) in length and may be subject to editing or abridgment.

The Impact of Red Wine on Blood Pressure Dizziness Continues

To the Editor:

We read with great interest the article of Chiva-Blanch et al recently published in your journal.[1] This randomized controlled trial evaluates the impact of dealcoholized red wine (DRW) on blood pressure (BP) in patients with high cardiovascular risk and concludes on its ability to lower BP. Although excited about the interesting results of this trial, we think several concerns exist that, if addressed in future studies, might lead to a better understanding of the findings and a wider acceptance of the conclusions.

First, office measurement has been used to evaluate the impact of various interventions on BP. Although not specified in the article, the patients were likely to have the interventions (ie, beverage intake) during evening hours. As such, there has been an interval of several hours between the intervention and the measurement of BP, which took place during the office visit the next day. It has been shown that repeated alcohol intake has a biphasic impact on BP (ie, initially decreasing, then increasing).[2] Because these depressor or pressor properties are affected by the differences in time intervals after the last drink, random measurement of BP could be misleading in this setting. The fact that alcohol's early BP-lowering effect has not been captured in this study makes it conceivable that a bias in favor of DRW has been created. Similarly, in the absence of a preplanned fixed interval between the beverage intake and BP measurement, the time interval is likely to have been variable, which could significantly affect the interpretability of the results. Ambulatory BP monitoring can be very helpful in this setting, providing time-sensitive information on changes in BP in relation to the time of intervention.

Second, the study has evaluated the effect of red wine on plasma concentration of NO, and the authors have concluded that DRW decreases BP through NO-mediated mechanisms. Obviously, BP-lowering effect would be of more importance in those patients who are hypertensive compared to those with normal BP. However, a closer look at the results (Online Table I in Chiva Blanch et al[1]) shows that NO concentration did not actually change in hypertensive subset of patients who received DRW (mean difference 2.2 μmol/L, CI 2.6–7.1). In the same intervention group, although the increase in NO concentration was found to be significant for nonhypertensive patients, the wide confidence interval (ie, 0.2–11.2) suggests that the data have been too variable to make a precise estimate. Besides, even in the alcohol-containing red wine group there was a statistically-significant correlation between changes in systolic BP and NO concentration (Online Figure I in Chiva Blanch et al[1]). In our opinion, the heterogeneity of these findings implies the presence of other unidentified mechanism(s) beyond NO to explain the impact of red wine on BP. The authors have appropriately compared the phenolic composition of the red wine before and after the dealcoholization process. However, it is noteworthy that this process, while only minimally changing phenolic composition, has been shown to modify other components of the wine (eg, antioxidants) with potential impact on endothelial function and BP.[3]

Third, objective assessment of adherence is obviously of utmost importance in a trial where a nutritional intervention is evaluated. Although the authors mention that diet and exercise were monitored and a number of markers of alcohol consumption have been used, the concern for lack of a more objective monitoring of the patients' compliance persists. For example, urinary ethylglucuronide, a biomarker of alcohol consumption, was present in the urine of patients after DRW intervention, although at lower levels compared to alcohol-consuming groups. Therefore, it cannot be ruled out that, unbeknownst to the investigators of this open-label trial, some patients in this group were actually consuming an unidentified amount of alcoholic beverages. Moreover, urinary measurement of sodium and potassium is an established means for objective evaluation of dietary sodium and potassium and, because of its simplicity, is even used in population-based cohort studies for this purpose.[4] Because 24-hour urine samples were collected in the study of Chiva-Blanch et al, it would have been interesting to also measure sodium and potassium in those samples. This inexpensive test not only could provide invaluable objective information on these dietary confounders of hypertension, but could also enable the investigators to further assess patients' adherence by comparing the patients' actual sodium and potassium consumption with their presumed intake (Online Table II in Chiva Blanch et al[1]).

Finally, Pearson correlation test was used to evaluate the association between serum NO level and BP for each intervention (Online Figure I in Chiva Blanch et al[1]). Because the changes were found to be in the opposite directions for all categories except for systolic BP in gin group, the r values need to be changed to negative (eg, $r = -0.598$ for systolic BP in DRW group).

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Disclosures

None.

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Letter to the Editor

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Reply to Iqbal and Kazory

We thank Drs Iqbal and Kazory[1] for their interest in our work and appreciate the concerns raised in their letter to address them in future studies.

Regarding the usefulness of office measurement of blood pressure (BP) in the detection of changes in BP in intervention studies, measurement of office BP is considered a reliable method according to the guidelines for management of arterial hypertension in a clinical setting[2] and it has been useful in large clinical trials.[3] To uniform the BP recordings in our study, a fixed preplanned interval between beverage intake and BP measurement was established (12 hours), as volunteers were asked to come at the same hour after each intervention period and were also asked to maintain their dietary pattern (ie, meal times) throughout the study, emphasizing the fact that the last week of the 1-month intervention was the most important in maintaining this pattern to avoid variability in the BP measurements. The biphasic impact of acute and repeated alcohol intake is a matter of interest, but it has been detected after the administration of higher doses of alcohol[3] (approximately twice the amount of alcohol we administered). In addition, our results agree with those reported in nonmedicated hypertensive patients[3] which found that BP during the interval of 8.00 AM to 3.00 PM in subjects consuming alcohol at dinner was not statistically different from the BP obtained after a 1-week period of alcohol washout. At any rate, we agree that ambulatory BP monitoring would be very helpful in providing information on the acute and chronic effects of moderate alcohol consumption.

Although BP lowering effects would be more relevant in hypertensive than in normotensive subjects, prevention of hypertension in high cardiovascular-risk patients is also important. As shown in Online Table I from our original article,[4] plasma NO concentrations increased, although not significantly, even in hypertensive patients. The lack of significance in the changes observed in hypertensive subjects could be explained by the fact that the effects of dealcoholized red wine (DRW) on endothelial NO synthesis could be masked by antihypertensive drug therapy such as angiotensin-converting-enzyme inhibitors, which have been shown to increase endothelial NO synthase.[6] As Iqbal and Kazory pointed out, after the DRW intervention the nonhypertensive population showed a significant increase in the NO plasma concentration and a significant decrease in BP. Although the data showed a great interindividual variability, the statistical method used took into account within-subject changes, and this allows detection of even subtle outcome variations. However, we agree that unidentified mechanisms beyond NO could also participate in the observed effects, as mentioned in the limitations section of our original article. In reference to the changes in the composition of DRW with respect to red wine, the dealcoholization process of the wine used in our study was not distillation but reverse osmosis, which is known to have little impact on antioxidant content[7] and minimally changed the phenolic composition of the red wine. Again, this process may have affected unknown compounds with a potential impact on endothelial function and BP.

We also agree with the importance of having adequate biomarkers of adherence to the dietary interventions. As Iqbal and Kazory[1] correctly point out, ethyl glucuronide was present in the urine of patients after the DRW intervention, although at lower levels compared with alcohol-consuming groups. This can be explained by the fact that DRW contains 0.42% ethanol, therefore, during the DRW period volunteers consumed ≈1 g ethanol/d. We concur with the adequacy of measuring urinary sodium and potassium as an objective assessment of dietary intake in studies evaluating BP. We determined 24-hour urinary excretion of sodium and potassium, and the results showed no significant differences before and after each intervention: −12 mEq/L (95% CI, −56, 32), −5 (−23, 13), and 5 (−31, 42) for urinary sodium, and −9 (−27, 10), −11 (−29, 7), and −5 (−21, 11) for urinary potassium after red wine, DRW, and gin periods, respectively. Neither showed differences between the interventions (P=0.515 and 0.769 for sodium and potassium, respectively, by repeated-measures ANOVA). Therefore, the BP changes observed could not possibly be ascribed to changes in dietary sodium intake.

Finally, we agree that in Online Figure I in our original article[5] and in the results section, the r values of the Pearson correlation should have a minus sign, except for systolic BP in the gin group.

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None.

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Absorption and metabolism of red wine polyphenols and their potential health benefits in cardiovascular function

Dear Sir:

In a recent issue of the Journal, Chiva-Blanch et al (1) reported the differential effects of the polyphenols and alcohol in red wine (RW) on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis in subjects at high risk of cardiovascular disease. We appreciate the authors' cautious deduction that both ethanol and nonalcoholic compounds contribute to the antiinflammatory effects of RW on the basis of a randomized clinical trial of RW and dealcoholized RW (DRW).

Although we have little doubt about the alcohol-independent cardiovascular effects of the phenolic compounds of RW, we were concerned about the actual concentrations of urinary polyphenols and their metabolites reported in the study. The authors reported that 24-h urinary excretion of total resveratrol metabolites increased in relation to baseline amounts from 1.24 μmol (95% CI: 0.91, 1.65 μmol) to 4.69 μmol (95% CI: 3.86, 5.53 μmol) and 8.33 μmol (95% CI: 6.86, 10.19 μmol) after ingestion of RW and DRW, respectively. The authors suggested that resveratrol metabolite concentrations were statistically higher after the DRW compared with RW intervention ($P = 0.002$). However, it seems clear from a great number of recent studies that the cardiovascular effects of polyphenols are dependent on their bioavailability, and alcohol in RW may improve polyphenol availability by increasing its intestinal absorption or by delaying its excretion (2, 3), indicating that RW intake should produce higher concentrations of urinary resveratrol metabolites than DRW ingestion. It is, therefore, reasonable to assume that the consumption of RW and DRW also should, at least, lead to similar concentrations of urinary resveratrol metabolites, despite the fact that the resveratrol content of RW is slightly higher than that of DRW as depicted in Table 1 of their article. More important, we also recognized that the observed significant increase ($P = 0.002$) in urinary resveratrol metabolites after DRW intake was not paralleled by the increase in the tested biomarkers related to atherosclerosis in comparison with RW ingestion (Tables 4 and 5 of their article). Therefore, the present data make sense if RW polyphenols, including resveratrol, are associated with the tested vascular health benefits.

One of the main limitations of Chiva-Blanch et al's study (1) is that it does not report the circulating metabolite concentrations after the consumption of RW and DRW. To elucidate the finding that polyphenols are responsible for the alcohol-independent cardiovascular effect of RW, plasma analysis for polyphenols and their metabolites is determinant. Several causality criteria for the assessment of any compound as a potential mediator of vascular function have been established previously, and these have to meet the condition that the test compound should be absorbed from a food matrix by humans and should be transported to the appropriate site or tissue (as quantifiable in circulation), and its circulating amounts temporally parallel the hypothesized vascular effects (4, 5). Although the study conducted by Chiva-Blanch et al (1) showed consistent effects of both RW and DRW on several intermediate markers for vascular function, it is still not fully known whether their action could be specifically related to RW polyphenols.

Another limitation of this study was that although the authors measured total urinary resveratrol metabolites as an indicator of polyphenolic absorption, 24-h urinary excretion results of total resveratrol metabolites were not corrected for creatinine (eg, nmol/g creatinine). It also must be noted that the authors omitted a necessary explanation as to whether the analysis of total resveratrol metabolites was to measure total resveratrol concentrations, which are calculated as the sum of resveratrol and its sulfated or glucuronidated metabolites after enzymatic hydrolysis with glucuronidase and sulfatase, as well as methylated resveratrol. It is well known that, in humans, ingested polyphenols are extensively metabolized and excreted as sulfated, glucuronidated, or methylated phase II conjugates (5–7), and authentic standards of resveratrol metabolites are not generally commercially available. It is also surprising that the study design was to elucidate whether the cardioprotective effects of RW intake are attributed to alcohol, polyphenols, or the synergistic effect of both, although the concentration-dependent effects of resveratrol metabolites and ethylglucuronide as the indicators of polyphenolic and alcohol absorption from RW, DRW, and gin were not adequately discussed in the article. The observed results did not well support the hypothesis raised by authors that the ethanol and polyphenols in RW were responsible for the regulation of soluble inflammatory mediators in high-risk patients.

Epidemiologic studies have shown that moderate intake of RW protects against cardiovascular diseases, and this effect has been attributed to polyphenols (2, 3, 8, 9). However, Chiva-Blanch et al (1) noted that there was inadequate experimental evidence to implicate specific polyphenols in RW. Data on the intestinal absorption of polyphenols from RW intake are sparse, and it is not yet known to what extent these components are bioavailable and whether alcohol plays a role in their absorption. Dietary intake of polyphenols is not guaranteed to equate with exposure at the tissue level, because polyphenols from food, including RW, are poorly absorbed in humans (8). Given this, the possibility that RW polyphenols affect vascular function is intriguing and deserves attention in future studies.

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Reply to X Yang and Y Zhao

Dear Sir:

We thank Yang et al for their interest in our work and appreciate the opportunity to respond to a number of the issues raised in their letter. The aim of our study (1) was to evaluate and compare the effects of moderate consumption of red wine (RW), gin, and dealcoholized RW (DRW) on the expression of soluble and leukocyte adhesion molecules as well as of proinflammatory cytokines related to the early stages of atherosclerosis in subjects at high risk of coronary heart disease. One of the concerns raised in the letter by Yang et al was the actual concentrations of urinary polyphenols and their metabolites. We reported 24-h urinary excretion of total resveratrol metabolites only as a measure of intervention compliance because resveratrol metabolites have been previously described as a marker of wine consumption by our research group (2). After our study was published, an error was identified in the calculation of total resveratrol metabolites in 24-h urinary excretion. Consequently, the results of the statistical analysis have been updated, and nonsignificant differences were observed after RW and DRW interventions. (The corrected values appear in an erratum in this issue.)

The comments by Yang et al with regard to the role of alcohol in RW on the availability of polyphenols raise important questions. In vitro studies cited by Yang et al found that alcohol from RW increased the absorption of quercetin and its 3-*O*-glucoside (3) and seems to contribute indirectly to the antioxidant capacity of wine by increasing the bioavailability of its phenolic compounds (4). However, the in vivo evidence supports similar bioavailability for catechins, malvidin-3-glucoside, and caffeic acid and production of 4-*O*-methylgallic acid after consumption of RW and DRW (5, 6). Nevertheless, the alcohol in RW delayed the elimination of (+)-catechin from the plasma compartment (6). Urinary catechin concentration after consumption of RW tended to be higher (20%) compared with that after the consumption of DRW ($P = 0.06$) (6). Other in vivo intervention studies that administered pure polyphenols (ie, resveratrol, catechin, or quercetin) in different matrices have shown contradictory results. For example, alcohol did not improve the bioavailability of total resveratrol and catechin when comparing white wine (11.5% ethanol content) with grape juice or vegetable juice/homogenate matrices but increased the absorption and excretion of quercetin (6). In addition, 5% alcohol did not improve the bioavailability of resveratrol when coadministered with quercetin (7). Therefore, the effect of alcohol on polyphenol absorption remains unclear, and further studies in this field are required.

We agree that the cardiovascular effects of RW polyphenols depend on their bioavailability. Total resveratrol represents 4.4% of total phenolics in the RW and DRW used in the study (our article's Table 1), and the resveratrol concentration of RW was not significantly higher than that of DRW. In terms of bioavailability, resveratrol is well absorbed but scarcely bioavailable because it is rapidly metabolized (7). Poorly absorbed proanthocyanidins and anthocyanins from wine reach the colon, where they are metabolized to phenolic acids by the microbiota (6). Therefore, knowledge of the whole profile of RW polyphenol metabolites as well as the effect of microbiota on bioavailability is key to understanding its health impact in further studies (6).

Yang et al highlighted 2 limitations of our study (1). In this long-term study, fasting blood samples were collected only at baseline and on the day after the last day of each intervention. When only fasting blood is sampled, care should be taken when interpreting data on biomarker intake (8). The relation between intake of a polyphenolic food constituent and the appearance of a metabolite in plasma depends on the elimination rate of this metabolite. Half-lives of polyphenols are ~2 h for anthocyanins and flavanones, 2–3 h for flavanols, and 11–28 h for flavonols such as quercetin (8). The 24-h urine accurately reflects the total polyphenol absorption, is a quantitative measure of the total amounts of polyphenol metabolites over a 24-h period, and monitors the daily intake more robustly than does a single plasma measurement (8). Therefore, we provided the urinary measure of biomarkers of wine consumption [sum of total resveratrol metabolites previously described by Zamora-Ros et al (2)] and ethylglucuronide as the biomarker of alcohol consumption in 24-h urine samples as an objective measure of adherence with the interventions.

The other limitation mentioned by Yang et al was that the 24-h urinary excretion of total resveratrol metabolites was not creatinine adjusted. The 24-h urinary excretion of polyphenols is considered to

be the most reliable and noninvasive method for assessing polyphenol biomarkers (8, 9). Creatinine is a usual correction method when spot urine has been collected (2). However, although creatinine is an indicator of renal function (9), its concentrations vary greatly depending on sex, age, physical activity, renal function, and diet (9).

With regard to the analysis of total resveratrol metabolites, the methodology was explained in detail in the article by Urpi-Sarda et al (10). This study analyzed glucuronide and sulfate conjugates of resveratrol without enzymatic hydrolysis of samples. This biomarker of wine consumption is defined as the sum of 7 phase II resveratrol metabolites, namely the following: *trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4′-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-sulfate, *trans*-resveratrol-3-*O*-sulfate, *cis*-resveratrol-4′-*O*-sulfate, and *cis*-resveratrol-3-*O*-sulfate (2). The methodology was quantitatively adapted to this study to analyze the 24-h urine samples collected on the last day of the run-in period and the last day of each intervention. As far as we know, authentic standards of some resveratrol metabolites have recently become commercially available in Canada (Toronto Research Chemicals) and in France (Bertin Pharma). In the present study, such metabolites were quantified with standards acquired from Toronto Research Chemicals and are as follows: *trans*- and *cis*-resveratrol-3-*O*-glucuronide (98% purity each), *cis*-resveratrol-4′-*O*-glucuronide (96% purity), and *trans*-resveratrol-3-*O*-sulfate (98% purity).

The present study was designed to determine whether ethanol or wine polyphenol interventions are responsible for the regulation of soluble inflammatory mediators. We agree with Yang et al that the effect of each RW polyphenol on vascular function is intriguing and deserves further investigation.

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Protein requirements and the indicator amino acid oxidation method

Dear Sir:

The process through which the dietary requirement for any nutrient is set is an important but challenging task, both conceptually and practically. After a thorough critical review of the literature, Dewey et al (1) proposed a mean requirement for protein for healthy school-age children of 0.69 $g \cdot kg^{-1} \cdot d^{-1}$ and a safe level of 0.86 $\cdot kg^{-1} \cdot d^{-1}$. The most recent international expert consultation on protein requirements by FAO/WHO proposed 0.75 and 0.92 $g \cdot kg^{-1} \cdot d^{-1}$, respectively (2). The calculations on which these requirements are based derive from studies of intakes that allow infants and young children to grow and develop normally. In their recent article, Elango et al (3) report data derived from metabolic studies, which leads them to conclude that the mean requirement should be 1.3 $g \cdot kg^{-1} \cdot d^{-1}$, with a safe protein requirement of 1.55 $g \cdot kg^{-1} \cdot d^{-1}$. This represents a substantial increase from the previous recommendations of 50–90% and carries very serious implications for policy, health care, food supplies, and the social welfare of large groups of the population. It is incumbent on those suggesting any revision of this order to be clear about how any proposed change compares with existing guidance. Any proposed change should be considered critically and discussed in the context of existing knowledge and with recognition of any potential limitations in the methods used to derive alternate values.

De: Circulation Research <circulation.research@circresearch.com>
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Asunto: Top Downloaded Online First Article in Circulation Research

August 15, 2012

Dear Dr. Estruch,

The editors of *Circulation Research* wish to congratulate you and your co-authors for having published one of the most downloaded recent articles in our journal. During Sept 2012, the PDF of your Online First article, "Dealcoholized Red Wine Decreases Systolic and Diastolic Blood Pressure and Increases Plasma Nitric Oxide," was downloaded and viewed over 1,700 times and the abstract was viewed another 5,000 times, placing it in the top tier for the month.

Thank you for submitting this outstanding article and for your contribution to *Circulation Research.*

Sincerely,

Roberto Bolli, MD

Editor in Chief, *Circulation Research*

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September 6, 2012 – Gemma Chiva-Blanch

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