UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE NUTRICIÓ I BROMATOLOGIA

RESVERATROL: MARCADOR BIOLÒGIC i DIETÈTIC DE CONSUM DE VI

RAUL ZAMORA ROS

2008

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE NUTRICIÓ I BROMATOLOGIA

PROGRAMA DE DOCTORAT

MEDICAMENTS, ALIMENTACIÓ I SALUT

BIENNI 2003-2005

RESVERATROL: MARCADOR BIOLÒGIC i DIETÈTIC DE CONSUM DE VI

Memòria presentada per Raul Zamora Ros per optar al títol de doctor per la Universitat de Barcelona

Cristina Andrés Lacueva

Rosa Mª Lamuela Raventós

Raul Zamora Ros

RAUL ZAMORA ROS

2008

Aquest treball ha estat financiat per:

Ministerio de Educación y Ciencia
AGL 2005-0559/ALI
AGL2006-14228-C03-02/01
Programa Ingenio Consolider FUN-C-FOOD (CDS 2007-063)

Ministerio de Sanidad y Consumo
RED G03/140
RETICS PI052443
RD06/0045/0003
CIBER 06/03 Fisiopatología de la Obesidad y la Nutrición (PREDIMED)

Caixa Penedès Obra Social
FBG302486
FBG302679

Consejo Regulador del Cava
FBG301442
FBG302069

AGAUR
Beca pre-doctoral 2004 FI 00270
Beca per a estades a l'estranger 2006 BE 00378
Projectes de recerca per potenciar grups de recerca consolidats:
2000SGR00043; 2001SGR00131; 2005SGR00156

AGRAÏMENTS

Aconseguir realitzar aquesta tesis i no perdre'm pel camí, ha estat possible gràcies a un gran esforç col·lectiu de persones i institucions a les quals voldria agrair sincerament. Gràcies a tot aquest suport he arribat a assolir aquesta gran fita personal, espero que només sigui una fita intermitja, dintre del meu caminar en el món científic.

En primer lloc voldria agrair a la meva mare i al meu pare tot el seu suport incondicional enfront aquest nou repte meu, ja que com tots els reptes, ha hagut moments de bon i no tan bon humor. Al meu germà que ha estat un dels pilars bàsics en el bon ambient casolà i en la consecució de la titulació de "dietètic". Voldria destacar als meus nebots, Nil i Robert, que han aconseguit fer-me sentir com el tiet més científic del món. I també vull agrair a les meves germanes el bon ambient i suport davant totes les situacions acadèmiques i personals.

Seguidament voldria donar les gràcies a les meves directores de tesi les Dres Rosa Lamuela i Cristina Andrés. A la Rosa per aconseguir donar-me l'oportunitat d'entrar a formar part del grup d'Antioxidants Naturals i haver apostat sempre per mi per participar en el PREDIMED. I com no a la Cristina, que tot i atabalar-me una mica de tant en tant, crec que ha aconseguit potenciar el meu rendiment i la meva curiositat per la ciència a uns nivells inimaginables respecte quan vaig entrar, ara ja fa uns anyets. També voldria agrair el vostre constant suport, i el tenir sempre oberta la porta per solucionar els meus dubtes i inquietuds i guiar-me dintre d'aquest camí que a vegades pot ser una mica tortuós.

A la Mireïa, companya de tesis i de dificultats en aquest fascinant món nostre de l'anàlisi del resveratrol. Com ja saps ,sense el teu ajut, aquesta tesi no hagués estat possible, però sobre tot hagués estat molt més avorrida. Ànims que la pròxima serà la teva.

A tots els companys de grup: Maite, per ser la meva mentora quan vaig arribar al grup; Elena, Lulú i Ana per fer que els dies d'experimental i d'ordinador fossin molt més agradables; *Alex, por esas grandes tardes de debate enfrente a datos estadísticos y a esos mojitos que nadie prepara como tu; Rafa, tu llegada ha sido como un ciclón de buen rollo y conocimiento*; Maria sense tu l'experimental, l'ordre de mostres i la integració de pics hagués estat molt i molt i molt més lenta; Gemma, les casualitats de la ciència ens han fet retrobar, gràcies per aquestes xerrades interminables; *Nacho,gracias por esta breve pero intensa relación con alguién de la "meseta"*. També voldria agrair a la Rosa Guash, la Maria Monagas, en Nasir, la Miriam, la Marta, la Rita i a tota la gent que ha passat pel grup durant aquests anys.

Per proximitat vull donar les gràcies a tot el grup de Vins: Joan només puc dir-te una cosa: ets únic i inigualable; Montse, Stefania i Paqui les grans companyes i amigues de Vins; i com no a en Joan Gallardo que també apunta bones maneres.

També volia donar les gràcies als altres grups del departament: Amines, Greixos I i II, amb noms que m'han deixat petjada com el Ricard i el José, companys d'aventures. Als companys de sempre: Alba, Gemma, Tomasso, Mari Luz, Marcos, Karina, Jorge, Isa i als presents: Carol i Mar per la comprensió i les bons moments en els últims i més complicats dies de la tesi, Jonathan, Carla, Miquel, Nuria, Adonis. Gràcies a tots per fer que el dia a dia al departament sigui més agradable, sobre tot els dinars i les escapades.

Vull donar les gràcies a tot el professorat del departament que m'ha donat suport en la realització de la tesis i que m'han permès participar en la docència del departament. En particular als Drs Francesc Guardiola i Abel Mariné per ser companys d'assignatura, ells han estat els qui m'han introduït una mica al "mundillo" de la docència. I aquí no puc oblidar a la Rosa i la Cristina que sempre han pensat en mi per col·laborar en les seves assignatures. Al Dr Pep Boatella per tots els seus sempre encertats comentaris i consells. A les Dres Susana Buxaderas, Elvira López i Maria Izquierdo, per les seves interessants i agradables converses.

També vull agrair molt especialment a l'Andreu Farran i la Pilar Cervera per ser les persones que em van iniciar en el CESNID en aquest apassionant món de la recerca i que em van animar a fer el doctorat. Guardo un gran record de totes les persones amb les que vaig col·laborar: Ana, Imma, Montses, Xavi, David, Alicia, Pruvi, Sr José,...

Al Dr Carlos Alberto González, coordinador de la cohort EPIC Espanya, per donar-me la possibilitat de començar a fer una col·laboració entre ambdós grups .També vull agrair a en Toni Berenguer per tota l'ajuda estadística rebuda en aquest projecte i en totes les altres consultes realitzades. I a la Paula pel suport en els projectes realitzats i per pensar en mi per participar en projectes futurs.

Non posso dimenticare la mia stanza a l'INRAN di Roma (Istituto Nazionale di Recerca per gli Alimenti e la Nutrizione) con i Dottori Mauro Serafini e Giuseppe Maiani. Mauro, grazie per la tua visione particolare sulla scienza e per la tua amicizia. Anche voglio ringraziare ai colleghi dal laboratorio, Francesca, Monia e Débora, senza loro la stanza no avrebbe stato uguale. Débora, gracias a ti y a Raffaello mis fines de semana en Roma fueron de todo menos aburridos. *Sara, grazie per essere la meglio padrona di casa che ho potuto avere.*

Només em resta agrair a totes les institucions públiques (*Ministerio de Sanidad y Consumo, Ministerio de Ciencia e Innovación*, Generalitat de Catalunya, AGAUR) i privades (Consell Regulador del Cava, i Caixa Penedès Obra Social) que han donat suport econòmic tan als projectes en els que he participat com a les beques que han fet possible la meva dedicació completa a la realització de la tesis. Sense aquest finançament la ciència no seria possible, per tant moltes gràcies.

ABREVIATURAS

ABAP = 2,2'azobis(2-amidinopropane)dihydrochloride

ADN = àcid desoxiribonucleic

ADP = adenosín difosfat

AP-1 = protein activator-1

API = ionització a pressió atmosfèrica

Apo = apolipoprotena

ATP = adenosín trifosfat

CBG = cytosolic-β-glucosidase

CD36 = scavenger receptor CD36

CID = cel·la de colisió

cGMP = guanosina monofosfat cíclica

Cmax = concentració plasmàtica màxima

COX = ciclooxigenasa

DAD = detector de feix de diodes (diode array detector)

ERK = extracelular signal-regulated kinase

E-RmRNASF = Estrogen-Regulated mRNA Stabilizing Factor

eNOS = enzima òxid nítric sintetasa endotelial

ET-1 = endotelina-1

ESI = ionització per electroesprai

EPIC = Euopean Prospective Investigation into Cancer an nutrition

FRAP = ferric-reducing ability of plasma

HDL = lipoproteïna d'alta densitat

HLB = hidrofílic-lipofílic-equilibrat

HPLC = cromatografia líquida d'alta eficacia

IC = interval de confiança

ICAM = molècula d'adhesió intracel·lular (intracelular adhesión molecule)

IGF = factor de creixement

IL = interleucina

iNOS = enzim òxid nítric sintetasa induïble

IMC = índex de massa corporal

JNK = C-Jun N-terminal kinasa

LC MS/MS = cromatografia líquida acoblada a un detector de masses en tàndem

LDL = lipoprotena de baixa densitat

LDLR- = receptor LDL negatiu

LFA-1 = lymphocyte function-associated antigen-1

LPH = lactase phlorizin hidrolase

MAPK = mitogen-activated protein kinasa

mARN = àcid ribonucleic missatger

MCP = monocyte chemoattractant protein

MRM = multiple reaction monitoring

MRP-2 = multidrug resistance-associated protein-2

MS = espectrometria de masses

MS/MS = espectrometria d e masses en tàndem

m/z = massa/càrrega

NAD = nicotinamida-adenín-dinucelòtid

NADH = nicotinamida-adenín-dinucelòtid fosfat deshidrogenasa

NAD(P) = nicotinamida-adenín-dinucelòtid fosfat

NFκβ = factor nuclear kappa β

NO = òxid nítric

NQO 1 = quinona oxidoreductasa-1

ODC = ornitina decarboxilasa

ORAC = oxygen radical absorbance capacity

p70 (S6K) = ribosomal protein p70(S6 kinasa)

PCR = proteïna C-reactiva

PDGF-A = platelet-derived growth factor

PGC-1α = peroxisome proliferator-activated receptor gamma coactivator-1α

PI3K = phosphoinositide 3-kinase

PKB = proteïna quinasa B

PKC = proteïna quinasa C

PREDIMED = PREvenció amb Dieta MEDiterrània

PV+ = valor predictiu positiu

PV- = valor predictiu negatiu

ROC = receiver operating characteristic

ROS = especies reactives de l'oxígen

SE = sensibilitat

SGLT-1 = sodium-glucose transport protein-1

SIRT= sirtuïna

SLe^x = Sialyl-$Lewis^x$

SP = Especificitat

SPE = extracció en fase sòlida

SR-A = scavenger receptors class A

TAC = capacitat antioxidant total

TCA = taules de composició dels aliments

TEAC = trolox equivalence antioxidant capacity

Tmax = temps en que s'arriba a la concentració màxima en plasma

TNFα = factor de necrosis tumoral-α

TPTZ = 2,4,6-tripyridyl-s-trizine

TRAP = total radical-trapping antioxidant parameter

UDP = uridina difosfat

UGT = UDP glucuronosil transferasa

VLA-4 = very late activation antigen-4

VLDL = lipoprotena de molt baixa densitat

ÍNDEX

INTERÈS

1. INTERÈS

Les bondats del vi ja foren descrites per Hipòcrates, pare de la medicina moderna, qui afirmava que "el vi és cosa admirablement apropiada per l'home, tant en l'estat de salut com en el de malaltia, si s'administra oportunament i amb la justa mesura, depenent de la constitució individual".

Tot i que al llarg de la història s'han descrit els efectes beneficiosos del vi, no va ser fins l'any 1992 quan Renaud i Lorgeril (Renaud and de Lorgeril, 1992) van publicar l'article sobre la "Paradoxa Francesa", en el qual el tema va adquirir una major rellevància científica. Els autors van portar a terme un estudi epidemiològic que va posar de manifest, com França un país amb un elevat risc cardiovascular degut al seu gran consum de greixos saturats, similar al d'altres països desenvolupats no mediterranis, presentava una mortalitat cardiovascular inferior a ells, més propera a altres països mediterranis. Els autors van proposar com a factor protector cardiovascular el consum moderat de vi, el que va millorar notablement el model matemàtic de regressió passant d'un coeficient de correlació de 0.73 a un de 0,87.

A partir d'aquesta observació s'han portat a terme nombrosos estudis clínics (Estruch et al., 2004;Badia et al., 2004;Sacanella et al., 2007;Vazquez-Agell et al., 2007) epidemiològics (Mukamal et al., 2003;Gronbaek, 2002) i revisions bibliogràfiques (Urquiaga and Leighton, 2005;Goldfinger, 2003;Burns et al., 2001) que intenten dilucidar quines són concretament els seus efectes beneficiosos, quins mecanismes es troben afectats i finalment quin component o components del vi (etanol, polifenols o sinergies entre ells) són els responsables d'aquestes accions.

Per arribar a conèixer els possibles efectes del vi, o dels seus components, és necessari estimar de forma precisa la ingesta dels mateixos. En assajos clínics controlats on l'investigador pot conèixer i controlar la majoria de les variables, aquest no és un factor tan determinant, però es tracta d'un factor limitant en estudis epidemiològics o en assajos clínics amb participants que fan vida normal.

Amb aquesta finalitat, en epidemiologia nutricional, existeixen las enquestes alimentàries i els biomarcadors nutricionals. Les enquestes són realitzades mitjançant entrevista personal o qüestionaris autoadministrats en els que els voluntaris han de recordar, normalment, la seva ingesta actual (últimes 24h) o habitual (últims 6 mesos o 1 any) tant de quin tipus d'aliment o beguda com de quantitat consumida (Arija Val and Fernández Ballart, 2002). Aquestes estimacions solen tenir biaixos, degut a que l'enquestat pot infraestimar la ingesta d'aliments

que considera poc saludables, com poden ser las begudes alcohòliques, o sobrevalorar el consum d'aliments que considera saludables, como poden ser fruites i verdures (Spencer et al., 2008). També és molt habitual el biaix anamnèsic, per no recordar de forma precisa la totalitat de la seva dieta.

Degut a que las enquestes alimentàries presenten diverses limitacions els investigadors cerquen opcions alternatives para estimar de forma més objectiva la ingesta dels participants d'un estudi. Una alternativa a aquests qüestionaris són els biomarcadors nutricionals, els quals presenten unes avantatges evidents respecte a les enquestes (Marshall, 2003;Potischman, 2003;Spencer et al., 2008), tot i que com totes les metodologies també tenen els seus punts febles. Els biomarcadors poden presentar tres avantatges importants respecte a les enquestes: i) és una mesura més precisa i objectiva; ii) les enquestes es transformen en components mitjançant les taules de composició d'aliments, les quals, en si mateixes presenten limitacions degut a la gran variabilitat de composició i de tipus d'aliments que hi ha en el mercat; iii) és una mesura que reflexa l'estatus nutricional de l'individu, que tenen en compte la biodisponibilitat dels components. Contràriament, les limitacions més importants dels biomarcadors són: el cost, molt més elevat que qualsevol tipus d'enquesta alimentària i la disponibilitat d'un biomarcador robust i validat per mesurar la ingesta d'un aliment o d'un component determinat.

Un bon marcador biològic ha de complir uns estrictes requisits per ser considerat com a tal, els que Spencer *et al* detallen en una recent revisió bibliogràfica (Spencer et al., 2008): i)metodologia d'anàlisi robusta; ii) sensible; iii) específic; iv) i coneixement sobre els seus paràmetres farmacocinètics.

La nostra hipòtesis de partida comença amb la selecció de potencials marcadors biològics de consumo de vi. Entre els possibles compostos destaca el resveratrol que és un component característic del raïm i dels seus productes derivats (Andres-Lacueva et al., 2002;Cantos et al., 2002b;Lamuela-Raventós et al., 1995;Romero Perez et al., 1996), i que es troba present únicament en petites quantitats en pocs aliments (cacauets, festucs i algunes fruites del bosc) (Burns et al., 2002;Rimando et al., 2004;Sobolev and Cole, 1999;Tokusoglu et al., 2005). Al 1997, la revista Science va publicar per primera vegada els efectes anticancerígens del resveratrol (Jang et al., 1997) i a continuació van sorgir diversos treballs que han atribuit al resveratrol, múltiples propietats beneficioses (Baur and Sinclair, 2006) (**Fig 1**) relacionades amb la seva potencial activitat antioxidant, antiinflamatòria, vasodilatadora, antiagregant plaquetària, anticancerígena i últimament s'ha demostrat que també podria actuar com a

mimètic de la restricció calòrica (Alarcón de la Lastra and Villegas, 2005;Baur and Sinclair, 2006;Delmas et al., 2005a;Delmas et al., 2006).

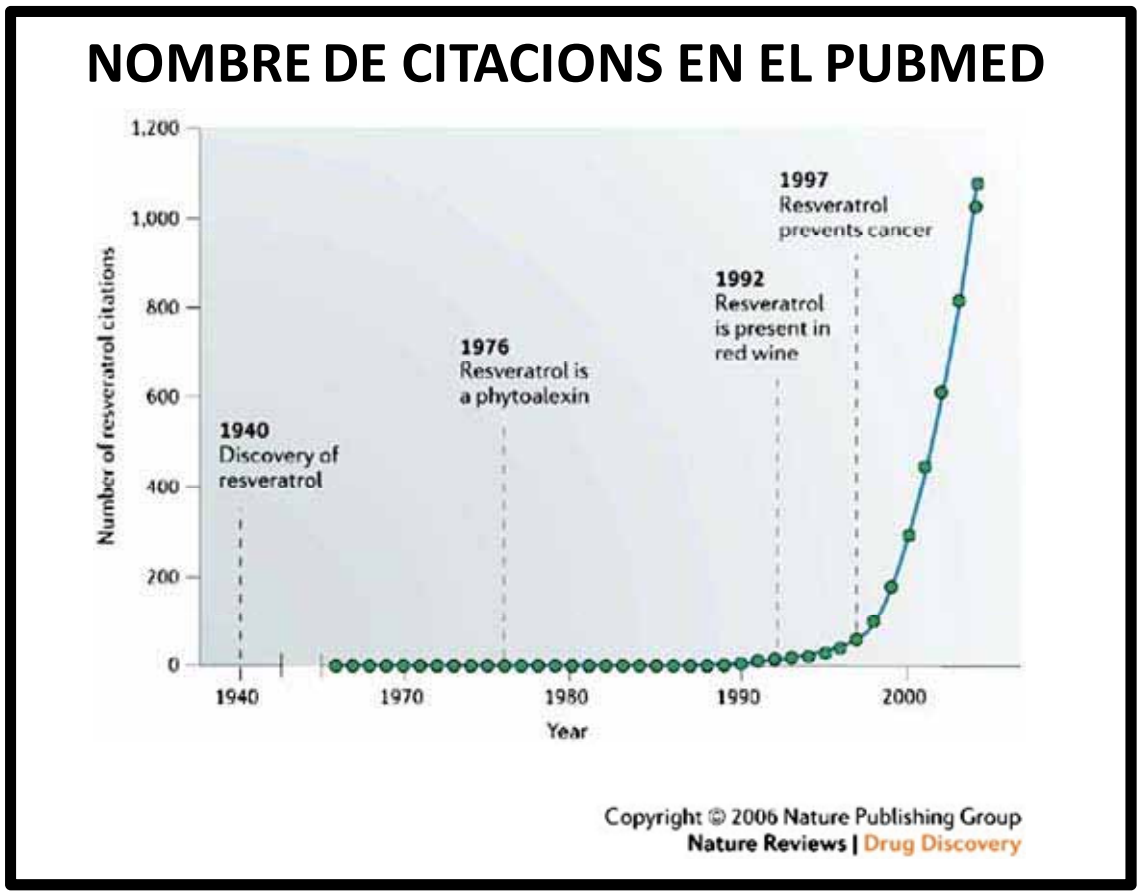


Figura 1. Nombre de publicacions aparegudes en el PubMed per any de la paraula "resveratrol", i els events més destacats en la història científica del mateix (Baur and Sinclair, 2006).

OBJECTIUS

2. OBJECTIUS

1. Determinar els metabòlits del resveratrol en teixits d'humans després d'un consum moderat de vi.
2. Establir un biomarcador del consum moderat de vi en humans.
3. Elaborar una taula de composició de resveratrol en aliments, específica per aliments d'origen espanyol.
4. Conèixer les fonts dietètiques més importants del consum de resveratrol i estimar la ingesta en la població adulta espanyola.
5. Valorar la utilitat del biomarcador de consum de vi en estudis clínics com a factor de acompliment del tractament.
6. Avaluar l'estat antioxidant de l'organisme i estudiar possibles mecanismes endògens de control.

ANTECEDENTS BIBLIOGRÀFICS

3. INTRODUCCIÓ

3.1. Compostos fenòlics

3.1.1. GENERALITATS

Els compostos fenòlics, químicament poden ser definits com a substàncies que tenen un mínim d'un anell aromàtic substituït amb almenys un grup hidroxil. Constitueixen el grup més nombròs i està àmpliament distribuït en el món vegetal, amb més de 8000 estructures conegudes (Harborne, 1989) i són productes del metabolisme secundari de les plantes que provenen de la via metabòlica del àcid siquímic i del acetat (Harborne, 1989).

Durant dècades els compostos fenòlics han estat coneguts per ésser responsables en part, de diverses propietats sensorials en aliments d'origen vegetal (Tomás-Barberán, 2003). Entre els pigments fenòlics destaquen les antocianines que aporten les tonalitats vermelloses, bleves i liles típiques de moltes fruites, hortalisses i derivats, com el vi negre. També és característica la astringència d'algunes fruites, hortalisses i derivats gràcies a la presencia dels tanins condensats (proantocianidines). Fins i tot alguns fenols senzills són responsables d'aromes característics d'algunes fruites (eugenol en plàtans).

Posteriorment aquest compostos fitoquímics van tenir la seva cabuda en els tratats de fisiologia vegetal, especialment por estar involucrats en el creixement i reproducció de plantes actuant com a fitoalexines (Bravo, 1998). Les fitoalexines són compostos sintetitzats per les plantes en resposta a infeccions o algun altre tipus d'estrès.

En el camp de la nutrició, l'interès d'aquests compostos fenòlics es basa en els possibles efectes beneficiosos per a la salut que la bibliografia els hi ha atribuït (Baur and Sinclair, 2006;Delmas et al., 2005a;Delmas et al., 2006;Fremont, 2000). Estudis epidemiològics han suggerit associacions entre la ingesta d'aliments rics en polifenols i la prevenció de malalties relacionades amb procesos oxidatius i de inflamació (malalties degeneratives, cardiovasculars, càncers) (Scalbert and Williamson, 2000;Arts and Hollman, 2005;Kampa et al., 2007).

3.1.2. CLASSIFICACIÓ

Les substàncies fenòliques constitueixen un grup molt nombrós de compostos que inclouen famílies de compostos amb estructures molt diverses, algunes simples com els àcids fenòlics i altres de molt complexes com els tanins. Els polifenols poden ser classificats en subgrups

segons els seus grups funcionals (**Taula 1**) o la seva estructura base química (**Taula 2**) (Harborne, 1989).

Taula 1: Classificació dels compostos fenòlics segons els seus grups funcionals.

Tipus	Grup	Exemples
No flavonoids	Alcohols fenòlics	Tirosol
	Àcids benzoics	Àcid gàlic, àcid protocatèquic, àcid vainíllic
	Aldehids benzoics	Vainillina, sirigaldehid
	Àcidos cinàmics	*Trans* -cafèic, *para* -cumàric, *trans* -ferúlic
	Ésters dels àcids cinàmics	Àcid cutàric, àcido caftàric, àcid fertàric, àcid clorogènic
	Aldehids cinàmics	Coniferaldehid, sinapaldehid
	Tanins hidrolitzables	Galotanins, elagitanins
	Fenols volàtils	Eugenol, 4-vinil-guaiacol
	Cumarines	Escopoletina
	Lignans	Pinoresinol
	Secoiridoids	Ligstròsids, oleuropeïna
	Estilbens	Resveratrol, Piceid, Viniferines
Flavonoids	Flavones	Apigenina, luteolina
	Flavonols	Quercetina, kampferol
	Flavanones	Naringenina, hesperidina
	Flavanols o flavan-3-ols	Catequina, epicatequina
	Antocianidines	Malvidina, pelargonidina
	Isoflavones	Genisteïna, daidzeïna
	Flavanonols	Dihidrofisetina, dihidroquercetina
	Proantocianidines o tanins condensats	Proantocianidina B2
	Xalcones	Buteïina, floretina

Taula 1: Classificació dels compostos fenòlics segons Harbone (Harborne, 1989)

Compostos fenòlics	Estructura base
Fenols simples	C_6
Àcids hidroxibenzoics	C_6-C_1
Acetofenones i àcids fenilacètics	C_6-C_2
Àcids hidroxicinàmics, cumarines, cromones	C_6-C_3
Lignans	$(C_6$-$C_3)_2$
Lignines	$(C_6$-$C_3)_n$
Naftoquinones	C_6-C_4
Benzofenones, xantones	C_6-C_1-C_6
Estilbens, antraquinones	C_6-C_2-C_6
Flavonoids	C_6-C_3-C_6
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Proantocianidines o tanins condensats	$(C_6$-C_3-$C_6)_n$

Dels subgrups principals (Tomás-Barberán, 2003), els que presenten les estructures més simples són els àcids hidroxicinàmics i els àcids hidroxibenzoic. Seguint l'ordre de complexitat estructural es troben els estilbens. A continuació apareixen els flavonoids, grup molt nombrós del que es conèixen més de 5000 compostos (Harborne, 1989). La seva estructura bàsica és la del 2-fenilbenzopirona (C6-C3-C6) (**Figura 2**) i es classifiquen en 8 subclasses: flavones, flavonols, flavanones, flavanols o flavan-3-ols, antocianidines, isoflavones, flavanonols i xalcones (Ross and Kasum, 2002). També s'han de destacar alguns lignans. Finalment, són també d'interès els tanins, polímers complexes classificats en base a la seva ruta de biosíntesi i a les seves propietats químiques en tanins condensats i tanins hidrolitzables. Els primers són també anomenats proantocianidines, i com el seu nom indica són polímers d'alt pes molecular de les antociandines. Els segons són polímers heterogenis formats per àcids fenòlics, en

particular àcid gàlic, i sucres simples. Són més petits que els tanins condensats i són hidrolitzats amb major facilitat.

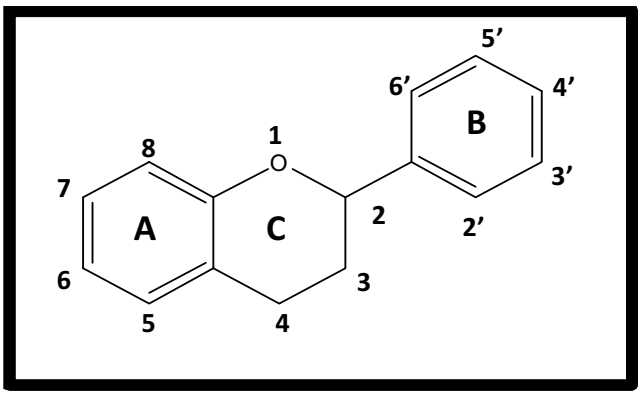


Figura 2. Esquelet base (C6-C3-C6) del que deriven tots els flavonoids.

Dado que este trabajo de tesis doctoral está focalizado en resveratrol, piceido y demás estilbenos, le dedicaremos un apartado especial a esta familia de compuestos.

3.2. Estilbens/Resveratrol

3.2.1. BIOSÍNTESI DEL RESVERATROL EN PLANTES

L'estructura química bàsica dels estilbens és la de 1,2-difeniletilé, d'ells el més important és el resveratrol (3,4',5-trihidroxiestilbé). Els estilbens presenten 2 estereoisòmers: les formes *cis* (*Z*) i les formes *trans* (*E*). Les formes *trans*- poden isomeritzar-se en les formes *cis*- quan ' exposen a les radiacions ultraviolades (Lamuela-Raventós et al., 1995).

El resveratrol és produït en algunes plantes superiors mitjançant l'enzim estilbeno sintetasa. Aquest transforma una molècula de *p*-coumaroil-CoA i tres molècules de malonil-CoA en el resveratrol. Els mateixos precursors son els emprats com a substrat per la xalcona sintetasa en la ruta metabòlica dels flavonoids (**Figura 3**).

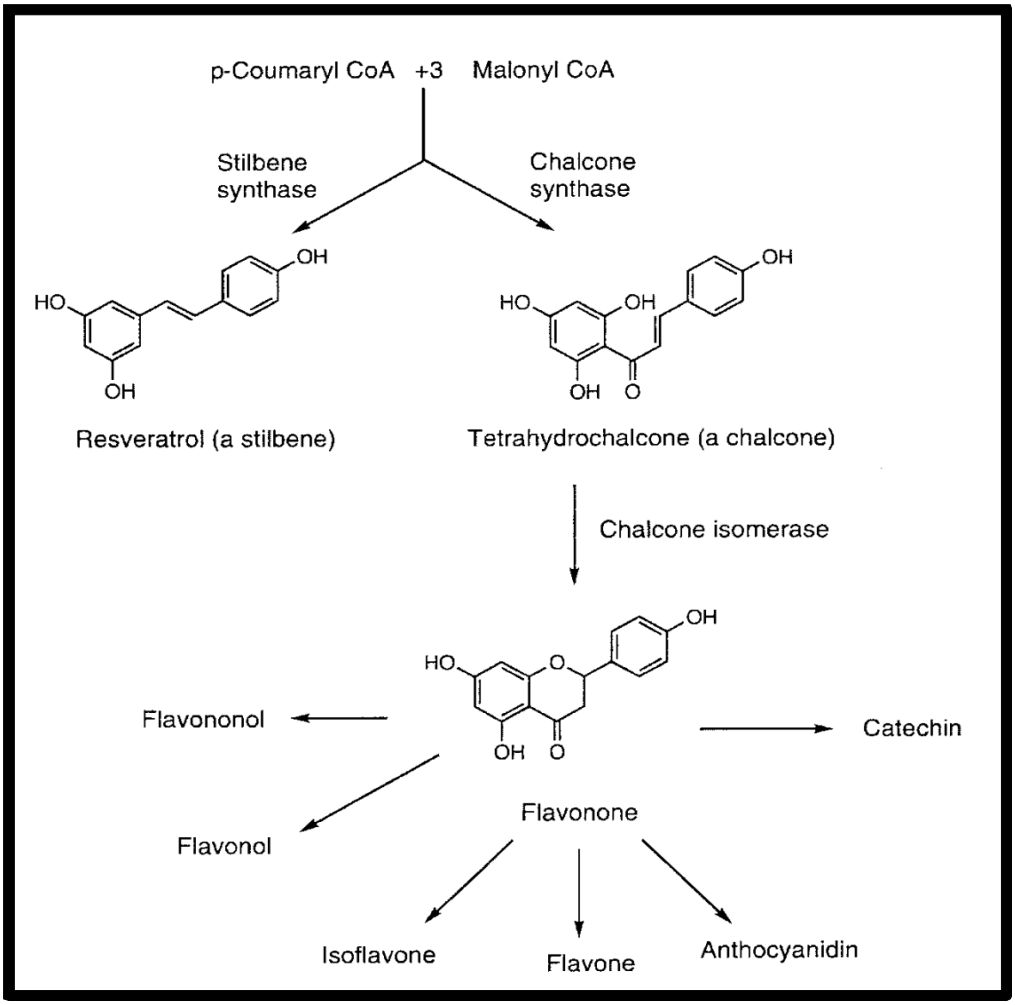
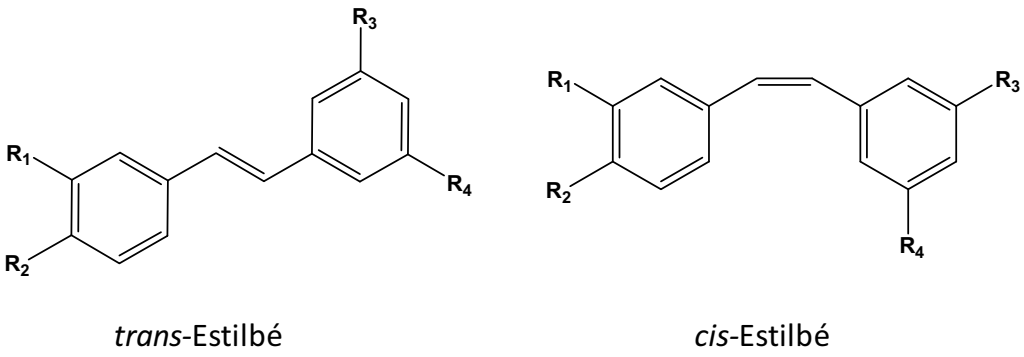


Figura 3. Ruta de biosíntesi del resveratrol i dels flavonoids en plantes (Shahidi and Naczk, 2004).

El piceid (resveratrol glucòsid) és el derivat majoritari del resveratrol present en plantes. La glucosidació està associada freqüentement a la necessitat d'incrementar l'emmagatzematge dels compostos polifenòlics.

D'altres estilbens, d'estructura similar al resveratrol, com el picetanol, pteroestilbé, rapontigenin, raponticin, pinosilvin, del quals es presenten les seves estructures químiques en la **Figura 4.** D'aquests també s'han estudiat per les seves potencials propietats beneficioses sobre malalties com la cardiovasculars o el càncer (Roupe et al., 2006)



trans-Estilbé *cis*-Estilbé

Estilbens	R_1	R_2	R_3	R_4
Resveratrol	H	OH	OH	OH
Piceid	H	OH	OH	*O*-Glucosa
Rapontigenina	OH	OCH_3	OH	OH
Raponticina	OH	OCH_3	OH	*O*-Glucosa
Picetanol	OH	OH	OH	OH
Pinosilvina	H	H	OH	OH
Pterostilbé	H	OH	OCH_3	OCH_3

Figura 4. Estructures químiques dels estilbens (formes *trans*- y *cis*-)

L'enzim estilbeno sintetasa no es troba únicament en el gènere *Vitis*, sinó que també es troba present en almenys 72 espècies en el regne vegetal, però d'aquestes només el raïm (Cantos et al., 2002b;Romero-Perez et al., 2001;Adrian et al., 2000), cacauets (Sobolev and Cole, 1999;Sanders et al., 2000;Lee et al., 2004;Chukwumah et al., 2007), festucs (Tokusoglu et al., 2005;Gentile et al., 2007) i algunes baies (Burns et al., 2002;Lyons et al., 2003;Rimando et al.,

2004;Wang et al., 2002c) es troben presents habitualment en la nostra dieta. En altres aliments i plantes també es pot localitzar resveratrol i/o piceid en quantitats variables: te itadori (arrel del tubercle *Polygonum cuspidatum* infusionada en te) (Burns et al., 2002), llúpol (*Humulus lupulus*) (Jerkovic et al., 2005;Callemien et al., 2005), alguna varietat de tomàquet (*Solanum lycopersicum*) (Ragab et al., 2006), Iuca (*Yucca schidigera*) (Oleszek et al., 2001), *Polygonum cuspidatum* (Gao et al., 2002), *Caragana sínica* (Shu et al., 2006), Ruibarbre (*Rheum undulatum*) (Matsuda et al., 2001), eucaliptus (*Eucaliptus sp.*) (Hillis et al., 1974), *Pterolobium hexapetallum* (Kumar et al., 1988), *Bauhinia racemosa* (Anjaneyulu et al., 1984), *Veratrum grandiflorum* (Hanawa et al., 1992). La producció d'aquesta fitoalexina està molt estimulada per l'estrès biòtic (Romero-Perez et al., 2001) i l'abiòtic (Rudolf et al., 2005;Chung et al., 2003) que pateix la planta o el fruit, incrementant la seva producció en cas d'infeccions fúngiques, exposició a radiacions UV (Cantos et al., 2001;Cantos et al., 2002a;Cantos et al., 2003) i al tractament amb ozó (Gonzalez-Barrio et al., 2006). La importància biològica a nivell vegetal dels estilbens, en general, i del resveratrol, en particular, es degut a la seva activitat antifúngica.

En l'actualitat s'està investigant la transferència del gen que codifica a l'estilbeno sintetasa a altres especies vegetals com el tabac (Hain et al., 1993), l' arròs (Stark-Lorenzen et al., 1997), el kiwi (Kobayashi et al., 2000), l'alfals (Hipskind and Paiva, 2000), l'ordi (Leckband and Lorz, 1998), el blat (Leckband and Lorz, 1998), la poma (Ruhmann et al., 2006;Szankowski et al., 2003), l'enciam (Liu et al., 2006), o el tomàquet (Nicoletti et al., 2007) per augmentar la resistència endògena a patògens d'origen fúngic. Aquestes plantes transgèniques encara es troben en fase experimental i, per aquesta raó, està prohibit el seu us en alimentació humana o animal.

3.2.2. FONTS ALIMENTÀRIES I CONSUM DE RESVERATROL

Els estilbens majoritaris en els aliments són el resveratrol i el piceid, en les seves formes *cis* i *trans*. Com s'ha comentat en l'apartat anterior, fins al moment s'ha detectat el resveratrol i/o el piceid en almenys 72 especies vegetals, sense tenir en compte les noves plantes transgèniques.

El resveratrol es va detectar per primer cop al 1988 en pells de raïms (Creasy and Coffee, 1988) i posteriorment en el vi negre (Goldberg et al., 1995;Lamuela-Raventós et al., 1995;Mattivi et al., 1995;Moreno-Labanda et al., 2004;Rodriguez-Delgado et al., 2002). En la dècada dels 90 la

presència de resveratrol es va ampliar a altres derivats del raïm com el most (Romero-Perez et al., 1999;Vinas et al., 2000), vi rosat (Romero Perez et al., 1996), blanc (Álvarez-Sala et al., 2000;Martinez-Ortega et al., 2000;Rodriguez-Delgado et al., 2002;Romero Perez et al., 1996) i cava (Andres-Lacueva et al., 2002;Pozo-Bayon et al., 2003). Entre 1993-1995 es descubreix el piceid en pells de raïm i en els productes vínics, comprovant que la forma glucosilada en molts casos és la majoritaria (Romero-Perez et al., 1999). Posteriorment els investigadors s'han centrat en apreciar diferències en el contingut d'estilbens deguts al raïm: varietats (Cantos et al., 2002b;Romero-Perez et al., 2001;Adrian et al., 2000), estat de maduració, climatologia, sòls (efecte *terroir*) (de Andres-de et al., 2007), grau d'infecció fúngica (Romero-Perez et al., 2001), i deguts a les diferents tecnologies utilitzades per a l'elaboració dels vins (Jeandet et al., 1995). D'aquesta forma, fins a finals de la dècada dels 90, les fonts alimentàries conegudes del resveratrol i piceid eren exclusivament el raïm i els derivats. Posteriorment el ventall d'aliments en que s'ha cercat i s'ha detectat el resveratrol i el piceid ha anat incrementant-se a alguna fruita seca com els cacauets (Sobolev and Cole, 1999;Sanders et al., 2000;Lee et al., 2004;Chukwumah et al., 2007), la crema de cacauet (Ibern-Gomez et al., 2000) i els fefstucs (Tokusoglu et al., 2005;Gentile et al., 2007), les fruites del bosc com els nabius, groselles o mores (Burns et al., 2002;Lyons et al., 2003;Rimando et al., 2004;Wang et al., 2002c), te itadori (arrel del tubercle *Polygonum cuspidatum* infusionada en te, és molt poc consumit) (Burns et al., 2002) o el llúpol (Jerkovic et al., 2005;Callemien et al., 2005). En tots els casos, a excepció del te (0.974mg/100g), les concentracions trobades són molt inferiors, entre 0.006-0.008mg/100g, a les descrites en el vi negre (0.847mg/100g).

La ingesta de resveratrol només ha estat estudiada per Levi *et al* (Levi et al., 2005) el qual investiga la relació entre el consum dietètic de *trans*-resveratrol i càncer de mama. D'aquest estudi únicament es disposen dels tertils, el segon tertil en el que està inclosa la mediana de la ingesta de raïm i vi aporten 72.3-126.4μg/d i 0.1-176.8μg/d respectivament. En aquesta tesis doctoral es presentaran les úniques dades disponibles de consum de resveratrol i piceid en humans en la cohort EPIC-Espanya (Zamora-Ros et al., 2007) on es va poder comprovar que el 98% del resveratrol prové de la ingesta de vi i la mitjana de la ingesta total és inferior als 137μg/d per a les dones i 686μg/d pels homes, perquè s'inclouen el resveratrol i el piceid (tant en les seves formes *cis* com *trans*).

3.2.3. BIODISPONIBILITAT

Existeixen pocs estudis sobre biodisponibilitat en humans del resveratrol, por aquest motiu són imprescindibles els treballs realitzats *in vitro*, *ex vivo* i en animals d'experimentació, per a obtenir un major coneixement sobre l'absorció, el transport, el metabolisme i l'excreció del mateix. La **figura 5** esquematitza el coneixement actual sobre la biodisponibilitat del resveratrol.

El resveratrol s'absorbeix en el intestí prim, en major quantitat en el jejú que a l'ili, en aquest últim s'absorbeix únicament un 38% de la quantitat que pot travessar el jejú (Kuhnle et al., 2000). Estudis de perfusió de resveratrol en l'intestí prim aïllat no s'observen diferencies entre les recuperacions a diferents dosis (Andlauer et al., 2000). En canvi en cèl·lules Caco-2 apareix una absorció dependent de la concentració a les tres primeres hores i particularment de forma lineal durant la primera hora (Kaldas et al., 2003;Maier-Salamon et al., 2006), tot i que després de tres hores d'incubació, la concentració de resveratrol assoleix una planúria (Henry et al., 2005;Maier-Salamon et al., 2006). A concentracions altes de resverarol, la concentració en cèl·lules Caco-2 s'incrementa, confirmant que els sistemes de transport no es saturen (Henry et al., 2005).

L'efecte matriu en l'absorció del resveratrol ha estat provat al ingerir el polifenol dissolt en tres diferents matrius (vi, suc de raïm o suc de verdures) (Goldberg et al., 2003) o barrejat amb diversos tipus d'àpats (estàndard, greixosa o magra) (Vitaglione et al., 2005) i s'ha demostrat que no presenta diferències significatives en la biodisponibilitat en humans. La forma glucosilada del resveratrol, el piceid, pot ser hidrolitzat per la β-glucosidasa ("*Lactase Phlorizin Hydrolase*") abans d'ésser absorbit pel enteròcit o pot entrar al enteròcit i allà ser hidrolitzat per la β-glucosidasa ("*Cytosolic- β-Glucosidasa*") (Henry-Vitrac et al., 2006). El piceid també pot ser absorbit directament como a tal, tot i que la quantitat i velocitat és 4 vegades menor que l'aglicona en cèl·lules Caco-2 (Henry et al., 2005). En estudis en humans, Meng *et al* conclou que el resveratrol estàndard s'absorbeix en major quantitat que el piceid aportat pel suc de raïm i per aquest motiu es troben diferents recuperacions en orina quan es comparen les mateixes dosis en les dos formes (Meng et al., 2004). El mecanisme de trasport del resveratrol en els enteròcits ha estat dilucidat mitjançant l'estudi en cèl·lules Caco-2, en les quals es va comprovar que el *trans*-resveratrol travessa la membrana apical per transport pasiu, en canvi el piceid utilitza el transportador actiu sodi-dependent SGLT1, tot i que el MRP2 també sembla

estar involucrat en la regulació del flux (Henry et al., 2005). El resveratrol i el piceid són ràpidament metabolitzats per el enteròcit transformant-los majoritàriament en les seves corresponents formes glucuronidades i sulfatades del resveratrol. Les formes sulfatades en les cèl·lules Caco-2 són majoritàries (Kaldas et al., 2003;Maier-Salamon et al., 2006), en canvi en els experiments ex vivo amb perfusions en intestí prim aïllat, són les formes glucuronidades les més abundants (Andlauer et al., 2000;Kuhnle et al., 2000).

A baixes concentracions de resveratrol (10μM) la conjugació és quasi total (84%) en cèl·lules Caco-2, mentrestant a altes dosis (200μM) la metabolització és solament del 7.6%, això pot ser degut a la saturació o a la inhibició de les vies enzimàtiques a altes concentracions d'estilbens (Maier-Salamon et al., 2006). S'ha comprovat que gran part de resveratrol, sobre tot a altes concentracions, s'acumula en els enteròcits (fins a un 60%) arribant a ser el major òrgan diana on exercir els seus potencials efectes beneficiosos (Kaldas et al., 2003;Maier-Salamon et al., 2006). El resveratrol no absorbit a nivell de l'intestí prim arriba al còlon, lloc on pot ésser metabolitzat per la microbiota intestinal i absorbit en forma de dihidroresveratrol, conjugant-se posteriorment en els seus respectius glucurònids i sulfats (Walle et al., 2004). Finalment el resveratrol no absorbit pot trobar-se en femtes en un porcentatge molt dispar entre 0.3 i 38.1% de la ingesta en humans (Walle et al., 2004).

Una vegada ja absorbit, el resveratrol és transportat via porta fins al fetge. Una vegada allà, el resveratrol aglicona és ràpidament metabolitzat en glucurònids i sulfats per hidrosolubilitzar la molècula i facilitar la futura excreció urinaria. Estudis en microsomes hepàtics han mostrat que les formes glucuronidades són majoritàries després de la incubació amb *trans*- i *cis*-resveratrol (Aumont et al., 2001;Brill et al., 2006). La glucuronidació en microsomes hepàtics és estèreo-selectiva, l'isòmer *cis* es conjuga entre 5 i 10 voltes més ràpid que l'isòmer *trans* (Aumont et al., 2001). També s'ha comprovat que la conjugació és regió-selectiva, la posició 3 es conjuga en major quantitat que la posició 4' en ambdós isòmers (Aumont et al., 2001). Els enzims hepàtics responsables de la glucuronidació del resveratrol són la UDP-glucuronosil transferasa família 1A (UGT1A), en particular la UGT1A1 pel *trans*-resveratrol-4'-*O*-glucurònid i la UGT1A9 pel *trans*-resveratrol-3-*O*-glucurónido. En estudis amb hepatòcits *in vitro*, els principals metabòlits són els glucurònids en humans mentre que en rata són els sulfats (Yu et al., 2002). En canvi de Santi *et al.* en biòpsies hepàtiques en humans va trobar que el resveratrol es millor substrat per les sulfotransferases (K_m 0.60μM) (de Santi et al., 2000c;de Santi et al., 2000b) que per les glucuronosil transferases (K_m 0.15μM) (de Santi et al., 2000a). Posteriorment el resveratrol pot arribar a ser excretat per la bilis, i ser reabsorbit a nivell intestinal. Aquesta

recirculació enterohepàtica provoca un segon augment en els nivells plasmàtics a partir de les 6 hores posteriors a la ingesta (Marier et al., 2002).

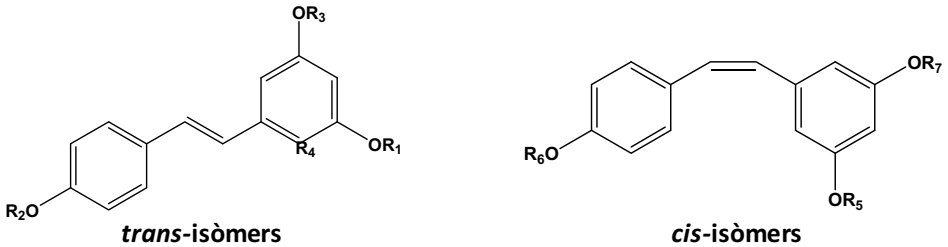
Finalitzada la seva etapa hepàtica, el resveratrol passa a circulació sistèmica, i pot ser transportat unit a cèl·lules sanguínies (Blache et al., 1997), eritròcits o plaquetes; i lipoproteïnes, LDL *in vitro* (Blache et al., 1997) i en humans (Urpi-Sarda et al., 2005;Urpi-Sarda et al., 2007), tot i que la major part (>50%) viatja unit a proteïnes plasmàtiques (Burkon and Somoza, 2008). La presència de resveratrol en plasma o sèrum en humans ha estat investigada en diversos estudis resumits en la **taula 3**. Les concentracions màximes (C_{max}) de resveratrol i dels seus metabòlits apareixen als 30-60 minuts després de la seva ingesta (Goldberg et al., 2003;Soleas et al., 2001), tot i que depenent del metabòlit poden aparèixer a les 3 hores (Boocock et al., 2007;Vitaglione et al., 2005) i fins i tot a les 6-8 hores (Burkon and Somoza, 2008). Cal destacar que únicament Vitaglione *et al.* Han estat capaços de detectar en plasma d'humans petites concentracions de resveratrol i dels seus metabòlits després de la ingesta de quantitats dietètiques de resveratrol (Vitaglione et al., 2005), altres autors no han detectat cap metabòlit en plasma o sèrum a dosis similars (Meng et al., 2004;Zamora-Ros et al., 2006). Els temps de vida mitjana del resveratrol i dels seus metabòlits en plasma oscil·la entre les 2.9 i les 11.5 hores tenint en compte el metabòlit i la dosis d'ingesta (Boocock et al., 2007), tot i que en un estudi es van arribar a detectar traces de radioactivitat a les 72 hores posteriors a la ingesta de ^{14}C-Resveratrol (Walle et al., 2004). El resveratrol-3-sulfat sembla ser el metabòlit més abundant (56%) , seguit de les 2 formes glucuronidades (39%) i només el resveratrol aglicó representa un 4.7% (Boocock et al., 2007). En un estudi recent s'ha ampliat el perfil metabòlic del resveratrol en plasma, sumant a les estructures esmentades amb anterioritat els diglucurònids i els disulfats (Burkon and Somoza, 2008) (**Figura 6)**.

Respecte a la posterior distribució del resveratrol en teixits. Els principals teixits diana, a part del tracte gastrointestinal (intestí prim i còlon), són els ronyons, el fetge i els pulmons, i en menor quantitat se pot localitzar en melsa, cor, cervell i testicles (Asensi et al., 2002;bd El-Mohsen et al., 2006;Vitrac et al., 2003).El resveratrol destaca per tenir la capacitat de travessar la barrera hematoencefàlica i poder exercir els seus efectes protectors a nivell del sistema nerviós central (Wang et al., 2002a).

La via d'excreció majoritària és l'orina (53-85%) i les femtes (0.3-38%) mesurades com a radioactivitat total a partir de la ingesta de resveratrol marcat (Walle et al., 2004). En canvi quan l'anàlisi es realitza emprant la medició directa del resveratrol i dels corresponents

metabòlits, les recuperacions en orina disminueixen fins el 5-37% de la dosis ingerida (Goldberg et al., 2003;Meng et al., 2004;Soleas et al., 2001;Urpi-Sarda et al., 2007;Walle et al., 2004;Zamora-Ros et al., 2006;Boocock et al., 2007;Burkon and Somoza, 2008). Les recuperacions del resveratrol ingerit en estudis en humans es troben resumides en la Taula 2. L'excreció urinària arriba al seu màxim a les 4 hores després del consum i representa aproximadament el 77% del total de l'excreció (Boocock et al., 2007). Tot i que el resveratrol i els seus metabòlits poden aparèixer en orina fins i tot 24 hores després del consum. El perfil metabòlic en orina ha anat ampliant-se paral·lelament al les millores i al desenvolupament de les tècniques analítiques utilitzades, actualment s'han identificat un total de 4 monoglucurònids (cis- i trans- en posicions 3 i 4'), 4 monosulfats (cis- i trans- en posicions 3 i 4'), 2 disulfats (trans-3,5- i trans-3,4'-), 2 diglucurònids (trans-2C/4'-O-diglucurònid i trans-2C/5-O-diglucurònid) resveratrol aglicó, dihidroresveratrol monoglucurònid i monosulfat (Figura 6 i 7). En orina, como sol succeir en plasma, semblen ser més abundants les formes sulfatades que las glucuronidades i en molt menor proporció l'aglicó.

METABÒLITS INTESTINALS



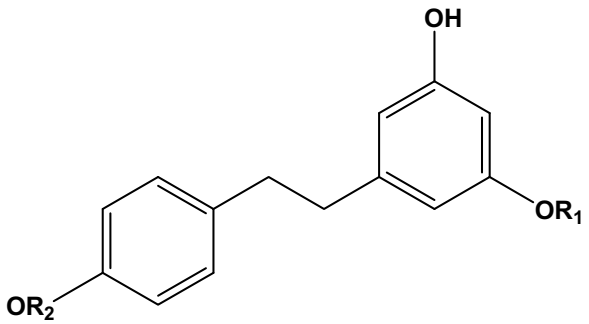
***trans*-isòmers** ***cis*-isòmers**

<u>*trans*-Isòmers</u>
***trans*-Resveratrol-3-*O*-glucurònid:** R1=àcid glucurònid; R2 yR3 y R4 =H
trans*-Resveratrol-4'-*O*-glucurònid*: R1=H; R2=àcid glucurònid; R3 y R4 =H
trans*-Resveratrol-2-C/4'-*O*-diglucurònid*: R1 y R3 =H; R2 y R4=àcid glucurònid
trans*-Resveratrol-2-C/5-*O*-diglucurònid*: R1 y R2 =H; R3 y R4=àcid glucurònid
***trans*-Resveratrol-3-sulfat:** R1=SO3H; R2=H; R3 y R4 =H
***trans*-Resveratrol-4'-sulfat:** R1=H; R2=SO3H; R3 y R4 =H
***trans*-Resveratrol-3,4'-disulfat:** R1 y R2 =SO3H; R3 y R4 =H
***trans*-Resveratrol-3,5-disulfat:** R1 y R3 =SO3H; R2 y R4= H

<u>*cis*-Isòmers</u>
***cis*-Resveratrol-3-*O*-glucurònid:** R5=àcid glucurònid; R6 y R7=H
cis*-Resveratrol-4'-*O*-glucurònid*: R5 y R7=H; R6=àcid glucurònid
***cis*-Resveratrol-3-sulfat:** R5=SO3H; R6 y R7=H
***cis*-Resveratrol-4'-sulfat:** R5 y R7=H; R6=SO3H

Figura 6. Estructura química dels metabòlits intestinals del resveratrol trobats en orina i/o plasma en estudis *in vivo.*

METABÒLITS DE LA MICROBIOTA



Dihidroresveratrol: R_1=H; R_2=H
Dihidroresveratrol-glucurònid: R_1=H o àcid glucurònid; R_2=H o àcid glucurònid
Dihidroresveratrol-sulfat: R_1=H o SO_3; R_2=H o SO_3

Figura 7. Estructura química dels metabòlits de la microbiota del resveratrol trobats en orina en estudis *in vivo* (Walle et al., 2004).

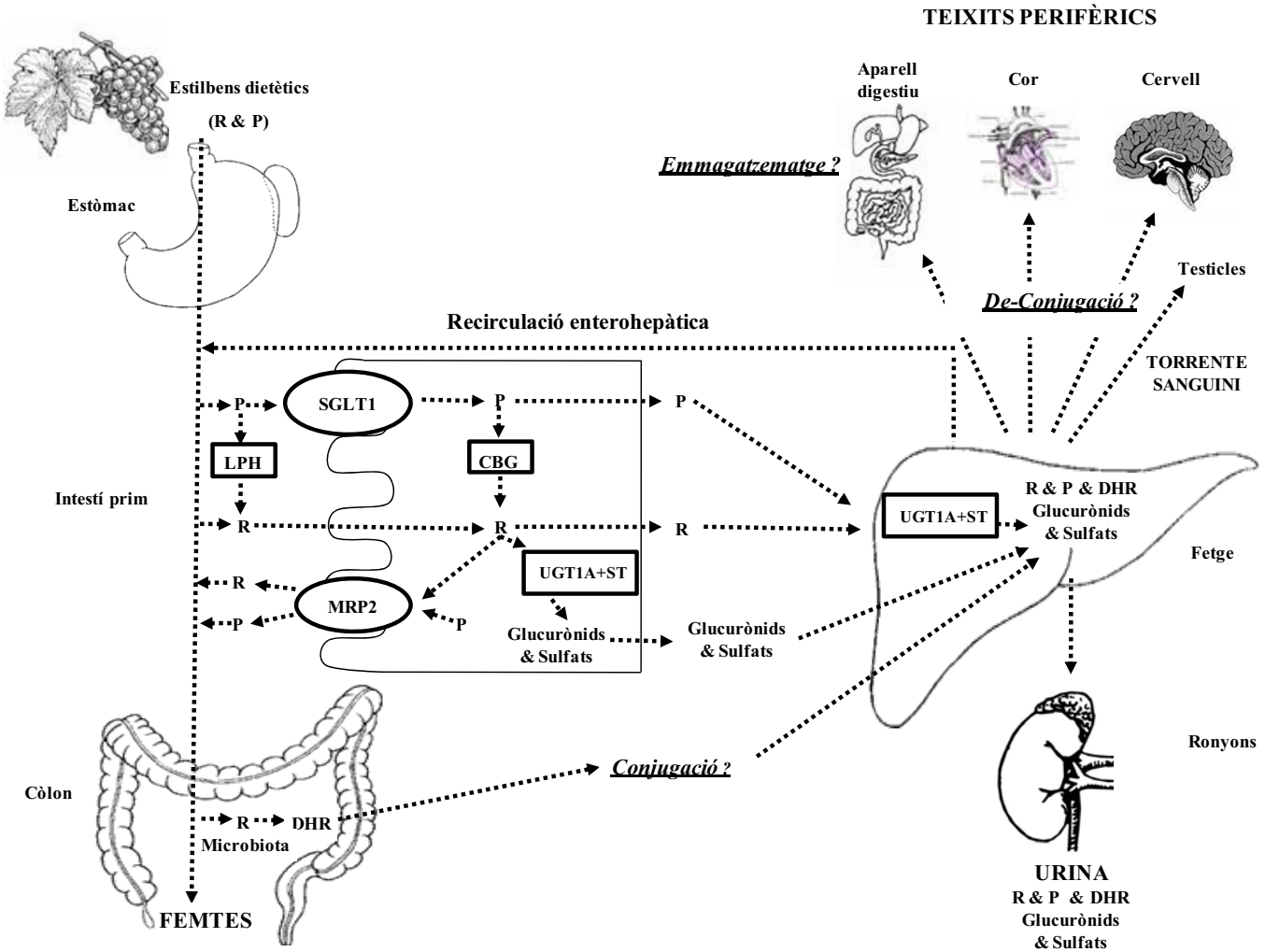


Figura 5. Esquema de l'absorció, metabolisme, distribució i excreció del resveratrol i piceid (R &P) de la dieta *in vivo.*
R:Resveratrol; P:piceid; DHR: dihidroresveratrol; LPH: lactase phlorizin hydrolase; CBG; cytosolic-β-glucosidasa; SGLT-1: sodium-Glucose transport proteins; MRP-2: multidrug resistance-associated protein 2; UGT: UDP glucuronosil transfersa; ST: sulfotransferasa

Taula 3. Estudis de biodisponibilitat de resveratrol en humans.

Nombre subjectes	Gènere	Edat	Font de Resveratrol	Dosis (mg/kg pes corporal)	C_{max} (μM/L)	t_{max} (h)	Excreció urinària (%)	Ref
10	Homes (45%)	19-61	Estàndard en 120mL de vi blanc	0.357mg/kg (25mg total)	Resv 0.031 Conjugats 338μg/L	0.5-1	Total 24.6% (24h)	(Soleas et al., 2001)
4	4 Homes (100%)	25-45	Estàndard disolt en 100mL suc vegetal	0.357mg/kg (25mg/70kg)	Resv 0.037 Conjugats 462.5μg/L	0.5	17.0 (24h)	(Goldberg et al., 2003)
4	4 Homes (100%)	25-45	Estàndard disolt en 100mL vi blanc	0.357mg/kg (25mg/70kg)	Resv 0.031 Conjugats 409.9μg/L	0.5	16.8 (24h)	(Goldberg et al., 2003)
4	4 Homes (100%)	25-45	Estàndard disolt en 100mL suc de raïm	0.357mg/kg (25mg/70kg)	Resv 0.035 Conjugats 416μg/L	0.5	16.0 (24h)	(Goldberg et al., 2003)
1	1 Home	30-50	Estàndard	0.03mg/kg	n.d.		Gluc 52 (24h)	(Meng et al., 2004)
1	1 Home	30-50	Estàndard	0.5mg/kg	n.d.		Gluc 34 (24h)	(Meng et al., 2004)
1	1 Home	30-50	Estàndard	1mg/kg	Gluc 1.86	1.5	Gluc 26 (24h)	(Meng et al., 2004)
1	1 Home	30-50	200mL suc de raïm	0.005mg/kg (0.32mg total)	n.d.		n.d. (24h)	(Meng et al., 2004)
1	1 Home	30-50	400mL suc de raïm	0.009mg/kg (0.64mg total)	n.d.		n.d. (24h)	(Meng et al., 2004)

1	1 Home	30-50	600mL suc de raïm	0.014mg/kg (0.96mg total)	n.d.		Cuantificado (24h)	(Meng et al., 2004)
1	1 Home	30-50	1200mL suc de raïm	0.027mg/kg (1.92mg total)	n.d.		Gluc 5.0(24h)	(Meng et al., 2004)
6	3 Homes (50%)	23-34	^{14}C-resveratrol ORAL	0.385mg/kg (25mg total)			53.4-84.9% (72h)	(Walle et al., 2004)
5		23-34	^{14}C-resveratrol INTRAVENÓS	0.023mg/kg (1.5 mg total)			42.3-83.2% (72h)	(Walle et al., 2004)
1			Estàndard ORAL	1.538mg/kg (100mg total)	Tr.		Gluc 13 (1) Sulf 24 (3) Total 37 (12h)	(Walle et al., 2004)
10	10 Homes (100%)	30 (25-40)	300mL vi negre Lambrusco + àpat	0.0034μg/kg	Gluc 0.096	1		(Vitaglione et al., 2005)
5	1 Homes (20%)	29 (24-38)	600mL vi negre Cabernet Franc	0.0329μg/kg	Gluc 0.687	0.5-2		(Vitaglione et al., 2005)
10	3 Homes (30%)	31 (24-54)	600mL vino tinto Aglianico + àpat	0.0075μg/kg	Resv 0.004 Gluc 0.150	0.5 1-2		(Vitaglione et al., 2005)
5	5 Homes (100%)	25-28	250mL vi negre	0.077mg/kg (5.4mg total)			t-4' Gluc 0.13 (0.19) t-3 Gluc 0.38 (0.59) c-4' Gluc 0.75 (1.2) c-3 Gluc 1.9 (1.9) t-4' Sulf 0.01 (0.03)	(Urpi-Sarda et al., 2007)

							t-3 Sulf 0.16 (0.67) c-4' Sulf 19.6 (17.4) c-3 Sulf 0.47 (2.2) Total 23.4 (4h)	
10	Homes (45%)	19-61	Estàndard	7.14mg/kg (0.5g total)	Rev 0.32 (0.16) Gluc 1 1.00 (0.35) Gluc 2 0.91 (0.36) 3-Sulf 3.69 (0.95)	0.833 (0.5-1.5) 2.00 (1.0-6.0) 1.50 (1.0-5.0) 1.50 (1.0-5.0)	0.04 (0.05) 2.0 (0.4) 8.9 (2.6) 11.4 (2.3) Total 22.34 (24h)	(Boocock et al., 2007)
10	Homes (45%)	19-61	Estàndard	14.29mg/kg (1g total)	Resv 0.51 (0.38) Gluc 1 1.17 (0.90) Gluc 2 1.66 (1.35) 3-Sulf 6.82 (21.39)	0.759 (0.5-4.0) 2.25 (1.0-6.0) 1.75 (1.0-5.1) 2.00 (1.0-5.0)	0.1 (0.1) 2.1 (1.1) 3.2 (1.7) 7.3 (3.1) Total 12.7 (24h)	(Boocock et al., 2007)
10	Homes (45%)	19-61	Estàndard	35.71mg/kg (2.5g total)	Resv 1.18 (0.65) Gluc 1 2.16 (0.81) Gluc 2 4.02 (2.88) 3-Sulf 9.05 (2.46)	1.375 (0.5-4.0) 2.375 (1.0-8.0) 2.00 (1.0-6.0) 2.00 (1.0-5.2)	0.1 (0.1) 1.7 (1.7) 3.1 (1.4) 5.2 (2.6) Total 10.1 (24h)	(Boocock et al., 2007)
10	Homes (45%)	19-61	Estàndard r	71.43mg/kg (5g total)	Resv 2.36 (1.71) Gluc 1 3.18 (1.47) Gluc 2 4.29 (2.85) 3-Sulf 13.94 (6.69)	0.833 (0.5-1.5) 2.00 (1.0-6.0) 1.50 (1.0-5.0) 1.50 (1.0-5.0)	0.1 (0.1) 0.5 (0.3) 3.0 (1.4) 5.0 (1.6) Total 7.7 (24h)	(Boocock et al., 2007)
9	9 Homes (100%)	23-41	Estàndard de piceid disolt en 100mL d'alcohol (15%) +	1.22mg/kg	t-3-Sulf 0.95 (0.16) t-3-4'-Disulf 0.33 (0.07) t-3-5-Disulf 0.94 (0.17)	1 6-8 6-8	4.53 1.71 7.18	(Burkon and Somoza,

	400mL de llet semidesnatada	3-Gluc 0.16 (0.04) 4'-Gluc 0.19 (0.05) (2) t-Digluc 0.35 (0.09)	6 6 6	2.99 0.69 2.65 Tot 13.6-35.7(48h)	2008)

*Calculat a partir de pesos de referència: 70kg per a homes i 60kg per a dones.

Resv: resveratrol; Gluc: glucurònids; Sulf: sulfats; Digluc: diglucurònids; Disulf: disulfats.

3.2.4. EFECTES BENEFICIOSOS

3.2.4.1. MALALTIA CARDIOVASCULAR

La malaltia cardiovascular, segons el Ministeri de Sanitat i Consum espanyol (2004) representa un 33.3% de la mortalitat total a Espanya, aquestes dades són extrapolables a la majoria de països desenvolupats (Ministerio de Sanidad y Consumo., 2004).

La importància del resveratrol com a possible compost protector enfront a la malaltia cardiovascular va sorgir a partir de diversos estudis epidemiològics, el primer i més destacable va ser portat a terme per Renaud i Lorgeril (1992) denominat la Paradoxa Francesa (Renaud and de Lorgeril, 1992). Al 1992 es va associar el vi com a factor protector de la mortalitat cardiovascular a França. Aquest país presenta un elevat consum de greixos saturats tot i que té una mortalitat cardiovascular menor que determinats països del nord d'Europa amb dietes similars en greixos saturats però amb menor consum de vi. Aquell mateix any es va descobrir la presència de resveratrol en el vi (Siemann and Creasy, 1992), i se li van associar part d'aquests possibles efectes cardioprotectors del vi. Posteriorment diversos estudis han confirmat aquests efectes beneficiosos del raïm i el vi enfront a la malaltia cardiovascular (Baur and Sinclair, 2006;Bradamante et al., 2004;Delmas et al., 2005a;Fremont, 2000). Els efectes estudiats corresponen a una disminució de l'agregació plaquetària, promoció de la vasorelaxació, reducció de la peroxidació lipídica i disminució de les concentracions sèriques de colesterol i triglicèrids.

RESVERATROL I ARTERIOSCLEROSI

L'aterosclerosi és la major causa de dany coronari i particularment de malaltia vascular isquèmica. Actualment es coneix que l'aterosclerosi és un procés inflamatori crònic en la paret de les grans arteries que ocorre en resposta a una agressió sobre l'endoteli. El procés arterioscleròtic és el resultat de la modificació de les reaccions normals entre els components sanguinis i els de la paret arterial. La placa d'ateroma té el seu origen en la placa lipídica que s'observa ja al néixer en les grandes arteries i es va transformant amb el temps en la placa d'ateroma. Inicialment provoca símptomes, però que sol manifestar-se quan venen associats als factors de risc de l'aterosclerosi, tals com diabetis, hipertensió, consum de tabac, nivells de colesterol LDL elevats (>160mg/dL) o de HDL baixos (<40mg/dL).

Els factors de risc provoquen esquinçades en la llum de las arteries de tamany mitjà i gros, en els que es depositen substàncies d'origen lipídic, es produeix inflamació i finalment una reducció de la llum de les arteries amb la conseqüent obstrucció al flux sanguini. El colesterol-

LDL es diposita dintre de les plaques d'ateroma quan la concentració de les liproproteïnes de baixa densitat o LDL és elevada. Les cèl·lules de la paret arterial interpreten aquest depòsit com una invasió activant al sistema inmune que provoca una resposta inflamatòria. Les cèl·lules immunitàries excitades són els monòcitos circulants que penetren en la paret de l'arteria, es transformen en macròfags i comencen a fagocitar partícules de LDL, transformant-se en cèl·lules escumoses. La inflamació forma també una càpsula de teixit fibrós entre la placa d'ateroma i l'arteria. Quan va avançant la placa d'ateroma, es produeix un estretament o estenosi de l'arteria, inicialment parcial, fins a evolucionar a una completa obstrucció. A més a més la placa d'ateroma és relativament fràgil i pot trencar-se, sagnar i formar un trombo o desprendre's de la paret de l'arteria i finalment provocar un trombo (**Figura 8**).

El resveratrol pot actuar en diversos estadis de l'aterogènesi: acumulació lipídica i oxidació de les LDL, infiltració de monòcits i limfòcits, proliferació i migració de les cèl·lules musculars llises i sobre l'agregació plaquetària (Baur and Sinclair, 2006;Bradamante et al., 2004;Delmas et al., 2005a;Fremont, 2000).

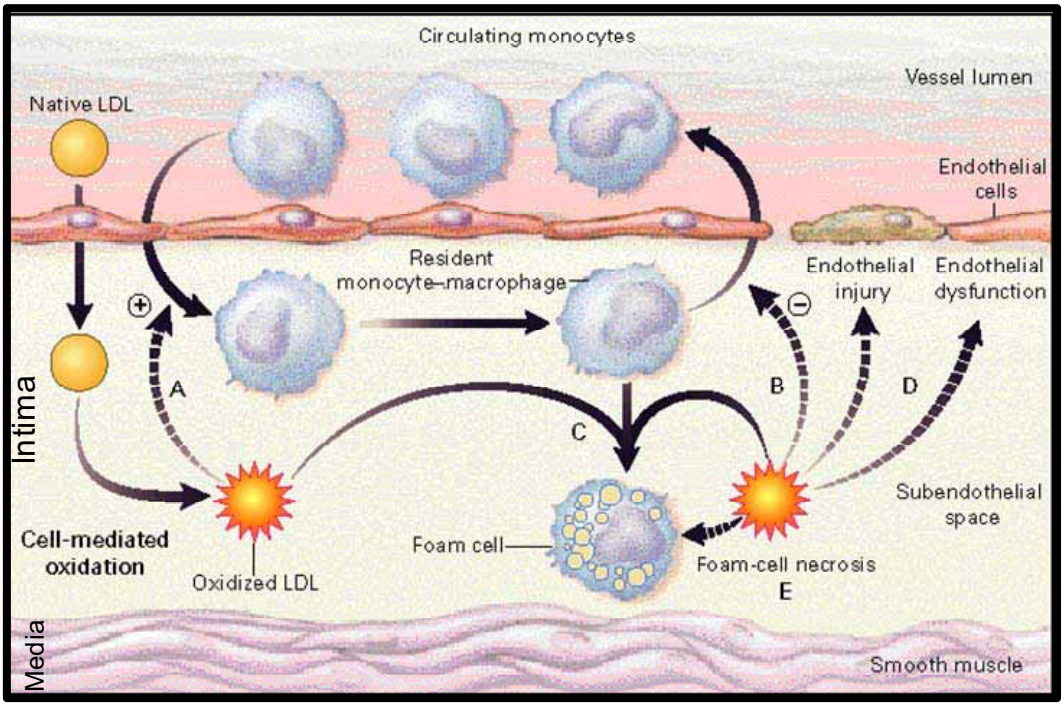


Figura 8: Esquema de la formació de la lesió ateromatosa segons Davies MJ (Davies, 1999)

RESVERATROL I LIPOPROTEÏNES, COLESTEROL I TRIGLICÈRIDS

Augments en els nivells de lipoproteïnes LDL i VLDL contribueixen a la promoció de la aterosclerosi. En estudis *in vivo*, el resveratrol ha demostrat no influenciar significativament i de forma directa en la disminució del colesterol i triglicèrids sèrics (Wang et al., 2002d), tot i que hi ha algun estudi en el que sí es podria observar una reducció de triglicèrids i VLDL (Zern et al., 2003). També s'ha de destacar que en rates hipercolesteromiants, s'ha observat una disminució, tant dels lípids totals (colesterol total i triglicèrids) com de las lipoproteïnes LDL i VLDL (Kollar et al., 2000). En conills amb dietes riques en colesterol, el tractament amb resveratrol va servir per reduir la placa d'ateroma (Miura et al., 2003).

Els mecanismes per els quals el resveratrol té la capacitat de disminuir les lipoproteïnes són diversos i estan actualment en estudi. En humans s'ha comprovat que el resveratrol pot arribar a les LDL després d'una ingesta de vi negre (Urpi-Sarda et al., 2005). El resveratrol redueix el contingut i la secreció de l'apolipoproteïna B (Pal et al., 2003), que és la responsable de la síntesi de les LDL i VLDL. El resveratrol també modula el ratio de secreció de triglicèrids sanguinis, provocant una reducció d'aquests, sense modificar els nivells de triglicèrids intracel·lulars. Aquesta situació podria afavorir una disminució dels nivells de VLDL, rics en triglicèrids amb un potencial efecte aterogènic (directament suplementant de colesterol als fibroblasts, produint alteracions endotelials i transformant els monòcits-macròfags en cèl·lules escumoses). S'ha descrit que el resveratrol podria presentar un potencial efecto estrogènic (Gehm et al., 2004;Bhat et al., 2001), degut a aquest efecte, el resveratrol podria també estimular l'expressió d'E-RmRNASF, que podria modular i bloquejar alguns aspectes del metabolisme hepàtic de les lipoproteïnes, les quals predisposen en última instància a l'aterosclerosi (Ratna and Simonelli, 2002).

RESVERATROL I ESTRÉS OXIDATIU

El segon factor en la formació de la placa d'ateroma és l'oxidació de la LDL en l'íntima fortament associat amb el risc de patir malaltia coronària i infart de miocardi. La peroxidació lipídica és una reacció en cadena induïda per diferents fonts de radicals lliures que estimulen l'agregació plaquetària i promouen l'activitat procoagulant en la superfície dels monòcits/macròfags.

Tot i que el resveratrol ha mostrat exercir un efecte antioxidant, aquest no seria el seu efecte més predominant i encara se desconeix si actuaria com agent quelant i antiradicalari (Frankel

et al., 1993;Fremont et al., 1999) o activant diverses rutes metabòliques o enzimàtiques amb marcada activitat antioxidant (Floreani et al., 2003).

Frankel *et al* (Frankel et al., 1993) van ser els primers investigadors que van demostrar que el resveratrol podria prevenir l'oxidació de les LDL *in vitro*, gràcies a la seva acció quelant sobre el coure (metall amb marcada acció pro-oxidant) i a la seva capacitat antiradicalària (Fremont et al., 1999;Belguendouz et al., 1997). A més a més d'aquesta acció directa, el resveratrol té l'habilitat de modular diversos sistemes enzimàtics presents en les cèl·lules endotelials i macròfags implicats en l'oxidació de les LDL. El resveratrol podria prevenir o disminuir l'activitat de la NAD(P)H oxidasa, hipoxantina/xantina oxidasa, mieloperoxidasa i augmentar l'activitat de la superòxid dismutasa, catalasa, glutation peroxidasa, glutation reductasa, glutation-S-transferasa i la NQO1 (Delmas et al., 2005a). L'acció d'aquests enzims consisteix en contribuir a la reducció de la formació intracel·lular en cèl·lules endotelials de les especies reactives de l'oxígen i a la inhibició de l'adhesió de leucòcits.

L'oxidació induïda per les cèl·lules endotelials o pels macròfags depèn dels lipoperòxids generats intracel·lularmente i posteriorment transferits a les LDL. Les lipooxigenases, especialment la lipooxigenasa 15, estan involucrades en aquest procés i iversos estudis han comprovat l'efecte inhibidor del resveratrol sobre les lipooxigenases (Pinto et al., 1999).

La ferrilmioglobina i el peroxinitrilo també són potents oxidants implicats en l'oxidació de les LDL. S'ha observat que el resveratrol podria provocar una disminució de l'acumulació d'hidroperòxids en les LDL promoguts per la ferrilmioglobina gràcies a la reducció del complex oxoferril amb la metilmioglobina. A més a més el resveratrol inhibeix les modificacions en les apoproteïnes de les LDL induïdes pel peroxinitrilo (Brito et al., 2002).

RESVERATROL I MACRÒFAGS

En condicions normals, els monòcits entren per diapèdesi en l'espai subendotelial, on es diferencien en macròfags. Quan apareix una disfunció endotelial, els monòcits circulants s'adhereixen a l'endoteli arterial, migrant directament a l'espai subendotelial, diferenciant-se en macròfags residents dintre de la matriu subendotelial. La LDL oxidada estimula l'expressió dels receptors CD36 i SR-A en monòcits, macròfags i cèl·lules musculars llises, que en situacions normals no es troben expressades. Aquests receptors internalitcen les LDL oxidades de forma específica, provocant una acumulació massiva d'èsters de colesterol fins que es

produeix la formació de la cèl·lula escumosa. Aquestes provoquen la placa lipídica que precedeix a estadis més avançats de lesió aterioscleròtica (Delmas et al., 2005a).

L'expressió del receptor SR-A és regulat per la prostaglandina E2, la qual és expressada per la ciclooxigenasa 2 en les cèl·lules de musculatura llisa i aquesta última es troba inhibida pel resveratrol (Mietus-Snyder et al., 2000). L'activitat del receptor SR-A en les cèl·lules de musculatura llisa també està incrementat per diversos factors del creixent com poden ser la IL 1, el TNFα, el factor de creixement epidèrmic, factor de creixent derivat de les plaquetes i el factor de creixement β transformat. El resveratrol té la capacitat de reduir l'activitat dels receptors SR-A en les cèl·lules de musculatura llisa mitjançant l'actuació sobre els factors nuclears enumerats anteriorment (Kaneuchi et al., 2003). Per aquesta raó la reducció de la interacció entre la LDL oxidada i aquests receptors dels macròfags contribueixen a prevenir un estadi molt prematur en l'aterogènesi.

RESVERATROL I LA FORMACIÓ DE CÈL·LULES ESCUMOSAS

La LDL oxidada estimula les cèl·lules endotelials perquè produeixin quimiocines i factors estimulants de granulòcits i macròfags, que tenen una activitat quimiotàxica directa sobre els monòcits de l'endoteli. El resveratrol redueix la producció de quimiocines responsables de la quimiotaxis i de l'acumulació de macròfags en la placa lipídica arterial, etapa inicial de la placa d'ateroma (Delmas et al., 2005a).

RESVERATROL I CÈL·LULES VASCULARS DE MUSCULATURA LLISA

La proliferació i migració de les cèl·lules vasculars de musculatura llisa contribueix al progressiu engrosament de la íntima i al desenvolupament de la paret arterial escleròtica. Les LDL oxidades promouen la proliferació de les cèl·lules vasculars de musculatura llisa, les quals acumulen grans quantitats d'èsters de colesterol que es convertiran en cèl·lules escumoses (Delmas et al., 2005a).

El resveratrol a baixes concentracions (6.25-12.5 μM) inhibeix la proliferació de les cèl·lules vasculars de musculatura llisa sense apoptosis, mitjançant els seus efectes antimitogènics bloquejant la transició G1$\rightarrow$S del cicle cel·lular i la síntesi d'ADN. No obstant elevades concentracions de resveratrol (25 μM) indueixen l'apoptosis en les cèl·lules vasculars de musculatura llisa estimulades del sèrum però no en les cèl·lules vasculars de musculatura llisa inactives (Mnjoyan and Fujise, 2003).

RESVERATROL I LA VASORELAXACIÓ

El resveratrol té la capacitat de modular la producció de vasoconstrictors (com l'endotelina-1) (Liu et al., 2003) i vasodilatadors endògens (com el NO) (Das et al., 2005), que són els responsables de la motilitat dels vasos sanguinis que estan afectats en l'aterosclerosi. En les cèl·lules vasculars de musculatura llisa l'estrès oxidatiu incrementa l'endotelina-1. El resveratrol inhibeix la secreció d'endotelina-1 mitjançant l'atenuació de l'activador de la proteïna-1, i interfirient en la via de l'ERK1/2 a través de la reducció de la formació de les especies reactives de l'oxígen (ROS) (Liu et al., 2003). El resveratrol també pot reduir l'expressió de l'endotelina-1 modulant varis del seus estimuladors com podrien ser l'angiotensina II (Miyazaki et al., 2008), trombina, PDGF-A i TNFα . Aquesta acció és deguda a l'atenuació per part del resveratrol de la fosforilació de diversos enzims, com el p70(S6K), PKB, ERK y ERK1/2, involucrats en la hipertrofia regulada per l'angiotensina II (Olson et al., 2005;Haider et al., 2002).

La vasorelaxació també pot ser dependent de la producció d'òxid nítric. El resveratrol modula els nivells de NO mitjançant la seva acció sobre la eNOS i la iNOS. En condicions normals les cèl·lules endotelials produeixen NO a baixes concentracions per a controlar la dilatació dels vasos sanguinis. No obstant, en situacions avançades d'arteriosclerosi, s'han trobat nivells de NO elevats. El resveratrol ha mostrat la seva capacitat per relaxar l'endoteli intacte, contret prèviament, de l'aorta de rates a través de l'augment del NO via eNOS (Andriambeloson et al., 1997;Flesch et al., 1998). Tot i que en arteries aïllades d'humans amb malaltia coronaria, la capacitat de vasorelaxació mitjançant mecanismes NO-dependents es perd, en canvi pot aparèixer una dilatació deguda a mecanismes NO-independents (Cruz et al., 2006). A nivell molecular, el resveratrol pot comporta-rse com un fitoestrogen sobreestimulant la eNOS i augmentant l'activitat promotora, l'estabilització del mARN eNOS i la pròpia activitat de la eNOS, modulada per l'activitat del factor transcriptor Sp1 (Wallerath et al., 2002).

La vasorelaxació també pot ser dependent de la ruta del cGMP, ja que el resveratrol incrementa el cGMP en las arteries coronaries, degut majoritàriament a l'activació de la guanilil ciclasa particulada (El-Mowafy, 2002). A nivell molecular, la cGMP/kinasa-G és un antiproliferatiu de la senyalització en les cèl·lules de musculatura llisa i a més a més dilata els vasos sanguinis a través de la reducció del calci intracel·lular. La vasorelaxació produïda pel resveratrol pot ser atribuïda a la capacitat que té d'estimular els canals K^+/Ca^{2+} (Li et al., 2000), i augmentar el NO a nivell endotelial.

RESVERATROL I L'AGREGACIÓ PLAQUETÀRIA

L'agregació plaquetària excessiva o inapropiada pot liderar la formació del trombo, i en conseqüència obstruir el vas sanguini. El resveratrol redueix l'agregació plaquetaria induïda per la trombina i l'ADP (Bertelli et al., 1995;Olas et al., 2002;Wang et al., 2002e). La trombina disminueix l'activitat endotelial de la ectonucleotidasa provocant alts nivells d'ADP i ATP que lideren l'activació endotelial plaquetària (Kaneider et al., 2004). A més a més, l'activació de les plaquetas per la trombina produeixen ROS, el resveratrol tindria la capacitat antiradicalària per a neutralitzar aquestes especies (Olas and Wachowicz, 2005). Un altre component involucrat en la agregació plaquetària seria el factor activador plaquetari (Fragopoulou et al., 2000) i el resveratrol podria inhibir l'efecte pro-agregant i pro-inflamatori, degut a la alliberació de tromboxans A_2 i leucotriens, estimulats pel factor activador plaquetari (Shigematsu et al., 2003).

La síntesi d'eicosanoids i leucotriens de l'àcid araquidònic està molt relacionada amb l'agregació plaquetaria i es troben involucrades diverses rutes metabòliques com la de la lipooxigenasa, la de la ciclooxigenasa i la de la prostaglandina H. El resveratrol inhibeix les lipooxigenasas prevenint la alliberació de substàncies pro-inflamatòries, i consecuentement bloquejant la síntesi d'hepoxilines, mediadores de la mobilització del calci, permeabilitat vascular i l'activació dels neutròfils. El resveratrol també modula la ruta de les ciclooxigenases, inhibint de forma preferent l'activitat de la COX1. Les 2 isoformes de les COX (COX1 i COX2) juguen un paper fonamental en la síntesi de prostaglandines que regulen l'homeòstasis vascular. Els tromboxans A_2, que es troben sintetitzats per la COX1 en les plaquetes, són uns potents inductors de l'agregació plaquetària i de la vasoconstricció. Mentre les prostaciclines, sintetitzades per la COX2 en les cèl·lules vasculars de l'endoteli, són un potent antiagregant plaquetari i vasodilatador. *In vivo* la capacitat antiagregant del resveratrol s'ha comprobat en conills en els que es bloqueja l'agregació plaquetària induïda per una dieta hipercolesterolèmica. També s'ha demostrat en ratolins genèticament hipercolesterolèmics (apoE-/-/LDLR-/-) reduint l'àrea arterioscleròtica i la mida del trombo en l'endoteli induït mitjançant làser.

RESVERATROL I MODULACIÓ DE FACTORS NUCLEARS

El resveratrol té la capacitat de modular la comunicació cel·lular i l'expressió de gens involucrats en el desenvolupament de la arteriosclerosi com la cascada MAPK. El resveratrol pot actuar inhibint la fosforilació de la proteïna quinasa C (Stewart et al., 1999), PI3K, Akt/PKB i ERK1/2/JNK/p38 que activen la cascada MAPK (Delmas et al., 2005b).

En referència a la capacitat de modificar l'expressió genètica, el resveratrol té la capacitat d'actuar sobre els factors nuclears com el NFκB, que està implicat tant en la iniciació i la progressió de l'arteriosclerosi, com també en processos cancerígens (Thurberg and Collins, 1998). Mitjançant l'atenuació de l'activitat d'alguns factors nuclears com l'AP-1, GATA o el ja anomenat anteriorment NFκB, el resveratrol pot modular l'expressió de diversos gens (iNOS, COX-2, ET-1, MCP-1, VCAM-1, ICAM-1, SR-A, IL-1, IL-6) (Tsai et al., 1999;Murias et al., 2004;Liu et al., 2003;Collins and Cybulsky, 2001) molt involucrats en l'arteriosclerosi i en la resposta inflamatòria (Delmas et al., 2005b).

RESVERATROL i INFLAMACIÓ

La inflamació presenta un rol molt important en el desenvolupament de la malaltia cardiovascular. Les ciclooxigenases tenen un paper crucial en la producció de molècules pro-inflamatòries, i el resveratrol és un inhibidor efectiu de l'activitat ciclooxigenasa *in vivo*. En rates amb una administració de resveratrol intravenós disminueix la inflamació associada a la formació de superòxid generat mitjançant isquèmia/reperfusió, producció d'oxidants per hipoxantina/xantina oxidasa o per l'activació del factor plaquetari. No obstant no afecta a la inflamació provocada pels leucotriens B_4, un mecanisme independent a la ruta del superòxid.

Per un altre banda, el resveratrol contribueix a la reducció de la resposta inflamatòria en l'arteriosclerosi quan els macròfags, cèl·lules de musculatura llisa o cèl·lules endotelials són activades i produeixen nombrosos productes pro-inflamatoris, com és el cas del TNFα, interleukina-6, la proteïna 1 quimioatraient dels monòcits (CMP-1) (Zhu et al., 2008). El resveratrol té la capacitat de disminuir la producció de citocines pro-inflamatòries, que regulen l'expressió de les molècules d'adhesió (VCAM-1 i ICAM-1). Aquesta inhibició ocorre en rates al mateix nivell en un interval de concentracions plasmàtiques molt ampli (d'un fins a 100 mmol/L), això suggereix que el resveratrol pot actuar com a un interferent ràpid de la senyal molecular en el mecanisme de l'expressió de les VCAM-1 i ICAM-1.

3.2.4.2. CÀNCER

La mortalitat deguda a malalties relacionades amb el càncer, segons el Ministeri de Sanitat i Consum espanyol (2004), representa un 26.1% de la mortalitat total, aquestes dades podrien ser extrapolades a la gran majoria de països desenvolupats (Ministerio de Sanidad y Consumo., 2004).

En 1997 Jang i col·laboradors van publicar els potencials efectes quimiopreventius i quimioterapèutics del resveratrol sobre els tres estadis de la carcinogènesi (iniciació, promoció i progressió) (Jang et al., 1997).

RESVERATROL I ANGIOGÈNESI

La creació de nous vasos sanguinis és una etapa essencial en el creixement de tumors de diàmetre superior a 2-3 mm (Baur and Sinclair, 2006). Dosis regulars de 2,5 a 100 mg/kg de pes corporal de resveratrol en rates inhibeix la neovascularització induïda per tumors prèviament induïts (Tseng et al., 2004) i cicatritzats (Brakenhielm et al., 2001). A dosis diàries de 48μg/kg de pes corporal en ratolins, el resveratrol té la capacitat d'inhibir la vascularització en assaigs de butxaca corneal ("*corneal micropocket"*) (Brakenhielm et al., 2001). El resveratrol pot modular diversos enzims i factors involucrats en l'angiogènesi com són les COX, ODC i la PKC (Baur and Sinclair, 2006;Delmas et al., 2005a). De tots aquests treballs es desprèn la hipòtesi de que el resveratrol podria tenir un paper important en la inhibició de la vascularització i el creixement dels tumors cancerígens.

RESVERATROL I ENZIMS

Estudis epidemiològics han evidenciat que la inhibició de la COX a llarg termini podria reduir significativament el risc de desenvolupar diversos tipus de càncer. La privació de la codificació del gen de la COX 2 protegeix a ratolins amb càncer colorectal. El resveratrol redueix l'activitat total de la COX en tumors i teixits normals *in vivo* mitjançant la inhibició de l'activitat de la COX 1 i la reducció de la COX 2 (Baur and Sinclair, 2006).

L'ornitina decarboxilasa (ODC) també té un paper important en la carcinogènesi a través de la inhibició de la PKC (Stewart et al., 1999). El resveratrol no inhibeix directament l'activitat de la ODC (Schneider et al., 2000), però redueix la seva expressió *in vivo* i pot prevenir la seva inducció per carcinògens (Afaq et al., 2003). Per aquest motiu el resveratrol podria enlentir el desenvolupament del tumor a través de múltiples mecanismes complementaris.

RESVERATROL I EL METABOLISME DE MEDICAMENTS

El metabolisme de fàrmacs està dividit en dos fases que involucren diferents tipus d'enzims. En general, els enzims de Fase I, consisteixen principalment en el citocrom P450s i en les flavin monooxigenases. Aquests enzims oxiden, redueixen o hidrolitzen molècules estranyes per convertir-les en molècules més polares que facilitaran la seva posterior excreció, tot i que aquests enzims, els citocroms P450s majoritàriament, també poden estar implicats en l'activació d'alguns pro-cancerígens. Els enzims de Fase II inclouen enzims conjugadors i antioxidants que són els encarregats de detoxificar les molècules més perilloses, incloent els productes tòxics provinents dels enzims de Fase I. Existeixen diversos agents quimiopreventius que incrementen l'activitat dels enzims de Fase II, considerats com una prometedora estratègia enfront la prevenció del càncer.

El resveratrol, *in vitro*, inhibeix l'activitat enzimàtica de diversos citocroms P450s (Chang et al., 2000;Yu et al., 2003a) i a més bloquegen la seva transcripció mitjançant antagonistes del seu receptor aril hidrocarboni (Ciolino et al., 1998;Ciolino and Yeh, 1999). No obstant la inhibició del citocrom P450 podria alterar la farmacocinètica d'altres fàrmacs. El resveratol s'ha mostrat com un potent inductor de l'expressió dels enzims en Fase II *in vitro* (Cao and Li, 2004). Per aquests motius, el resveratol es confirma com un inhibidor dels gens que codifiquen els enzims de Fase I i com activador dels enzims de Fase II mitjançant cADN array i la PCR transcriptasa reversa usant fetges de rates tratades amb resveratrol. Gràcies a la seva activitat sobre els enzims metabolitzadors de medicaments, el resveratrol pot prevenir l'activació de pro-carcinògens mentre simultàniament incrementa la capacitat d'eliminar molècules potencialment perilloses.

RESVERAROL I ALTERACIÓ EN EL CICLE CEL·LULAR I APOPTOSI

Un altre mecanisme pel qual el resveratrol podria lluitar contra la formació de tumors cancerígens és la inhibició del cicle cel·lular i inducció de l'apoptosi. L'efecte antiproliferatiu i pro-apoptòtic del resveratrol en línies cel·lulars tumoroses està ampliament documentat (Aggarwal et al., 2004) i es basa en la inhibició del cicle cel·lular de proteïnes (Yu et al., 2003b) i l'augment de l'apoptosi en models tumorals *in vivo* (Fulda and Debatin, 2006). Altres estilbens com el pteroestilbè també poden actuar inhibint el creixement o activant l'apoptosi cel·lular en el melanoma B16 (Ferrer et al., 2005;Ferrer et al., 2007), aquests efectes podrien ser deguts per la mediació dels estilbens sobre la generació de NO, un potencial regulador biològic de l'apoptosi (Chung et al., 2001).

RESVERATROL I ESTRÈS OXIDATIU

Les espècies reactives de l'oxigen participen en la iniciació i la progressió del càncer, alterant l'ADN i altres macromolècules (Hansen et al., 2007;Sgambato et al., 2001). El resveratrol pot actuar directament en la modulació dels enzims antioxidants, que pertanyen als enzims de Fase II, a més el resveratrol pot tenir activitat antioxidant de *per se,* tot i que no seria la seva activitat principal. *In vivo*, el resveratrol incrementa la capacitat antioxidant del plasma i disminueix la peroxidació lipídica (Wenzel et al., 2005;Sengottuvelan et al., 2006), no obstant existeix una gran dificultat en valorar si l'efecte antioxidant del resveratrol és degut a la seva activitat o a la seva capacitat de modular els enzims de Fase II.

3.2.4.3. RESVERATROL I ENVELLIMENT

El envelliment és un procés natural que avança de forma inalterable en tots els organismes vius. Els mecanismes bioquímics involucrats en l'envelliment són encara confusos, un dels factors més conegut i rellevant és el genètic. No obstant, actualment, els factors medioambientals, en els que s'inclou l'alimentació estan augmentant el seu protagonisme, ja que podrien estar involucrats en l'expressió i/o alteració gènica. Un exemple que evidència aquesta situació va ser demostrat en la illa de Okinawa, on existeixen varies famílies japoneses amb una elevada esperança de vida. Part d'aquesta població va emigrar a Brasil i progressivament van anar modificant els seus hàbits alimentaris (canviant una dieta basada en peix i hortalisses per una dieta amb una ingesta calòrica superior al 30% i amb un consum alt de carns grasses) provocant una disminució en la seva su esperança de vida.

Observacions epidemiològiques (Willcox et al., 2007a;Willcox et al., 2006;Willcox et al., 2007b;Willcox et al., 2007c) i assaigs amb animals (Sinclair, 2005;Ingram et al., 2004) han mostrat que una reducció d'entre un 30 i un 40% de la ingesta calòrica habitual (*ad libitum*) és la forma més robusta i reproduïble per retardar el envelliment i les malalties relacionades amb aquest procés i d'aquesta manera augmentar l'esperança de vida (Roth et al., 2005;Roth et al., 2000). La restricció calòrica redueix l'arteriosclerosi (Rajala and Scherer, 2003), la inflamació (Chung et al., 2002), els efectes de l'envelliment (Kim et al., 2002a;Kim et al., 2002b;Kim et al., 2002c;Yu and Chung, 2001), la resistència insulínica en el teixit adipòs (Bergamini et al., 2003), i la autofagia lisosomal (Bergamini et al., 2003). Aquest fenomen està caracteritzat per un gen silenciador, disminuint l'expressió de gens metabòlics com és el cas dels que codifiquen

l'hormona del creixement-IGF1 (Al-Regaiey et al., 2005;Masternak et al., 2006). Sota els efectes de la restricció calòrica, el consum alterat d'oxigen modifica la ratio NAD^+/NADH i lidera l'activació NAD^+-depenent de la sirtuïna (Howitz et al., 2003).

La sirtuïna és una proteïna denominada reguladora silenciosa de la informació, i pertany a la família de les deacetilases NAD^+-dependent. En mamífers, set gens de la sirtuïna han esta identificats (SIRT1-7) (Baur et al., 2006b). La funció principal de la SIRT 1 sembla ser la de promoure la supervivència i la resistència al estrès en temps d'adversitat, i es creu que intervé en la majoria dels efectes beneficiosos de la restricció calòrica (Guarente and Picard, 2005). Degut a les seves prometedores funcions, diversos autors han realitzat un exhaustiu cribat entre més de 20000 molècules, per identificar 25 molècules capaces d'augmentar, *in vitro*, l'activitat de la SIRT 1 (Howitz et al., 2003). El resveratrol ha resultat ser un potent inductor de l'activitat de la SIRT 1.

Posteriorment, el resveratrol s'ha estudiat en diversos tipus d'organismes per comprovar el seu potencial efecte sobre les sirtuïnes. El resveratrol va perllongar l'esperança de vida (**Figura 9**) en llevats (*Saccharomyces cerevisiae*) (Howitz et al., 2003), en organismes invertebrats: *Caenorhabditis elegants* (Wood et al., 2004;Viswanathan et al., 2005) i *Drosophila melanogaster* (Wood et al., 2004;Bauer et al., 2004); i més recentment en vertebrats: *Nothobranchius furzeri* (Valenzano et al., 2006) i en ratolins amb dietes hipercalòriques (Baur et al., 2006b) (**Figura 10**) a través de la via de la Situina-2 (Sir2)-dependent. Tot i que, actualmente alguns autors estan posant en dubte el seu efecte (Garber, 2008).

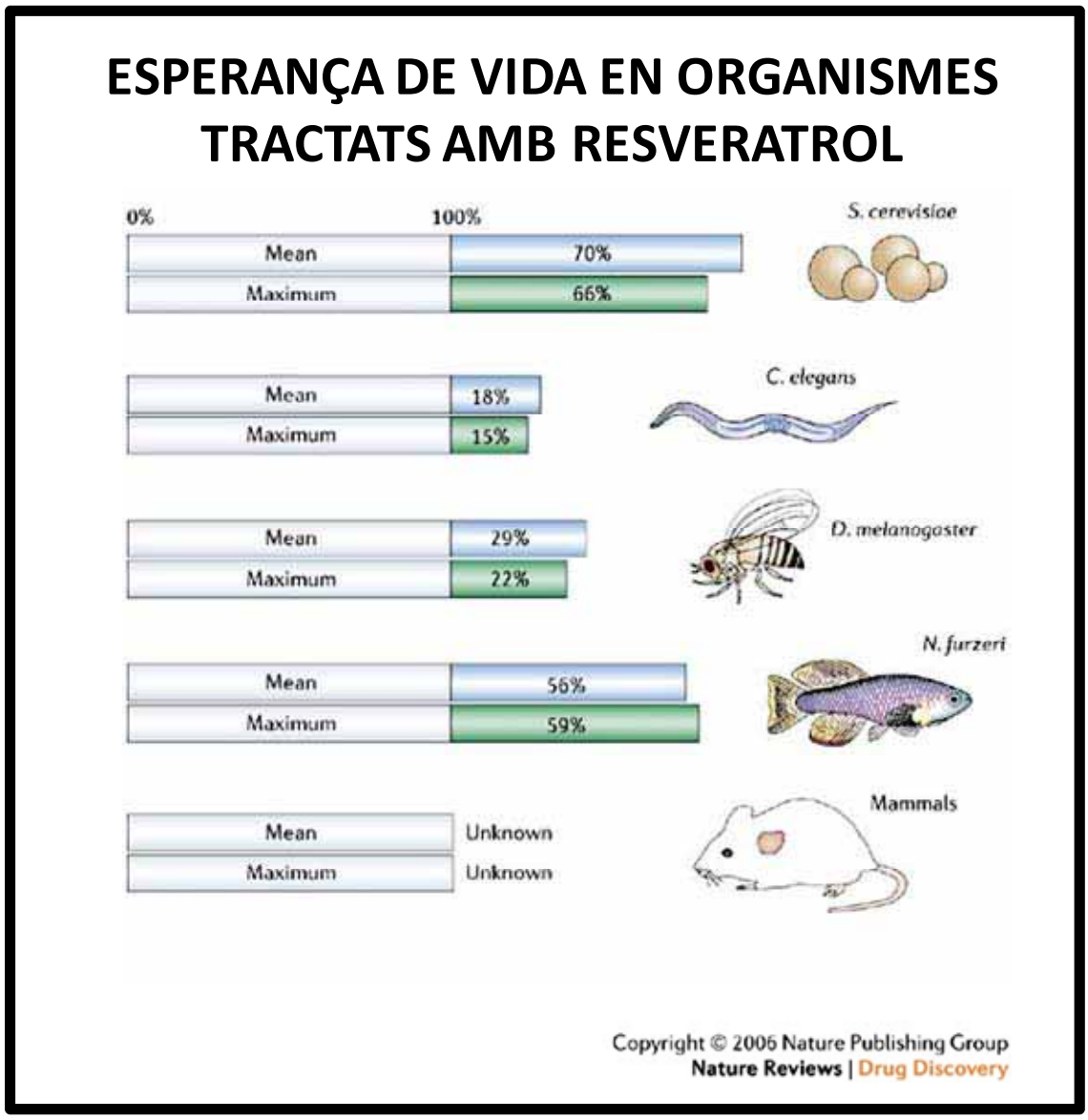


Figura 9. Esperança de vida en organismes tractats amb resveratrol (Baur and Sinclair, 2006).

La SIRT 1 regula diversos processos metabòlics com la producció de glucosa i insulina (Milne et al., 2007), el metabolisme dels lípids (Baur et al., 2006b) i la supervivència cel·lular (Guarente and Picard, 2005). L'envelliment també es caracteritza per estar incrementades les alteracions en el metabolisme de proteïnes, l'oxidació lipídica i el dany de DNA. A més a més de la regulació de la SIRT 1 pel resveratrol, aquest és un potent modulador d'enzims antioxidants capaços de reduir les altres alteracions involucrades en l'envelliment.

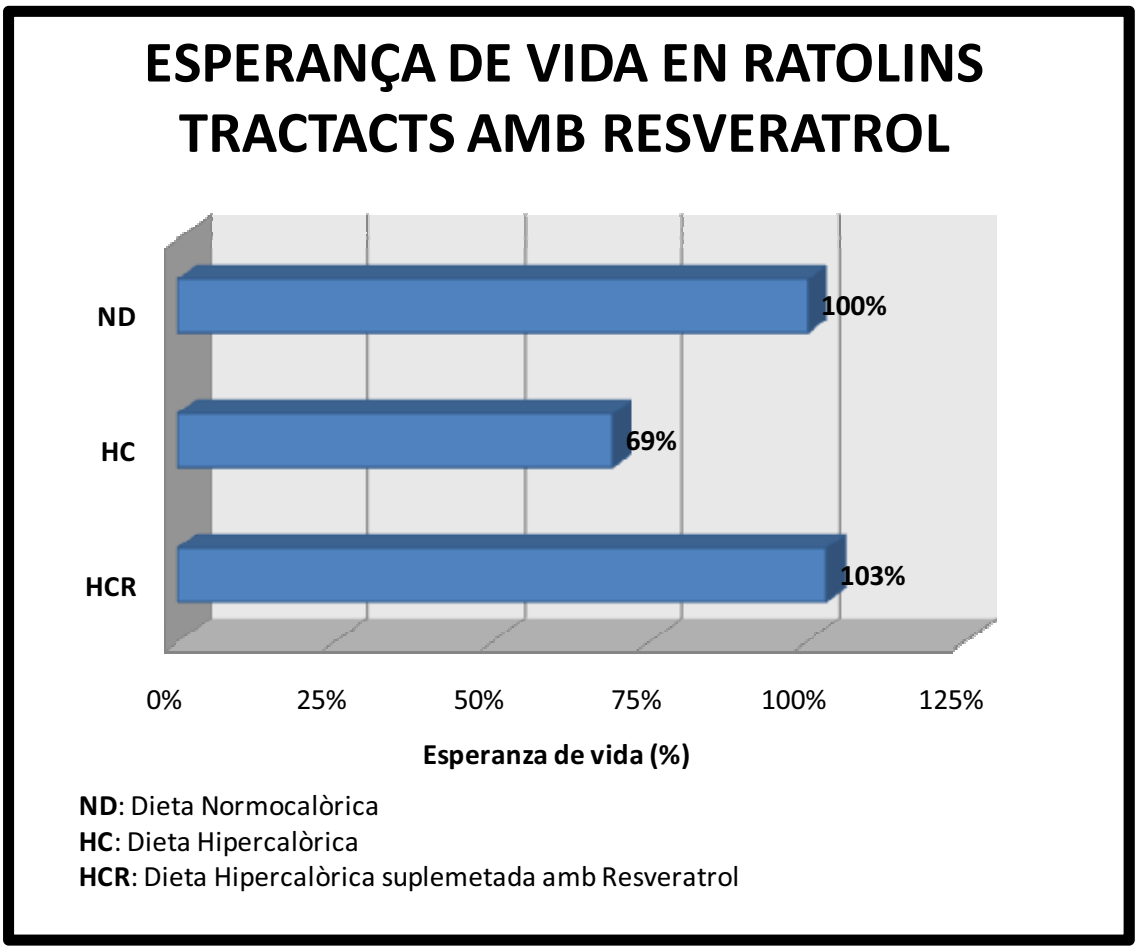


Figura 10. Esperança de vida en ratolins tractats amb resveratrol (Baur et al., 2006a).

3.2.4.4. ISQUÈMIA I DANY CEREBRAL

La malaltia cerebrovascular representa un 9.1% de la mortalitat total a Espanya, segons el Ministeri de Sanidad i Consum (2004), en dones arriba fins un 11.3% en canvi en homes no arriba al 8% (Ministerio de Sanidad y Consumo., 2004).

Diversos estudis han mostrat com el resveratrol podria actuar protegint del dany cerebral. En rates suplementades diàriament amb resveratrol intravenós durant 21 dies, van mostrar una millora motora i una disminució significativa del volum de la zona afectada per la isquèmia després de l'oclusió de l'arteria cerebral media (Sinha et al., 2002). A jerbs, un tipus de rosegador, se'ls va inyectar resveratrol durant o immediatament a continuació d'una isquèmia cerebral global transitòria, seguida per una segona dosis a les 24 hores, disminuint o retardant la mort de cèl·lules neuronals i l'activació de les cèl·lules glials en el hipocamp (Wang et al.,

2002b). Un tercer estudi ha mostrat que el resveratrol administrat de forma intravenosa va reduir significativament el volum isquèmic i el contingut d'aigua cerebral a dosis molt baixes (100ng a 1μg/kg de pes) després de l'oclusió de l'arteria cerebral mitja en rates (Wang et al., 2003). Aques resultats suggereixen que el resveratrol té la capacitat d'atravessar la barrera hematoencefàlica i d'exercir els seus efectes beneficiosos, fins i tot a dosis molt baixes.

3.2.4.5. OBESITAT I DIABETIS

La restricció calòrica produeix uns perfils metabòlics interessants pel tractament de malalties relacionades amb l'envelliment com poden ser el sobrepes/obesitat (Baur et al., 2006b) i la diabetis tipus II (Facchini et al., 2001;Milne et al., 2007).

El sobrepes i l'obesitat s'han convertit en la gran epidèmia del segle XXI, provocant greus problemes de morbi-mortalitat (Li et al., 2005). En el treball de Baur *et al.* (Baur et al., 2006b) en ratolins alimentats amb dietes hipercalòriques, suplementades o no amb resveratrol (22.4mg/kg d), i comparats amb una dieta normocalòrica seguits durant 2 anys. Els ratolins normocalòrics van mantenir el pes corporal sense canvis significatius durant tot l'estudi, mentre que els ratolins amb dietes hipercalòriques van incrementar molt el pes. Els ratolins amb la dieta hipercalòrica suplementada amb resveratrol també van augmentar de pes però mai va arribar a ser tan elevat com amb la dieta no suplementada amb resveratrol. Tot i que el més destacable de l'estudi va ser que la supervivència dels ratolins suplementats amb resveratrol va ser igual a la dels ratolins normocalòrics i molt més elevada que els ratolins que van seguir una dieta hipercalòrica sense adició de resveratrol. Això va ser degut a un increment de la sensibilitat de la insulina, una reducció dels nivells del factor del creixement-1 (IGF-1), a un augment de la proteïna quinasa AMP-activada i l'activitat de la PGC-1α, un major nombre de mitocondries i la millora de la funció motora. Aquest estudi mostra com el resveratrol pot reduir varies de les conseqüències negatives del seguiment d'una dieta hipercalòrica, obrint una nova línia d'investigació en relació al tractament del sobrepes i de l'obesitat.

La diabetis és un desordre metabòlic que properament assolirà proporcions d'epidèmia, l'Organització Mundial de la Salut ha predit que en l'any 2030, aproximadament uns 370 milions de persones en tot el món patiran aquesta malaltia (Amos et al., 1997;Wild et al., 2004). La diabetis, eventualment, lidera el risc de patir patologies tan freqüents com la malaltia cardio- i cerebro-vascular, problemes renals, ceguera, complicacions neurològiques i mort

prematura (Harris et al., 1987;Patlak, 2002). Existeixen dos tipus de diabetis: la tipus I i la tipus II, molt més freqüent que l'anterior. La diabetis tipus II es caracteritza per un complex mecanisme fisiopatològic, on la seva principal característica és el dèficit relatiu de la producció d'insulina i una deficient utilització perifèrica pels teixits de la glucosa, la denominada resistència a la insulina.

En estudis amb ratolins amb dietes hipercalòriques, el resveratrol va protegir als ratolins enfront la resistència a la insulina (Lagouge et al., 2006;Baur et al., 2006b). En rates diabètiques el resveratrol va tenir un efecte hipoglucemiant (Chi et al., 2007;Su et al., 2006). Recentment s'ha trobat que aquest polifenol disminueix la secreció d'insulina de les illetes pancreàtiques de rates normals (Szkudelski, 2006;Szkudelski, 2007) i que té la capacitat de modificar la concentració d'insulina plasmàtica (Chi et al., 2007;Su et al., 2006;Baur et al., 2006b;Chen et al., 2007).

D'una altra banda, els activadors de la SIRT1 milloren l'homeòstasi de la glucosa i la sensibilitat a la insulina en teixits metabòlicament molt importants, com són l'hepàtic, el muscular i l'adipós (Bordone and Guarente, 2005;Cohen et al., 2004;Heilbronn et al., 2005;Nisoli et al., 2005;Imai et al., 2000). El resveratrol sembla actuar com a mimètic de la restricció calòrica exercint els seus efectes beneficiosos en la funció metabòlica i mitocondrial (Baur et al., 2006b;Howitz et al., 2003;Lagouge et al., 2006;Jarolim et al., 2004;Wood et al., 2004) en mamífers. Per aquests motius poden ser un tractament molt esperançador contra la diabetis tipus II, entre d'altres malalties relacionades amb l'envelliment (Milne et al., 2007).

3.2.5. ANÀLISI DE RESVERATROL I DELS SEUS METABÒLITS EN MOSTRES BIOLÒGIQUES

3.2.5.1. MÈTODE D'EXTRACCIÓ EN FASE SÒLIDA DE RESVERATROL I DELS SEUS METABÒLITS EN MOSTRES BIOLÒGIQQUES

En estudis nutricionals, on la concentració dels principis actius estudiats poden considerar-se molt baixos respecte a estudis farmacològics o fins i tot de toxicitat, es fa necessari la posada a punt de mètodes analítics validats de gran sensibilitat.

Degut a la complexitat i a la baixa concentració dels analits estudiats (resveratrol i els seus metabòlitos) en les mostres biològiques de l'estudi, previ a l'anàlisi cromatogràfic, aquestes mostres han de patir una etapa preparativa.

Les tècniques preparatives tenen tres funcions bàsiques: a) Reduir compostos interferents que podrien dificultar la posterior identificació i quantificació dels analits d'interès; b) Concentrar els analits, ja que com succeeix en el cas del resveratrol en mostres biològiques es troben en quantitats molt baixes (nano i picomolars); c) Canviar la matriu de la mostra, per facilitar la detecció per espectrometria de masses, ja que la matriu pot provocar supressió iònica i conseqüentment impossibilitar la detecció dels nostres analits.

L'extracció en fase sòlida (SPE) acompleix les tres funcions que anteriorment s'han descrit en el mateix pas analític, per aquest motiu es un dels mètodes de preparació més utilitzats per mostres biològiques.

Addicionalment el volum de mostra necessari per realitzar el SPE pot adaptar-se a les característiques de l'estudi i quan es tracta d'estudis clínics o epidemiològics, on es disposen de quantitats molt limitades degut al gran valor de les mostres biològiques es necessiten mètodes molt eficients, com és el cas del SPE.

De cartutxs de SPE al mercat existeixen cada vegada una varietat més gran, aquests ofereixen una amplia gamma de possibilitats depenent de la naturalesa química dels analits estudiats i de les substàncies interferents. Com en qualsevol cromatografia, la interacció dels analits i dels interferents amb la fase estacionària (adsorbent del cartutx) i la fase mòbil (solvents) proporcionarà uns resultats més o menys adequats. En aquesta memòria s'expondrà el funcionament dels cartutxs SPE del tipus HLB® de Waters, ja que són els emprats en l'apartat experimental.

3.2.5.1.1. CARTUTXS DE SPE CON RELLENO HLB®.

El farcit HLB® està format per un sorbent Hidrofílic-Lipofílic-Equilibrat. Està fabricat amb una relació característica de monòmers N-vinilpirrolidona hidrofílica i divinilbenzè lipofílic. És el cartutx habitual de fase reversa, tot i que degut a la seva composició millorada presenta unes unions polars neutres que ofereixen una millor retenció dels analits polars. El mètode general recomanat perr utilitzar aquests cartutxs es detalla a continuació de forma més amplia.

- Acondicionament i activació del cartutx. Etapa en la que el cartutx es renta per eliminar possibles substàncies interferents. L'activació també serveix per preparar l'adsorbent del cartutx pel seu ús.
- Càrrega de la mostra. La mostra s'introdueix en el cartutx, depenent del cartutx i de lo concentrada que estigui la mostra, es pot carregar més o menys quantitat de mostra. Aquesta mostra prèviament pot haver estar tractada amb àcid (per hidrolitzar enllaços proteïna-polifenols) i posteriorment centrifugada (per eliminar interferents de pes molecular elevat). Si la mostra biològica està molt concentrada és de gran utilitat diluir la mostra prèviament a realitzar la càrrega en el cartutx per evitar colmatacions. En aquesta fase també s'ha d'introduir el patró intern que al final servirà per a la quantificació.
- Rentat del cartutx. Depenent del mètode poden haver-hi una o varies etapes de rentat. Aquestes serveixen per eliminar el màxim nombre d'interferents sense eluir els analits.
- Elució. En aquesta etapa, després d'haver eliminat un gran nombre d'interferents s'elueix mitjançant solvents amb més afinitat per l'analit que pel farcit del cartutx. Aquesta etapa ha de ésser el més exhaustiva possible per recuperar la màxima quantitat d'analit en el mínim volum. Amb l'analit també eluirem els interferents de la mostra de naturalesa química similar a els nostres analits d'interès. No obstant, una gran part dels interferents els haurem eliminat en l'etapa del rentat i alguns més que poden ser retinguts en el cartutx després de l'elució.
- Evaporació i reconstitució. Una vegada ja hem eluit els analits, es pot evaporar el solvent, fins una certa quantitat de dissolvent o fins a sequedat. Posteriorment s'ha de reconstituir els analits amb la menor quantitat possible de fase mòbil. D'aquesta forma s'aconsegueix concentrar als analits i canviar la matriu de l'analit.

3.2.5.2. MÈTODE D'ANÀLISI MITJANÇANT CROMATOGRAFIA LÍQUIDA ACOBLADA A ESPECTROMETRIA DE MASSES EN TÀNDEM

La cromatografía líquida d'alta eficàcia (CLAE o HPLC) és la tècnica de separació per excel·lència en l'anàlisi del resveratrol en aliments (Andres-Lacueva et al., 2002;Cantos et al., 2002b;Lamuela-Raventós et al., 1995;Wang et al., 2002c) i en mostres biològiques (Boocock et al., 2007;Meng et al., 2004;Urpi-Sarda et al., 2005;Urpi-Sarda et al., 2007), tot i que alguns autors prefereixen l'ús de la cromatografia de gasos (Goldberg et al., 2003;Soleas et al., 2001).

ELs detectors acoplaTs a l'HPLC poden ser diversos i han anat canviant a mesura que ha evolucionat la instrumentació analítica. Quan la mostra a analitzar es troba en concentracions elevades es poden emprar els detectors de ultravioleta-visible i és preferible si es disposa d'un diode d'array. Els isòmers *trans*- del resveratrol aglicó i del glucòsid són habitualment detectats a 306 nm. No obstant els dos isòmers *cis*- són detectats a la longitut d'onda de 285 nm (Romero-Perez et al., 2001). Degut a que el límit de detecció i de quantificació que ens proporciona aquesta tècnica de detecció espectrofotomètrica són relativament alts o no suficientment sensibles per a detectar analits a concentracions baixes, pocs autors escullen aquesta metodologia en l'anàlisi de mostres biològiques (Juan et al., 1999;Walle et al., 2004;Zhu et al., 1999). Actualment la majoria de determinacions en mostres biològiques es realitzen amb detectors d'espectrometria de masses, simple quadrupol (MS) (Vitaglione et al., 2005;Yu et al., 2002) i triple quaprupol (MS/MS) (Boocock et al., 2007;Meng et al., 2004;Urpi-Sarda et al., 2005;Urpi-Sarda et al., 2007;Yu et al., 2002). Cada cop és més freqüent l'ús d'aquesta tecnologia, fins i tot en l'anàlisi d'aliments, per verificar la identificació i per quantificar compostos a molt baixes concentracions (Callemien et al., 2005).

Els espectròmetres de masses treballen amb molècules ionitzades, de les quals identifiquen els ions en funció de la seva relació massa/càrrega (m/z). Un espectròmetre de masses presenta tres components fonamentals: la font d'ionització, l'analitzador de massa i el detector.

La font d'ions és l'element de l'espectròmetre que ionitza el material a ésser analitzat (l'analit). Després els ions són transportats pels camps magnètics o elèctrics a l'analitzador total. Les fonts d'ionització més freqüents en l'actualitat són les que treballen a pressió atmosfèrica (API), i entre elles destaca la d'ionització per electroespray (ESI). Aquesta tècnica genera els ions en la fase mòbil abans de que la mostra a analitzar arribi a l'espectròmetre de masses (Fenn et al., 1989).

L'analitzador de massa és la peça més flexible de l'espectròmetre de massa. Utilitza un camp elèctric o magnètic per afectar la trajectòria o la velocitat de les partícules carregades d'una certa manera. D'espectròmetres de masses existeixen de quatre tipus: a) quadrupol; b) temps de vol; c) trampa d'ions; d) transformacions de Fourier. Del primer tipus es poden subclassificar bàsicament, segons el nombre de quadrupols, en dos tipus: a) quadrupol simple (que actua de quadrupol analitzador); b) quadrupol triple, que presenta els tres quadrupols en línia, el primer (Q1) i el tercer (Q3) actuen com analitzadors, mentre que el segon actua com a cel·la de col·lisió (CID) en mètodes tàndem. Els ions precursors analitzats en Q1 s'acceleren i col·lisionen en la CID per fragmentar-se donant com a resultar els ions productes que seran escanejats en Q3.

En el cas del triple quadrupol es poden realitzar els quatre tipus d'experiments en tàndem (**Figura 11**) que es resumeixen en la **Taula 4** i que es detallen a continuació:

- *Product Ion Scan*: es determinen els ions fragmentats que procedeixen d'un ió precursor concret. És la tècnica utilitzada per confirmar la identitat d'un compost determinat.

- *Precursor Ion Scan:* es busquen els ions precursors d'un únic ió producte. És la técnica utilitzada per identificar la família de compostos (metabòlits) que provenen d'un analit (aglicó).

- *Neutral Loss Scan*: es mostren tots els parells d'ions (ió precursor i ió producte) que perden un fragment neutre constant. És la tècnica usada per identificar d'una barreja de compostos aquells que tenen un fragment de molècula conegut (per exemple: molècules que tenen un glucurònid o un glucòsid).

- *Multiple Reaction Monitoring (MRM)*: es fixen tan les masses dels ions precursors i ions productes a analitzar. És la tècnica més emprada per la quantificació de compostos, ja que s'obtenen els límits de detecció i per tant els de quantificació més baixos.

L'element final de l'espectròmetre és el detector. El detector registra la càrrega induïda o la corrent produïda quan un ió passa a prop o colpeja una superfície. En un instrument d'exploració la senyal és produïda en el detector durant la trajectòria de la mateixa (en què *m/z*) i produirà un espectre de massa, un expedient del *m/z*'s en el qual els ions estan presents.

Taula 4. Experiments d'espectrometria de masses en tàndem (MS/MS) que es poden realitzar en múltiples etapas.

Experiment	Q1	Q3
Product Ion Scan	Estàtic, selecció de l'ió precursor	Escanejat
Precursor Ion Scan	Escanejat	Estàtic, selecció dels ions producte
Neutral Loss Scan	Escanejat, sincronitzat amb Q3	Escanejat, sincronitzat amb Q1
Multiple Reaction Monitoring	Estàtic, selecció de l'ió precursor	Estàtic, selecció dels ions producte

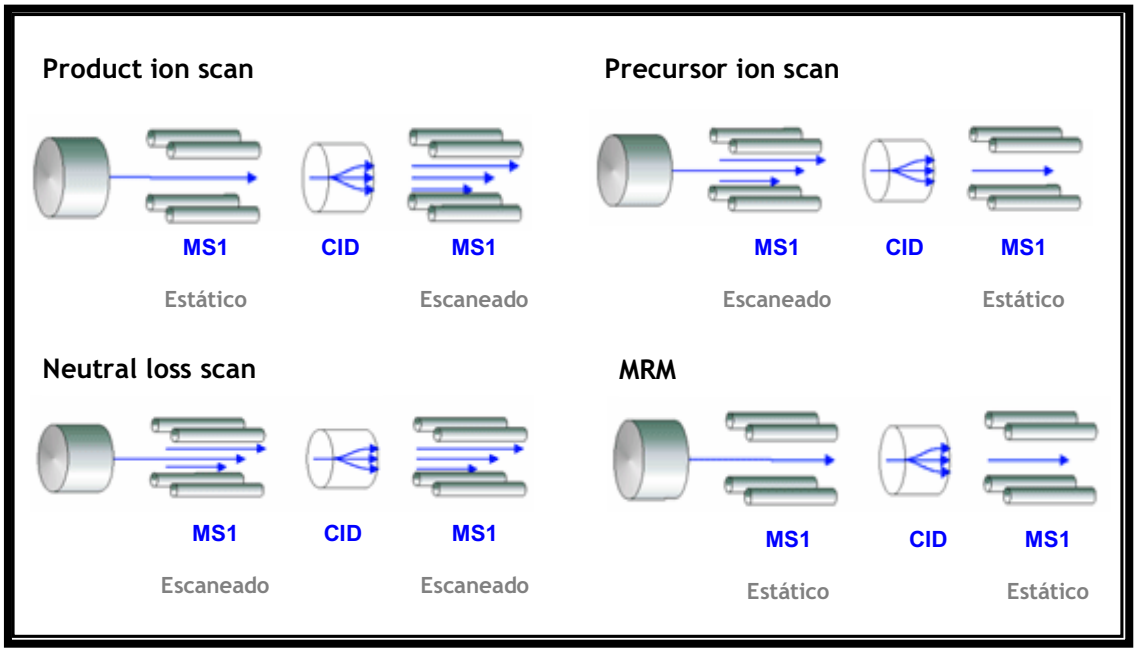


Figura 11. Esquema dels experiments de MS/MS que es poden realitzar en múltiples etapas.

3.3. BIOMARCADORS

Segons l'Institut Nacional del Càncer un marcador biològic és una molècula biològica que es troba en la sang, altres líquids o teixits corporals, i és un signe d'un procés normal o anormal, o de una afecció o malaltia. Un marcador biològic pot usar-se per determinar la resposta del cos a un tractament per una malaltia o afecció.

Un biomarcador també pot ser utilitzat en epidemiologia per mesurar l'exposició a diverses substàncies medioambientals. En particular, el biomarcador nutricional és un compost extern, com podrien ser els components dels aliments o una variant de la substància externa processada per l'organisme, un metabòlit analitzat en les mostres biològiques dels participants que serveix per determinar la seva exposició o ingesta a aquest aliment o component.

El principal objectiu de l'epidemiologia nutricional és avaluar els efectes de la dieta en el risc de patir malalties. Per comprendre fiablement com influeix la dieta és necessari conèixer de forma precisa la ingesta d'aliments dels participants. La valoració de la dieta es pot realitzar mitjançant biomarcadors nutricionals o a partir d'enquestes alimentàries.

3.3.1. ENQUESTES ALIMENTÀRIES

Per a conèixer el consum alimentari individual o poblacional es disposa de les enquestes alimentàries que estimen el consum durant un període de temps determinat. Existeixen diferents classificacions de les enquestes, però una de les més habituals és en funció del període de temps que es vol avaluar:

- Mesures de la ingesta dietètica de dies concrets o actual
    - Registre alimentari: mètode prospectiu que consisteix en sol·licitar a la persona entrevistada que anoti en un formulari diàriament durant varis dies (els més freqüents són els de 3 dies), els aliments i begudes que va consumint tant en a casa com fora.
    - Recordatori de 24 hores: mètode retrospectiu que consisteix en demanar al subjecte que recordi tots els aliments i begudes ingerides el dia anterior a l'entrevista.

- Mesures de la ingesta habitual

    - Qüestionari de Freqüència de Consum: mètode retrospectiu en el que se li administra al subjecte una llista tancada d'aliments i begudes sobre la que es sol·licita la freqüència y la porció de consum durant un periode de temps determinat (6 mesos, 1 any).

    - Història dietètica: mètode retrospectiu que inclou un o varis recordatoris de 24 hores, amb la finalitat de conèixer la ingesta actual, i un qüestionari de freqüència de consum per conèixer la ingesta habitual.

En el capítol del llibre d'Arija Val & Fernández Ballart apareix una completa revisió de les avantatges i limitacions de l'ús de les diverses enquestes alimentàries (Arija Val and Fernández Ballart, 2002). Les avantatges i limitacions de les enquestes estan en gran part influenciades pel mètode d'administració de les mateixes, aquestes poden realitzar-se mitjançant entrevista, ja sigui en persona o per via telefònica o autoadministrada. L'entrevista dóna millors resultats perquè l'entrevistador pot facilitar i afavorir la resposta del qüestionari, no obstant pot influenciar de forma diferent en la resposta dels participants introduint en l'estudi un biaix degut a l'entrevistador (Delgado et al., 2008).

3.3.2. AVANTATGES DELS MARCADORS BIOLÒGICS RESPECTE A L'ESTIMACIÓ DIETÈTICA o MARCADORS DIETÈTICS.

Beaton *et al.* Va declarar que: "Sempre existeix un error en les estimacions dietètiques i el desafiament actual és entendre, estimar, i tenir en compte aquest error estructural durant l'anàlisi de les dades" (Beaton et al., 1997). Per conèixer aquest error és necessari disposar de biomarcadors que reflexin la ingesta real dels individus. Aquests biomarcadors poden arribar a substituir les estimacions dietètiques tradicionals (Kristal et al., 2005). Tot i que en algunes situacions, les estimacions dietètiques són encara imprescindibles degut a limitacions econòmiques i a la falta de biomarcadors útils(Kelemen, 2006).

Existeixen tres raons principals per decantar-se per l'ús de biomarcadors nutricionals respecte a les enquestes dietètiques convencionals (Potischman, 2003;Kelemen, 2006).

1. Els biomarcadors nutricionals són més precisos que les estimacions dietètiques. Les enquestes, sobre tot els qüestionaris de freqüència de consum, ofereixen uns inconvenients a l'hora d'identificar correctament l'aliment, degut a la gran gamma

d'aliments presents en el mercat, a l'amplia varietat de tècniques culinàries disponibles i finalment a les diferències en ingredients i en preparació de receptes culinàries. Un altre factor limitant és la quantificació d'aquests aliments, tot i que es faciliti mitjançant mesures casolanes i llibres de fotos sobre aquestes porcions. També cal destacar que existeix la tendència entre els participants a sobreestimar el consum d'aliments saludables (per exemple fruites i verdures) i infravalorar el consum d'aliments considerats menys saludables (aliments rics en greixos saturats i begudes alcohòliques, entre d'altres) (Spencer et al., 2008).

2. Les enquestes d'aliments són transformades a components gràcies a les bases de dades o taules de composició d'aliments (TCA). Les TCA presenten moltes limitacions en quant a nombre d'aliments, variabilitat en composició dels aliments, mètodes analítics utilitzats i nombre de components disponibles, sobre tot en el cas de components minoritaris com poden ser els polifenols. També s'ha de tenir en compte la complexitat de saber quines són les TCA més adequades per l'estudi i com utilitzar les TCA per part de l'investigador (Farran Codina and Zamora-Ros, 2006).

3. Els biomarcadors proporcionen una mesura més propera de l'estat nutricional que les estimacions dietètiques. Això és degut a que els biomarcadors integren en la mesura, la biodisponibilitat i el metabolisme del component. En canvi les TCA només informen de la quantitat present en l'aliment.

La funció principal dels biomarcadors nutricionals, com s'ha comentat anteriorment, és la de conèixer de forma menys subjectiva que les enquestes alimentàries el consum d'un component o grup de components (es tracti d'un nutrient o un no nutrient) o d'un aliment o un grup d'aliments.

Una funció secundària dels biomarcadors nutricionals és la de valorar l'acompliment d'una intervenció nutricional. Comprovar objectivament que els participants realment han seguit el tractament o la suplementació, en aquest cas dietètica, de forma correcta. En aquest cas les enquestes alimentàries no podrien substituir l'ús de biomarcadors. Per exemple, en l'estudi PREDIMED es suplementa la dieta habitual amb oli d'oliva verge o fruits secs, per verificar que els participants que segueixen aquestes suplementacions ho estan seguint correctament les pautes se'ls analitza en el grup de l'oli d'oliva verge, l'hidroxitirosol i el tirosol en orina (polifenols característics de l'oli d'oliva), també s'analitza l'àcid oleic en

plasma (àcid gras característic de l'oli d'oliva), i en el grup dels fruits secs l'àcid α-linolènic plasmàtic (característic de les nous) (Estruch et al., 2006).

3.3.3. CARACTERÍSTIQUES DELS BIOMARCADORS NUTRICIONALS O MARCADORS BIOLÒGICS

La gran avantatja que presenten els biomarcadors nutricionals és que l'exposició es mesura de forma més objectiva que en les enquestes dietètiques. Com en qualsevol mesura analítica correcte biomarcador ha de complir els clàssics requisits d'exactitud, reproducibilitat, fiabilitat i validesa (Marshall, 2003).

A més a més qualsevol biomarcador nutricional per ser considerat útil ha de satisfer els següents 4 criteris (Spencer et al., 2008):

1. Disposar d'un mètode analític qualitativa i quantitativament robust. És necessari treballar amb la metodologia adequada per garantitzar la validesa dels resultats. En components minoritaris, com és el cas dels polifenols, la sensibilitat i especificitat de la tècnica adquireixen gran importància. Els avenços en tècniques d'anàlisi químic, com és el cas de LC-MS/MS, estan permitint l'estudi d'aquests components en mostres biològiques (Liu et al., 2002;Day and Williamson, 2001).

2. Les concentracions del biomarcador en la mostra biològica (teixit o biofluid) han de ser sensibles als canvis en el consum del compost d'interès. Aquesta és la característica més important que necessita complir un biomarcador nutricional. En aquest apartat és important considerar la capacitat que té el biomarcador de discriminar als consumidors dels no consumidors, en el caso que sigui preceptiu, com en qualsevol prova diagnòstica, ha de presentar uns bons paràmetres de sensibilitat, especificitat i unos valors predictius elevats, minimitzant els falsos positius i negatius. També cal destacar la capacitat de detectar petits increments en l'exposició, mostrant una bona correlació entre mesura i exposició.

3. El biomarcador ha de ser específic de la ingesta del component d'interès. Per aquest motiu qualsevol variació en la seva concentració ha de ser el resultat en un canvi en el consum del component d'interès. En aquest apartat apareixen 2 tipus de biomarcadors nutricionals: a) Biomarcador de consum d'un aliment específic o d'un grup d'aliments (exemples de polifenols i vitamines com biomarcadors nutricionals es poden observar a la **Taula 5**); b) Biomarcador de consum d'un component o grup de components específics (exemples **Taula 6**).

El cas ideal seria que un únic aliment tingués el component d'interès, d'aquesta forma les variacions en el marcador serien degudes exclusivament a les variacions en el consum d'aquest aliment. Aquesta possibilitat és molt complicada, tot i que alguns components són específics d'uns aliments molt concrets. Aquest és el cas d'alguns polifenoles, i el consum d'aliments rics en aquestes substàncies com el te, cafè, vi, soja, ceba (Spencer et al., 2008). Per millorar l'especificitat d'els marcadors és necessari conèixer més detalladament la composició dels aliments, sobre tot en components minoritaris. Paral·lelament és important que apareguin més estudis de consum d'aquests components para conèixer quines són les fonts dietètiques majoritàries en la població d'estudi.

Els biomarcadors d'un component se suposa que són més fàcils de trobar, tot i que canvis en el consum no tenen necessàriament que reflexar-se directament en el biomarcador (Marshall, 2003). Tots els marcadors d'aliment no deixen d'estar inclosos dintre d'aquest apartat, degut a que quan es mesuren les isoflavones en plasma s'està mesurant l'exposició d'isoflavones, tot i que com aquestes són casi exclusives dels productes de la soja i derivats, es pot extrapolar al consum d'aquets grup d'aliments.

4. La interpretació del biomarcador és més complexa que la de la enquesta alimentària degut a que el biomarcador també té en compte la biodisponibilitat del component. Per aquest motiu els estudis fàrmaco o nutri-cinètics són imprescindibles per aprofundir en el coneixement de l'absorció, metabolisme i excreció del component. En el cas dels polifenols, depenent de la dosis, del temps transcorregut des de la ingesta i de l'individu poden aparèixer diferents perfils metabòlics del mateix polifenol. Per aquest motiu els avenços en les tècniques analítiques són imprescindibles per poder disposar del perfil total de metabòlits del polifenol. Una altra possibilitat, cada vegada menys emprada, és la hidròlisi, normalment enzimàtica, de les mostres biològiques. L'inconvenient és que es perd una informació que pot ser molt valuosa sobre quins metabòlits i la quantitat que es troben presents en la mostra biològica.

   Una limitació de l'ús dels biomarcadors és el temps de vida mitjana dels components en la mostra biològica. Existeixen múltiples exemples, depenent del teixit o biofluid seleccionat, de biomarcadors d'ingesta a curt termini (2-6 hores) com podrien ser els polifenols en plasma, d'ingesta a mig termini (1-5 dies) com podrien ser els polifenols en orina, i de llarg termini (setmanes o mesos) per exemple el seleni en ungles

(Marshall, 2003). Depenent de la nostra investigació ens interessarà més seleccionar un teixit/biofluid o un altre, perquè la informació que ens oferirà el biomarcador serà totalment diversa: a) un biomarcador a curt termini ens informarà d'una ingesta aguda (consum puntual); i b) un biomarcador a mig/llarg termini ens informarà d'una ingesta regular o habitual (consum continuat en el temps).

Una altra limitació important és la gran variabilitat interindividual que existeix en la majoria de respostes metabòliques quan es dóna la mateixa quantitat/dosis de component, aquesta és una situació molt freqüent en els estudis de biodisponibilitat de polifenols (Urpi-Sarda et al., 2005;Urpi-Sarda et al., 2007;Manach et al., 2004).

Taula 5. Biomarcador de consum de components o grup de components en estudis en humans.

Component	Biomarcador	Teixit/Biofluid	N	Enquesta alimentària	Correlació (r)	Referència
Àcids grassos	AG Poliinsaturats	Teixit adipós subcutani	321	2 recordatoris 24h	0.5	(Plakke et al., 1983)
	AG Monoinsaturats				0.22	
	AG Saturats				0.24	
Greix làctic	Àcid pentadecanoic (C15:0)	Teixit adipós subcutani	81	Registre 1 setmana	0.63	(Wolk et al., 1998)
				FFQ	0.40	
Energia	Aigua doplement marcada ($^{2}H_{2}{}^{18}O$)	Orina 15 dies	81	4 Recordatoris 24h	0.41	(Davies et al., 1994)
Nitrogen	Nitrogen	Orina 24h	160	FFQ	0.187	(Kipnis et al., 2001)
			8	28 dies en cambra metabòlica	0.16-0.70	(Bingham and Cummings, 1985)
			156	16 dies registre amb pesades	0.69	(Bingham et al., 1997)
				FFQ	0.24	
				Recordatori 24h	0.10	
				Registre 7 días	0.65	
Potassi	Potassi	Orina 24h	156	16 dies registre amb pesades	0.76	(Bingham et al., 1997)

				FFQ	0.25	
				Recordatori 24h	0.51	
				Registre 7 dies	0.66	
Vitamina C	Vitamina C	Plasma	127	16 dies registre amb pesades	0.49	(Bingham et al., 1997)
				FFQ	0.26	
				Recordatori 24h	0.26	
				Registre 7 dies	0.22	
Carotens	Carotens	Plasma	156	16 dies registre amb pesades	0.2-0.69	(Bingham et al., 1997)
				FFQ	0.03-0.42	
				Recordatori 24h	0-0.19	
				Registre 7 dies	0.07-0.34	
			307	FFQ	0.11-0.52	
Alfa-tocoferol	Alfa-tocoferol	Plasma	307	FFQ	0.41-0.52	
Flavonol	Flavonol	Plasma	10	Registre 7 dies	0.75	(Noroozi et al., 2000)
		Orina 24h			0.73	
Vitamina K	Vitamina K	Orina 24h	9	30 dies en cambra metabòlica	0.70	(Harrington et al., 2007)

Daidzeïna	Daidzeïna	Plasma	96	FFQ	0.37	(Frankenfeld et al., 2003)
Genisteïna	Genisteïna	Plasma	96	FFQ	0.43	(Frankenfeld et al., 2003)

Taula 6. Biomarcador de consum d'un aliment o grup d'aliments en estudis humans.

Aliment	Biomarcador	Teixit/Biofluid	N	Enquesta alimentària	Correlació (r)	*P*	Referència
Fruita i Hortalisses	Flavonoids totals	Orina 24h	94	Recordatori 3 dies	0.35	<0.001	(Nielsen et al., 2002)
	Naringenina				0.30	0.004	
	Hesperitina				0.38	<0.001	
	Tamarixetina				0.27	0.01	
	Isohamnetina				0.28	0.008	
Fruita	Carotenoids	Plasma	161	FFQ	0.30	<0.01	(Bogers et al., 2003)
	Vitamina C				0.25	<0.01	
	Ploretina	Orina 24h	94	Recordatori 3 dies	0.29	0.006	(Nielsen et al.,
	Kamferol	Orina puntual	53	Recordatori 2 dies	0.30	0.03	

	Lignans	Orina 24h	98	FFQ/Recordatori 2 dies	0.27	0.008	2002) (Mennen et al., 2006) (Lampe et al., 1999)
Hortalisses	Carotenoids	Plasma	161	FFQ	0.32	<0.01	(Bogers et al., 2003)
	Vitamina C				0.34	<0.01	
	Quercetina	Orina 24h	94	Recordatori 3 dies	0.28	0.007	
	Enterolactona	Orina 24h	53	Recordatori 2 dies	0.31	0.02	(Nielsen et al., 2002) (Mennen et al., 2006)
Suc de fruites	Hesperitina	Orina 24h	94	Recordatori 3 dies	0.32	0.002	(Nielsen et al., 2002)
	Isohamnetina	Orina puntual	53	Recordatori 2 dies	0.30	0.03	
	Naringenina				0.44	0.001	(Mennen et al., 2006)
	Hesperitina				0.39	0.004	
	Àcid gàlic				0.33	0.02	
	4-O-metilgàlic				0.37	0.006	

	Naringenina	Orina 24h			0.37	0.007	
Cafè	Àcid clorogènic	Orina puntual	53	Recordatori 2 dies	0.63	<0.001	(Mennen et al., 2006)
	Àcid cafeic				0.29	0.03	
	Àcid isoferúlic	Orina 24h	344	FFQ	0.18-0.26	<0.001	(Hodgson et al., 2004b)
Te	Ácido m-cumárico	Orina puntual	53	Recordatori 2 dies	0.44	0.001	(Mennen et al., 2006)
	Ácido clorogénico				0.31	0.03	
	Ácido gálico				0.45	<0.001	
	4-O-metilgálico				0.54	<0.001	
	4-O-metilgálico	Orina 24h	344	FFQ	0.50-0.57	<0.001	(Hodgson et al., 2004a)
Vi	Àcid gàlic	Orina puntual	53	Recordatori 2 dies	0.45	<0.001	(Mennen et al., 2006)
	4-O-metilgàlic				0.37	<0.006	
	Àcid cafeic	Orina 24h			0.38	0.005	
	Àcid gàlic				0.70	<0.001	
	4-O-metilgàlic				0.52	<0.001	

Poma	Ploretina	Orina puntual	53	Recordatori 2 dies	0.60	<0.001	(Mennen et al., 2006)
	Àcid m-cumàric	Orina 24h			0.36	0.009	
	Isohamnetina				0.31	0.02	
	Kamferol				0.45	<0.001	
	Ploretina				0.35	0.01	
Raïm	Naringenina	Orina puntual	53	Recordatori 2 dies	0.31	0.02	(Mennen et al., 2006)
Fruites cítriques	Hesperitina	Orina puntual	53	Recordatori 2 dies	0.52	<0.001	(Mennen et al., 2006)
	Naringenina				0.56	<0.001	
	Hesperitina	Orina 24h			0.46	<0.001	
	Naringenina				0.37	0.007	
Llegums	Isoflavones	Orina 24h	19	Recordatori 3 dies	0.668	<0.01	(Adlercreutz et al., 1991)
	Lignans				0.492	<0.05	
Soja i derivats	Isoflavones	Orina 24h	60	FFQ	0.5	<0.001	(Chen et al., 1999)
	Isoflavones	Orina 24h	98	FFQ/Recordatori 5 dies	0.39	<0.001	(Lampe et al., 1999)

Soja bullida	Isoflavones Lignans	Orina 24h	19	Recordatori 3 dies	0.76 085	<0.001 <0.001	(Adlercreutz et al., 1991)
Derivats soja	Isoflavones	Orina 24h	19	Recordatori 3 dies	0.59	<0.01	(Adlercreutz et al., 1991)
Proteïna soja	Isoflavones	Orina del matí		Recordatori 3 dies	0.61	<0.001	(Maskarinec et al., 1998)
Blat i cibada integrals	Alkilresorcinols	Plasma	30	3 dies registre amb pesadas	0.58	<0.001	(Landberg et al., 2008)

PART EXPERIMENTAL

4. PART EXPERIMENTAL

Amb la finalitat d'aconseguir els objectius d'aquesta tesi es va plantejar una metodologia de treball dividida en els següents tres apartats:

4.1. METODOLOGIA

4.1.1. Disseny dels estudis

Per a la realització d'aquesta tesi s'han utilitzat dades i/o mostres de 3 assags clínics i d'un estudi de cohorts, resumits en les publicacions i que a continuació es detallen més ampliamente.

4.1.1.1. ESTUDIS CLÍNICS

4.1.1.1.1. CAVA versus GINEBRA

Per a la realització d'aquest primer treball, es va dissenyar un estudi clínic prospectiu, aleatoritzat, creuat, controlat i a simple cec portat a terme conjuntament amb l'Hospital Clínic de Barcelona (Vazquez-Agell et al., 2007). Es van reclutar 20 homes sans, d'entre 25 i 50 anys, normopes o amb un lleuger sobrepes [IMC= 25.2 (1.3) kg/m^2], dels quals cap d'ells va declarar cap dels següents criteris d'exclusió: diabètic, fumador, hipertens, amb nivells de colesterol LDL plasmàtic superior a 160mg/dL, HDL<40mg/dL, i absència de qualsevol patologia cardiovascular, història familiar de malaltia prematura cardiovascular, malaltia cerebrovascular, malaltia vascular perifèrica, infecció por VIH, malalties hepàtiques degudes a l'alcohol, càncer o alguna infecció aguda greu. A més cap voluntari va consumir medicaments, ni suplements vitamínics durant el període de realització de l'estudi. Tots els participants eren consumidors moderats de begudes alcohòliques al menys durant els 5 anys previs a l'inici d'aquest estudi.

Les dos intervencions van consistir en la ingesta de 30g d'alcohol al dia durant 4 setmanes, en forma de cava (300mL/d) o de ginebra (100mL/d) per avaluar les diferències entre els efectes beneficiosos sobre la salut de dos begudes alcohòliques amb un contingut mig en polifenols (cava) i l'altre sense polifenols (ginebra). Abans de cada període d'intervenció es va seguir un període de rentat (*wash-out*) de 4 setmanes en el que estava prohibit la ingesta d'alcohol. Durant les 16 setmanes de l'estudi als voluntaris se'ls va proporcionar una llista d'aliments i begudes a restringir, entre elles el cafè, te, ceba i l'oli d'oliva verge (aliments molt rics en polifenols que podrien interferir o emmascarar els efectes en la part mèdica de l'estudi). Les

mostres de sang i orina de primera hora del matí es va obtenir el primer i últim dia de cada període d'intervenció. Aquest dia, els voluntaris eren entrevistats per una dietista que recollia mitjançant un qüestionari de freqüència de consum validat la ingesta d'aliments i mitjançant el qüestionari de Minesota l'exercici físic realitzat (Elosua et al., 1994). El disseny de l'estudi s'exposa esquemàticament en la **Figura 12**. Dels 20 participants únicament es va determinar el resveratrol urinari en 10 d'ells (10).

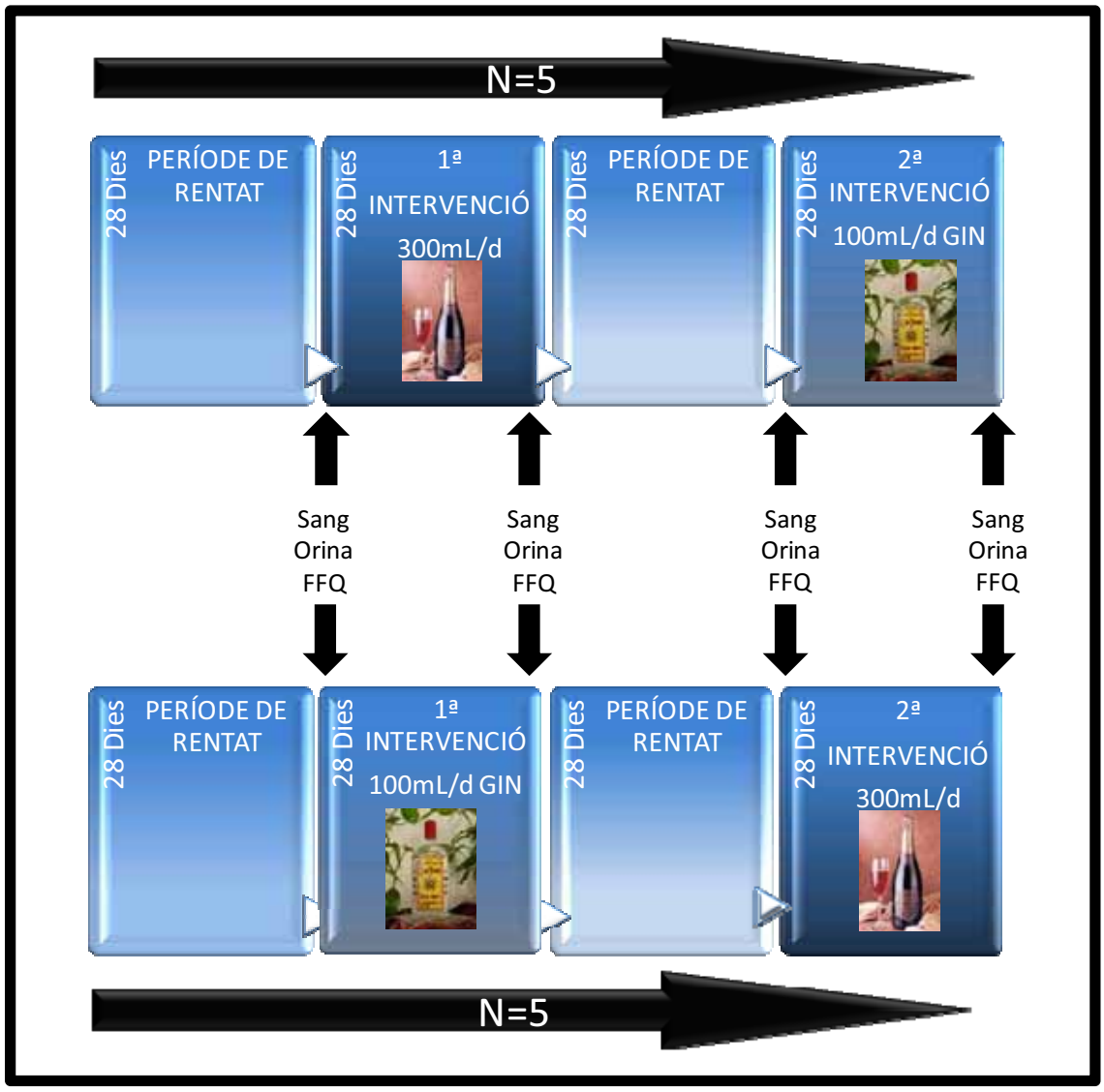


Figura 12. Disseny de l'estudi cava vs ginebra

4.1.1.1.2. VI BLANC versus VI NEGRE

El disseny d'aquest segon treball va ser molt similar a l'anterior, es va realitzar també un assaig clínic prospectiu, aleatoritzat, creuat, controlat i a simple cec elaborat conjuntament amb l'Hospital Clínic de Barcelona (Sacanella et al., 2007). Es van reclutar 35 dones sanes, d'entre 23 i 50 anys, normopes o amb un lleuger sobrepes [IMC= 24.1 (4.0) kg/m^2], les quals no van declarar cap dels següents criteris d'exclusió: diabètica, fumadora, hipertensa, LDL> 160mg/dL, HDL<35mg/dL, i absència de qualsevol patologia cardiovascular, història familiar de malaltia prematura cardiovascular. A més cap voluntària va consumir medicaments, anticonceptius orals ni suplements vitamínics durant el període de realització de l'estudi. Totes les participants eren consumidores moderades de begudes alcohòliques com a mínim durant els últims 5 anys previs a l'inici d'aquest estudi.

Les dos intervencions van consistir en la ingesta de 20g d'alcohol (equivalents a un consum moderat d'alcohol) al dia durant 4 setmanes, en forma de vi blanc (200mL/d) o de vi negre (200mL/d) per avaluar les diferències entre els efectes beneficiosos sobre la salut de dos begudes alcohòliques amb un contingut mig i alt de polifenols, respectivament. Abans de cada període d'intervenció es va seguir un període de rentat (*wash-out*) de 4 setmanes en el que estava prohibida la ingesta de qualsevol tipus d'alcohol. Durant les 16 setmanes de l'estudi a les voluntàries se'ls va proporcionar una llista d'aliments i begudes riques en polifenols que han de restringir el seu consum. Totes les intervencions es van iniciar amb el primer dia de la menstruació, degut a que és el moment del cicle menstrual amb menor presència de progesterona, a més d'aquesta forma totes les dones eren avaluades en la mateixa data del cicle i per tant el possible efecte hormonal es contraresta. Les mostres de sang i d'orina de primerahora del matí es van obtenir el primer i últim dia de cada intervenció. Aquest dia, les voluntàries eren entrevistades per una dietista, que recollia mitjançant un qüestionari de freqüència de consum validat la ingesta d'aliments i amb el qüestionari de Minesota el exercici físic realizat (Elosua et al., 1994). El disseny de l'estudi s'exposa de forma més detallada a la **Figura 13**. De les 35 participants només es va determinar el resveratrol urinari en 10 d'elles.

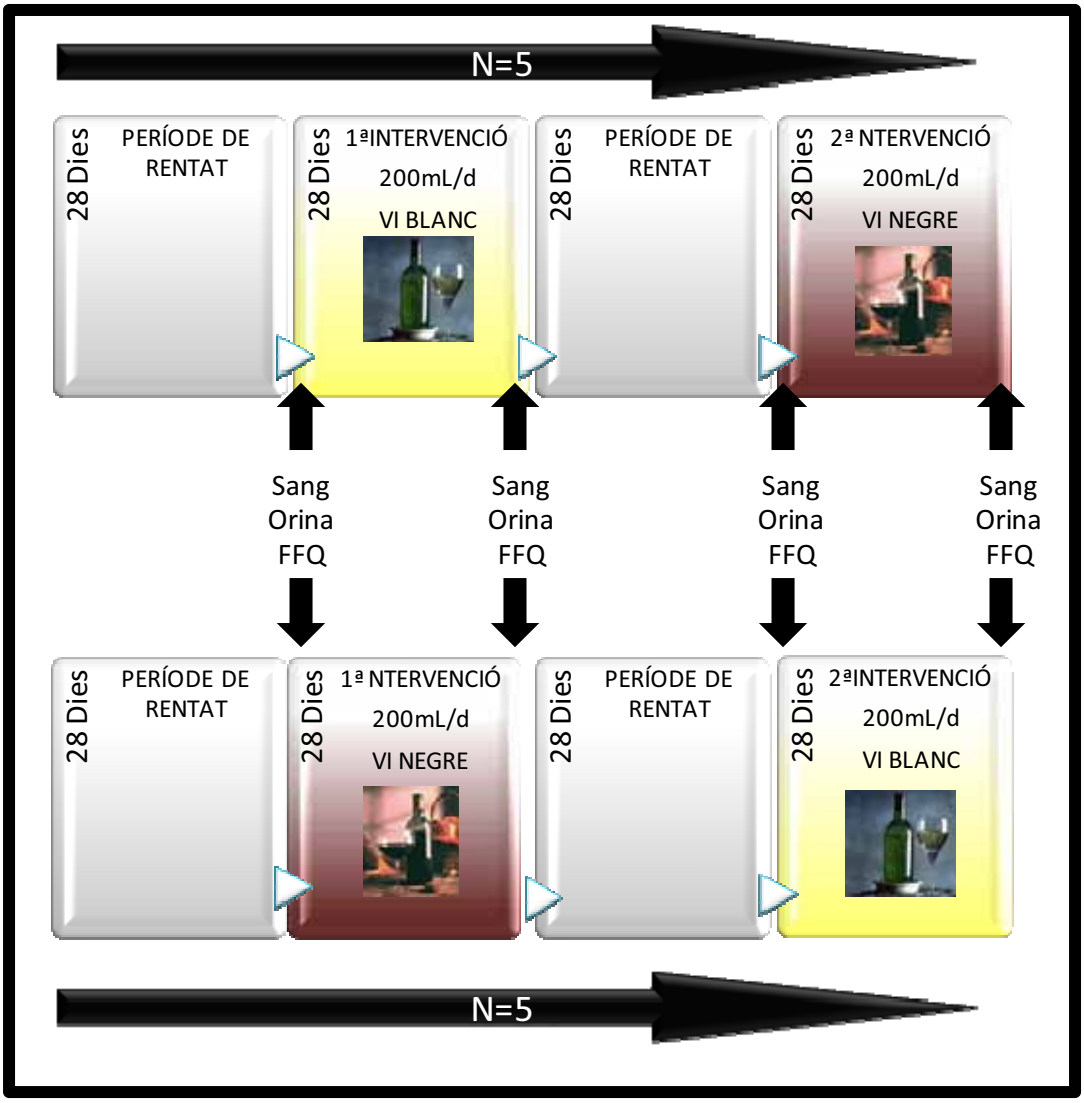


Figura 13. Disseny de l'estudi vi blanc vs vi negre

4.1.1.1.3. ESTUDI PREDIMED

L'estudi PREDIMED (PREvenció amb Dieta MEDiterrània) és una xarxa temàtica de centres del Ministeri de Sanitat i Consum iniciada en Octubre 2003, i que engloba a 16 grups d'investigadors en 8 comunitats autònomes. Es tracta d'un gran assaig clínic d'intervenció a 5 anys, multicèntric, amb grups paral·lels, aleatoritzat i controlat, que avalua l'eficàcia d'una Dieta Mediterrània tradicional suplementada amb oli d'oliva verge o fruits secs, en comparació amb una Dieta Baixa en Greix, en la prevenció primària de malalties cardiovasculars en persones d'alt risc (www.predimed.org) (Estruch et al., 2006).

Fins la data s'han reclutat més de 6000 participants d'alt risc cardiovascular, tot i que vol arribar la xifra de 9000, distribuïts uniformement entre les tres intervencions. Els participants

han de complir els següents criteris d'inclusió per poder ser admesos en l'estudi: homes d'entre 55 i 80 anys o dones d'entre 60 i 80 anys, que siguin diabètics o que tinguin 3 o més dels següents factors de risc cardiovascular: fumador, amb hipertensió arterial, nivells LDL> 160mg/dL, HDL<40mg/dL, IMC>25kg/m^2, o història familiar de malaltia cardiovascular prematura. A més a més els participants no eren acceptats en l'estudi si tenien algun dels següents criteris d'exclusió: història de malaltia cardiovascular, malalties cròniques severes, addicció a drogues o alcohol, alergia o intolerància a fruits secs o a l'oli d'oliva, o finalment baixa disposició a canviar d'hàbits dietètics.

Els voluntaris són distribuïts aleatòriament en una de les tres intervencions: dieta mediterrània suplementada amb oli d'oliva verge (1L/setmana per a tota la família), dieta mediterrània suplementada amb 30g/d de fruits secs (50% nous, 25% ametlles i 25% avellanes) o el grup control, en aquest cas val ésser pacients con amb alt risc cardiovascular es segueix la pauta de l'Associació de Cardiòlegs Americans, aquesta dieta es pot resumir com una dieta en general baixa en greix. El segon objectiu de l'estudi PREDIMED consisteix en valorar la ingesta de vi com a factor cardiosaludable. Degut a que cap beguda alcohòlica pot ser recomenada a un abstemi, l'efecte del vi serà estudiat de forma observacional. Per aquest motiu els participants de qualsevol de les 3 intervencions poden consumir vi, l'única recomanació realitzada per la dietista és la de reduir el consumo del vi fins que aquest estigui dintre de la moderació: 2-3 copes/d per als homes i 1-2 copes/d per a les dones. Als voluntaris que no bebien vi no se'ls va recomanar la seva ingesta. En la **Figura 14** s'esquematitza el disseny de l'estudi.

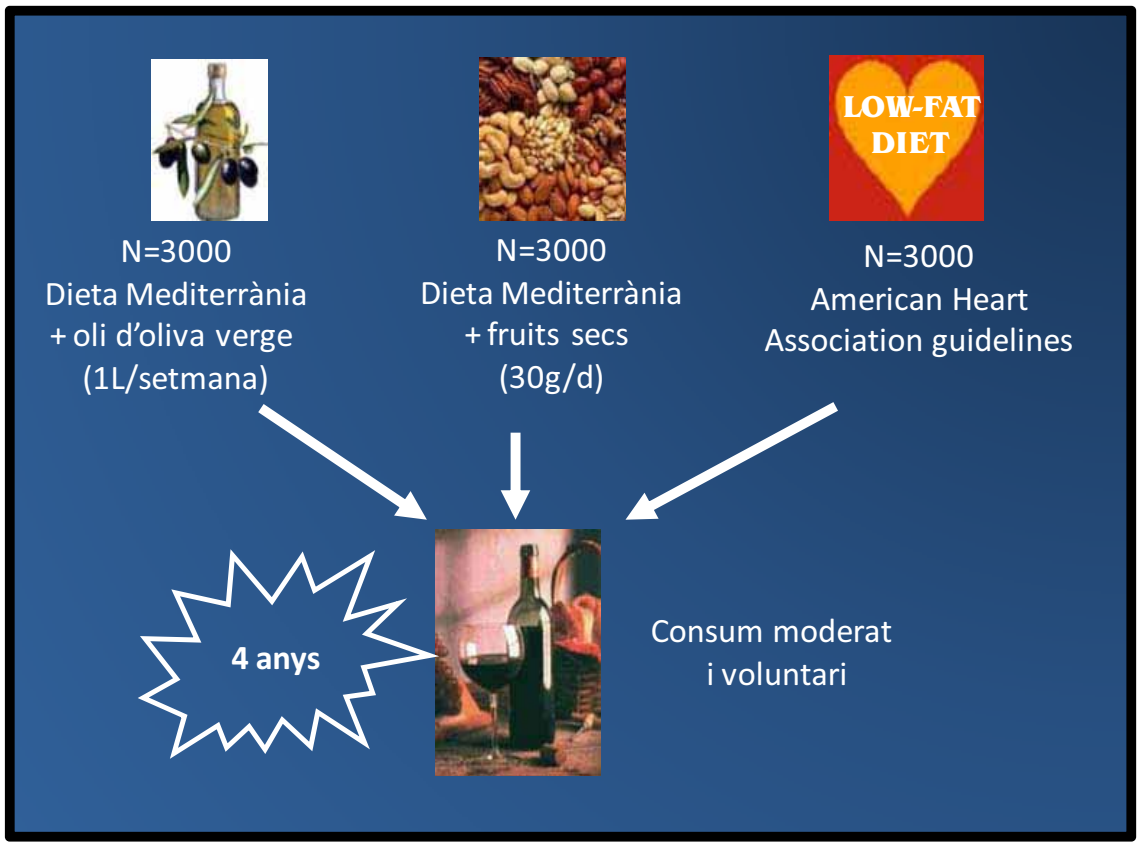


Figura 14. Esquema de les intervencions en l'estudi PREDIMED.

Els participants són entrevistats per dietistes entrenades, a l'inici, 3 mesos (prova pilot, que només es va realitzar amb una submostra de 772 participants), i posteriorment els seguiments són anuals fins els 4 anys de duració de l'estudi. En aquestes entrevistes s'omplena un qüestionari general, on entre d'altres s'obtenen les dades personals, història mèdica i fàrmacs prescrits, un qüestionari de freqüència de consum validat, una enquesta de 14 ítems per valorar l'adherència a la dieta mediterrània, i el qüestionari de Minesota adaptat i validat per recollir la informació sobre l'exercici físic (Elosua et al., 1994). A més als voluntaris se'ls mesura la pressió arterial i la freqüència cardíaca, es va realitzar mesures antropomètriques (pes, alçada, perímetre de la cintura) i també se'ls hi va extreure mostres d'orina i sang. En la visita inicial també es recull mostres d'ungles dels peus per realitzar l'anàlisi de metalls. Les mostres d'orina i sang són alicuotades i congelades a -80ºC fins al seu anàlisi. La **Taula 7** resumeix totes les recollides d'informació i de mostres durant els 5 anys de duració de l'estudi PREDIMED.

Taula 7. Qüestionaris, mesures i recollida de mostres dels participants de l'estudi PREDIMED

Visita	**1**	**2**	**3**	**4**	**5**	**6**
Temps (anys)	0	0.4	1	2	3	4
Qüestionari. Inclusió	**X**					
Qüestionari General	**X**					
Qüestionari Seguiment		**X**	**X**	**X**	**X**	**X**
FFQ	**X**	**X**	**X**	**X**	**X**	**X**
Mediterranean score	**X**	**X**	**X**	**X**	**X**	**X**
Qüestionari Activitat física	**X**	**X**	**X**	**X**	**X**	**X**
Mesures antropomètriques	**X**	**X**	**X**	**X**	**X**	**X**
Mesures pressió arterial	**X**	**X**	**X**	**X**	**X**	**X**
Electrocardiograma	**X**	**X**	**X**	**X**	**X**	**X**
Anàlisi de sang	**X**	**X**	**X**	**X**	**X**	**X**
Anàlisi d'orina	**X**	**X**	**X**	**X**	**X**	**X**
Anàlisi ungles dels peus	**X**					

4.1.1.2. ESTUDIS DE COHORTS

4.1.1.2.1. ESTUDI EPIC-ESPANYA

L'estudi EPIC (*European Prospective Investigation into Cancer and nutrition*) (www.iarc.fr/epic) és un estudi de cohorts, prospectiu, multicèntric, coordinat per l'Agència Internacional d'Investigació sobre càncer (IARC, International Agency for Research on Cancer) de l'Organització Mundial de la Salut (WHO). El reclutament dels participants es va iniciar al 1993 i el van portar a terme 23 centres de 10 països europeus (Alemanya, Dinamarca, Espanya, França, Grècia, Holanda, Itàlia, Noruega, Regne Unit i Suècia). En l'estudi europeu s'han reclutat 519978 voluntaris sans, la majoria d'entre 35 i 69 anys, dels que un 70.5% són dones.

La cohort espanyola (www.epic-spain.com) està formada per 40885 voluntaris (61.7% de dones) reclutats en 5 àrees geogràfiques (Astúries, Granada, Guipúscoa, Múrcia i Navarra). Aquest estudi va sorgir amb l'objectiu de integrar l'epidemiologia amb les investigacions de laboratori, amb els factors genètics i metabòlics, i aprofundir en el coneixement científic de la nutrició i el càncer.

Als participants se'ls recollí la informació sobre la ingesta habitual en l'any anterior, a través d'una entrevista personalitzada mitjançant l'aplicació d'un qüestionari informatitzat del mètode de la història dietètica, dissenyat i validat especialment per aquest estudi. El qüestionari va ser estructurat en àpats, i de cada individu es va recollir la informació sobre el consum habitual d'aliments en cada ocasió d'ingesta en l'any previ a l'entrevista. Aquest contenia una llista bàsica de més de 600 aliments i begudes, i aproximadament 150 receptes o plats regionals consumits en les comunitats participants. La porció habitual de consum era quantificada amb un manual de trenta cinc fotos, a més d'unitats i mesures casolanes.

També es va realitzar una exploració física que consistia en la medició del pes, talla dret i assentat, la circumferència de la cintura i el perímetre de la cadera. En una submostra de la cohort es van realitzar dos mesures de la pressió arterial. El qüestionari utilitzat va incloure informació sobre característiques socio-demogràfiques, història ocupacional, activitat física laboral i en el temps d'oci, història reproductiva i consum hormonal en dones, consum de tabac, així com els antecedents clínics i quirúrgics. A més de tots els participants de la cohort es va extreure una mostra de sang.

Com en qualsevol estudi prospectiu el seguiment és un apartat clau dintre del projecte. Aquesta fase té com a objectiu conèixer l'aport de cada subjecta al temps d'observació en la

cohort, conèixer i avaluar canvis en l'exposició, conèixer l'estatus vital de l'individu i identificar els casos de càncer. En l'any 1996 es va iniciar un seguiment actiu de tots els individus que formen la cohort i es va finalitzar al 1999, amb un seguiment proper al 98% (**Figura 14**). Per portar-ho a terme es va elaborar un qüestionari de seguiment informatitzat realitzant-se via telefònica. A més a més s'utilitzen d'altres mètodes de seguiment, com el creuament de diversos registres que es realitza anualment de forma informatitzada. La font principal per a la identificació dels difunts i el coneixement de la causa és el Registre de Mortalitat de l'Institut Nacional de Estadística (INE) i els Registres de Mortalitat autonòmics. La identificació dels nous casos de càncer es realitza a través dels Registres de Càncer poblacionals de cada una de las regions participants.

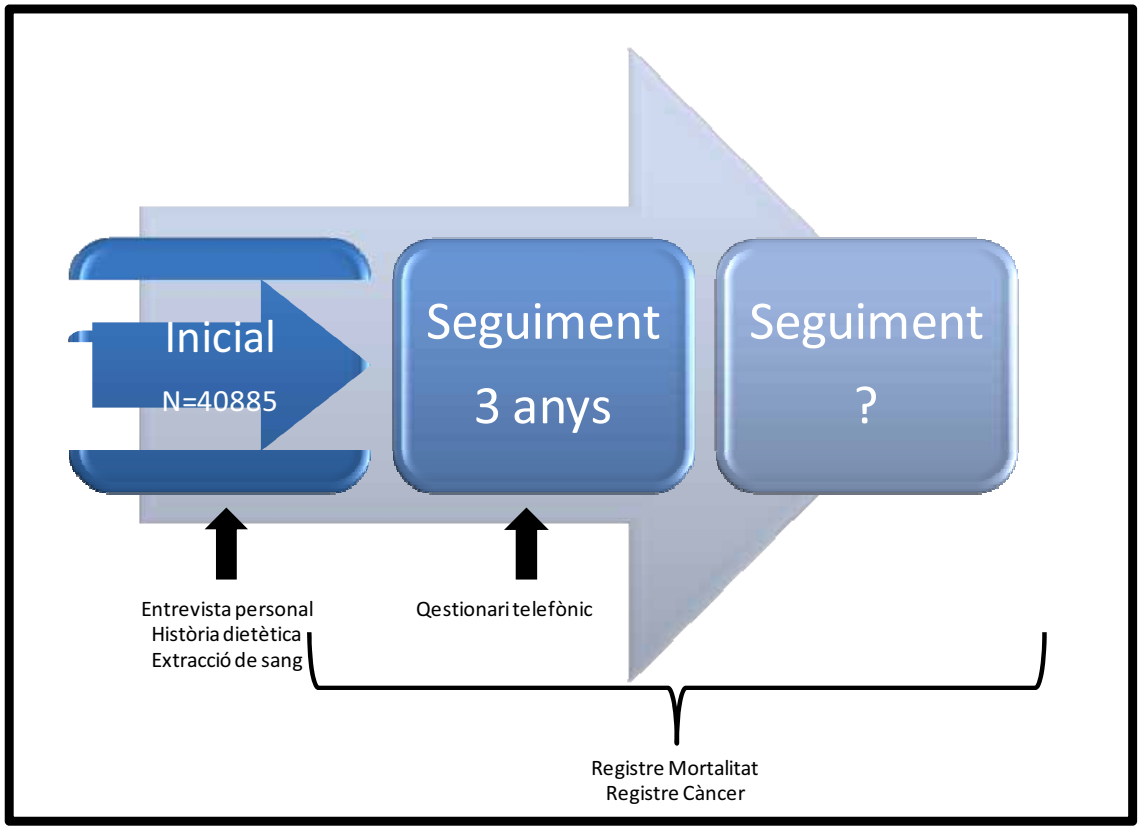


Figura 14. Esquema del disseny de l'estudi EPIC-Espanya

4.1.2. Metodologia analítica: Determinació de resveratrol en mostres biològiques

La metodologia utilitzada i les millores introduïdes per a la determinació del resveratrol i els seus metabòlits en mostres biològiques, plasma i orina i la seva adaptació a estudis epidemiològics amb un gran nombre de mostres han estat publicats pel nostre grup d'investigació en revistes analítiques de prestigi internacional (Urpi-Sarda et al., 2005;Urpi-Sarda et al., 2007).

En la primera publicació d'aquesta tesi es va emprar la metodologia posada a punt per Urpí *et al.* al 2005 (Urpi-Sarda et al., 2005) i que va ser millorada 2 anys més tard amb la finalitat d'augmentar la sensibilitat, el perfil metabòlic i l'aplicabilitat del mètode (Urpi-Sarda et al., 2007). Ambdues utilitzen una preparació prèvia de la mostra, una extracció en fase sòlida en cartutxos Oasis HLB® de Waters, i finalment una cromatografia líquida d'alta eficàcia acoblada a un detector de masses en tàndem (LC-MS/MS).

Les millores en el mètode van consistir en una adaptació del SPE de cartutxos individuals a plaques de 96 pouets. Gràcies a aquesta millora el mètode pot ser aplicat a estudis epidemiològics, amb un gran nombre de mostres, como és el cas del PREDIMED. Una atra millora clau en el mètode va consistir en assolir una bona resolució dels pics dels sulfats del resveratrol, augmentant qualitativament el perfil metabòlic del resveratrol biològic i reduint el temps de cromatografia de 38 a 10 minuts.

Les orines (1mL) dels voluntaris, un cop descongelades, es centrifuguen a 12500rpm a 10ºC durant 5 minuts i s'acidulen amb HCl 200mmol/L. Les mostres de sèrum (500μL) descongelades són acidificades amb àcid orto-fosfòric, agitades en vòrtex durant 1 minut i després centrifugades en les mateixes condicions que l'orina. En aquesta etapa previa s'aconsegueix reduir les interferències de gran pes molecular. A continuació es procedeix a realitzar l'extracció en fase sòlida. Els cartutxos (plaques de 96 pouets) s'activen i s'acondicionen amb 1mL de metanol i 1mL d'aigua acidulada (2M àcid acètic). Es procedeix amb la càrrega de 1mL de l'orina, a la que s'afegeix el patró intern (hexestrol). Posteriorment es renta el cartutx per eliminar el màxim possible d'interferències, en el nostre cas amb 1mL d'aigua acidulada (2M àcid acètic) i 1mL d'una solució aquosa acidulada (2M àcid acètic) amb metanol al 15%. A continuació es procedeix a l'elució amb 0.5mL de metanol acidulat (1M àcid acètic) i 1.5mL d'acetat d'etil acidulat (1M àcid acètic), incorporat en dos etapes (0.75 + 0.75mL). Amb la intenció de concentrar la mostra, els 2mL d'eluit es van evaporar fins a sequedat sota una corrent de nitrogen. Finalment es reconstitueix amb 100μL de fase de

reconstitució amb taxifolina, el segon patró intern utilitzat. La fase de reconstitució està formada pels dissolvents a les mateixes concentracions de les condicions inicials de la cromatografia posterior. En la **Figura 14** s'esquematitza totes les etapes de la SPE.

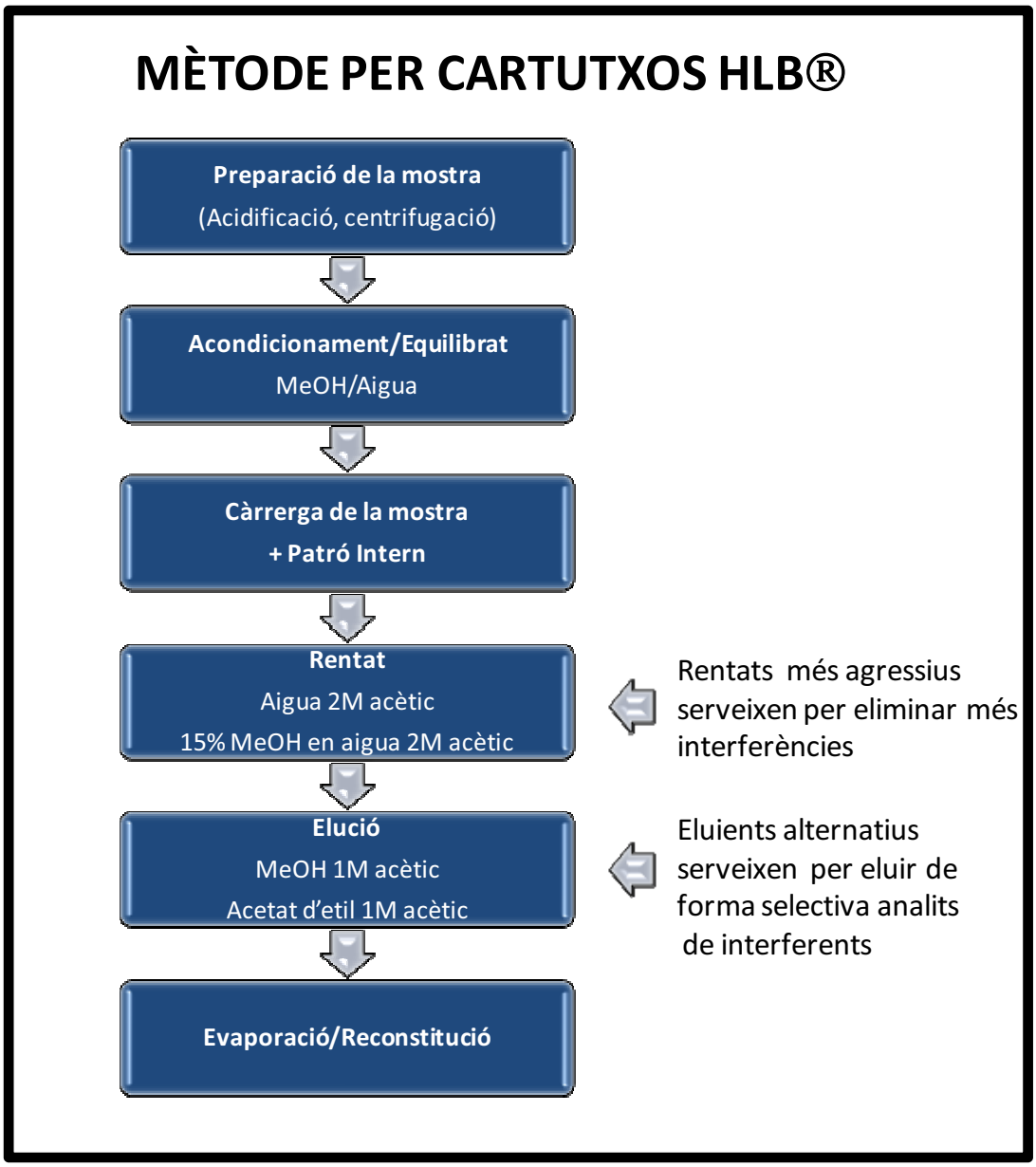


Figura 15. Mètode general per a cartutxos Oasis HLB® (Urpi-Sarda et al., 2007)

A continuació les mostres s'analitzen als Serveis Científico-Tècnics de la Universitat de Barcelona on s'injecten en el cromatògraf (Perkin-Elmer s200) acoplat a un espectròmetre de masses triple quadrupol (API 3000, Applied Biosystem) equipat amb una font d'ionització Turbo Ion Spray. La columna cromatogràfica emprada va ser una Luna C18, 50x2.0mm i.d., 5µm (Phenomenex) mantinguda a una temperatura de 40ºC. El volum d'injecció va ser de 15µL

i el flux de 550μL/minut. La fase mòbil A era aigua acidulada (0.05% àcid acètic) i la fase B 70% acetona, 30% acetonitril i 0.04% àcid acètic. El gradent utilitzat va ser lineal i està detallat a la **Taula 8**. La columna necessitava 6 minuts per reequilibrar-se.

Taula 8. Gradent cromatogràfic en la LC-MS/MS per la determinació de resveratrol i els seus metabòlits en mostres biològiques (Urpi-Sarda et al., 2007)

T (min)	Fase A (%)	Fase B (%)
0	85	15
1	85	15
1.5	60	40
2.5	0	100
4.5	0	100
4.8	85	15
10	85	15

Dels experiments disponibles en espectrometria de masses en tàndem, es va seleccionar el mode MRM (multiple reaction monitoring) per a la detecció i quantificació del resverartol i dels seus metabòlits. Es van monitoritzar 6 transicions *m/z* per cada anàlisi en mode negatiu, que corresponen al resveratrol (227/185), glucòsids del resveratrol (389/227), glucurònids del resveratrol (403/227), sulfats del resveratrol (307/227), i als patrons interns utilitzats taxifolina (303/285) i hexestrol (269/134).

4.1.3. Determinació de la capacitat antioxidant total (TAC) en mostres de plasma

Els mètodes de determinació de l'activitat antioxidant es basen en comprovar com un agent oxidant indueix dany oxidatiu a un substrat oxidable, dany que és inhibit o reduït en presència d'un antioxidant. Aquesta inhibició és proporcional a l'activitat antioxidant del compost o de la mostra. La TAC es defineix com el nombre de mols de radicals lliures neutralitzats per 1L de

mostra o de solució patró (Serafini and Del Rio, 2004). En aquesta mesura s'inclou tant l'activitat antioxidant singular de cada compost, com també les interaccions sinèrgiques entre les molècules redox presents en matrius complexes (Serafini et al., 2006).

A la bibliografia apareixen un gran nombre d'assaigs per mesurar la TAC classificats en 2 grups (Huang et al., 2005):

a) Assaigs basats en la transferència d'àtoms d'hidrògen, són reaccions competitives, en les que l'antioxidant o el substracte competeixen pels radicals peroxils generats tèrmicament a través de la descomposició de compostos azoics. En aquest grup s'inclouen: el TRAP, l'ORAC, la inhibició de l'auto-oxidació induïda de les LDL, i la decoloració de la crocina, entre d'altres.

b) Assaigs basats en la transferència d'electrons, són aquells que mesuren la capacitat d'un antioxidant en la reducció d'un oxidant, el qual canvia de color quan es redueix. Dintre d'aquest grup es pot destacar tècniques com el FRAP, el TEAC, el DPPH, o la capacitat de reduir Cu^{2+}, entre d'altres.

Actualment no hi ha un consens en la comunitat científica sobre quina de les tècniques existents per mesurar la TAC aporta una informació més adequada, això és degut a la gran diversitat de reaccions que es poden incloure sota la definició de la TAC. Per aquest motiu a la majoria d'estudis es realitzen varies tècniques analítiques per obtenir una informació més completa, en el nostre cas s'ha optat per seleccionar un assaig corresponent a cada un dels grups exposats anteriorment: TRAP i FRAP.

4.1.3.1. ANÀLISI DE TRAP EN MOSTRES DE PLASMA

L'assaig utilitza un compost que té la capacitat de perdre la seva fluorescència a l'oxidar-se, la R-ficoeritrina. A ella se li afegeix la mostra en la que hi ha presents una quantitat variable d'antioxidants que capten els radicals peroxil i alcoxil produïts per la descomposició en calent de l'ABAP. Aquesta disminució en la fluorescència es monitoritza obtenint la fase-lag (*lag-phase*) (una planúria provocada per la presència d'antioxidants que són els que capten els radicals produïts per l'ABAP) (**Figura 16**). Aquesta fase-lag és proporcional al contingut d'antioxidants de la mostra.

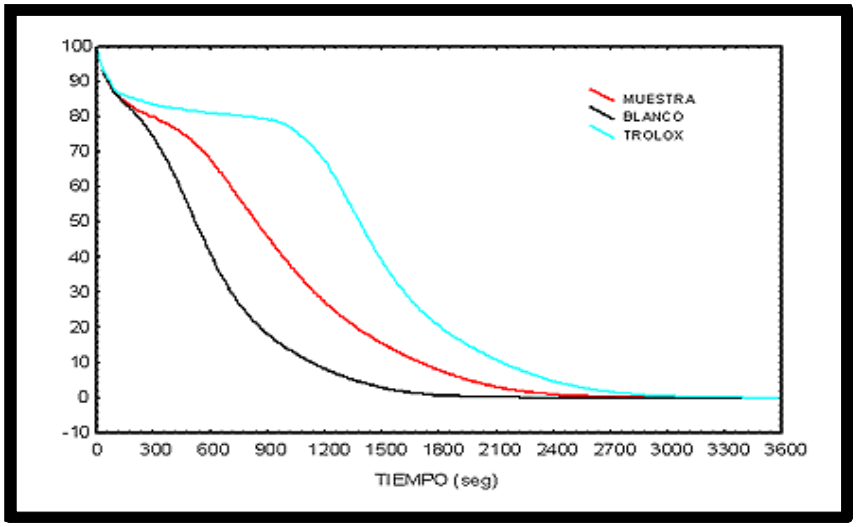


Figura 16. Fluorescència relativa de R-ficoeritrina incubada amb 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) enfront al temps en presència d'un tampó fosfat (blanc), trolox (20mM) o una mostra (Fernandez-Pachon et al., 2006).

La metodologia aplicada en aquest treball es basa en la tècnica publicada per Ghiselli *et al* (Ghiselli et al., 1995), adaptada a plaques de 96 pouets per poder ser utilitzada en estudis epidemiològics amb un gran nombre de mostres.

El protocol s'inicia amb la descongelació de les mostres de plasma. A continuació s'inicia la preparació de la placa de 96 pouets afegint 15μL de R-ficoeritrina (4.3μg/mL), 50μL de plasma diluït 1:20 en PBS i 75μL de PBS. Seguidament la placa s'introdueix en un bany a 38ºC a les fosques durant 15min. Finalment s'addicionen 60μL d'ABAP (50mM) i s'introdueix en el fluorímetre durant 1 hora a 38ºC, on es realitzen 80 lectures (1 mesura cada 45 segons) a una longitud d'onda d'excitació de 495nm i una d'emissió de 570nm.

Una vegada finalitzada les lectures les dades s'exporten a KaleidaGraph® (Synergy Software, Pensilvania, USA) on es preparen les dades i posteriorment s'exporten a una aplicació realitzada en Excel on es calcula la fase-lag de cada mostra.

El tiempo de fase-lag es transforma a concentració mitjançant la comparació amb la relació temps de fase-lag i concentració dels diferents patrons realitzats amb Trolox.

4.1.3.2. ANÀLISI DE FRAP EN MOSTRES DE PLASMA

L'assaig consisteix en barrejar un reactiu oxidant (Fe^{2+}) i la nostra mostra, la qual conté les substàncies antioxidants, aquestes s'oxiden i es redueixen amb el ferro (Fe^{3+}), produint un canvi de color mesurable colorimètricament. El grau de canvio de color és proporcional a la concentració d'antioxidants presents en la mostra.

La tècnica utilitzada per a la determinació del FRAP en mostres de plasma és una adaptació de la publicada per Benzie & Strain (Benzie and Strain, 1996). Les millores tornen a estar encaminades en la aplicabilitat de la metodologia a un gran nombre de mostres.

El protocol s'inicia amb la descongelació de les mostres de plasma. A continuació s'inicia la preparació de la placa de 96 pouetes afegint 30μL de aigua bidestil·lada, 10μL de plasma diluït 1:2 en aigua i 160μL de reactiu pel FRAP*. Seguidament la placa s'introdueix en l'espectrofotòmetre a 37ºC durant 30min de incubació i posteriorment es realitza la lectura a 595nm.

*El reactiu pel FRAP es realitza a diari amb una solució de tampó d'acetat sòdic (0.3M), TPTZ (10mM) en àcid clorhídric (40mM) i clorur fèrric ($FeCl_3$, 20mM) en unes proporcions de 10:1:1 respectivament.

Les dades d'absorbància són transformats a concentracions mitjançant la extrapolació amb rectes de calibració realitzades amb sulfat ferrós ($FeSO_4x7H_2O$).

4.1.4. Metodologia estadística per la determinació de marcadors biològics.

Per la determinació dels metabòlits del resveratrol com a marcadors biològics del consum de vi s'ha utilitzat principalment dos grups de proves estadístiques:

- Correlacions
- Proves diagnòstiques

4.1.4.1. CORRELACIONS

La correlació indica la força i la direcció d'una relació lineal entre dos variables aleatòries quantitatives (Doménech, 2006a). Es considera que dos variables quantitatives estan correlacionades quan els valors d'una d'elles varien sistemàticament respecte als valors homònims de l'altre.

Depenent de la distribució de les variables es pot utilitzar dos coeficients de correlació, el de Pearson quan les dos variables segueixen una distribució Normal (proves paramètriques) i el de Spearman quan una o les dos variables no segueixen una distribució Normal (proves no paramètriques).

4.1.4.2. PROVES DIAGNÒSTIQUES

Les proves diagnòstiques són les eines de l'estadística per a classificar als subjectes segons presentin o no la característica determinada. S'utilitzen habitualment en medicina per a comprovar la utilitat d'un test per a classificar als subjectes en malalts i no malalts (Doménech, 2006b).

En el cas d'aquest treball la prova diagnòstica és d'utilitat per distingir entre els bevedors i els no bevedors de vi, mitjançant el càlcul del punt de tall òptim en la variable (resveratrol urinari) per efectuar aquesta classificació.

La característica més important d'una prova diagnòstica és l'exactitud, també anomenada eficàcia o rendiment diagnòstic, i mesura la capacitat de la prova per distingir entre dos estats o grups. L'exactitud diagnòstica de les proves s'acostuma a avaluar mitjançant la sensibilitat i l'especificitat.

Sensibilitat (SE): Proporció de diagnòstics positius (resveratrol urinari superior al punt de tall) obtinguts a l'aplicar la prova en una població de persones consumidores de vi. (**Figura 17**)

Especificitat (SP): Proporció de diagnòstics negatius (resveratrol urinari inferior al punt de tall) obtinguts al realitzar la prova en una població de persones no consumidores de vi (**Figura 17**).

Les raons de verosimilitud relacionen els conceptes de sensibilitat i especificitat. La més utilitzada és la raó de verosimilitud pels positius (LR+), i es calcula divididint la sensibilitat entre el complement de la especificitat. Anàlogament es defineix la raó de verosimilitud pels negatius (LR-), i es calcula dividint el complement de l'especificitat entre la sensibilitat.

A més de l'exactitud diagnòstica d'una prova s'ha d'avaluar el seu comportament quan s'utilitza en diferents contextos, són els anomenats valors predictius.

Valor predictiu positiu (PV+): Proporció de subjectes que consumeixen vi en el conjunt de subjectes amb resultat positiu a la prova (resveratrol urinari superior al punt de tall) (**Figura 17**).

Valor predictiu negatiu (PV-): Proporció de subjectes que no consumeixen vi en el total de subjectes amb resultat negatiu a la prova (resveratrol urinari inferior al punt de tall) (**Figura 17**).

Valor global (*Efficiency*): Proporció total de subjectes classificats correctament per la prova. (**Figura 17**).

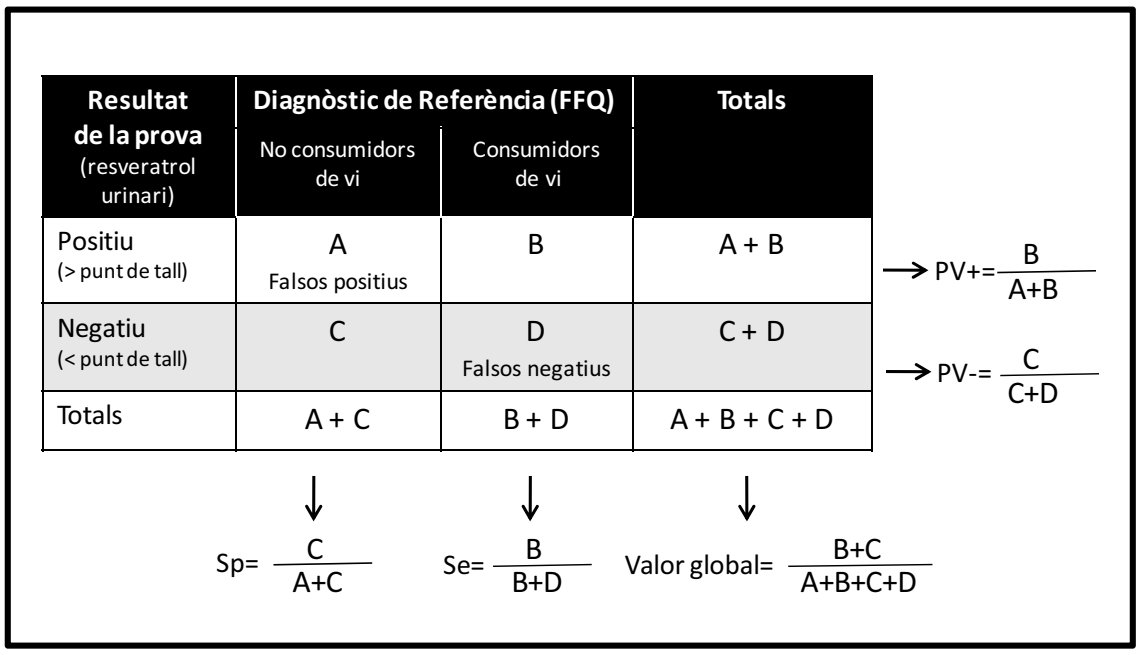


Figura 17. Índexos d'exactitud i valors predictius d'una prova diagnòstica.

La sensibilitat i l'especificitat ofereixen una mesura de l'exactitud de la prova diagnòstica en un determinat punt de tall de la variable (resveratrol urinari). Una mesura global de l'exactitud de la prova per a tots sls possibles punts de tall s'obté mitjançant la corba ROC (**Figura 18**). Per construir aquest gràfic es necessari calcular la sensibilitat (eix de les abcisses) i el complement de la especificitat (eix de les ordenades). Una prova amb la sensibilitat i especificitat perfecta, seria igual a un, on la corba ROC es veuria representada pels costats esquerre i superior del gràfic. No obstant una prova sense discriminació, sensibilitat i especifitat igual a 0.5, correspondria a una corba ROC representada per la diagonal principal del gràfic. L'àrea sota la corba que s'obté és un valor comprés entre 0.5 i 1 que pot ser utilitzat com a mesura d'exactitud global.

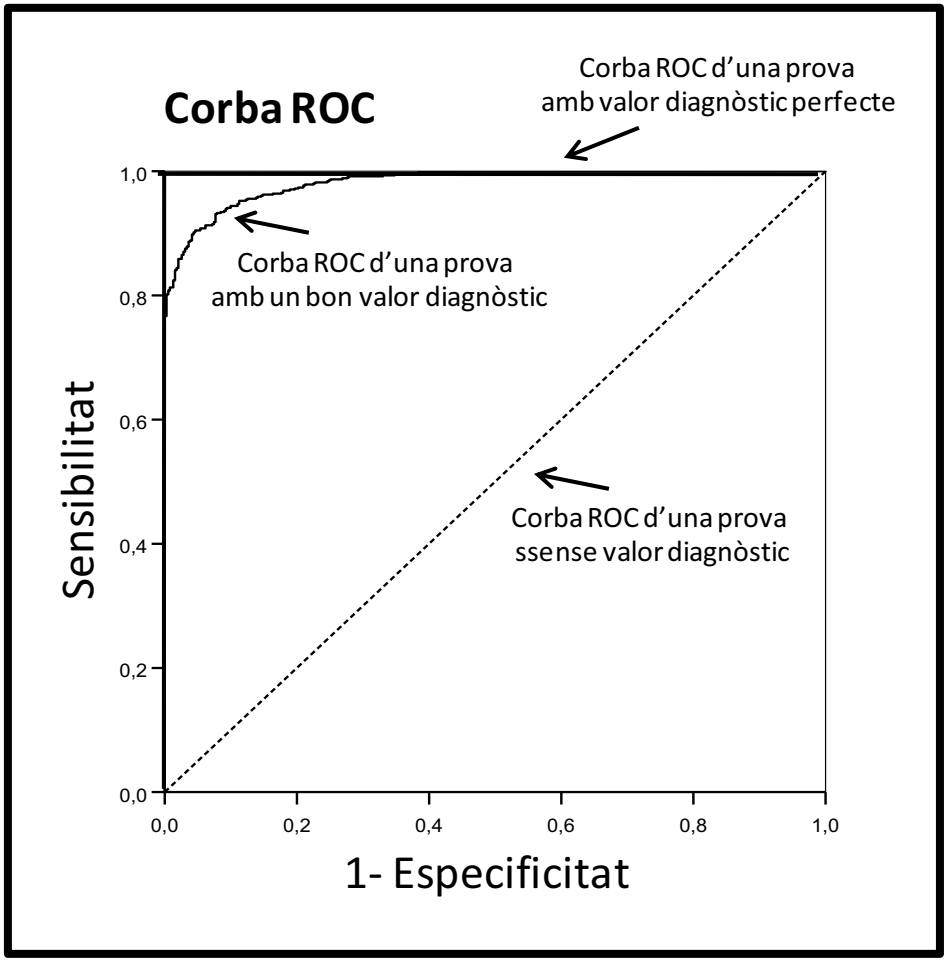


Figura 18. Curves ROC de tres proves amb diferent poder diagnòstic.

4.2. Resultats

En aquesta secció s'exposen els resultats obtinguts gràcies al treball experimental realitzat en la present tesi doctoral. Aquests resultats s'han concretat en 3 publicacions en revistes del *Science CItation Index.* Addicionalment i en relació als resultats obtinguts en la primera d'elles s'ha publicat també una carta de discussió. També s'exposen els resultats publicats en un estudi en col·laboració amb altra tesi doctoral presentada en l'Hospital Clínic de Barcelona, on es va realitzar el treball conjuntament.

Previ a cada publicació hi ha un resum on es detallen els objectius, el disseny de l'estudi, la metodologia utilitzada, els resultats obtinguts i les principals conclusions.

4.2.1. Avaluació diagnòstica dels metabòlits del resveratrol en orina com a biomarcadors del consum moderat de vi.

Article 1: Zamora-Ros R, Urpí-Sardà M, Lamuela-Raventós RM, Estruch R, Vázquez-Agell M, Serrano-Martínez M, Jaeger W, Andres-Lacueva C. Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. *Clinical Chemistry.* **2006**; 52 (7):1373-80.

Resum:

El principal objectiu de la epidemiologia nutricional és avaluar els efectes de la dieta en el risc de patir malalties. Per comprendre fiablement com influencia la dieta és necessari conèixer de forma precisa i real la ingesta d'aliments dels participants de l'estudi. La valoració de la dieta es pot realitzar mitjançant biomarcadors nutricionals o a partir d'enquestes alimentàries. Els biomarcadors nutricionals són mesures més fiables de l'exposició dietètica que les dades aportades per les enquestes alimentàries. Així doncs l'objectiu del present estudi ha estat proposar i avaluar la suma dels metabòlits del resveratrol presents a l'orina com a potencial biomarcador del consum de vi en humans, després de la ingesta moderada de cava, vi blanc o negre, com representants de begudes amb diferent contingut de resveratrol. A més a més s'avalua la efectivitat del marcador biològic en una cohort no controlada.

Per a la realització de l'estudi es porten a terme dos assaigs clínics aleatoritzats i creuats. En el primer estudi es van reclutar 10 voluntaris homes sans que va consumir 30g d'alcohol diari en forma de cava o ginebra durant 28 dies (estudi clínic descrit amb més deteniment en l'apartat 4.1.1.1.1.). Abans de cada una de les dos intervencions els voluntaris van seguir un període de rentat (*washout*) de 4 setmanes. En el segon estudi es van reclutar 10 dones sanes les quals van consumir 20g d'alcohol diari en forma de vi blanc o vi negre durant 28 dies (apartat 4.1.1.1.2.), també van seguir els seus respectius períodes de rentat de 4 setmanes. Addicionalment es van incloure en l'estudi 52 mostres dels primers voluntaris de la cohort PREDIMED. L'estudi PREvenció amb Dieta MEDiterrània (PREDIMED) és un gran assig clínic, multicèntric, amb grupo paral·lels, controlat, aleatorizat i amb 4 anys de seguiment que encara ara s'està portant a terme per avaluar els efectes de la dieta mediterrània en la prevenció primària de la malaltia cardiovascular (www.predimed.org) descrit amb més detall en l'apartat 4.1.1.1.3.

La determinació dels metabòlits de resveratrol en les mostres d'orina i plasma es va realizar mitjançant cromatografia líquida acoblada a espectrometria de masses en tàndem (LC-MS/MS) després d'una extracció en fase sòlida de la mostra (Urpi-Sarda et al., 2007). Per valorar l'exactitud diagnòstica del biomarcador es va utilitzar la corba ROC (*Receiver Operating Characteristic*).

Es van observar increments positius significatius en la suma de metabolitos de resveratrol urinari [72.4 (95% IC, 48.5-96.2; *P*=0.005), 211.5 (166.6-256.3; *P*=0.005), y 560.5nmol/g de creatinina (244.9-876.1; *P*=0.005)] després del consum de cava, vi blanc, o vi negre, respectivament. No va haver-hi canvis significatius després dels 4 períodes de rentat o de la intervenció amb ginebra. En la cohort PREDIMED, la dosis de vi dietètic ha estat correlacionada positivament amb la suma de metabòlits del resveratrol urinari (r=0.654; *P*<0.001). Utilitzant un punt de tall de 90nmol/g creatinina, el nostre biomarcador té la capacitat de diferenciar els consumidors de vi dels no consumidors amb una sensibilitat del 72% (60%-84%); i una especificitat del 94% (87%-100%).

Com a conclusió d'aquest treball podem afirmar que la suma de metabòlits del resveratrol en orina pot ser un biomarcador útil per conèixer la ingesta real de vi en estudios epidemiològics i d'intervenció.

Clinical Chemistry 52:7
1373–1380 (2006)

Endocrinology and Metabolism

Diagnostic Performance of Urinary Resveratrol Metabolites as a Biomarker of Moderate Wine Consumption

Raul Zamora-Ros,[1] Mireia Urpí-Sardà,[1] Rosa M. Lamuela-Raventós,[1] Ramón Estruch,[2] Mónica Vázquez-Agell,[2] Manuel Serrano-Martínez,[3] Walter Jaeger,[4] and Cristina Andres-Lacueva[1*]

Background: **Nutritional biomarkers may be better measures of dietary exposure than self-reported dietary data. We evaluated resveratrol metabolites, potential biomarkers of wine consumption, in humans after moderate consumption of sparkling, white, or red wines.**
Methods: **We performed 2 randomized, crossover trials and a cohort study. In the first study, 10 healthy men consumed 30 g of ethanol/day as sparkling wine or gin for 28 days. In the second trial, 10 healthy women consumed 20 g of ethanol/day as white or red wine for 28 days. We also evaluated 52 participants in a study on the effects of a Mediterranean diet on primary prevention of cardiovascular disease (the PREDIMED Study). We used liquid chromatography–tandem mass spectrometry to analyze urinary total resveratrol metabolites (TRMs) and predictive values and ROC curve analyses to assess the diagnostic accuracy.**
Results: **We observed significant increases in TRMs [72.4 (95% confidence interval, 48.5–96.2; $P = 0.005$), 211.5 (166.6–256.3; $P = 0.005$), and 560.5 nmol/g creatinine (244.9–876.1; $P = 0.005$)] after consumption of sparkling, white, or red wine, respectively, but no changes after the washout or gin periods. In the cohort study, the reported daily dose of wine consumption correlated directly with TRMs ($r = 0.654$; $P < 0.001$). Using a cutoff of 90 nmol/g, we were able to use TRMs to differentiate wine consumers from abstainers with a sensitivity of 72% (60%–84%); and a specificity of 94% (87%–100%).**
Conclusions: **Resveratrol metabolites in urine may be useful biomarkers of wine intake in epidemiologic and intervention studies.**

Epidemiologic studies have shown a negative correlation between moderate wine consumption and cardiovascular disease *(1)*. In addition to ethanol, wine contains several minor compounds, such as polyphenols, that contribute to the differences observed between wine and distillates *(2, 3)*. To date, no studies have been performed to determine biomarkers of wine consumption. Resveratrol (3,5,4′-trihydroxystilbene) and piceid (resveratrol-3-*O*-β-glucoside) are phenolic compounds present mainly in grapes and wine *(4)*, and these compounds may have a role in the prevention of cancer, cardiovascular disease *(1)*, and neurodegenerative diseases *(5)*. In addition, they may be useful as biomarkers of wine consumption.

Biomarkers for epidemiologic and clinical assays have 3 distinct advantages over dietary data obtained by food frequency questionnaires (FFQs)[5] *(6, 7)*. One advantage is that biochemical markers of the intake of some nutrients are more precise than dietary assessment. Another advantage is that dietary data obtained by FFQ are often inadequate because of insufficient reporting of food com-

[1] Nutrition and Food Science Department-CeRTA, Pharmacy School, and [2] Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain.
[3] Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona, Spain.
[4] Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna, Austria.
* Address correspondence to this author at: Nutrition and Food Sciences Department-CeRTA, Pharmacy School, University of Barcelona, Av. Joan XXIII, s/n, 08028 Barcelona, Spain. Fax 34-93-403-59-31; e-mail candres@ub.edu.
Received December 23, 2005; accepted April 11, 2006.
Previously published online at DOI: 10.1373/clinchem.2005.065870

[5] Nonstandard abbreviations: FFQ, food frequency questionnaire; LC-MS/MS, liquid chromatography–mass spectrometry; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; and TRMs, total resveratrol metabolites.

position. The third advantage is that biomarker analysis provides a more proximal measure of specific nutrient intake than do FFQ data because it is an integrated measure of the bioavailability and metabolism of the component.

Recent advances in analytical techniques have improved the effectiveness and expanded the possibilities of biomarker analyses. Tandem mass spectrometry increases the sensitivity and selectivity of measurement of the metabolites of some nutrients *(8, 9)*. Resveratrol metabolites could be the best nutritional biomarkers for wine consumption because *trans*-resveratrol-3-*O*-glucuronide has been reported to be the main resveratrol metabolite in human blood *(10)*, urine *(11)*, LDL *(12)*, and target organs *(13)*. Other phenolic metabolites previously used as biomarkers of food consumption include 4′-*O*-methylgallic acid (the main gallic acid metabolite) for tea *(14)*, isoferulic acid for coffee *(14)*, and isoflavonoids for soy *(15)*.

The aim of this study was to determine the concentrations of resveratrol metabolites in blood and urine in 2 different studies after 4 weeks of wine consumption and to evaluate their usefulness as potential biomarkers of wine intake in intervention studies. In addition, we analyzed baseline data from a cohort included in a large intervention study to assess the diagnostic performance of this biomarker in real-life conditions.

Material and Methods

STUDY PARTICIPANTS

Clinical trials. The 2 intervention studies were open, prospective, randomized, crossover, single-blinded clinical trials.

The sparkling wine study (January to June 2005) included 10 healthy men [mean (SD) age, 28.2 (7.3) years; body mass index, 25.2 (1.3) kg/m^2], and the wine study (September to December 2004) included 10 healthy women [mean (SD) age, 38.1 (9.2) years; body mass index, 24.1 (4.0) kg/m^2]. All participants in both studies were healthy, and none reported any prior relevant disease.

COHORT STUDY

The PREDIMED (PREvención con DIeta MEDiterránea) Study is a large, parallel group, multicenter, controlled, randomized 4-year clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease (http://www.predimed.org). In the present study, we analyzed the baseline data of 52 consecutively admitted trial participants (30 men and 22 women admitted April to July 2005). Exclusion and inclusion criteria have been described previously by Estruch et al. *(16)*. Twenty-nine participants (55.8%) reported a mean (SD) daily intake of 118.3 (112.3) mL of wine. Seven (13.5%) reported intermittent drinking, mostly during weekends, consuming a mean of 98.0 (28.7) mL of wine per week, and 16 participants (30.7%) did not drink. All but 2 (93%) of the daily drinkers reported to preferentially consume red wine, although 24% also reported drinking lower amounts of white wine and sparkling wine. The Institutional Review Board of the Hospital Clinic of Barcelona approved the 3 study protocols, and written informed consent was obtained from each participant.

STUDY DESIGN

Clinical trials. Both studies were carried out over a 16-week period. During the first 4 weeks, the participants did not drink any alcoholic beverages (first washout period). During the next 4 weeks, they underwent the first intervention, after which they underwent a second 4-week washout period. During the final 4 weeks, the participants underwent the second intervention.

In the sparkling wine study, the interventions consisted of the intake of 30 g of ethanol/day as sparkling wine (300 mL/day) or as gin (100 mL/day) in a random order during dinner. In the wine study, the volunteers consumed 20 g of ethanol/day as red wine (200 mL/day) or white wine (200 mL/day), also in a random order during dinner.

In both studies, diet was monitored before and after each intervention period by use of a 3-day food-and-drink recall questionnaire, which had been validated previously in our country *(17)*. We converted the reported consumption into nutritional data with the Professional Diet Balancer software (Cardinal Health Systems, Inc.). The clinical investigators and laboratory technicians did not know the sequence of the intervention.

Reports from the participants and the number of empty bottles returned showed adherence. We did not observe significant differences between nutrient intake, anthropometric variables, and energy expended in physical activity before and after the evaluated interventions.

Urine and serum samples were collected the morning after the interventions and washout periods after overnight fasting and were coded with random numbers and stored at −80 °C until analyses, which were performed with no knowledge of the clinical data.

Cohort study. At baseline, participants completed a 137-item validated FFQ *(18)* and the validated Spanish version *(19)* of the Minnesota Leisure Time Physical questionnaire. Data collected included information on drinking habits, such as amount, frequency, and type of alcohol intake. We took samples of fasting blood and morning urine from all participants. Energy and nutrient intakes were calculated from Spanish food composition tables *(20)*. Urine samples were coded and stored at −80 °C until analyses. The clinical investigators and laboratory technicians were blinded to clinical data.

Reported daily consumption of the key food items and nutrients, as well as estimated energy expenditure from physical activity, were similar in the participants who drank wine daily, those who drank intermittently, and those who did not drink any kind of wine.

MEASUREMENT OF TOTAL RESVERATROL IN BEVERAGES BY HPLC WITH A DIODE ARRAY DETECTOR

We concentrated 5 mL of sparkling wine, white wine, or gin, under reduced pressure and protected against exposure to ultraviolet light, to a final volume of 2 mL. Wines were injected directly into the HPLC according to the previously described method *(21)*. Results are reported as milligrams of total resveratrol consumed per day.

QUANTIFICATION OF RESVERATROL METABOLITES FROM HUMAN SAMPLES

We used liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described elsewhere *(12)* to analyze resveratrol metabolites extracted from urine and serum samples by solid-phase extraction. Briefly, urine samples (5 mL) were loaded on Oasis HLB cartridges (60 mg; Waters) that had been equilibrated. The cartridges were washed, and resveratrol metabolites were eluted with acidified methanol solution and ethyl acetate. The organic extract was evaporated under N_2. The samples were redissolved with 100 μL of the mobile phase used for the LC initial conditions with taxifolin as internal standard and then analyzed in the LC-MS/MS system.

We identified and quantified resveratrol metabolites in urine and serum with an LC system (Perkin-Elmer s200) coupled to a triple-quadrupole mass spectrometer (API 3000; Perkin-Elmer Sciex) as described elsewhere *(12)*. The intra- and interassay CVs for *trans*-resveratrol were 2.4% and 4.8%, respectively, and the analyses were performed in duplicate. All results for urinary resveratrol metabolites were corrected for urinary creatinine and are reported as nanomoles per gram of creatinine in the morning urine *(11, 12)*. Urinary creatinine was assayed with the standard Jaffe (alkaline picrate) kinetic method *(22)*. Serum (500 μL) was treated with 20 μL of *ortho*-phosphoric acid, vortex-mixed for 1 min, and processed by the same procedure.

STATISTICAL ANALYSIS

We used the standard statistical methods of the SPSS Statistical Analysis System, Ver. 11.5 (SPSS). Descriptive statistics with the mean (SD) were used for the baseline characteristics of the participants. Because the data were skewed (Kolmogorov and Levene tests), we used the Wilcoxon test for related samples to compare changes in outcome variables in response to each intervention period in both clinical trials. To exclude the presence of a carryover effect, we compared the observed outcome variables before both intervention periods. To compare groups in the cohort study, we used the 2-tailed *t*-test and ANOVA when indicated. We used Pearson correlations to examine associations between wine consumption and urinary excretion of resveratrol metabolites. To assess the accuracy of urinary resveratrol metabolite measurement for differentiating between wine consumers and nonconsumers, we calculated the sensitivity, specificity, positive (PPV) and negative predictive values (NPV), the likelihood ratio, and the ROC curve for the 2 randomized, crossover trials and the cohort study. With ROC curve analysis, we calculated a cutoff point that provided optimized sensitivity and specificity for the identification of wine consumers. Within- and between-group differences are expressed as means and 95% confidence intervals (CIs). All statistical tests were 2-tailed, and the significance level was 0.05.

Results

RESVERATROL CONCENTRATIONS IN BEVERAGES

The amount of total resveratrol consumed per day in the clinical trials was 0.357, 0.398, and 2.56 mg for sparkling, white, and red wine, respectively. The content of resveratrol in gin was below the detection limits.

CLINICAL TRIALS

Sparkling wine study. After 28 days of dietary supplementation with 300 mL/day of sparkling wine, *cis*- and *trans*-resveratrol-3-*O*-glucuronides were found in the urine of all participants, whereas only very low concentrations of these metabolites were detected in the urine after the washout periods and the gin period. The mean concentrations of resveratrol metabolites in urine before and after each intervention are shown in Fig. 1. The amount of total resveratrol metabolites (TRMs) identified in this study increased by 72.4 nmol/g (95% CI, 48.5–96.2 nmol/g; $P = 0.005$) after sparkling wine consumption, whereas the concentration of these metabolites did not vary significantly after the gin period (Fig. 1). The order of interventions did not affect the results. No positive results were obtained when urine was checked for resveratrol aglycone, piceid, and sulfoconjugates. Serum concentrations of resveratrol and its metabolites were below the limits of detection in all participants evaluated.

White and red wine study. After 28 days of dietary supplementation with white or red wine (200 mL/day), *trans*- and *cis*-resveratrol-3-*O*-glucuronide were found in the urine of all participants, whereas only very low concentrations of these metabolites were detected in urine after the washout periods (Fig. 2). According to the TRM results, both metabolites increased by 211.5 nmol/g (95% CI, 166.6–256.3 nmol/g; $P = 0.005$) after white wine consumption and by 560.5 nmol/g (244.9–876.1 nmol/g; $P = 0.005$) after red wine intake. The differences between the changes observed after white and red wine intake significantly favored red wine [349.6 nmol/g (86.8–612.3 nmol/g); $P = 0.005$]. For all participants, no free resveratrol, piceid, or sulfoconjugates were detected in the urine, and resveratrol and its metabolites were below the limits of detection in serum.

BOTH CLINICAL TRIALS

We used ROC curves to assess the effectiveness of urinary resveratrol metabolite measurement as a biomarker for wine intake. The optimal cutoff point was 90 nmol/g,

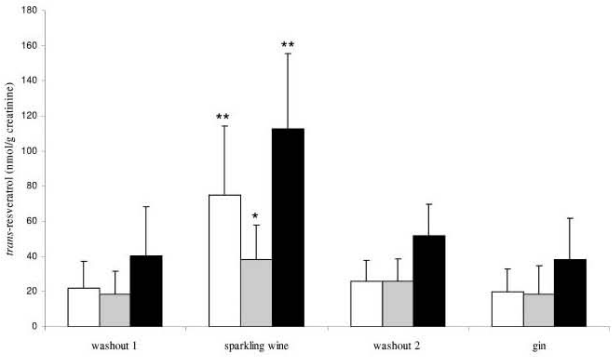


Fig. 1. Concentrations of urinary resveratrol glucuronides after washout period 1, sparkling wine intervention, washout period 2, and gin intervention.

Results are expressed as nmol of *trans*-resveratrol/g creatinine. Values are the means (SD; *error bars*) of duplicate measurement of samples from 10 volunteers. □, *trans*-resveratrol-3-glucuronide; ▨, *cis*-resveratrol-3-glucuronide; ■, total resveratrol glucuronides. Significant differences: *, $P < 0.05$; **, $P = 0.005$ for difference from results obtained during the previous washout period (Wilcoxon test).

which allowed differentiation of the washout and gin periods from the wine periods (Fig. 3): area under the curve = 0.985 (95% CI, 0.928–0.999); sensitivity = 93% (88%–99%); specificity = 98% (94%–100%); likelihood ratio = 46.7 (35.8–57.6); PPV = 95.6% (91.1%–100%); NPV = 85.3% (77.5%–93.1%).

COHORT STUDY

Participants who reported wine consumption had significantly higher urinary concentrations of *trans*- and *cis*-resveratrol-3-*O*-glucuronide than those who did not consume wine (Fig. 4). The mean (SD) urinary TRM concentration was 282.7 (305.2) nmol/g for participants who reported moderate daily wine consumption, a value that differed significantly from that measured in those who did not consume wine [mean difference, 242.2 nmol/g (95% CI, 125.0–359.3 nmol/g); $P = 0.001$] and those who consumed wine intermittently [171.4 nmol/g (44.5–298.2 nmol/g); $P = 0.01$; Fig. 3]. Mean (SD) urinary TRM concentrations were 111.3 (69.1) nmol/g for participants who reported intermittent wine consumption, a value that differed from the concentration observed in abstainers [70.8 nmol/g (6.4–135.2 nmol/g); $P = 0.035$]. The reported daily wine consumption correlated directly with urinary concentrations of resveratrol glucuronides ($r = 0.654$; $P < 0.001$).

The cutoff of 90 nmol/g enabled differentiation of moderate wine drinkers from those who did not drink wine with an area under the ROC curve (Fig. 5) of 0.863 (95% CI, 0.739–0.942), a sensitivity of 72% (60%–84%), a specificity of 94% (87%–100%), a PPV of 96.4% (91.3%–100%), an NPV of 59.1% (45.7%–72.5%), and a likelihood ratio of 11.6 (2.9–20.3). The percentage of false negatives was higher in those who consumed wine intermittently than in those who consumed it daily (43% and 24%, respectively); consequently, the sensitivity was higher in those who consumed moderate amounts of wine daily (76%) than in those who consumed wine intermittently (57%).

In all participants, no free resveratrol, piceid, or sulfoconjugates were detected in the urine, and resveratrol and its metabolites were below the limit of detection in the serum.

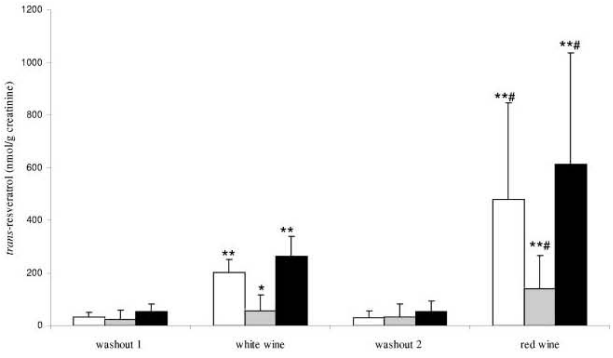


Fig. 2. Concentrations of urinary resveratrol glucuronides after washout period 1, white wine intervention, washout period 2, and red wine intervention.

Results are expressed as nmol of *trans*-resveratrol/g creatinine. Values are the means (SD; *error bars*) from duplicate measurements of samples from 10 volunteers. □, *trans*-resveratrol-3-glucuronide; ▨, *cis*-resveratrol-3-glucuronide; ■, total resveratrol glucuronides. Significant differences: *, $P < 0.05$; **, $P = 0.005$ for differences from results obtained during the previous washout period (Wilcoxon test); #, $P = 0.005$ for difference from values after white wine intake (Wilcoxon test).

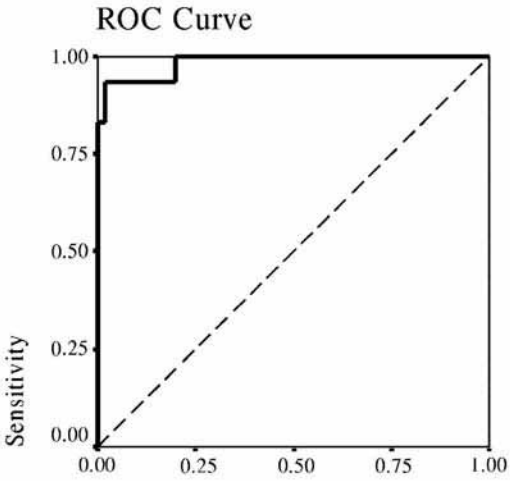


Fig. 3. ROC curve of urinary TRM concentrations for wine consumption periods vs washout and gin consumption periods in the clinical studies.

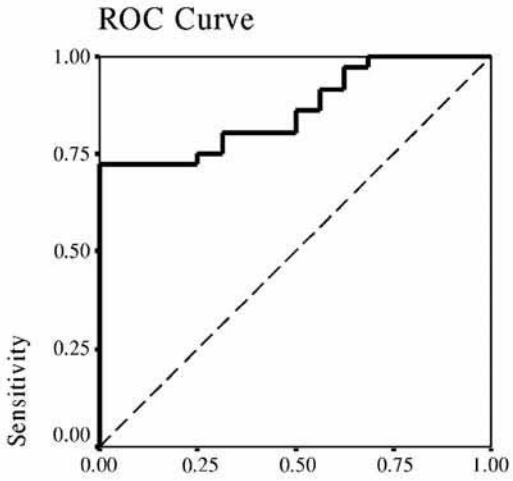


Fig. 5. ROC curve of urinary TRM for discrimination of wine consumers from non–wine consumers in the PREDIMED Study.

Discussion

An ideal biomarker should be specific, have an adequate half-life, and provide good correlation between the measured value and exposure *(7)*. The results of the current study indicate that resveratrol metabolites fulfill the criteria to be considered as a biomarker of wine intake.

The 2 intervention clinical trials, which included men and women, allowed assessment of a urinary concentration of resveratrol metabolites of 90 nmol/g as a cutoff to differentiate wine drinkers from non–wine drinkers: this cutoff had a sensitivity and specificity >90% and a PPV >95%. The usefulness of this biomarker was then tested in a cohort of 52 consecutively admitted participants in a large-scale feeding clinical trial, the PREDIMED Study. In real-life conditions, this biomarker had a sensitivity of 73%, a specificity of 93%, and a PPV of 96%. However, the NPV was 60% because of a high percentage of false negatives among intermittent drinkers. Thus, urinary concentrations of resveratrol metabolites are particularly useful as biomarkers of wine intake for moderate and regular drinkers, just as other phenolic compounds have been shown to be useful biomarkers for the intake of fruits, vegetables, tea, or coffee *(14, 23)*. We selected resveratrol as a marker of wine intake because it is a characteristic polyphenol of grape and wine products. Although a few other foods contain resveratrol *(24–27)*, the quantities in those foods are much lower than those observed in grape and wine products *(4, 21, 28)*.

Resveratrol metabolites were detected in morning urine after moderate and regular wine intake. Resveratrol metabolism has been investigated extensively in preclinical studies using animals *(11, 29, 30)*, but few studies have been performed in humans *(10, 12, 30–33)*. In the current trials, resveratrol intake ranged from 0.0040 mg/kg (0.35 mg of total resveratrol) for those who drank sparkling wine to 0.041 mg/kg (2.56 mg of total resveratrol) for those who consumed red wine; these values are

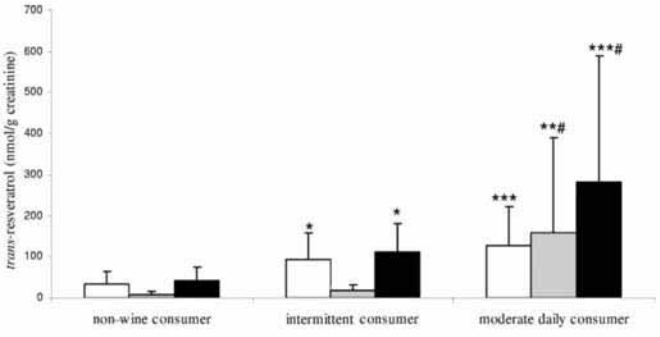


Fig. 4. Concentrations of urinary resveratrol glucuronides among non–wine consumers, intermittent consumers, and moderate daily consumers.

Results are expressed as nmol of *trans*-resveratrol/g creatinine. Values are the means (SD; *error bars*) from 52 participants. □, *trans*-resveratrol-3-glucuronide; ▨, *cis*-resveratrol-3-glucuronide; ■, sum of the resveratrol glucuronides. Significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for difference from results obtained for samples from non–wine consumers (unpaired *t*-test). #, $P < 0.01$ for difference from values for intermittent wine consumers (unpaired *t*-test).

similar those reported in the literature (*10, 30*). These amounts cover the usual range of resveratrol intake from wine products (*34, 35*). To achieve high amounts of resveratrol, supplements must be taken (*36*). In the current trials, we were able to identify resveratrol metabolites in urine ~10 h after moderate intake of sparkling wine. Meng et al. (*30*) did not detect any resveratrol metabolites in the urine of volunteers after a comparable single dose of resveratrol, but the authors were able to quantify resveratrol metabolites in urine samples after a higher single dose (0.014 mg/kg). Because the accumulation of a metabolite increases after several days of ingestion (*6, 37*), our results suggest that urine analysis may be useful for determining regular wine intake. The metabolically active compounds could enter the urine when their concentrations in plasma increase and exceed the relevant renal threshold (*38*). Taking into account the bioactivity of wine phenols and comparing a single dose vs regular and moderate intake, Fisher and Hollenberg (*39*) observed an increased vascular response indicated by endothelial nitric oxide release over time. Furthermore, inclusion of resveratrol intake in a complex meal could increase or decrease the bioavailability of polyphenols (*40*).

We also observed interindividual variability in our intervention studies (Figs. 1 and 2). Similar results have been described previously for resveratrol in LDL particles (*12*), anthocyanins (*41*), isoflavones (*42*), and olive oil phenols such as tyrosol and hydroxytyrosol (*43*). Despite this variability among individuals, however, we observed a highly significant correlation between wine intake and urinary concentrations of the metabolites. Higher concentrations of resveratrol metabolites were found after higher resveratrol intake (when red wine was consumed). Likewise, a lower concentration of resveratrol metabolites was found after lower resveratrol intake (sparkling or white wine). After the washout periods as well as after gin intervention, very low concentrations of resveratrol glucuronide were detected in urine, possibly from previous intake of food with very low resveratrol content or interference from compounds with a similar structure, such as estrogens. Similar results have been observed after washout periods in studies investigating quercetin (*44*) and other polyphenols (*45*), as well as for hydroxytyrosol, a metabolite of dopamine, in an study of olive oil (*46*). Mean (SD) TRM concentrations did not differ significantly in response to various periods of no wine intake in our studies [43.4 (23.5), 51.5 (36.1), and 40.5 (33.6) nmol/g for sparkling, white, and red wine, respectively] as part of the larger PREDIMED Study.

Urinary resveratrol glucuronide is the main metabolite of resveratrol in humans (~97%) (*30–32*) and rodents (>90%) (*29, 30*), except in the study by Walle et al. (40%) (*33*). In studies of low resveratrol intake, only the glucuronidate form was detected (*30*). In the current studies, 2 monoglucuronides, *trans*- and *cis*-resveratrol-3-*O*-glucuronide, were identified after moderate wine consumption, findings similar to those of previous studies (*11, 47*). Resveratrol sulfates were not detected in morning urine after wine intake. However, further investigation is needed in this regard. The sulfate form has been reported in human urine after administration of high resveratrol doses (*33*). In other studies, the free form of resveratrol was not detected in human urine after moderate wine consumption but was found after high doses were consumed (*31, 32*).

In the present study, resveratrol metabolites were also measured in serum. In these samples, however, no resveratrol metabolites were detected an average of 10 h after the consumption of wine. In previous studies, resveratrol has been detected in plasma after a minimum resveratrol intake of 0.357 mg/kg in a single dose (*31, 32*). Furthermore, the half-life of resveratrol in human plasma is 2 h, with the highest concentrations being recorded at 30 min (*31, 32*). Thus, plasma concentrations of resveratrol are not a useful marker for regular intake because plasma concentrations increase only after very recent intake.

In summary, we identified resveratrol metabolites in human urine after moderate wine intake, suggesting that these metabolites can be used as a biomarker of moderate wine intake in regular drinkers. This biomarker can also be used to exclude moderate wine drinking in abstainers but may be less effective in intermittent drinkers. Therefore, resveratrol metabolites may be used as a measure of compliance in interventional studies as well as an objective measure of wine consumption in epidemiologic studies.

This study was supported by a grant from the Instituto de Salud Carlos III (Red de Grupo G03/140) from the Ministry of Health, and partially by Grants AGL2004-08378-C02-01/02/ALI and AGL 2005-0559/ALI from the Ministry of Education and Science (MEC) of the Spanish government. R.Z.R. was supported by the Departament d'Universitats, Recerca i Societat de la Informació, and M.U.S. by an FPI fellowship from MEC. We are indebted to Drs. Isidre Casals and Olga Jauregui from the Scientific and Technical Services (University of Barcelona, Barcelona, Spain), and to Dr Maite Ibern for project management. We also thank Dr. Antonio Cherubini for reading the manuscript and providing helpful comments.

References

1. Szmitko PE, Verma S. Cardiology patient pages: red wine and your heart. Circulation 2005;111:e10–1.
2. Badia E, Sacanella E, Fernandez-Sola J, Nicolas JM, Antunez E, Rotilio D, et al. Decreased tumor necrosis factor-induced adhesion of human monocytes to endothelial cells after moderate alcohol consumption. Am J Clin Nutr 2004;80:225–30.
3. Estruch R, Sacanella E, Badia E, Antunez E, Nicolas JM, Fernandez-Sola J, et al. Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial; effects of wine on inflammatory markers. Atherosclerosis 2004;175:117–23.

4. Lamuela-Raventós R, Romero-Pérez A, Waterhouse A, de la Torre-Boronat M. Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceic isomers in Spanish red vitis vinifera wines. J Agric Food Chem 1995;43:281–3.
5. Dore S. Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. Neurosignals 2005;14:61–70.
6. Potischman N. Biologic and methodologic issues for nutritional biomarkers. J Nutr 2003;133(Suppl 3):875S–80S.
7. Marshall JR. Methodologic and statistical considerations regarding use of biomarkers of nutritional exposure in epidemiology. J Nutr 2003;133(Suppl 3):881S–7S.
8. Liu DQ, Xia YQ, Bakhtiar R. Use of a liquid chromatography/ion trap mass spectrometry/triple quadrupole mass spectrometry system for metabolite identification. Rapid Commun Mass Spectrom 2002;16:1330–6.
9. Day AJ, Williamson G. Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma. Br J Nutr 2001;86(Suppl 1): S105–10.
10. Vitaglione P, Sforza S, Galaverna G, Ghidini C, Caporaso N, Vescovi PP, et al. Bioavailability of *trans*-resveratrol from red wine in humans. Mol Nutr Food Res 2005;49:495–504.
11. Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, et al. Human, rat, and mouse metabolism of resveratrol. Pharm Res 2002;19: 1907–14.
12. Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas M, et al. Uptake of diet resveratrol into the human low density lipoprotein: identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. Anal Chem 2005;77:3149–55.
13. Vitrac X, Desmouliere A, Brouillaud B, Krisa S, Deffieux G, Barthe N, et al. Distribution of [^{14}C]-*trans*-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. Life Sci 2003;72:2219–33.
14. Hodgson JM, Chan SY, Puddey IB, Devine A, Wattanapenpaiboon N, Wahlqvist ML, et al. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. Br J Nutr 2004;91:301–6.
15. Seow A, Shi CY, Franke AA, Hankin JH, Lee HP, Yu MC. Isoflavonoid levels in spot urine are associated with frequency of dietary soy intake in a population-based sample of middle-aged and older Chinese in Singapore. Cancer Epidemiol Biomarkers Prev 1998; 7:135–40.
16. Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutiérrez V, Covas MI, et al. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. Ann Intern Med 2006;in press.
17. Schroder H, Covas MI, Marrugat J, Vila J, Pena A, Alcantara M, et al. Use of a three-day estimated food record, a 72-hour recall, and a food-frequency questionnaire for dietary assessment in a Mediterranean Spanish population. Clin Nutr 2001;20:429–37.
18. Martín-Moreno JM, Boyle P, Gorgojo L, Maisonneuve P, Fernández-Rodríguez JC, Salvinini S, et al. Development and validation of a food frequency questionnaire in Spain. Int J Epidemiol 1993;22: 512–9.
19. Elosua R, Marrugat J, Molina L, Pons S, Pujol E, Arquer A, et al. Validation of the Minnesota leisure-time physical activity questionnaire in Spanish men. Am J Epidemiol 1994;139:1197–209.
20. Mataix J. Tabla de composición de Alimentos Españoles, 4th ed. Granada, Spain: University of Granada, 2003:560pp.
21. Andres-Lacueva C, Ibern-Gomez M, Lamuela-Raventos RM, Buxaderas S, de la Torre-Boronat M. Cinnamates and resveratrol content for sparkling wine characterization. Am J Enol Vitic 2002; 53:147–50.
22. Jaffé M. Über den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt und uber eine neue Reaction des Kreatinins. Z Physiol Chem 1886;10:391–400.
23. Krogholm KS, Haraldsdottir J, Knuthsen P, Rasmussen SE. Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables. J Nutr 2004;134:445–51.
24. Burns J, Yokota T, Ashihara H, Lean ME, Crozier A. Plant foods and herbal sources of resveratrol. J Agric Food Chem 2002;50:3337–40.
25. Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. Resveratrol, pterostilbene, and piceatannol in vaccinium berries. J Agric Food Chem 2004;52:4713–9.
26. Romero-Perez AI, Ibern-Gomez M, Lamuela-Raventos RM, Torre-Boronat MC. Piceid, the major resveratrol derivative in grape juices. J Agric Food Chem 1999;47:1533–6.
27. Sobolev VS, Cole RJ. *trans*-Resveratrol content in commercial peanuts and peanut products. J Agric Food Chem 1999;47: 1435–9.
28. Romero Perez AI, Lamuela Raventos RM, Waterhouse AL, de LaTorre Boronat MC. Levels of *cis*- and *trans*-resveratrol and their glucosides in white and rose *Vitis vinifera* wines from Spain. J Agric Food Chem 1996;44:2124–8.
29. Asensi M, Medina I, Ortega A, Carretero J, Bano MC, Obrador E, et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med 2002;33:387–98.
30. Meng X, Maliakal P, Lu H, Lee MJ, Yang CS. Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. J Agric Food Chem 2004;52:935–42.
31. Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 2003;36:79–87.
32. Soleas GJ, Yan J, Goldberg DM. Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. J Chromatogr B Biomed Sci Appl 2001;757: 161–72.
33. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 2004;32:1377–82.
34. Mattivi F, Reniero F, Korhammer S. Isolation, characterization, and evolution in red wine vinification of resveratrol monomers. J Agric Food Chem 1995;43:1820–3.
35. Soleas GJ, Goldberg DM, Diamandis EP, Karumanchiri A, Yan J, Ng E. A derivatized gas-chromatographic mass-spectrometric method for the analysis of both isomers of resveratrol in juice and wine. Am J Enol Vitic 1995;46:346–52.
36. Gescher AJ, Steward WP. Relationship between mechanisms, bioavailibility, and preclinical chemopreventive efficacy of resveratrol: a conundrum. Cancer Epidemiol Biomarkers Prev 2003;12: 953–7.
37. de Boer V, Dihal AA, van der WH, Arts IC, Wolffram S, Alink GM, et al. Tissue distribution of quercetin in rats and pigs. J Nutr 2005;135:1718–25.
38. Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van OB. Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr 2005;82:497–503.
39. Fisher ND, Hollenberg NK. Flavanols for cardiovascular health: the science behind the sweetness. J Hypertens 2005;23:1453–9.
40. Lamuela-Raventos RM, Romero-Perez AI, Andres-Lacueva C, Tornero A. Review: health effects of cocoa flavonoids. Food Sci Technol Int 2005;11:159–76.
41. Wu X, Cao G, Prior RL. Absorption and metabolism of anthocya-

nins in elderly women after consumption of elderberry or blueberry. J Nutr 2002;132:1865–71.
42. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.
43. Bonanome A, Pagnan A, Caruso D, Toia A, Xamin A, Fedeli E, et al. Evidence of postprandial absorption of olive oil phenols in humans. Nutr Metab Cardiovasc Dis 2000;10:111–20.
44. Manach C, Donovan JL. Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Radic Res 2004;38:771–85.
45. Ito H, Gonthier MP, Manach C, Morand C, Mennen L, Remesy C, et al. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. Br J Nutr 2005;94:500–9.
46. Miro-Casas E, Covas MI, Farre M, Fito M, Ortuno J, Weinbrenner T, et al. Hydroxytyrosol disposition in humans. Clin Chem 2003;49:945–52.
47. Aumont V, Krisa S, Battaglia E, Netter P, Richard T, Merillon JM, et al. Regioselective and stereospecific glucuronidation of *trans*- and *cis*-resveratrol in human. Arch Biochem Biophys 2001;393:281–9.

4.2.1.1. Els marcadors inflamatoris d'arteriosclerosi disminueixen després del consumo moderat de cava en homes amb baix risc cardiovascular.

Artícle 2: Vázquez-Agell M, Sacanella E, Tobias E, Monagas M, Antúnez E, Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, Fernández-Solá J, Nicolás JM, Estruch R. Inflammatory markers of aterosclerosis are decreased after moderate consumption of cava (sparkling wine) in men with low cardiovascular risk. *Journal of Nutrition.* **2007**;137: 2279-2284

Resum general del treball en col·laboració amb la tesi doctoral de la Dra Mònica Vázquez (Hospital Clínic de Barcelona):

L'arteriosclerosi és una malaltia crònica inflamatòria de baix grau de la paret arterial. Diversos estudis han mostrat que les begudes alcohòliques riques en polifenols, com el vi negre, tenen un major efecte antiinflamatori que les begudes alcohòliques sense polifenols, com la ginebra. No obstant es desconeix l'efecte antiinflamatori de begudes alcohòliques amb un contingut intermig en polifenols, com pot ser considerat el cava.

Per realitzar aquest assaig clínic creuat i aleatoritzat s'han reclutat 20 homes sans, amb una edat mitjana de 34±9 anys. Els participants van rebre 30g d'alcohol diari en forma de cava (300mL/d) o ginebra (100mL/d) durant 28 dies (estudi clínic descrit en l'apartat 4.1.1.1.). Prèviament als 2 períodes d'intervenció els voluntaris van tenir una etapa de rentat durant 2 setmanes, que va consistir en no consumir cap tipus de beguda alcohòlica. Els marcadors inflamatoris i l'expressió de les molècules d'adhesió mesurats en leucòcits van ser analitzades abans i després de cada intervenció.

L'expressió dels LFA-1, VLA-4, SLe^x i CD40 en monòcits va disminuir després de la intervenció amb cava (P<0.05), mentre que només SLe^x es va reduir després del període amb ginebra (P=0.036). Els marcadors circulants d'arteriosclerosi incloent VCAM-1, E-Selectina i P-Selectina van disminuir després d'ambdues intervencions (P<0.05). En canvi únicament la CRP d'alta sensibilitat, ICAM-1, IL-6, MCP-1 i CD40L van disminuir després de la ingesta de cava (P<0.05). Els efectes amb el cava van ser més importants en CD40L, ICAM-1 i mCP-1 circulant i en l'expressió de monòcits de CD40, LFA-1 i VLA-4 que els ocorreguts amb la ginebra (P<0.05). La conclusió que es pot extreure d'aquest treball és que ambdues begudes tenen propietats antiinflamatòries, tot i que el cava presenta un major efecte protector, possiblement degut al seu contingut en polifenols.

Resum general del treball realitzat en col·laboració amb la tesi doctoral de la Dra Mònica Vázquez (Hospital Clínico de Barcelona):

En aquest treball realitzat en col·laboració, la participació que forma part d'aquesta tesi doctoral ha consistit en la valoració del acompliment de la intervenció, en els períodes de rentat, de ginebra i de cava. Les dades provenen del primer assaig clínic (apartat 4.1.1.1.1.). En el que es pot apreciar que després dels períodes de rentat i ginebra únicament hi ha nivells bassals de resveratrol i dels seus metabòlits en orina, no obstant hi ha un augment significatiu d'aquests després de la intervenció amb cava (*P*<0.05) (**Figura 17**).

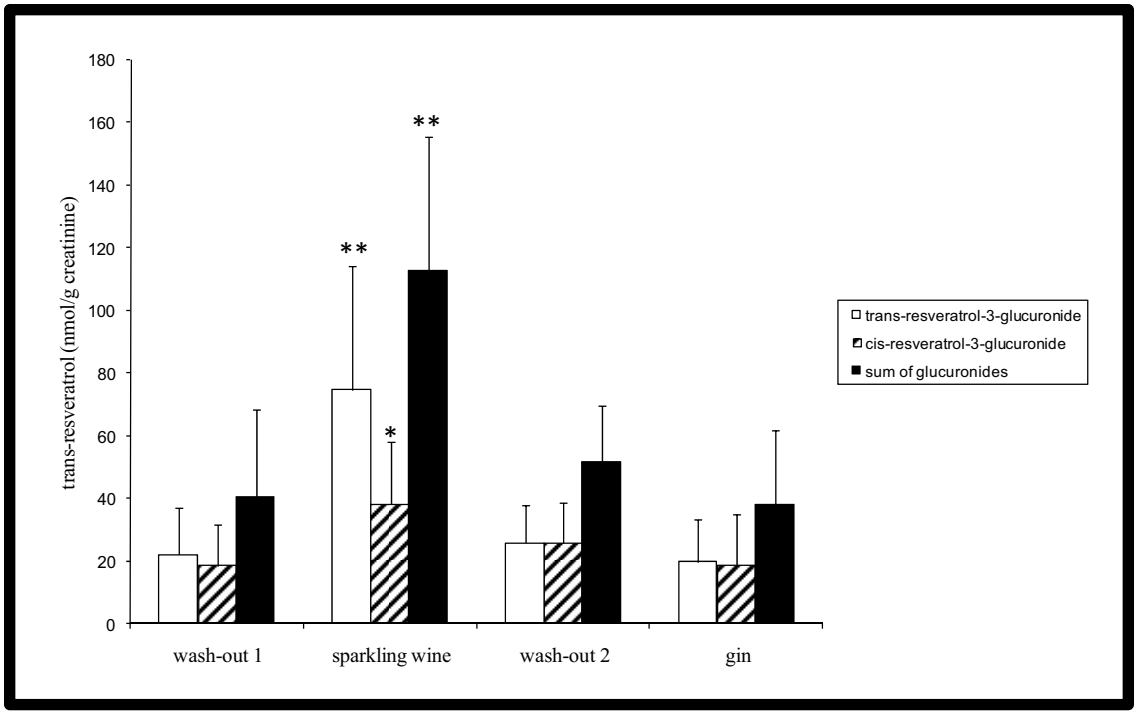


Figura 17. Concentracions dels metabòlits del resveratrol en l'estudi del cava vs ginebra. Diferències significatives:***P*=0.005; **P*<0.05.

The Journal of Nutrition
Nutrition and Disease

Inflammatory Markers of Atherosclerosis Are Decreased after Moderate Consumption of Cava (Sparkling Wine) in Men with Low Cardiovascular Risk[1,2]

Mònica Vázquez-Agell,[3,4] Emilio Sacanella,[3–5]* Ester Tobias,[3] María Monagas,[3,4] Emilia Antúnez,[3–5] Raúl Zamora-Ros,[6] Cristina Andrés-Lacueva,[6] Rosa M[a] Lamuela-Raventós,[6] Joaquim Fernández-Solá,[3–5] José M[a] Nicolás,[3–5] and Ramon Estruch[3–5]

[3]Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona 08036, Spain; [4]CIBER 06/03: Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Madrid 28029, Spain; [5]Department of Internal Medicine, Hospital Clínic of Barcelona, Barcelona 08036, Spain; and [6]Department of Nutrition and Food Science-XaRTA, Pharmacy School, University of Barcelona, Barcelona 08007, Spain

Abstract

Atherosclerosis is considered a low-grade inflammatory disease. Polyphenol-rich alcoholic beverages (red wine) have shown a more pronounced antiinflammatory effect than polyphenol-free alcoholic beverages (gin). However, no studies to our knowledge have evaluated the antiinflammatory effects of alcoholic beverages with medium-level polyphenol content such as cava (sparkling wine). We enrolled 20 healthy men (aged 34 ± 9 y) in a randomized crossover study to receive 30 g ethanol/d as cava or gin for 28 d. Before both interventions, subjects abstained from alcohol for 2 wk. Inflammatory biomarkers of atherosclerosis and expression of adhesion molecules on peripheral leukocytes were measured before and after each intervention. Likewise, dietary intake and exercise were also evaluated. Expression of lymphocyte function-associated antigen-1 (LFA-1), very late activation antigen-4 (VLA-4), Sialyl-Lewis[x] (SLe[x]), and CD40 on monocytes decreased after cava intake (all $P < 0.05$), whereas only SLe[x] was reduced after gin intake ($P = 0.036$). Circulating markers of atherosclerosis including vascular cell adhesion molecule-1, E-selectin, and P-selectin decreased after both interventions (all $P < 0.05$). High-sensitivity C-reactive protein, intercellular adhesion molecule-1 (ICAM-1), IL-6, monocyte chemoattractant protein-1 (MCP-1), and CD40L were diminished only after cava intake (all $P < 0.05$). The effects of cava on circulating CD40L, ICAM-1, and MCP-1, and monocyte surface expression of CD40, LFA-1, and VLA-4 were greater than those of gin (all $P < 0.05$). In conclusion, both cava and gin showed antiinflammatory properties; however, cava had a greater protective effect, probably due its polyphenol content. J. Nutr. 137: 2279–2284, 2007.

Introduction

Many epidemiological studies involving subjects of different gender, race, and age have shown that moderate alcohol consumption is associated with a reduced risk of cardiovascular disease (1–4). Because part of the atheroprotective effects associated with moderate alcohol intake has been attributed to changes in serum lipoproteins, coagulation, and platelet aggregation, other alternative mechanisms have been proposed (5–7).

Up to the 1980s, atherosclerosis was considered to be the result of lipid accumulation in the arterial wall. Nonetheless, better knowledge of the atheroma plaque formation has led to the conclusion that atherosclerosis is, indeed, a chronic low-grade inflammatory disease of the arterial wall (8). Large epidemiologic studies have reported a significant association between moderate alcohol consumption and lower levels of inflammatory biomarkers related to atherosclerosis (9–14), suggesting that antiinflammatory effects of alcoholic beverages may play a role in their protective effect against cardiovascular disease (15). Because this effect was independent of the type of alcoholic beverage (liquor, beer, and wine), some researches attributed this action to ethanol itself (16). However, other epidemiologic studies have found significant differences in the effects of wine and other alcoholic beverages on global mortality, cardiovascular mortality, and incidence of cancer, in favor of wine (17,18). The heterogeneity in the results obtained in these epidemiologic studies may be due to the fact that it is very difficult to adequately monitor diet and physical activity in such studies. Because almost all alcoholic beverages (some spirits, beer, and wine) contain ethanol and nonalcoholic compounds (mainly polyphenols), it seems

[1] Supported by the Spanish Ministries of Education and Science and Health (PI020611, PI041837, PI051519, G03/140, CB06/03, ALI/AGL 2006-14228-C03-02/01, and 2005-0550).
[2] Author disclosures: M. Vázquez-Agell, E. Sacanella, E. Tobias, M. Monagas, E. Antúnez, R. Zamora-Ros, C. Andrés-Lacueva, R. M. Lamuela-Raventós, J. Fernández-Solá, J. M. Nicolás and R. Estruch, no conflicts of interest.
* To whom correspondence should be addressed. E-mail: esacane@clinic.ub.es.

0022-3166/07 $8.00 © 2007 American Society for Nutrition.
Manuscript received 12 June 2007. Initial review completed 26 June 2007. Revision accepted 20 July 2007.

difficult to differentiate the effects of both compounds in epidemiologic studies.

Other types of evidence may be obtained regarding the biologic plausibility of this hypothesis. Clinical trials measuring the effects of moderate alcoholic beverage intake in surrogate markers of atherosclerosis in humans may be used to explain the mechanisms by which alcoholic beverages could exert their positive effects. In this sense, previous studies have concluded that polyphenol-rich alcoholic beverages, such as red wine, exert a higher antiinflammatory effect than ethanol itself (19). However, up to now, no clinical trials to our knowledge have analyzed the effects of medium-level polyphenol-content beverages, such as cava (sparking wine) compared with those observed after the administration of a polyphenol-free alcoholic beverage, such as gin. We embarked, therefore, upon a prospective, randomized crossover clinical trial to evaluate the effects of moderate intake of cava vs. gin on adhesion molecules, chemokines, and other inflammatory biomarkers related to the early stages of atherosclerosis.

Participants and Methods

Participants and study design. We selected 30 healthy men, between 25 and 50 y, who were working in the Department of Internal Medicine of our institution and reported a daily ethanol intake ranging from 10 to 30 g over the last 5 y. Five declined participation, 3 did not meet lipid criteria, and 2 suffered from hypertension. Thus, we finally included 20 volunteers who gave informed consent to a protocol approved by the Institutional Review Board of the Hospital Clinic. None reported any of the following exclusion criteria: diabetes mellitus, tobacco smoking, hypertension, LDL cholesterol levels > 4.14 mmol/L, HDL cholesterol levels < 1.04 mmol/L, coronary heart disease (CHD),[7] family history of premature CHD, cerebrovascular disease, peripheral vascular disease, HIV infection, alcoholic liver disease, malnutrition, or neoplastic or acute infection disease. In addition, no subjects were receiving any medication or taking any vitamin supplements. Participants received free cava and gin but no monetary compensation.

The study was an open, prospective, randomized, crossover, and single-blinded clinical trial in which subjects received 30 g ethanol/d as cava (0.3 L/d) or gin (0.1 L/d) for 28 d in a random order. Before both interventions, the subjects abstained from alcohol for 2 wk (washout periods 1 and 2). We followed the dietary intake and physical activity of the participants throughout the study. Before and after each intervention period, we withdrew blood samples after overnight fasting and coded them with random numbers to perform biochemical tests and immunological studies. Finally, at the end of each intervention, 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 alcoholic beverage intake.

Alcoholic beverages. We used a monovarietal cava made from white grapes of *Vitis vinifera* cv. Chardonnay (12% alcoholic strength) and gin (40% alcoholic strength) in this study. We selected these beverages on the basis of their polyphenolic content (medium level for the cava and negligible for the gin). Total phenolic compounds were determined by the Folin-Ciocalteu reagent (20). In addition, individualized phenolic compounds were determined by HPLC as previously described (21) (**Table 1**).

Diet and exercise monitoring. The participants in the study followed an isocaloric Mediterranean-type diet, which was designed according to their personal preferences. Participants were not allowed to consume onions, virgin olive oil, and green and black tea, which are rich in polyphenols and antioxidants. Other foods with high polyphenol content, ascorbic acid, α-tocopherol, and/or β-carotene, such as cocoa, chocolate, orange and tomato juices, nuts, some fruits (oranges, lemons, strawberries, grapes, melon, apples, and apricots), some vegetables (spinach, turnips, carrots, parsley, peppers, garlic, and tomatoes), and soybean products were restricted, providing a similar antioxidant content for all the participants throughout the study. Before and after each intervention, we used a 3-d food recall questionnaire, validated in our population (22), to assess the dietary intake and converted this information into nutritional data using the Professional Diet Balancer software (Cardinal Health Systems). We monitored physical activity with the Minnesota Leisure Time Physical Activity Questionnaire (23).

TABLE 1 Total phenolic and individual phenolic compound concentrations of Chardonnay cava

Phenolics by HPLC-DAD	Gallic acid
	mg/L
Total phenolic content (Folin-Ciocalteu)	202
Hydroxybenzoic acids	
Gallic acid	5.00
Protocatechuic acid	0.05
Hydroxycinnamic acids	
trans-Caftaric acid	13.59
2-S-glutathionylcaftaric acid	9.25
trans-Caffeic acid	1.60
trans-p-Coumaric acid	0.63
trans-Ferulic acid	0.37
Stilbenes	
trans-Resveratrol	0.14
cis-Resveratrol	0.13
trans-Piceid	ND[1]
cis-Piceid	0.92
Phenolic alcohols	
Tyrosol	11.14
Flavonols	
(+)-Catechin	0.40
(−)-Epicatechin	0.20
Quercetin-3-glucuronide	0.33

[1] ND, not detected.

Laboratory analysis. At the end of each 4-wk period (run-in, intervention 1, wash-out, and intervention 2), we obtained blood samples from fasting and a spot urine specimen. Immunophenotyping of peripheral blood mononuclear cells (PBMC) were performed. Serum and EDTA-plasma samples were stored at −80°C for analysis of inflammatory molecules at the end of the study. Analyses determined in frozen samples of plasma as was homocysteine by fluorescence polarization immunoassay (Siemens Medical Solutions Diagnostics) or whole serum as appropriate were: high-sensitivity C-reactive protein (hsCRP) by particle-enhanced immunonephelometry; soluble adhesion molecules [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin), monocyte chemoattractant protein-1 (MCP-1), and CD40L by standard ELISA (Bender MedSystems). We used high sensitivity immunoassays for IL-6 and TNFα to detect low serum concentrations of these molecules (80 pg/L and 310 pg/L, respectively). Intra- and inter-assay variation coefficients for hsCRP, ICAM-1, VCAM-1, E-selectin, P-selectin, CD40L, MCP-1, TNFα, and IL-6 ranged from 1.8 to 5.4% and from 0.9 to 9.9%, respectively.

We performed all analyses in duplicate. As a measure of intervention compliance, we measured urinary resveratrol metabolites by HPLC-MS/MS before and after each intervention, as previously reported (24).

[7] Abbreviations used: CHD, coronary heart disease; hsCRP, high-sensitivity C-reactive protein; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; MCP-1, monocyte chemoattractant protein-1; PBMC, peripheral blood mononuclear cell; SLe^x, Sialyl-Lewis^x; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

PBMC immunophenotyping. PBMC were isolated from whole blood by density gradient centrifugation over Ficoll-Hypaque (Pharmacia) (25). We analyzed the expression of adhesion molecules on PBMC surface via double direct immunofluorescence using commercial monoclonal antibodies. Cell counting and fluorescence analysis were performed in a FACScan Clinical Cytometer (Becton-Dickinson) using the CellQuest software. The adhesion molecules studied were: very late activation antigen-4 (VLA-4) (Cytognos), lymphocyte function-associated antigen-1 (LFA-1) (Bender MedSystems), Mac-1 (Bender MedSystems), and Sialyl-Lewis^x (SLe^x) (Beckman Coulter). CD40 (Caltag Laboratories), another related molecule, was also measured. We identified monocytes and T-lymphocytes separately using anti-CD14 and anti-CD2 (Caltag Laboratories) monoclonal antibodies, respectively.

Statistical analysis. We performed statistical analysis using the SPSS Statistical Analysis system 11.0. Values in the text are expressed as means ± SD, unless otherwise indicated. Values with a skewed distribution (hsCRP, VCAM-1, ICAM-1, and IL-6) were transformed to their natural logarithm for analyses. We compared changes in outcome variables in response to each intervention treatment with the 2-tailed paired *t* test. To exclude the presence of a carryover effect for the 2 interventions, we compared the outcome variables observed before the cava and gin treatments and did not observe differences in any of the variables analyzed (see above). Within- and between-group differences are expressed as mean percent difference (95% CI). Differences were considered significant at $P < 0.05$.

Results

Baseline characteristics, intervention compliance, diet, exercise monitoring, and side effects. Of 30 eligible subjects, we excluded 10 from the study for the reasons explained above. Thus, we included the remaining 20 healthy men (34 ± 9 y, range 25–50 y) in the study and randomly assigned them to 1 of the 2 interventions (cava or gin). All subjects completed both phases of the study. Prior to participating in the study, they reported a daily ethanol intake of 16.8 ± 12.6 g during a period of 17 ± 10 y. We evaluated the compliance of intervention by analyzing participants' reports and recounts of empty bottles returned. In addition, as an objective measure of intervention compliance, we determined resveratrol metabolites in urine. The urine concentration of total resveratrol metabolites increased by 72.4 nmol/g (95% CI = 48.5–96.2 nmol/g; $P = 0.005$) after the cava intervention compared with the corresponding wash-out period, whereas we did not find any significant changes after the gin intervention. Based on these data, all participants were compliant. Self-reported diets were close to the planned diets and none of the subjects consumed a significant quantity of polyphenol-rich foods during the study, so the nutritional intake including total energy, carbohydrates, fat, protein, and vitamins was similar before and after each intervention period in all the subjects. Only 1 participant reported a 1-d violation (onion) 17 d before assessment. In addition, we did not find any significant differences in physical activity throughout the study (**Table 2**). None of the participants reported any side effects during the both phases of the study.

Expression of cell adhesion molecules on leukocyte cell surface. Expression of cell adhesion molecules on leukocyte cell surface before and after each intervention is reported in **Table 3**. Adhesion molecule expression did not differ before cava and gin intervention. Changes in T-lymphocyte surface molecules were minimal. We detected a reduction in LFA-1 expression after cava intake by 16% ($P = 0.001$) and an upregulation of LFA-1 expression after gin intake by 19% ($P = 0.034$); however, the rest of molecules did not differ on T-lymphocytes. Changes were more prominent on monocyte surface. After cava intake, there were reductions of 11–21% in LFA-1 ($P = 0.048$), VLA-4 ($P = 0.015$), SLe^x ($P = 0.01$), and CD40 ($P = 0.033$) expression. On the other hand, after the gin intake period, only SLe^x declined ($P = 0.036$). The following molecules were significantly downregulated more by cava than by gin: LFA-1 in T-lymphocytes ($P = 0.001$) and monocytes ($P = 0.021$), VLA-4 in monocytes ($P = 0.008$), and CD40 in monocytes ($P = 0.029$).

Changes in homocysteine and circulating inflammatory markers. Plasma homocysteine concentrations were similar before and after cava (10.9 ± 2.7 μmol/L to 11.5 ± 3.6 μmol/L) or gin (10.5 ± 2.6 μmol/L to 11.2 ± 2.9 μmol/L) intervention periods. Serum adhesion molecule concentrations did not differ before both the interventions. Some circulating adhesion

TABLE 2 Dietary intake, body weight, and exercise energy output in men before and after cava and gin interventions[1]

	Cava			Gin		
	Before	After	Difference % (95% CI)[2]	Before	After	Difference % (95% CI)
Dietary intake						
Energy[3], kcal/d	2248 ± 693	2236 ± 468	−3 (−13 to 7)	2506 ± 673	2255 ± 668	−8 (−18 to 2)
Cholesterol, mg/d	374 ± 104	396 ± 115	13 (−7 to 33)	431 ± 123	395 ± 150	−4 (−21 to 12)
SFA, mg/d	32.2 ± 11.8	30.8 ± 10.1	−0.1 (−14 to 14)	37.2 ± 14.7	33.2 ± 12.0	−4 (−20 to 10)
Monounsaturated fatty acids, mg/d	47.5 ± 16.8	48.3 ± 14.3	8 (−7 to 23)	55.6 ± 16.6	48.9 ± 13.3	−6 (−23 to 11)
PUFA, mg/d	13.8 ± 6.0	15.8 ± 5.9	29 (−5 to 64)	17.2 ± 6.4	15.8 ± 6.0	−1 (−20 to 18)
Vitamin A, mg/d	400 ± 200	463 ± 201	32 (−1 to 65)	455 ± 241	445 ± 228	−18 (−54 to 10)
Vitamin E, mg/d	10.2 ± 4.1	12.0 ± 5.3	26 (−1 to 53)	12.0 ± 4.5	11.1 ± 5.5	−1 (−26 to 24)
Vitamin C, mg/d	130 ± 69.4	131 ± 53.3	1 (−31 to 33)	135 ± 81.6	134 ± 82.7	−1 (−61 to 59)
Polyphenols, mg/d	227 ± 59	225 ± 72	−15 (−56 to 26)	240 ± 85	231 ± 88	−18 (−46 to 10)
Exercise						
Energy output, kcal/d	231 ± 133	227 ± 155	−1 (−21 to 19)	289 ± 238	214 ± 160	−12 (−46 to 23)
Body weight, kg	78.0 ± 12.3	78.5 ± 12.4	−0.6 (−1.2 to 0.1)	78.2 ± 10.1	78.1 ± 10.1	−0.02 (−0.6 to 0.6)

[1] Values are means ± SD, $n = 20$.
[2] 95% CI of mean differences (%) after the intervention, $n = 20$.
[3] 1 kcal = 4.184 kJ.

TABLE 3 Expression of inflammatory molecules on leukocyte cell surfaces of men before and after cava and gin interventions[1]

	Cava			Gin		
	Before	After	Difference	Before	After	Difference
T-Lymphocytes	AU[2]		% (95% CI)[3]	AU[2]		% (95% CI)[3]
LFA-1	139.2 ± 25.1	115.8 ± 23.9**	−16 (−24 to −8)***	122.6 ± 29.4	139.3 ± 26.7*	19 (3 to 34)
VLA-4	29.2 ± 5.0	26.9 ± 6.8	−2 (−16 to 11)	25.9 ± 5.3	27.6 ± 2.3	11 (−2 to 24)
SLe^x	12.2 ± 4.4	13.1 ± 4.1	17 (−5 to 39)	12.5 ± 4.6	13.9 ± 7.5	21 (−16 to 57)
CD40	16.9 ± 3.2	18.9 ± 4.3	16 (−0.6 to 32)	17.4 ± 5.6	16.4 ± 5.2	−0.6 (−19 to 18)
Monocytes						
LFA-1	138.2 ± 16.8	121.1 ± 24.1*	−11 (−21 to −2)***	121.7 ± 24.9	130.9 ± 29.1	12 (−4 to 28)
Mac-1	63.1 ± 21.2	58.6 ± 14.2	−2 (−17 to 21)	58.7 ± 12.6	55.1 ± 17.2	−4 (−17 to 9)
VLA-4	39.7 ± 9.8	31.7 ± 5.9*	−17 (−26 to −7)***	34.6 ± 10.5	36.7 ± 10.8	12 (−6 to 30)
SLe^x	63.0 ± 24.4	44.1 ± 14.7**	−21 (−37 to −5)	50.6 ± 14.3	37.9 ± 10.5*	−20 (−32 to −8)
CD40	43.8 ± 17.1	32.1 ± 8.8*	−19 (−33 to −4)***	34.4 ± 11.6	36.2 ± 16.7	18 (−18 to 53)

[1] Values are means ± SD. Asterisks indicate different from baseline: *$P < 0.05$; **$P < 0.01$; ***Different between cava and gin intervention period, $P < 0.05$.
[2] Arbitrary units.
[3] 95% CI of mean differences (%) after the intervention, $n = 20$.

molecules and other inflammatory biomarkers changed after each intervention (**Fig. 1**). After the cava period, concentrations of CD40L ($P = 0.015$), VCAM-1 ($P = 0.001$), hsCRP ($P = 0.049$), IL-6 ($P = 0.008$), P-selectin ($P = 0.01$), E-selectin ($P = 0.02$), ICAM-1 ($P = 0.013$), and MCP-1 ($P = 0.01$) diminished from 11 to 27%. After gin consumption, we observed reductions of 13–18% in serum E-selectin ($P = 0.012$), P-selectin ($P = 0.028$), and VCAM-1 ($P = 0.034$) concentrations and 21% higher MCP-1 levels ($P = 0.026$). The effect of cava was greater than that of gin for CD40L ($P = 0.030$), ICAM-1 ($P = 0.015$), and MCP-1 ($P = 0.001$).

Discussion

In this 3-mo feeding intervention trial, consumption of 30 g ethanol/d as cava was associated with a downregulation of the expression of LFA-1, VLA-4, SLe^x, and CD40 in monocytes and of LFA-1 in T-lymphocytes. In addition, serum concentrations of hsCRP, VCAM-1, ICAM-1, E-selectin, P-selectin, IL-6, MCP-1, and CD40L also decreased after cava intake. Gin intake also exerted an antiinflammatory effect, because serum concentrations of VCAM-1, E-selectin, and P-selectin decreased and, also, SLe^x was downregulated in monocyte surface. However, the effects of cava on inflammatory biomarkers of atherosclerosis were significantly greater than those of gin.

The epidemiological association between moderate alcohol consumption and lower prevalence of atherosclerosis may have several pitfalls, because even in prospective cohort studies it is difficult to assess the type and amount of ethanol intake exactly and to control the effects of the diet consumed and physical activity performed on the variables studied (26). In fact, some investigators have suggested that the lower risk of CHD in

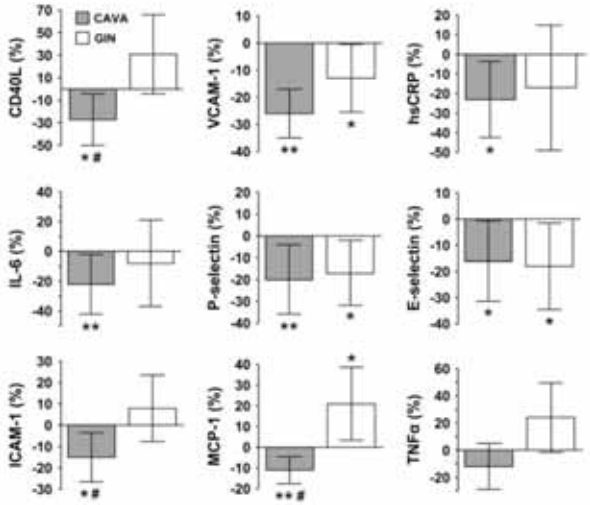


FIGURE 1 Percent changes from baseline in circulating adhesion molecules and other inflammatory biomarkers in healthy men after cava and gin consumption. Values are means and 95% CI, $n = 20$. Asterisks indicate different from baseline: *$P < 0.05$; **$P < 0.01$. #Different from gin, $P < 0.05$.

moderate drinkers may be also related to their characteristic lifestyle or the consumption of a healthier diet (27). It should be taken into account that fruits and vegetables contain large amounts of polyphenolic compounds, such as flavonoids, and some studies have suggested that flavonoid intake may explain the low mortality rates from CHD reported in Western countries (28,29). On the other hand, physical activity increases HDL cholesterol serum concentrations (30) and may reduce cardiovascular mortality by itself. Thus, the issue of nutrition and exercise may be solved in only well-designed clinical trials in which the intervention could be monitored by biochemical analysis. To avoid this problem, in this trial, we monitored nutritional intake and exercise performed throughout the study by means of validated scales, but we did not detect any differences in these variables between the periods of the study. We also assessed protocol compliance by personnel interviews, counting empty bottles, and measuring concentrations of resveratrol metabolites in urine after each intervention (24). After analyzing all these data, we concluded that protocol compliance was nearly 100% in all the subjects. Therefore, the changes observed in inflammatory variables analyzed in the study should be attributed to the consumption of cava or gin.

The mechanisms by which moderate alcohol consumption may prevent atherosclerosis are not completely known. Beyond changes in lipid profile, coagulation, and fibrinolitic system observed in alcohol drinkers, the involvement of other alternative mechanisms may completely explain the protective effect of alcoholic beverages (7,13,31). Thus, the possible antiinflammatory effects of alcoholic beverages in the arterial wall have become a matter of research (15).

Epidemiological studies suggest that moderate alcohol intake is associated with reduced levels of circulating inflammatory predictive markers of atherosclerosis (9,14,32). In this sense, a reduction in C-reactive protein, α_1-globulins, α_2-globulins, IL-6, sTNF-R1, sTNF-R2, and fibrinogen have been observed in moderate drinkers compared with nondrinking subjects. Likewise, moderate amounts of red wine inhibit the expression of MCP-1 and neointimal hyperplasia after a balloon injury in cholesterol-fed rabbits (33), whereas in vitro studies show that ethanol inhibits MCP-1 expression in IL-1β-activated human endothelial cells (34). Previous clinical trials performed by our group revealed that moderate consumption of red wine exerted greater antiinflammatory effects than ethanol itself (gin). In addition, red wine prevented nuclear factor-κB activation in PBMC, a process that activates genes involved in immune and inflammatory responses (35,36). These antiinflammatory effects have been attributed to the high polyphenol content of red wines. The results of this study also confirm that moderate consumption of cava, a medium-level polyphenol content beverage, is able to reduce the expression of adhesion molecules that participate in the passage of monocytes and T-lymphocytes into the arterial wall.

The interaction of T-lymphocytes and monocytes with endothelium through adhesion molecules is the first event in atheroma plaque formation. This process may involve several steps such as rolling, tethering, firm adhesion, and transmigration of circulating mononuclear cells in which different adhesion molecules participate (8). Selectins and SLe^x exert their function during the rolling phase, whereas integrins, ICAM-1, and VCAM act during firm adhesion and transmigration (37). Our results suggest that moderate consumption of cava and gin may have an effect in the initial phases of the atherosclerosis process. Until now, no studies to our knowledge have reported the antiinflammatory effect of cava consumption in human beings. These effects may contribute, with others previously reported (such as reduction of LDL oxidation in vitro or decrease of aortic fatty streak formation in hamsters) (38,39), to the overall beneficial effect of wine against atherosclerosis.

In summary, our study suggests that alcoholic beverages with medium-level polyphenol content such as cava induce greater reductions of inflammatory markers of atherosclerosis (adhesion molecules, cytokines, and CD40/CD40L system) compared with alcoholic beverages with negligible levels of polyphenols, such as gin. Therefore, these data suggest that some of the atheroprotective effect of alcoholic beverages could be partially mediated by their antiinflammatory activity in the vascular wall.

Literature Cited

1. Doll R, Peto R, Hall E, Wheatley K, Gray R. Mortality in relation to consumption of alcohol: 13 years' observations on male British doctors. BMJ. 1994;309:911–8.
2. Mostwyler J, Hennekens C, Buring J, Gaziano J. Mortality and light to moderate alcohol consumption after myocardial infarction. Lancet. 1998;352:1882–5.
3. Rotondo S, Iacoviello L, de Gaetano G. Light to moderate alcohol consumption and the risk of stroke among American male physicians. Ital Heart J Suppl. 2000;1:569–70.
4. Di Castelnuovo A, Rotondo S, Iacoviello L, Donati MB, De Gaetano G. Meta-analysis of wine and beer consumption in relation to vascular risk. Circulation. 2002;105:2836–44.
5. Gaziano JM, Buring JE, Breslow JL, Goldhaber SZ, Rosner B, VanDenburgh M, Willett W, Hennekens CH. Moderate alcohol intake, increased levels of high-density lipoprotein and its subfractions, and decreased risk of myocardial infarction. N Engl J Med. 1993;329:1829–34.
6. Nigdikar SV, Williams NR, Griffin BA, Howard AN. Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo. Am J Clin Nutr. 1998;68:258–65.
7. Estruch R. Wine and cardiovascular disease. Food Res Int. 2000;33:219–26.
8. Libby P. Inflammation in atherosclerosis. Nature. 2002;420:868–74.
9. Imhof A, Froehlich M, Brenner H, Boeing H, Pepys MB, Koenig W. Effect of alcohol consumption on systemic markers of inflammation. Lancet. 2001;357:763–7.
10. Sacanella E, Badia E, Nicolas JM, Fernandez-Sola J, Antunez E, Urbano-Marquez A, Estruch R. Differential effects of moderate or heavy alcohol consumption on circulating adhesion molecule levels. Thromb Haemost. 2002;88:52–5.
11. Sierksma A, van der Gaag MS, Kluft C, Hendriks HF. Moderate alcohol consumption reduces plasma C-reactive protein and fibrinogen levels: a randomized, diet-controlled intervention study. Eur J Clin Nutr. 2002;56:1130–6.
12. Van der Gaag M, Ubbink J, Sillanaukee P, Nikkari S, Hendriks H. Effect of consumption of red wine, spirits, and beer on serum homocysteine. Lancet. 2000;355:1522.
13. Imhof A, Woodward M, Doering A, Helbecque N, Loewel H, Amouyel P, Lowe GD, Koenig W. Overall alcohol intake, beer, wine, and systemic markers of inflammation in Western Europe: results from three MONICA samples (Augsburg, Glasgow, Lille). Eur Heart J. 2004;25:2092–100.
14. Pai JK, Hankinson SE, Thadhani R, Rifai N, Pischon T, Rimm EB. Moderate alcohol consumption and lower levels of inflammatory markers in US men and women. Atherosclerosis. 2006;186:113–20.
15. Stewart SH. Alcohol and inflammation: a possible mechanism for protection against ischemic heart disease. Nutr Metab Cardiovasc Dis. 2002;12:148–51.
16. Rimm EB, Giovannucci EL, Willett WC, Colditz GA, Ascherio A, Rosner B, Stampfer MJ. Prospective study of alcohol consumption and risk of coronary disease in men. Lancet. 1991;338:464–8.
17. Gronbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G, Sorensen TI. Type of alcohol consumed and mortality from all causes, coronary heart disease and cancer. Ann Intern Med. 2000;133:411–9.

18. Theobald H, Johansson SE, Engfeldt P. Influence of different types of alcoholic beverages on self-reported health status. Alcohol Alcohol. 2003;38:583–8.
19. Estruch R, Sacanella E, Badia E, Antúnez E, Nicolás JM, Fernández-Solá J, Rotilio D, De Gaetano G, Rubin E, et al. Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial. Effects of wine on inflammatory markers. Atherosclerosis. 2004;175:117–23.
20. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolibdicphosphotungstic acid reagent. Am J Enol Vitic. 1965;16:144–58.
21. Ibern-Gómez M, Andrés-Lacueva C, Lamuela-Raventós RM, Buxaderas S, Singleton VL, de la Torre-Boronat MC. Browning of cava (sparkling wine) during aging in contact with lees due to the phenolic composition. Am J Enol Vitic. 2000;51:29–36.
22. Schröder H, Covas MI, Marrugat J, Vila J, Pena A, Alcantara M, Masia R. Use of a 3-days estimated food record, a 72-hours recall and food frequency questionnaire for dietary assessment in a Mediterranean Spanish population. Clin Nutr. 2001;20:429–37.
23. Elosua R, Marrugat J, Molina L, Pons S, Pujol R. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. Am J Epidemiol. 1994;139:1197–209.
24. Zamora-Ros R, Urpi-Sardá M, Lamuela-Raventós RM, Estruch R, Vázquez-Agell M, Serrano-Martínez M, Jaeger W, Andres-Lacueva C. Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. Clin Chem. 2006;52:1373–80.
25. Sacanella E, Estruch R, Gaya A, Ferrer K, Fernandez-Sola J, Alonso JR, Nicolas JM, Urbano-Marquez A. Upregulated expression of VLA proteins and CD29 in peripheral blood lymphocytes of chronic alcoholics without ethanol-related diseases. Alcohol Clin Exp Res. 1999;23:371–5.
26. Borodulin K, Laatikainen T, Salomaa V, Jousilahti P. Associations of leisure time physical activity, self-rated physical fitness, and estimated aerobic fitness with serum C-reactive protein among 3803 adults. Atherosclerosis. 2006;185:381–7.
27. Ruidavets JB, Bataille V, Dallongeville J, Simon C, Bingham A, Amouyel P, Arveiler D, Ducimetiere P, Ferrieres J. Alcohol intake and diet in France, the prominent role of lifestyle. Eur Heart J. 2004;25:1153–62.
28. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet. 1993;342:1007–11.
29. Knekt P, Jarvinen R, Reunanen A, Maatela J. Flavonoid intake and coronary mortality in Finland: a cohort study. BMJ. 1996;312:478–81.
30. Arquer A, Elosua R, Covas MI, Molina L, Marrugat J. Amount and intensity of physical activity, fitness, and serum lipids in pre-menopausal women. Int J Sports Med. 2006;27:911–8.
31. Lucas DL, Brown RA, Wassef M, Giles TD. Alcohol and the cardiovascular system research challenges and opportunities. J Am Coll Cardiol. 2005;45:1916–24.
32. Avellone G, Di Garbo V, Campisi D, De Simone R, Raneli G, Scaglione R, Licata G. Effects of moderate Sicilian red wine consumption on inflammatory biomarkers of atherosclerosis. Eur J Clin Nutr. 2006;60:41–7.
33. Feng AN, Chen YT, Ding YZ, Lin SJ. Red wine inhibits monocyte chemotactic protein-1 expression and modestly reduce neointimal hyperplasia after balloon injury in cholesterol-fed rabbits. Circulation. 1999;100:2254–9.
34. Cullen JP, Sayeed S, Jin Y, Theodorakis NG, Sitzmann JV, Cahill PA, Redmond EM. Ethanol inhibits monocyte chemotactic protein-1 expression in interleukin-1{beta}-activated human endothelial cells. Am J Physiol Heart Circ Physiol. 2005;289:H1669–75.
35. Blanco-Colio LM, Valderrama M, Alvarez-Sala LA, Bustos C, Ortego M, Fernandez-Presa MA, Cancelas P, Gomez-Gerique J, Millan J, et al. Red wine intake prevents nuclear factor-kappa B activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. Circulation. 2000;102:1020–6.
36. Blanco-Colio LM, Muñoz-García B, Martín-Ventura JL, Alvarez-Sala LA, Castilla M, Bustamante A, Lamuela-Raventós RM, Gómez-Gerique J, Fernández-Cruz A, et al. Ethanol beverages containing polyphenols decrease nuclear factor kappa-B activation in mononuclear cells and circulating MCP-1 concentrations in healthy volunteers during a fat-enriched diet. Atherosclerosis. 2007;192:335–41.
37. Toborek M, Kaiser S. Endothelial cell functions. Relationship to atherogenesis. Basic Res Cardiol. 1999;94:295–314.
38. Satué-Gracia MT, Andrés-Lacueva C, Lamuela-Raventós RM, Frankel EN. Spanish sparkling wines (cavas) as inhibitors of in vitro human low-density lipoproteins oxidation. J Agric Food Chem. 1999;47:2198–202.
39. Auger C, Rousnet JM, Vanderlinde R, Bornet A, Décordé K, Lequeux N, Cristol JP, Teissedre PL. Polyphenols-enriched Chardonnay white wine and white sparkling wine Pinot Noir red wine identically prevent early atherosclerosis in hamsters. J Agric Food Chem. 2005;53:9823–9.

4.2.1.2. El resveratrol urinari com a nou biomarcador del consum moderat de vi

Carta d'opinió: Zamora-Ros R, Lamuela-Raventós RM, Estruch R, Andres-Lacueva C. Resveratrol, anew biomarker of wine intake?. *British Journal of Nutrition.* 2008. **Acceptada.**

Resum

En un recent *review* de la revista *British Journal of Nutrition*, Spencer *et al.*(Spencer et al., 2008) han realitzat una meticulosa revisió presentant i avaluant els punts forts i dèbils dels biomarcadors nutricionals de la ingesta de polifenols. En aquesta revisió es repeteix la idea de que els biomarcadors ofereixen uns resultats més reals de la ingesta respecte a les estimacions dietètiques, en aquest cas de polifenols, que les estimacions obtingudes mitjançant enquestes alimentàries. Els autors han identificat quins criteris han d'acomplir aquests biomarcadors: i) metodologia robusta; ii) sensibilitat; iii) especificitat; iv) biodisponibilitat. A més els autors han recopilat un conjunt d'exemples de potencials biomacadors, i en aquesta carta d'opinió el nostre grup suggereix la inclusió del resveratrol com biomarcador del consum moderat de vi. Això és degut a que als articles 1 d'aquesta tesi es mostren resultats sobre la sensibilitat i especificitat, respectivament, del resveratrol com biomarcador del consum de vi. A més la metodologia d'anàlisis per LC-MS/MS és una tècnica suficientment robusta i recentment Boocock *et al. (Boocock et al., 2007)* han estudiat els paràmetres farmacocinètics en humans del resveratrol.

Per aquests motius, el nostre grup proposa el resveratrol i els seus metabòlits en orina com a biomarcador del consum moderat de vi, perquè acompleix amb tots els requisits considerats per Spencer *et al*.

BJN 2687—20:15, 6/5/2008——299569

British Journal of Nutrition (2008), **not known**, 1 2
doi: 10.1017/S0007114508995696

British Journal of Nutrition
NS

Letter to the Editor

Resveratrol, a new biomarker of moderate wine intake?

In a recent study published in the *British Journal of Nutrition*, Spencer *et al.*[1] reviewed the strengths and the limitations of the biomarkers of dietary polyphenol intake, since nutritional biomarkers may be a better measure of dietary exposure than self-reported dietary data. These authors identified the criteria that must be considered in the development of such biomarkers as the following: (i) robust methodology; (ii) sensitivity; (iii) specificity; (iv) bioavailability. Different polyphenols were reviewed as potential biomarkers by the authors; we suggest that resveratrol should also be considered. We analysed resveratrol metabolites as potential biomarkers of wine consumption in two randomised cross-over trials and a cohort study[2]. Using a cut-off of 90 nmol/g, we were able to use urinary total resveratrol metabolite concentration to differentiate wine consumers from abstainers with a sensitivity of 72 % (60–84 %) and a specificity of 94 % (87–100 %). In these trials, urinary resveratrol was specific, as wine has been reported as the most important source of dietary resveratrol (98·4 %)[3], has an adequate half-life and provided a good correlation between these measured values and the dietary data reported (r 0·654; $P<0·001$). In addition, there is a robust analytical technique[4,5] using LC-MS-MS to determine urinary resveratrol metabolites and their pharmacokinetic parameters have been recently studied by Boocock *et al.*[6].

Taking these points into consideration, we want to propose urinary resveratrol metabolites as a biomarker of grape product consumption; this would be a new nutritional biomarker which accomplish and fulfil the criteria of Spencer *et al.*[1].

Q2 We declare no conflict of interest.

Raul Zamora-Ros, Rosa M. Lamuela-Raventós and Cristina Andres-Lacueva*
Nutrition and Food Science Department-Xarta, INSA, AGL2006-14228-C03-02/01 and INGENIO-CONSOLIDER Program, Fun-c-food CSD2007-063, Pharmacy School, University of Barcelona, Av. Joan XXIII, s/n 08028 Barcelona, Spain,
Q1
Fax +34 93 403 59 31,
**email* candres@ub.edu

Ramón Estruch
Department of Internal Medicine, Hospital Clínic, University of Barcelona, 08036 Barcelona, Spain
Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, 08036 Barcelona, Spain
CIBER 06/03: Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, 28029 Madrid, Spain

References

1. Spencer JPE, Abd El Mohsen MM, Minihane A-M & Mathers JC (2008) Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *Br J Nutr* **99**, 12–22.
2. Zamora-Ros R, Urpí-Sardà M, Lamuela-Raventós RM, Estruch R, Vázquez-Agell M, Serrano-Martínez M, Jaeger W & Andres-Lacueva C (2006) Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. *Clin Chem* **52**, 1373–1380.
3. Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, *et al.* (2007) Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. *Br J Nutr* (Epublication ahead of print version 21 Q3 December 2007).
4. Urpí-Sardà M, Jáuregui O, Lamuela-Raventós RM, Jaeger W, Miksits M, Covas MI & Andres-Lacueva C (2005) Uptake of diet resveratrol into the human low density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. *Anal Chem* **77**, 3149–3155.
5. Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos R, Cherubini A, Jauregui O, de la Torre R, Covas MI, Estruch R, Jaeger W & Andres-Lacueva C (2006) Rapid and highly sensitive method to characterize the metabolism of resveratrol in humans. *Clin Chem* **53**, 292–299.
6. Boocock DJ, Faust GE, Patel KR, *et al.* (2007) Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiol Biomarkers Prev* **16**, 1246–1252.

BJN 2687—20:15, 6/5/2008——299569

Author Queries

JOB NUMBER: 2687
JOURNAL: BJN

Q1 Is the fax number correct?

Q2 Please confirm there are no conflicts of interest.

Q3 Reference 3. Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventt RM, *et al.* Note to CUP – please update year of publication and add volume and page number if/when printed

NS British Journal of Nutrition

4.2.2. Concentració de resveratrol i dels seus derivats en aliments i l'estimació de la seva ingesta dietètica en una població espanyola: cohort que pertany a l'estudi prospectiu europeu sobre dieta i càncer (EPIC-Espanya).

Article 3: Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, Berenguer T, Jakszyn P, Martínez C, Sánchez MJ, Navarro C, Chirlaque MD, Tormo MJ, Quirós JR, Amiano P, Dorronsoro M, Larrañaga N, Barricarte A, Ardanaz E, González CA. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. *British Journal of Nutrition.***2008**; 100: 188-196.

Resum:

Els efectes beneficiosos del resveratrol respecte a malalties relacionades amb processos oxidatius i/o inflamatoris es troben àmpliament descrits en la bibliografia. A més en els últims anys, han aparegut estudis on al resveratrol se li han atribuït propietats relacionades amb l'antienvelliment, ja que té la capacitat d'augmentar l'esperança de vida d'organismes simples i mamífers petits. L'objectiu del presente treball és estimar la ingesta dietètica del resveratrol i del piceid (R&P) presents en els aliments, i la identificació de les principals fonts dietètiques d'aquest compostos en una població adulta espanyola.

Per assolir amb l'objectiu, en primer lloc es va tenir que recopilar una base de dades de composició d'aliments espanyols (BDCA) específica per R&P. Finalment es va aconseguir una BDCA amb 160 ítems que es van agrupar en un llistat de 18 aliments genèrics. La població espanyola estudiada va ser la cohort de 40685 subjectes, d'entre 35 i 64 anys de regions del nord i del sud de Espanya, inclosos en l'estudi prospectiu europeu sobre dieta i càncer (EPIC-España). La ingesta habitual d'aliments va ser estimada mitjançant una entrevista personal emprant una versió informatitzada d'un qüestionari validat de la història dietètica.

La mediana i la mitjana de la ingesta de R&P de la població adulta espanyola és de 100 i 933 μg/dia, respectivament. Tot i que aproximadament el 32% de la població espanyola no consumeix R&P. Dels 4 estilbens estudiats (R&P, formes *cis* i *trans*) el majoritari va ser el *trans*-piceid (53.6%), seguit pel *trans*-resveratrol (20.9%), *cis*-piceid (19.3%) i en últim lloc el *cis*-resveratrol (6.2%). La font dietètica més abundant de R&P va ser el vi (98.4) seguit pel raïm i els sucs de raïm (1.6%), mentre que els cacauets, festucs i baies contribueixen amb menys d'un 0.01%.

Així doncs, com a conclusió d'aquest treball es pot observar que el patró d'ingesta de R&P és similar al patró d'ingesta de vi. Aquest ha estat el primer treball científic que estima el consum de R&P en un país mediterrani.

British Journal of Nutrition (2008), 100, 188–196
doi:10.1017/S0007114507882997

British Journal of Nutrition

Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort

Raul Zamora-Ros[1], Cristina Andres-Lacueva[1]*, Rosa M. Lamuela-Raventós[1], Toni Berenguer[2], Paula Jakszyn[2], Carmen Martínez[3], María J. Sánchez[3], Carmen Navarro[4], María D. Chirlaque[4], María-José Tormo[4], Jose R. Quirós[5], Pilar Amiano[6], Miren Dorronsoro[6], Nerea Larrañaga[6], Aurelio Barricarte[7], Eva Ardanaz[7] and Carlos A. González[2]

[1]*Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Av. Joan XXIII, s/n. 08028, Barcelona, Spain*
[2]*Unit of Epidemiology, Catalan Institute of Oncology, Institute of Biomedical Research of Bellvitge, L'Hospitalet de Llobregat, Spain*
[3]*Andalusian School of Public Health, Granada, Spain*
[4]*Servicio de Epidemiología, Consejería de Sanidad, Murcia, Spain*
[5]*Consejería de Sanidad y Servicios Sociales de Asturias, Oviedo, Spain*
[6]*Dirección de Salud de Guipúzcoa, San Sebastián, Spain*
[7]*Instituto de Salud Pública de Navarra, Pamplona, Spain*

(Received 12 September 2007 – Revised 25 October 2007 – Accepted 6 November 2007 – First published online 21 December 2007)

Resveratrol has been shown to have beneficial effects on diseases related to oxidant and/or inflammatory processes and extends the lifespan of simple organisms including rodents. The objective of the present study was to estimate the dietary intake of resveratrol and piceid (R&P) present in foods, and to identify the principal dietary sources of these compounds in the Spanish adult population. For this purpose, a food composition database (FCDB) of R&P in Spanish foods was compiled. The study included 40 685 subjects aged 35–64 years from northern and southern regions of Spain who were included in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. Usual food intake was assessed by personal interviews using a computerised version of a validated diet history method. An FCDB with 160 items was compiled. The estimated median and mean of R&P intake were 100 and 933 μg/d respectively. Approximately, 32 % of the population did not consume R&P. The most abundant of the four stilbenes studied was *trans*-piceid (53·6 %), followed by *trans*-resveratrol (20·9 %), *cis*-piceid (19·3 %) and *cis*-resveratrol (6·2 %). The most important source of R&P was wines (98·4 %) and grape and grape juices (1·6 %), whereas peanuts, pistachios and berries contributed to less than 0·01 %. For this reason the pattern of intake of R&P was similar to the wine pattern. This is the first time that R&P intake has been estimated in a Mediterranean country.

Resveratrol: Food composition databases: Intake: Wine: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain

Resveratrol (3,5,4′-trihydroxystilbene) is the parent compound of a family of molecules, including glycosides (piceid) and polymers (viniferins), existing in *cis* and *trans* configurations classified as stilbenes[1]. The essential structural skeleton comprises two aromatic rings linked by a methylene bridge (Fig. 1).

Resveratrol and piceid (R&P) are mainly present in grape and wine derivatives and their composition is affected by grape cultivar, degree of maturity at harvest, fungal pressure, climate and wine-making technology[2,3]. Secondary food sources of stilbenes are peanuts, pistachios and berries[4–7]. Recently R&P were also detected in the skin of tomatoes, although the concentrations are 3000 times lower than those found in red table grapes, and R&P have not been found in all kinds of tomato[8]. The importance of R&P food sources depends on food composition and the amount of consumption of them (standard serving size: grapes, 150 g; wine, 125 ml; berries and peanuts, 30 g). The total qualitative and quantitative R&P profile is also affected by the source: *trans*-piceid is mainly present in red and white wines and grape juice; *cis*-piceid in rosé and sparkling wines and *trans*-resveratrol in grapes, berries, peanuts and pistachios[4,7,9–11]. Until now, viniferins have only been described in grape derivatives[12,13].

Resveratrol is of great interest in nutrition and medicine due to its potential health benefits, such as anti-carcinogenic[14,15], neuroprotector[16] and antioxidant effects[17], as a modulator

Abbreviations: EPIC, European Prospective Investigation into Cancer and Nutrition; FCDB, food composition database; R&P, resveratrol and piceid.

* **Corresponding author:** Dr Cristina Andrés-Lacueva, fax +34 93 403 59 31, email candres@ub.edu

BJN British Journal of Nutrition

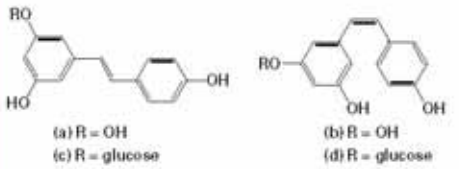


Fig. 1. Structures of resveratrol and derivatives: (a) *trans*-resveratrol; (b) *cis*-resveratrol; (c) *trans*-piceid; (d) *cis*-piceid.

of lipid and lipoprotein metabolism, as an antiplatelet aggregator[18] and its oestrogenic activity[19]. Indeed, it has been hypothesised that resveratrol uses the same pathways activated by energy restriction[20–22]. The biological effects have been studied mainly *in vitro*, although there is also growing *in vivo* evidence[20]. Some effects required a high concentration of resveratrol in tissues, although chemopreventive and chemotherapeutic anticancer effects are an exception[14,15]. In this case, resveratrol, at micromolar concentrations, affects the activity of transcriptional factors involved in proliferation and stress responses and leads to the modulation of survival and apoptotic factors in carcinogenesis[14,15]. In atherosclerotic and neurodegenerative diseases, the effects of resveratrol are not only due to its antioxidant and scavenging activities, but also to its participation in the modulation of signal transduction pathways and in the activation of several enzymes at micromolar concentrations[14,23,24].

The pharmacological effects are consistent with the resveratrol concentration in plasma, LDL and urine after oral administration in human subjects[25–32]. The biological effect of resveratrol will ultimately depend on the cellular effects of the circulating metabolites that are effectively absorbed (glucuronides and sulfates)[30] and not on the native forms in food[33,34]. However, other authors have speculated that resveratrol metabolites may become deconjugated at the target sites of action, thereby releasing aglycone to elicit biological activity[15,35].

Intake values of *trans*- and *cis*-resveratrol and piceids are not available either since there is no complete food composition database (FCDB) of R&P. The aim of the present study was to compile composition data of R&P in common Spanish foods and to evaluate major food sources and their daily intake in the Spanish adult population.

Materials and methods

Population

Dietary data and other lifestyle factors from 41 440 subjects, aged 29–69 years, who participated in the European Prospective Investigation into Cancer and Nutrition (EPIC) in Spain, were studied. Participants were healthy volunteers, blood donors principally, recruited between October 1992 and July 1996 in five Spanish regions: three from the North (Asturias, Navarra and Gipuzkoa) and two from the South (Murcia and Granada)[36]. After the exclusion of 755 subjects because of implausible dietary information, the final population studied consisted of 40 885 subjects (15 448 men and 25 237 women) aged 35–64 years. The mean ages at recruitment were 50·8 and 48·4 years for men and women, respectively.

Dietary information

Usual food intake during the preceding year, taking into account seasonal variations, was estimated by personal interview using a computerised diet history questionnaire. This was developed and validated specifically for the EPIC study in Spain[37,38]. The questionnaire was structured according to occasion of food intake (breakfast, lunch, dinner). Trained interviewers gathered data on preparation method, average frequency of consumption per week, and usual portion size for each food consumed at least twice per month (or once per month for seasonal foods). Portion sizes were reported in natural units, household measures or with the aid of a manual of thirty-five sets of photographs prepared specifically for the study. The questionnaire included a list of more than 600 foods and beverages and about 150 regional recipes. For each food described, the final amount consumed was calculated, taking into account the cooking method used and the edible part consumed.

Food composition database

A literature search was conducted in MEDLINE (United States National Library of Medicine, 2006) and in the Food Science and Technology Abstracts (International Food Information Service, 2006) to identify sources of resveratrol compounds in Spanish foods in published food composition data. The search terms included resveratrol, piceid, food composition, food, wine, berry, peanuts, pistachios and tomato. Review papers that did not contain new primary data were excluded. However, the citations used in these reviews were cross-checked with initial literature searching to identify any additional references.

The following information was extracted from each publication: (1) food information: name, food description, scientific name and country of the study; (2) measurement information: value, type of value (mean, median, range, other), number of samples, sampling method and analytical method; (3) bibliographic reference. With this information we assessed the data quality for inclusion in the Database following the key points originally developed in the EU-AIR NETTOX Project[39].

The appropriate methods of analysis were HPLC diode array or GC/MS. When *cis*- and *trans*-piceid were quantified by spectrophotometric method and were expressed as resveratrol, conversion factors were applied: × 1·57 and × 1·75 for *trans*- and *cis*-piceid, respectively. These factors were calculated using the relationship between the molar absorptivities of *trans*-resveratrol (UV λ (10 % ethanol) nm (ε) 306 (31 800/M/cm))[40] and *trans*-piceid (UV λ (10 % ethanol) nm (ε) 306 (20 100/M/cm)) (R Zamora-Ros *et al.*, unpublished results), and the relationship between *cis*-resveratrol (UV λ (10 % ethanol) nm (ε) 286 (13 100/M/cm))[40] and *cis*-piceid (UV λ (10 % ethanol) nm (ε) 286 (7500/M/cm)) (R Zamora-Ros *et al.*, unpublished results), respectively.

Units of measurements and modes of expression varied across the studies. To standardise, values were converted into mg/100 g fresh weight. Data for similar foods were aggregated as weighted means, taking into account the number of samples, sampling plan and frequency of consumption of Spanish foods[41].

When we did not find Spanish food values for important sources of resveratrol such as peanuts, pistachios or berries we selected foreign food values. Other unknown values were estimated using a biologically similar food or calculating

British Journal of Nutrition

recipes. Despite the use of other countries' and estimated values, data were still not available for some foods.

Statistical analyses

Distributions were expressed as means, standard deviations, medians, and as 25th and 75th percentiles, and were measured separately for men and women. Because R&P intakes were skewed toward higher values, we used median values to compare results. The average estimates of dietary intakes were standardised by sex and age of the Spanish population aged 35–64 years[42]. The contribution of each food to the total intake of individual and total R&P was calculated as a percentage.

To assess the differences in R&P intake with respect to the categories of age, region, educational level, tobacco smoking, BMI and energy intake, estimations of the proportion of consumers and R&P median intake among consumers were calculated using linear regression analysis, respectively. All these models were adjusted by sex, age, region, BMI and energy intake (kJ/d). To perform the linear regression analysis, a Box–Cox transformation of the response variable was necessary to observe the assumptions of the model, and the inverse transformation was applied to the resulting estimates to interpret them as medians[43]. Data were analysed with the R language and environment for statistical computing and graphics[44].

Results

Food composition database

Resveratrol values from fifty-four studies were used to compile the final food database. The compilation included 160 food items with information on the concentrations of *trans*- and *cis*-resveratrol, *trans*- and *cis*-piceid and sum of R&P. Table 1 summarises the resveratrol content from all references compiled for all the common Spanish foods considered. Red wine (0·847 mg/100 g) and itadori tea (0·974 mg/100 g) were the highest sources of R&P, but itadori tea is not consumed in Spain. Intermediate sources of R&P (0·08–0·547 mg/100 g) corresponded to other kinds of wine, grapes, grape juice and peanut butter. Lowest sources of R&P (< 0·01 mg/100 g) were peanuts, pistachios and berries.

Estimated resveratrol intake

Table 2 shows the mean and median values and percentiles of *trans*- and *cis*-resveratrol, *trans*- and *cis*-piceid and total reveratrol intake by sex in the studied population. Average intake of R&P was 933 μg/d, with a median of 100 μg/d. As indicated by the median and percentiles, the distribution was skewed to higher values. A total of 13 175 participants (39·0 and 20·0 % of total women and men standardised by sex and age of the Spanish population respectively) had a total resveratrol intake of 0 μg/d (non-consumers). *trans*-Piceid contributed 53·7 % of total resveratrol intake, *trans*-resveratrol 20·8 %, *cis*-piceid 19·3 % and *cis*-resveratrol 6·2 %.

Table 3 shows the differences in R&P intake according to sex, age, geographic area, energy intake, BMI, education and tobacco smoking. Medians and percentages of consumers were adjusted by sex, age, BMI, region and energy consumption. R&P consumption was lower in quantity and percentage of women consumers (137 μg/d and 61·0 %) than in men (686 μg/d and 80·0 %). Mean of R&P and percentage of

Table 1. Food composition data sources for resveratrol content (mg/100 g fresh weight)

Food item	*trans*-resveratrol	*cis*-resveratrol	*trans*-piceid	*cis*-piceid	Total resveratrol	References
Wine, not specified	0·114	0·037	0·303	0·105	0·558	Calculated*
Red wine	0·181	0·044	0·495	0·127	0·847	Lamuela-Raventós *et al.*[10], Moreno-Labanda *et al.*[46], Goldberg *et al.*[60], Rodriguez-Delgado *et al.*[61]
Rosé wine	0·041	0·041	0·071	0·154	0·307	Romero Perez *et al.*[11]
White wine	0·010	0·016	0·26	0·022	0·074	Romero Perez *et al.*[11], Rodriguez-Delgado *et al.*[61], Alvarez-Sala *et al.*[62], Martinez-Ortega *et al.*[63]
Sparkling wine	0·005	0·014	0·018	0·055	0·092	Andres-Lacueva *et al.*[64], Pozo-Bayon *et al.*[65]
Fortified wine	0·110	0·095	0·141	0·040	0·386	de Lima *et al.*[66], Goldberg *et al.*[67]
Grapes, not specified	0·150	–	0·067	–	0·223	Cantos *et al.*[9,68,69]
Red grapes	0·250	tr	0·060	–	0·310	Cantos *et al.*[9,68,69]
White grapes	0·068	tr	0·025	–	0·093	Cantos *et al.*[9,68]
Must	0·070	0·012	0·465	–	0·547	Vinas *et al.*[70]
Grape juice	0·010	tr	0·036	0·043	0·088	Martinez-Ortega *et al.*[62], Roldan *et al.*[71], Romero-Perez *et al.*[72]
Sangria†	0·091	0·022	0·248	0·063	0·424	Recipe
Peanuts, toasted	0·006	–	–	–	0·006	Sobolev & Cole[73], Lee *et al.*[47]
Pistachios, toasted	0·007	–	–	–	0·007	Tokusoglu *et al.*[7]
Peanut butter	0·065	nd	0·014	nd	0·080	Ibern-Gomez *et al.*[72]
Cranberry juice	tr	–	–	–	tr	Zhang & Zuo[74]
Berries‡	0·008	–	–	–	0·008	Rimando *et al.*[8], Lyons *et al.*[75]
Itadori tea (infusion)	0·068	nd	0·906	nd	0·974	Burns *et al.*[4]

tr, Traces; nd, not detected.
* Calculated from consumption of Spanish population: 57 % red wine, 25 % white wine and 18 % rosé wine.
† Recipe of sangria (typical Spanish beverage): 50 % of red wine and 50 % of orange juice, fruit mix (peaches, oranges, lemons, etc.) and sugar.
‡ Berries included: blueberry, bilberry, sparkleberry, deerberry, cranberry, lingonberry and partridgeberry.

Table 2. Estimated resveratrol intake (μg/d) in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain by sex

	trans-resveratrol*	cis-resveratrol*	trans-piceid*	cis-piceid*	Total resveratrol*
Men (*n* 15 448)					
Mean	337	101	878	313	1629
SD	442	121	1213	380	2076
Median	165	61	356	181	902
Percentiles, 25th–75th	14–502	0–162	14–1309	4–497	48–2504
Women (*n* 25 237)					
Mean	51	15	123	46	235
SD	121	35	325	111	571
Median	0	0	0	0	0
Percentiles, 25th–75th	0–42	0–9	0–56	0–27	0–148
Total (*n* 40 685)					
Mean	194	58	501	180	933
SD	354	89	965	310	1675
Median	31	5	36	16	100
Percentiles, 25th–75th	0–228	0–81	0–545	0–246	0–1219

*Adjusted by sex and age of Spanish population aged 35–64 years.

consumers tended to increase in the older age categories. Individuals from the northern regions consumed more resveratrol than from the southern regions (513 *v.* 125 μg/d), although the percentage of consumers was approximately the same (88·3 *v.* 69·4 %). Increasing intake of R&P and percentage of consumers seemed to be correlated with higher energy intake. Individuals with a BMI between 25 and 30 kg/m^2 had the highest intake of total resveratrol (333 μg/d) and the obese group had the smallest percentage of consumers (66·3 %). Individuals with a high level of education (technical and professional, secondary school or university degree) had a higher intake of R&P than those with only primary education or no education (340–346 *v.* 298–308 μg/d), and the proportion of consumers was also higher in this group (69·8–72·4 % *v.* 68·0–68·6 %). There was a decrease in R&P intake and percentage of consumers in non-smokers (259 μg/d and 67·1 %) when compared with current (369 μg/d and 71·0 %) and former smokers (342 μg/d and 69·4 %).

Sources of resveratrol

Table 4 shows the major contributors to R&P intake. The richest source was red wine (82·6 %). As grouped foods, the main contributors were wines (98·4 %), grapes (1·1 %), must and juices (0·5 %) and, finally, peanuts and pistachios (< 0·01 %). For *trans*-piceid, the major contributors were wines (98·7 %), must and juices (0·7 %) and grapes (0·6 %). For *trans*-resveratrol, we identified the following food items: wines (95·9 %), grapes (3·8 %), must and juices (0·3 %) and peanuts, pistachios and berries (0·03 %). For *cis*-isomers, we observed the next ranking: wines (99·9 and 99·7 %) and must and juices (0·1 and 0·3 %) for *cis*-resveratrol and *cis*-piceid, respectively.

Discussion

The present study represents the first attempt to compile the available literature for R&P in common Spanish foods. After developing an FCDB, we estimated dietary intakes and food sources of R&P in Spanish adults.

Previous papers have compared results of the *trans*-resveratrol content but have not compiled data from *trans*- and *cis*-piceid and *cis*-resveratrol[4,20]. R&P are characteristic components of *Vitis vinifera* L. and are present in grape derivatives. R&P is not unique to *Vitis* because it is also present in at least seventy-two other plant species[45], but only berries, peanuts and pistachios are components of the human diet. The high variability in R&P food composition, red wines ranged between 2·86[10] and 32·33 mg R&P per 100 ml[46], was solved with weighted means, adjusted according to Spanish food consumption[41]. Another consideration in the FCDB was the potential losses in R&P from foods during cooking. The data available from the study by Lee *et al.*[47] suggest that average losses during toasting peanuts are approximately 30 %. The most common method for the measurement of R&P is HPLC coupled to a UV detector. Until 2004–5, due to the non-availability of a commercial standard, the piceid results were expressed as equivalents of resveratrol, underestimating 1·57- and 1·75-fold for *trans*- and *cis*-piceid, respectively. We, therefore, applied a correction factor to minimise this error. FCDB also reported a quality index for each value to guarantee the individual quality data and the global control of FCDB. However, further investigation is required to analyse new sources of R&P, because to date many foods have not yet been studied. In a recent study, R&P were found in the tomato skin, but in very small concentrations (0–18·4 parts per million of dried tomato skin)[8]. This value was not used in this FCDB because not all kinds of tomato contain R&P, the concentration is very low (3000 times lower than in red table grape skin) and, at this moment, only one paper reported this compound in American varieties of tomato[8] and not in European varieties[48].

The median and the mean of the estimated daily intake of R&P were 100 and 933 μg/d respectively, and were standardised according to the age and sex structure of the Spanish population aged 35–64 years. The median of intake was significantly higher in males, in oldest age, current smokers, highest educational levels, Northern region and highest energy intake. The large discrepancies between the mean and median values were due to the fact that more than 32 % of the participants did not consume R&P, and there was a

NS British Journal of Nutrition

Table 3. Estimated intake (μg/d) and percentage of consumers of total resveratrol in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort by age and selected demographic and lifestyle factors*

	Subjects (*n*)	Consumers (%)	Adjusted (%)	Percentage lower 95 %	Percentage upper 95 %	Median adjusted	Lower 95 %	Upper 95 %
Sex								
Female	25237	57·1	61·0	60·3	61·8	137	131	144
Male	15448	84·7	80·0	79·0	88·9	686	664	709
Age (years)								
35–44	13877	65·7	67·3	66·3	68·3	271	259	283
45–54	16107	68·8	69·5	68·7	70·4	324	312	336
55–64	10701	68·2	70·8	69·8	71·9	365	349	382
Region								
North Spain	24752	69·5	68·3	67·5	69·0	513	498	528
South Spain	15933	64·8	69·4	68·5	70·3	125	119	131
Energy intake (kJ/d)								
Q1 (1350–6900)	8137	45·6	53·2	51·4	55·0	130	115	146
Q2 (6900–8410)	8137	59·2	63·7	62·4	65·0	202	189	217
Q3 (8410–9960)	8137	69·1	70·6	69·5	71·7	266	253	280
Q4 (9960–12050)	8137	77·4	74·9	73·9	76·0	375	357	392
Q5 (12050–42680)	8137	86·8	79·8	78·5	81·0	520	492	550
BMI (kg/m^2)†								
< 25	8965	67·7	70·5	69·2	71·7	298	281	317
25–30	19390	70·5	70·2	69·4	71·0	333	321	346
> 30	12219	63·0	66·3	65·2	67·3	296	281	311
Highest school level†								
None	13936	64·0	68·0	66·9	69·0	298	283	313
Primary completed	15846	67·2	68·6	67·6	69·5	308	296	321
Technical/professional	3344	77·2	72·4	70·4	74·3	345	314	378
Secondary school	2611	70·1	69·8	67·8	71·8	346	313	381
University degree	4674	72·3	72·0	70·5	73·6	340	315	365
Smoking status†								
Former smoker	7180	75·7	69·4	68·1	70·8	342	324	360
Current smoker	9951	74·2	71·0	69·9	72·1	369	352	387
Never a smoker	22558	61·2	67·1	66·3	67·9	259	249	269

* Adjusted by age and sex of Spanish population aged 35–64 years; differences between categories for all variables $P<0·001$.
† The values reported are calculated by the number of subjects with valid information. The number of subjects with missing information was as follows: BMI *n* 111; highest school level, *n* 274; smoking status, *n* 996.

Table 4. Consumption of total resveratrol in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort by food items

Food item	Proportion of intake (%)	Cumulative percentage
Red wine	82·63	82·63
Rosé wine	12·19	94·83
Wine, not specified	2·92	97·75
Grapes, not specified	1·13	98·88
Must	0·39	99·27
White wine	0·29	99·56
Vermouth	0·21	99·77
Fruit juice	0·11	99·88
Sparkling wine	0·05	99·93
Fortified wine	0·04	99·97
Txacoli (typical Basque wine)	0·02	99·99
Sherry wine	<0·01	99·99
Peanuts, toasted	<0·01	99·99
Pistachio, toasted	<0·01	100

skewed distribution toward higher values in the consumers. This distribution of R&P intake was similar to that of wine, because more than 98 % of R&P intake was due to wine. The pattern of wine consumption in the EPIC European cohort was described by Sieri *et al.*[49]. A typical high wine consumer, and consequently high R&P consumer, was an older man, a resident of northern Spain, with a high educational level, smoker, with excess weight but not obese and a high energy intake[49,50]. In Spain, as in Portugal, the pattern of alcohol consumption is changing: the prevalence of wine drinkers is decreasing, and younger generations are shifting from wine to beer and spirits[51].

To our knowledge, only one case–control study estimated *trans*-resveratrol intake for women in the Swiss Canton of Vaud[32]. One limitation to comparison of the results is that Levi *et al.*[32] did not include a complete description, only reporting tertiles. On the other hand, they only used grapes and white and red wine, without taking into account other sources of *trans*-resveratrol such as grape juice, other kinds of wine, peanuts, berries, etc. Taking into account that *trans*-resveratrol only corresponded to 21 % of the four stilbenes investigated in the present study, the median of intake of total individuals was 31 μg/d (0 μg/d for women), and the sources of R&P were 29 μg/d for wine (98·3 %) and 0·5 μg/d for grapes (1·2 %). However, in the study by Levi *et al.* the distribution in food sources was very different, because the second tertiles for wine and grapes were 0·1–176·8 μg/d and 72·3–126·4 μg/d, respectively[32]. This great difference in resveratrol intake from grapes can be due to using other food composition data. Furthermore, in populations with other dietary patterns, the contribution of berries and peanut butter may be different.

In human subjects, the proportion of nutritional resveratrol absorbed ranged from 16 to 25 % of intake, measured in urine by MS techniques[25,27]. Piceid may be absorbed directly, as reported for the rat small intestine[53], and/or hydrolysed by glycosidases before absorption[54], contributing to the biologically available resveratrol dose. Biomarkers of resveratrol intake, such as urinary resveratrol metabolites, can be used as an alternative to evaluate resveratrol status and to assess relationships between resveratrol and disease[32]. The use of biomarkers avoids problems associated with an FCDB[55]. In a recent study, resveratrol metabolites in urine were used as a biomarker of moderate wine consumption in intervention and epidemiological studies[32]. However, not all epidemiological studies are able to undertake the measurement of biomarkers due to a lack of resources or expertise. For this reason, estimation of resveratrol intake from dietary questionnaires and records using adequate food composition data is also required[56].

R&P have been shown to have health benefits in *in vitro* studies, and against cancer, cardiovascular and neurodegenerative diseases. Levi *et al.* found a significant inverse association between *trans*-resveratrol and breast cancer from grapes (OR 0·64 and 0·55) but not from wine[32]. Polyphenols in wine may play an active role in limiting the initiation and progression of atherosclerosis[57]. Localised accumulation of resveratrol in epithelial cells along the aerodigestive tract, and potentially active resveratrol metabolites, may also produce cardiovascular effects. Moreover, resveratrol has been considered to be a energy restriction mimetic *in vitro* and in lower organisms and mice, because it interacts with a variety of enzymes, such as sirtuin, involved in regulating stress responses and longevity[20–23]. So, long-term consumption of a low concentration of polyphenol, such as resveratrol, or a synergic effect with other phenolic compounds or other micronutrients in the Mediterranean diet could be sufficient to cause beneficial effects against these alterations and could constitute a potential arm for prevention of chronic diseases and new therapeutic strategies[23]. It is, therefore, of interest to study the relationship between R&P intake and the risk of chronic diseases in an epidemiological context. However, wine polyphenols are a complex mixture of flavonoids and non-flavonoids (where resveratrol would be included) and the relative contribution of each single one or synergistic contribution of them is still unclear and further investigation should be considered.

One limitation of the present study was that the EPIC-Spain cohort is based on a non-representative sample of the general population. However, the number of volunteers was very large, the participation rate was relatively high, and the subjects came from different social backgrounds and different geographical areas. In addition, the pattern of dietary intake was very similar to that observed in population-based surveys carried out in the Spanish regions included in the present study[38,39].

We conclude that R&P and especially *trans*-piceid are common components of the Mediterranean diet. Clearly, wine is the major contributor of R&P in this population (>98 %); the contribution of non-grape derivatives is lower than 0·01 %. This is the first attempt to compile the existing published scientific data on the R&P content of foods. This database allowed the quantification of intakes that can be used to investigate the role of R&P in health benefits to increase lifespan.

Acknowledgements

The present study is part of the EPIC-Spain Study. The EPIC Study in Spain has received financial support from the Health Research Fund (FIS, 02/0652) of the Spanish Ministry of

NS British Journal of Nutrition

Health, the ISCIII (RETIC-RD06/0020 and the CIBER in Epidemiology and Public Health) and the Spanish Regional Government of Andalusia, Asturias, Basque Country, Murcia and Navarra and the Catalan Institute of Oncology. Some authors are partners of ECNIS, a network of excellence of the EU 6FP. AGL2006-14228-C03-02/ALI and Program Ingenio CONSOLIDER CSD2007-063 also contributed to the present study. R. Z.-R. was supported by the Departament d'Universitats, Recerca i Societat de la Informació (Catalunya). The authors are not aware of any conflict of interest.

References

1. Soleas GJ, Diamandis EP & Goldberg DM (1997) Resveratrol: a molecule whose time has come? And gone? *Clin Biochem* **30**, 91–113.
2. Gonzalez-Barrio R, Beltran D, Cantos E, Gil MI, Espin JC & Tomas-Barberan FA (2006) Comparison of ozone and UV-C treatments on the postharvest stilbenoid monomer, dimer, and trimer induction in var. 'Superior' white table grapes. *J Agric Food Chem* **54**, 4222–4228.
3. Romero-Perez AI, Lamuela-Raventos RM, Andres-Lacueva C & Torre-Boronat MC (2001) Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J Agric Food Chem* **49**, 210–215.
4. Burns J, Yokota T, Ashihara H, Lean ME & Crozier A (2002) Plant foods and herbal sources of resveratrol. *J Agric Food Chem* **50**, 3337–3340.
5. Rimando AM, Kalt W, Magee JB, Dewey J & Ballington JR (2004) Resveratrol, pterostilbene, and piceatannol in Vaccinium berries. *J Agric Food Chem* **52**, 4713–4719.
6. Sobolev VS & Cole RJ (1999) *Trans*-resveratrol content in commercial peanuts and peanut products. *J Agric Food Chem* **47**, 1435–1439.
7. Tokusoglu O, Unal MK & Yemis F (2005) Determination of the phytoalexin resveratrol (3,5,4′-trihydroxystilbene) in peanuts and pistachios by high-performance liquid chromatographic diode array (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS). *J Agric Food Chem* **53**, 5003–5009.
8. Ragab AS, Van FI, Jaakuwski B, Park JH & Bobzin SC (2006) Detection and quantitation of resveratrol in tomato fruit (*Lycopersicon esculentum* Mill.). *J Agric Food Chem* **54**, 7175–7179.
9. Cantos E, Espin JC & Tomas-Barberan FA (2002) Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. *J Agric Food Chem* **50**, 5691–5696.
10. Lamuela-Raventós R, Romero-Pérez A, Waterhouse A & de la Torre-Boronat M (1995) Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceic isomers in Spanish red *Vitis vinifera* wines. *J Agric Food Chem* **43**, 281–283.
11. Romero Perez AI, Lamuela Raventos RM, Waterhouse AL & de la Torre Boronat MC (1996) Levels of *cis*- and *trans*-resveratrol and their glucosides in white and rosé *Vitis vinifera wines* from Spain. *J Agric Food Chem* **44**, 2124–2128.
12. Vitrac X, Bornet A, Vanderlinde R, Valls J, Richard T, Delaunay JC, Merillon JM & Teissedre PL (2005) Determination of stilbenes (δ-viniferin, *trans*-astringin, *trans*-piceid, *cis*- and *trans*-resveratrol, ε-viniferin) in Brazilian wines. *J Agric Food Chem* **53**, 5664–5669.
13. Landrault N, Larronde F, Delaunay J, Castagnino C, Vercauteren J, Mérillon J, Gasc F, Cros G & Teissedre P (2002) Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development. *J Agric Food Chem* **50**, 2046–2052.
14. Delmas D, Lancon A, Colin D, Jannin B & Latruffe N (2006) Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets* **7**, 423–442.
15. Signorelli P & Ghidoni R (2005) Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J Nutr Biochem* **16**, 449–466.
16. Dore S (2005) Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. *Neurosignals* **14**, 61–70.
17. Fremont L (2000) Biological effects of resveratrol. *Life Sci* **66**, 663–673.
18. Olas B & Wachowicz B (2005) Resveratrol, a phenolic antioxidant with effects on blood platelet functions. *Platelets* **16**, 251–260.
19. Juan ME, Gonzalez-Pons E, Munuera T, Ballester J, Rodriguez-Gil JE & Planas JM (2005) *trans*-Resveratrol, a natural antioxidant from grapes, increases sperm output in healthy rats. *J Nutr* **135**, 757–760.
20. Baur JA & Sinclair DA (2006) Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat Rev Drug Discov* **5**, 493–506.
21. Ingram DK, Zhu M, Mamczarz J, Zou S, Lane MA, Roth GS & deCabo R (2006) Calorie restriction mimetics: an emerging research field. *Aging Cell* **5**, 97–108.
22. Baur JA, Pearson KJ, Price NL, *et al.* (2006) Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**, 337–342.
23. Delmas D, Jannin B & Latruffe N (2005) Resveratrol: preventing properties against vascular alterations and ageing. *Mol Nutr Food Res* **49**, 377–395.
24. Orallo F (2006) Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. *Curr Med Chem* **13**, 87–98.
25. Goldberg DM, Yan J & Soleas GJ (2003) Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin Biochem* **36**, 79–87.
26. Meng X, Maliakal P, Lu H, Lee MJ & Yang CS (2004) Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. *J Agric Food Chem* **52**, 935–942.
27. Soleas GJ, Yan J & Goldberg DM (2001) Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. *J Chromatogr B Biomed Sci Appl* **757**, 161–172.
28. Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas M & Andres-Lacueva C (2005) Uptake of diet resveratrol into the human low density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. *Anal Chem* **77**, 3149–3155.
29. Vitaglione P, Sforza S, Galaverna G, Ghidini C, Caporaso N, Vescovi PP, Fogliano V & Marchelli R (2005) Bioavailability of *trans*-resveratrol from red wine in humans. *Mol Nutr Food Res* **49**, 495–504.
30. Walle T, Hsieh F, DeLegge MH, Oatis JE & Walle UK (2004) High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab Dispos* **32**, 1377–1382.
31. Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, Hirschelman WH, Pezzuto JM, Mehta RG & van Breemen RB (2002) Human, rat, and mouse metabolism of resveratrol. *Pharm Res* **19**, 1907–1914.
32. Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Vazquez-Agell M, Serrano-Martinez M, Jaeger W & Andres-Lacueva C (2006) Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. *Clin Chem* **52**, 1373–1380.
33. Koga T & Meydani M (2001) Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells. *Am J Clin Nutr* **73**, 941–948.

NS British Journal of Nutrition

British Journal of Nutrition

34. Moon JH, Tsushida T, Nakahara K & Terao J (2001) Identification of quercetin 3-*O*-β-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. *Free Radic Biol Med* **30**, 1274–1285.
35. Boocock DJ, Patel KR, Faust GES, Normolle DP, Marczylo TH, Crowell JA, Brenner DE, Booth TD, Gescher A & Steward WP (2007) Quantitation of *trans*-resveratrol and detection of its metabolites in human plasma and urine by high performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* **848**, 182–187.
36. Gonzalez CA, Navarro C, Martinez C, *et al.* (2004) The European Prospective Investigation into Cancer and Nutrition (EPIC) (article in Spanish). *Rev Esp Salud Publica* **78**, 167–176.
37. Anonymous (1997) Relative validity and reproducibility of a diet history questionnaire in Spain. I. Foods. EPIC Group of Spain. European Prospective Investigation into Cancer and Nutrition. *Int J Epidemiol* **26**, Suppl. 1, S91–S99.
38. Anonymous (1997) Relative validity and reproducibility of a diet history questionnaire in Spain. II. Nutrients. EPIC Group of Spain. European Prospective Investigation into Cancer and Nutrition. *Int J Epidemiol* **26**, Suppl. 1, S100–S109.
39. Moller A, Rhodes M, Preece R, Walker A, Atkins D, Bailey G, van Klaveren J & Vittozzi L (1998) Proposal for the structure and organisation of a future European database Report no. 3. EU-AIR NETTOX (1995–1997). http://www.foodcomp.dk/basis/ (accessed 15 December 2006).
40. Trela BC & Waterhouse AL (1996) Resveratrol: isomeric molar absorptivities and stability. *J Agric Food Chem* **44**, 1253–1257.
41. Ministerio de Agricultura Pesca y Alimentación (2004) *La Alimentación en España. 2003 (Food in Spain.2003)*. Madrid: Ministerio de Agricultura Pesca y Alimentación.
42. Instituto Nacional de Estadística (2005) Instituto Nacional de Estadística (National Statistics Institute) http://www.ine.es (accessed 15 December 2006).
43. Flanders WD, DerSimonian R & Freedman DS (1992) Interpretation of linear regression models that include transformations or interaction terms. *Ann Epidemiol* **2**, 735–744.
44. R Development Core Team (2006) *A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
45. Alarcón de la Lastra C & Villegas I (2005) Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. *Mol Nutr Food Res* **49**, 405–430.
46. Moreno-Labanda J, Mallavia R, Pérez-Fons L, Lizama V, Saura D & Micol V (2004) Determination of piceid and resveratrol in Spanish wines deriving from Monastrell (*Vitis vinifera* L.) grape variety. *J Agric Food Chem* **52**, 5396–5403.
47. Lee S, Lee S, Kim M, Chun J, Cheong Y & Lee J (2004) Analysis of *trans*-resveratrol in peanuts and peanut butters consumed in Korea. *Food Res Intern* **37**, 247–251.
48. Nicoletti I, De Rossi A, Giovinazzo G & Corradini D (2007) Identification and quantification of stilbenes in fruits of transgenic tomato plants (*Lycopersicon esculentum* Mill.) by reversed phase HPLC with photodiode array and mass spectrometry detection. *J Agric Food Chem* **55**, 3304–3311.
49. Sieri S, Agudo A, Kesse E, *et al.* (2002) Patterns of alcohol consumption in 10 European countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) project. *Public Health Nutr* **5**, 1287–1296.
50. Guallar-Castillón P, Rodríguez-Artalejo F, Díez-Gañan L, Banegas Banegas JR, Lafuente Urdinguio P & Herruzo Cabrera R (2001) Consumption of alcoholic beverages and subjective health in Spain. *J Epidemiol Community Health* **55**, 648–652.
51. Marques-Vidal P & Dias CM (2005) Trends and determinants of alcohol consumption in Portugal: results from the national health surveys 1995 to 1996 and 1998 to 1999. *Alcohol Clin Exp Res* **29**, 89–97.
52. Levi F, Pasche C, Lucchini F, Ghidoni R, Ferraroni M & La Vecchia C (2005) Resveratrol and breast cancer risk. *Eur J Cancer Prev* **14**, 139–142.
53. Kuhnle G, Spencer JPE, Chowrimootoo G, Schroeter H, Debnam ES, Srai SKS, Rice-Evans C & Hahn U (2000) Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem Biophys Res Commun* **272**, 212–217.
54. Henry-Vitrac C, Desmouliere A, Girard D, Merillon JM & Krisa S (2006) Transport, deglycosilation, and metabolism of *trans*-piceid by small intestinal epithelial cells. *Eur J Nutr* **45**, 376–382.
55. Potischman N (2003) Biologic and methodologic issues for nutritional biomarkers. *J Nutr* **133**, Suppl. 3, 875S–880S.
56. Kelemen LE (2006) Food frequency questionnaires: not irrelevant yet. *Cancer Epidemiol Biomarkers Prev* **15**, 1054.
57. Szmitko PE & Verma S (2005) Cardiology patient pages. Red wine and your heart. *Circulation* **111**, e10–e11.
58. Aranceta J, Perez C, Eguileor I, Gonzalez de Galdeano J, Matxix J & Saez de Buruaga J (1990) *Nutritional Survey. Technical Documents of Public Health*, ser. A, no. 9. Vitoria, Spain: Government of the Basque Country.
59. Matxix J, Llopis J, Martinez de Victoria E, Montellano MA, Lopez M & Aranda P (1999) *Evaluation of the Nutritional Status in the Autonomous Community of Andalucia*. Granada, Spain: Public Health Service and Institute of Nutrition and Food Technology of the University of Granada.
60. Goldberg DM, Karumanchiri A, Ng E, Yan J, Diamandis EP & Soleas GJ (1995) Direct gas-chromatographic mass-spectrometric method to assay *cis*-resveratrol in wines: preliminary survey of its concentration in commercial wines. *J Agric Food Chem* **43**, 1245–1250.
61. Rodríguez-Delgado M, González G, Pérez-Trujillo J & García-Montelongo F (2002) *Trans*-resveratrol in wines from the Canary Islands (Spain). Analysis by high performance liquid chromatography. *Food Chem* **76**, 371–375.
62. Álvarez-Sala L, Slowing-Barrillas K, Gómez-Serranillos P, Torres-Segovia F, Valderrama-Rojas M & Millán-Núñez J (2000) Variabilidad del contenido de polifenoles de distintos tipos de vino y su potencial aplicación al conocimiento de sus efectos biológicos (Variability of the content of polyphenols of different types from wine and potential application from the knowledge of their biological effects). *Med Clin (Barc)* **114**, 331–332.
63. Martinez-Ortega MV, Carcía-Parrilla MC & Troncoso AM (2000) Resveratrol content in wines and musts from the south of Spain. *Nahrung* **44**, 253–256.
64. Andres-Lacueva C, Ibern-Gomez M, Lamuela-Raventos RM, Buxaderas S & de la Torre-Boronat M (2002) Cinnamates and resveratrol content for sparkling wine characterization. *Am J Enol Vitic* **53**, 147–150.
65. Pozo-Bayon MA, Hernandez MT, Martin-Alvarez PJ & Polo MC (2003) Study of low molecular weight phenolic compounds during the aging of sparkling wines manufactured with red and white grape varieties. *J Agric Food Chem* **51**, 2089–2095.
66. de Lima M, Waffo-Téguo P, Teissedre P, Pujolas A, Vercauteren J, Cabanis J & Mérillon J (1999) Determination of stilbenes (*trans*-astringin, *cis*- and *trans*-piceid, and *cis*- and *trans*-resveratrol) in Portuguese wines. *J Agric Food Chem* **47**, 2666–2670.
67. Goldberg DM, Yan J, Ng E, Diamandis EP, Karumanchiri A, Soleas G & Waterhouse AL (1995) A global survey of *trans*-resveratrol concentrations in commercial wines. *Am J Enol Vitic* **46**, 159–165.
68. Cantos E, Espin JC & Tomas-Barberan FA (2002) Postharvest stilbene-enrichment of red and white table grape varieties using UV-C irradiation pulses. *J Agric Food Chem* **50**, 6322–6329.
69. Cantos E, Tomas-Barberan FA, Martinez A & Espin JC (2003) Differential stilbene induction susceptibility of seven red wine grape varieties upon post-harvest UV-C irradiation. *Eur Food Res Technol* **217**, 253–258.

70. Vinas P, Lopez-Erroz C, Marin-Hernandez JJ & Hernandez-Cordoba M (2000) Determination of phenols in wines by liquid chromatography with photodiode array and fluorescence detection. *J Chromatogr A* **871**, 85 93.
71. Roldan A, Palacios V, Caro I & Perez L (2003) Resveratrol content of *Palomino fino* grapes: influence of vintage and fungal infection. *J Agric Food Chem* **51**, 1464 1468.
72. Romero-Perez AI, Ibern-Gomez M, Lamuela-Raventos RM & Torre-Boronat MC (1999) Piceid, the major resveratrol derivative in grape juices. *J Agric Food Chem* **47**, 1533 1536.
73. Ibern-Gomez M, Roig-Pérez S, Lamuela-Raventos RM & de la Torre-Boronat M (2000) Resveratrol and piceid levels in natural and blended peanut butters. *J Agric Food Chem* **48**, 6352 6354.
74. Zhang K & Zuo YG (2004) GC-MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. *J Agric Food Chem* **52**, 222 227.
75. Lyons MM, Yu C, Toma RB, Cho SY, Reiboldt W, Lee J & van Breemen RB (2003) Resveratrol in raw and baked blueberries and bilberries. *J Agric Food Chem* **51**, 5867 5870.

NS British Journal of Nutrition

4.2.3. Metabòlits del resveratrol en orina com a biomarcador del consumo de vi en estudis epidemiològicos: l'estudi PREDIMED.

Article 4: Zamora-Ros R, Urpí-Sardà M, Lamuela-Raventós RM, Estruch E, Martínez-González MA, Salas-Salvadó J, Arós F, Andres-Lacueva C. Resveratrol metabolites in urine is as biomarker of wine intake in free-living subjects: the PREDIMED Study. **En procés de revisió.**

Resum:

Diversos estudis, tant clínics com epidemiològics, han mostrat els efectes cardio-saludables del consum moderat de vi. L'estimació del consum d'aliments o de nutrients sempre és un factor crític en epidemiologia nutricional. Les dades dietètiques obtingudes mitjançant enquestes alimentàries són menys fiables que les dades aportades pels biomarcadors nutricionals.

Aquest estudi s'ha portat a terme amb 1000 participants seleccionats aleatòriament del temps inicial de l'estudi PREDIMED, els voluntaris van ser admesos des de Octubre 2003 a Juny del 2005. La suma dels metabòlits del resveratrol (TRMs) en orina de primera hora del matí van ser analitzats per cromatografia líquida acoblada a espectrometria de masses en tàndem (LC-MS/MS) després d'una neteja mitjançant una extracció en fase sòlida (Urpi-Sarda et al., 2007).

Els nivells de TRMs han estat correlacionats directament amb la quantitat diària de vi declarada (r=0.895; *P*<0.001). Usant el punt de tall de 411.38nmol/g de creatinina, els TRMs són capaços de discriminar entre els consumidors i els no consumidors de vi amb una sensibilitat del 93.27% (91.53%-94.74%); i una especificitat del 92.07% (90.22%-93.67%).

Les conclusions que es poden extreure del present estudi són que els TRMs en l'orina del matí acompleixen tots els criteris de valoració d'un marcador biològic per ser considerat un bon biomarcador del consum de vi en grans estudis epidemiològics. Per aquesta raó, aquest biomarcador pot proporcionar una eina addicional per investigar les relacions entre la ingesta de vi i el seu potencial efecte en la salut.

Resveratrol metabolites in urine as biomarker of wine intake in free-living subjects: the PREDIMED Study[1-3]

*Raul Zamora-Ros, Mireia Urpí-Sardà, Rosa M. Lamuela-Raventós, Ramon Estruch, Miguel Ángel Martínez-González, Mònica Bulló, Fernando Arós and Cristina Andres-Lacueva**

[1]From the Nutrition and Food Science Department-XaRTA INSA, Pharmacy School, University of Barcelona, Barcelona, Spain (RZ-R, MU-S, RML-R, and CA-L); Internal Medicine Department, Hospital Clínic, Barcelona, and CIBER 06/03:Fisiopatología de la Obesidad y la Nutrición, Spain (RE, MB);Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra-Clinica Universitaria, Pamplona, Spain (MAM-G); Human Nutrition Unit, School of Medicine, University Rovira i Virgili, Reus (Tarragona), Spain (MB) and Department of Cardiology, Hospital Txagorritxu, Vitoria, Spain (FA).

[2] This study was supported by grants from the Spanish Ministry of Science and Innovation (Red de Grupo G03/140 and RETICS RD06/0045; Ingenio-CONSOLIDER program, FUN-C-Food CSD2007-063; AGL2006-14228-C03-02/01 and 2005-0559; CIBEROBN is an initiative of the Instituto de Salud Carlos III (ISCIII), Spain. RE has a sabbatical grant from ISCIII. R.Z.R. was supported by *Departament d'Universitats, Recerca i Societat de la Informació* (DURSI), *Generalitat de Catalunya*, and M.U.S from FPI fellowship program.

[3]Reprints not available. Address correspondence to: C Andres-Lacueva, Department of Nutrition and Food Science, Pharmacy School, University of Barcelona, Av/Joan XXIII

s/n. 08028, Barcelona, Spain; phone 34-93-403-48-40, fax 34-93-403-59-31, E-mail: candres@ub.edu

Running title: Resveratrol as biomarker of wine intake

ABSTRACT

Background: Several clinical and epidemiological studies have shown that moderate wine consumption may exert a protective effect against coronary heart disease. However, the epidemiological assessment of wine consumption has usually been obtained using self-reported questionnaires with less reliable information to assess total intake accurately than nutritional biomarkers. A reliable biomarker for wine consumption is, therefore, needed.

Objectives: To validate urinary resveratrol metabolites (RMs) as a biomarker of wine consumption in a large cohort of free-living subjects.

Design: Consecutive 1000 subjects entering a substudy of the PREDIMED trial (PREvención con DIeta MEDiterránea) were evaluated. Data were collected in a validated semiquantitative food frequency questionnaire and information was compiled on drinking habits. RMs were measured in morning urine by LC-MS/MS after sample cleanup by solid-phase extraction.

Results: 45.8% participants reported a mean daily intake of 182.1 g of wine, whereas 15.1% were non wine consumers and 39.1% reported an intermittent consumption of wine (less than 3 glasses a week). Urinary RMs values correlated directly with reported daily amounts of wine consumed ($r = 0.895$; $P < 0.001$). Using a cut-off of 411.4 nmol/g creatinine, urinary RMs could discriminate wine consumers from non wine consumers and intermittent consumers with a sensitivity of 93.3% (95% confidence interval, CI 91.5-94.7%); and a specificity of 92.1% (CI 90.2-93.7%).

Conclusions: Urinary RMs fulfill criteria to be considered as nutritional biomarker of wine consumption in a large sample of free-living subjects. This biomarker would

provide an additional tool to investigate the relationship between wine and health benefits more precisely.

(Word count: 249)

INTRODUCTION

Results of several epidemiological studies have supported the healthy effects of the Mediterranean food pattern (1). Wine is one of the most representative foods of this pattern and it has been reported that wine intake may explain why Southern European countries have a low prevalence of coronary heart disease (CHD), despite exhibiting relatively high prevalence of cardiovascular risk factors. For example, the French population consumes large amounts of saturated fat, but the incidence of CHD is low, known as the French Paradox (1;2). The exact mechanisms of the beneficial effects of moderate wine consumption are still uncertain; part of these effects have been attributed to ethanol itself and part to its polyphenol content. Other potential benefits attributed to moderate wine consumption in epidemiological studies include a reduced risk of ischemic stroke (3), hypertension (4), diabetes (5), dementia (6) and several causes of mortality (7). However, epidemiological studies have yielded widely variable results (4-6), probably due to the fact that it is very difficult to accurately assess dietary habits (including alcoholic beverage consumption) and physical activity in these studies. In other words, there are many uncertainties associated with the dietary assessment methods currently used in epidemiological studies (8). Additionally, the appropriate use of biomarkers in epidemiologic and clinical studies requires their validity to first be verified in samples of free-living populations. There are several advantages of biomarkers over self-reported dietary data, since they reflect a more objective assessment of the nutrient intake (9-11). Therefore, for a better insight into the health

effects of moderate wine drinking, reliable biological markers for wine intake are needed. Furthermore, the availability of biomarkers can be used to reduce bias in the assessment of associations between dietary habits and disease risk.(12).

Wine, especially red wine, is the richest identified dietary source of stilbenes, the most representative of which is resveratrol. The amounts of resveratrol and piceid in red wine are 4-fold and more than 100 fold higher than in grape and other stilbene dietary sources (peanuts, pistachios and berries) respectively (13). In the EPIC-Spanish cohort, the most important source of total resveratrol (sum of *cis*- and *trans*-resveratrol and piceid) was wine (>98%), with an estimated daily stilbene intake of 933μg (13). After a recent study in which we reported that urinary excretion of resveratrol metabolites (RMs) may be used as a potential biomarker of wine consumption in clinical trials (14), in the current work we assessed the feasibility of this biomarker of wine consumption from a food frequency questionnaire (FFQ) in a large cohort of free-living subjects.

SUBJECTS AND METHODS

Subjects

The present study is a cross-sectional assessment of the first 1,000 consecutively admitted participants recruited from October 2003 to July 2005 in a substudy of the PREDIMED (PREvención con DIeta MEDiterránea) Trial (www.predimed.org). This is a large, parallel group, multicenter, controlled, randomized 5-year clinical trial designed to evaluate the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. Full details of the study protocol have been published elsewhere (15). The Institutional Review Board of all participant centers approved the study

protocol, and has been registered in the Current Controlled Trials, London (ISRCTN 35739639).

Eligible participants are community-dwelling men, 55 to 80 years of age, and women, 60 to 80 years of age; with an absence of prior CHD; and the presence of type 2 diabetes or at least three or more of the following CHD risk factors: current smoking, hypertension (blood pressure>140/90 mmHg, or treatment with antihypertensive drugs), LDL cholesterol ≥160 mg/dL (or treatment with hypolipidemic drugs), low HDL cholesterol (≤40 mg/dL), body mass index (BMI) ≥25 kg/m^2, or family history of premature CHD.

Dietary assessment

The baseline examination included administration of a validated 137-item FFQ (16). Data reported included information on drinking habits, such as amount, frequency, and type of alcohol intake. Energy and nutrients; and resveratrol and piceid intakes were calculated from Spanish food tables (17) and compiled Spanish food composition data (13), respectively.

Samples and analytical methods

Morning urine samples were collected from all participants. Urine samples were coded and stored at –80ºC until analyses. The clinical investigators and laboratory technicians were blinded to clinical data. Resveratrol metabolites (RMs) in urine samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (18;19). Briefly, 1 mL of urine with the internal standard was loaded onto a previously equilibrated Oasis HLB 96-wells SPE plate (30mg; Waters). Urinary RMs were eluted with acidified methanol solution and ethyl acetate. After evaporation, the samples were

reconstituted with 100 μL of the mobile phase and then analyzed in the LC (Perkin-Elmer s200) coupled to a triple-quadrupole mass spectrometer (API 3000; Perkin-Elmer Sciex). All results for urinary RMs were corrected for creatinine and were reported as nanomols per gram of creatinine in the morning urine (14). Urinary creatinine was measured by the standard Jaffe (alkaline picrate) kinetic method (20;21).

Statistical analysis

Descriptive statistics with the mean (SD) were used for the baseline characteristics of the participants. Chi-square tests and analyses of variance (ANOVA) were used to compare qualitative traits and means of quantitative variables, respectively, between wine consumption groups. As urinary resveratrol data was skewed (Kolmogorov and Levene tests), median (interquartile range) were used to describe this variable, and comparisons between groups of wine consumption were performed using non-parametric tests (Kruskal-Wallis and Mann-Whitney with Bonferroni adjustment). Spearman's rank correlation was calculated to estimate the association between urinary RMs excretion and dietary wine intake or dietary resveratrol and piceid consumption. Using ROC curve analysis, a cut-off point providing optimized sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for the identification of wine consumers were calculated. All statistical test were 2-tailed, and the significance level was $P<0.05$. Statistical analysis was performed using the SPSS 14.0.

RESULTS

We analyzed the baseline data of 1,000 high cardiovascular-risk participants (479 men and 521 women, mean (SD) aged 66.6 (6.2)) (Table 1). Participants of moderate daily wine group were significantly higher in males, current smokers, high education level and had low chronic diseases with a similar pattern than other epidemiological studies

(7;22;23).The mean (SD) daily alcohol intake of the evaluated subjects was 10.86 (16.33) g/day, mainly as some form of wine. 45.8% of participants were moderately daily consumers of wine [182.1 (151.9) mL/d], 15.1% drank intermittently (less than 3 glasses a week) [12.16 (6.21) mL/d] and the remaining 39.1% did not drink any kind of wine. Among the wine drinkers, most of them preferentially drank red wine (76.7%) and only a few, white wine (11.9 %) and rosé wine (11.4%). The participants who reported to drink only beer and/or spirits (4.8%) were included in the group of non wine consumers.

Total urinary RMs were calculated as the sum of individual metabolites (*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*-sufate and *cis*-resveratrol-3-*O*-glucuronide). The mdian (interquartile range) urinary RMs amounts were 120.7 (205.5), 599.8 (381.3) and 1401.3 (1242.6) nmol/g for volunteers who reported non wine, intermittent and daily wine consumption, respectively (Fig 1). Compared with the participants classified as moderate daily wine consumers, urinary RMs concentrations increased significantly for the intermittent wine consumers ($P<0.001$) and for the non wine consumers ($P<0.001$). Significant differences were also observed when intermittent and non wine consumers were compared ($P<0.001$). The reported wine consumption was correlated directly with the urinary RMs concentrations ($r = 0.895$; $P <0.001$) (Fig 2). The estimated resveratrol and piceid consumption from FFQ was correlated directly with the urinary RMs concentration ($r = 0.890$; $P <0.001$).

According to ROC curve analysis, the optimal cutoff point for urinary RMs was 411.38 nmol/g which allowed differentiation of non-wine consumers from wine consumers

with an area under the ROC curve (Fig 3) of 0.983 (95% CI, 0.973-0.990), a sensitivity of 93.3% (CI 91.5-94.7%), a specificity of 92.1% (CI 90.2-93.7%), a positive predictive value (PPV) of 94.8% (CI 93.2-96.1%), and a negative predictive value (NPV) of 89.8% (CI 87.8-91.6%).

DISCUSSION

We report the first data on resveratrol as a nutritional marker of moderate wine consumption in a large sample of free-living subjects, previously described in two controlled clinical trials and in a small cohort (14). Spencer *et al.*(11) established the optimal criteria of potential compounds to serve as useful nutritional biomarkers: i) robust methodology; ii) sensitivity; iii) specificity; iv) bioavailability, characteristics also confirmed by van Damm and Hu (24). Since the validated LC-MS/MS methodology carried out in this study allowed us to improve the resveratrol metabolite profile in biological samples, parameters such as selectivity, sensitivity, recovery, linearity, precision and stability were maximally optimized (18). Moreover, the solid phase extraction adaptation into 96 wells and 10 min of chromatographic time allowed us to analyze a large number of samples, essential in epidemiological studies.

Although some differences were observed between resveratrol dietary intake and concentrations of urinary RMs in controlled, crossover and randomized clinical trials (14), in the current study we showed that RM concentration in urine correlated significantly with calculated resveratrol intake obtained from FFQ in large free-living populations (r: 0·890, $P< 0.001$). Moreover, urinary RMs also correlated highly significantly with reported wine consumption (r: 0·895, $P< 0.001$), which indicates the usefulness of such determination as a biomarker of wine consumption.

However, other premises should be taken into account. The best specific dietary biomarker would only be modified by one food. Since this situation hardly ever occurs, the major food sources of polyphenols should be analyzed. Resveratrol may be found in red wine, but also in grape, nuts and berries. Nevertheless, the amounts of resveratrol and piceid in red wine are 4-fold, 120-fold, 105-fold higher than in grape, peanuts or pistachios, and berries, respectively (13). In our study, resveratrol and piceid sources corresponded to wine (96.4%), grape (3.6%) and other foods (<0.1%). Thus, wine is the most important dietary source of resveratrol (13). Other important limitation to assess the specificity of a nutritional biomarker may be the wide variability in nutrient composition of a same food. It is well-known that resveratrol content in wines may vary up to 10-fold (25;26) due to grape variety and wine making. However other polyphenols, well-established as good nutritional biomarkers (11), such as quercetin in onions and lettuce, also vary 7-fold and 82-fold, respectively, due to variety, part of plant (outer vs inner leaves) and cooking process (27).

In the current study, specificity and sensitivity were also evaluated with a ROC curve that was used to distinguish between non-wine and wine consumers in a large free-living population. All diagnostic parameters were higher than in our previous two controlled clinical trials and the small cohort, probably due to the large number of participants included in the present cohort and the different technique used to determine RMs in urine.

Resveratrol bioavailability in humans has been investigated previously in several clinical trials (14;18;28-34). All pharmacokinetic parameters of resveratrol have only been studied by Boocock *et al* (28) and they reported that RMs remained in urine at least 12-24h after intake. In fact, Walle *et al* still detected radioactivity in plasma at 72h

after oral or intravenous ^{14}C-labeled resveratrol dose (34). On the other hand, we observed interindividual differences, as described by other authors, for resveratrol concentrations in urine (14;18;30;34), plasma (31;33) and low-density lipoproteins (19). However, the large number of participants included in the cohort studied allows us to extrapolate the results to other similar free-living populations.

Blood and spot urine are more useful than urine 24-h urine for large epidemiological studies. Although excretion of flavonoids in 24-h urine showed better correlation coefficients than data obtained in morning urine (r=0.86 versus r=0.56) as a biomarker of fruit and vegetables (35), the Spearman's rank correlation obtained in the current study between reported wine consumption and morning urinary resveratrol was as high (r=0.895), permitting us to propose the general use of the of RMs determination in morning urine as a biomarker of wine consumption. Furthermore, Mennen *et al.* reported that concentrations of several polyphenols in spot urine correlated with those obtained in 24-h urine (36). Other accepted food intake biomarkers, such as overnight urinary isoflavones for soy intake (r=0.52) (37); 4-*O*-methylgallic acid in urine 24h (r=0.5 and 0.57) for usual and current tea intake, respectively; and isoferulic acid in urine 24h (r=0.18 and 0.26) for current and usual coffee intake, respectively (38), have shown lower correlation coefficients. In plasma, some nutritional biomarkers were also positively evaluated, such as alkylresorcinols as markers of whole grain wheat and rye intake (r= 0.58) (39).

In conclusion, our data support the use of RMs in morning urine as a specific and accurate biomarker of moderate wine consumption in a large free-living population. This biomarker would constitute an additional tool to investigate the relationship between wine consumption and health benefits more precisely.

REFERENCES

1. Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 1992;339:1523-6.

2. Rimm EB, Ellison RC. Alcohol in the Mediterranean diet. Am J Clin Nutr 1995;61:1378S-82S.

3. Mukamal KJ, Ascherio A, Mittleman MA et al. Alcohol and risk for ischemic stroke in men: the role of drinking patterns and usual beverage. Ann Intern Med 2005;142:11-9.

4. Sesso HD, Cook NR, Buring JE, Manson JE, Gaziano JM. Alcohol consumption and the risk of hypertension in women and men. Hypertension 2008;51:1080-7.

5. Beulens JW, Stolk RP, van der Schouw YT, Grobbee DE, Hendriks HF, Bots ML. Alcohol consumption and risk of type 2 diabetes among older women. Diabetes Care 2005;28:2933-8.

6. Mehlig K, Skoog I, Guo X et al. Alcoholic beverages and incidence of dementia: 34-year follow-up of the prospective population study of women in Goteborg. Am J Epidemiol 2008;167:684-91.

7. Gronbaek M, Becker U, Johansen D et al. Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. Ann Intern Med 2000;133:411-9.

8. Nielsen SE, Freese R, Kleemola P, Mutanen M. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. Cancer Epidemiol Biomarkers Prev 2002;11:459-66.

9. Marshall JR. Methodologic and statistical considerations regarding use of biomarkers of nutritional exposure in epidemiology. J Nutr 2003;133 Suppl 3:881S-7S.

10. Potischman N. Biologic and methodologic issues for nutritional biomarkers. J Nutr 2003;133 Suppl 3:875S-80S.

11. Spencer JP, bd El Mohsen MM, Minihane AM, Mathers JC. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. Br J Nutr 2008;99:12-22.

12. Spiegelman D, Schneeweiss S, McDermott A. Measurement error correction for logistic regression models with an "alloyed gold standard". Am J Epidemiol 1997;145:184-96.

13. Zamora-Ros R, ndres-Lacueva C, Lamuela-Raventos RM et al. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. Br J Nutr 2007;1-9.

14. Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM et al. Diagnostic Performance of Urinary Resveratrol Metabolites as a Biomarker of Moderate Wine Consumption. Clin Chem 2006;52:1373-80.

15. Estruch R, Martínez-González MA, Corella D et al. Effects of a Mediterranean-Style Diet on Cardiovascular Risk Factors: A Randomized Trial. Ann Intern Med 2006;145:1-11.

16. Martín-Moreno JM, Boyle P, Gorgojo L et al. Development and validation of a food frequency questionnaire in Spain. Int J Epidemiol 1993;22:512-9.

17. Mataix J. Tabla de composición de Alimentos Españoles. Granada, Spain: 2003.

18. Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos RM et al. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. Clinical Chemistry 2007;53:292-9.

19. Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM et al. Uptake of diet resveratrol into the human low density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. Anal Chem 2005;77:3149-55.

20. Jaffé M. Über den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt und uber eine neue Reaction des Kreatinins. Z Physiol Chem 1886;10:391-400.

21. Roura E, ndres-Lacueva C, Estruch R, Lamuela-Raventos RM. Total polyphenol intake estimated by a modified Folin-Ciocalteu assay of urine. Clin Chem 2006;52:749-52.

22. Waterhouse AL, German JB, Walzem RL, Hansen RJ, Kasim-Karakas SE. Is it time for a wine trial? Am J Clin Nutr 1998;68:220-1.

23. Mukamal KJ, Conigrave KM, Mittleman MA et al. Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men. N Engl J Med 2003;348:109-18.

24. van Dam RM, Hu FB. Are alkylresorcinols accurate biomarkers for whole grain intake? Am J Clin Nutr 2008;87:797-8.

25. Lamuela-Raventós R, Romero-Pérez A, Waterhouse A, de la Torre-Boronat M. Direct HPLC analysis of cis- and trans-Resveratrol and piceic isomers in spanish red vitis vinifera wines. J Agric Food Chem 1995;43:281-3.

26. Romero Perez AI, Lamuela Raventos RM, Waterhouse AL, de laTorre Boronat MC. Levels of cis- and trans-resveratrol and their glucosides in white and rose Vitis vinifera wines from Spain. J Agric Food Chem 1996;44:2124-8.

27. Crozier A, Plant Molecular Sci.Group BBDoB&MBIoB. Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuces, and celery. Journal of Agricultural & Food Chemistry 1997;45:590-5.

28. Boocock DJ, Faust GE, Patel KR et al. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 2007;16:1246-52.

29. Burkon A, Somoza V. Quantification of free and protein-bound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides - Two novel resveratrol metabolites in human plasma. Mol Nutr Food Res 2008;52:549-57.

30. Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 2003;36:79-87.

31. Meng X, Maliakal P, Lu H, Lee MJ, Yang CS. Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. J Agric Food Chem 2004;52:935-42.

32. Soleas GJ, Yan J, Goldberg DM. Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. J Chromatogr B Biomed Sci Appl 2001;757:161-72.

33. Vitaglione P, Sforza S, Galaverna G et al. Bioavailability of trans-resveratrol from red wine in humans. Mol Nutr Food Res 2005;49:495-504.

34. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High Absorption but Very Low Bioavailability of Oral Resveratrol in Humans. Drug Metab Dispos 2004;32:1377-82.

35. Krogholm KS, Haraldsdottir J, Knuthsen P, Rasmussen SE. Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables. J Nutr 2004;134:445-51.

36. Mennen LI, Sapinho D, Ito H, Galan P, Hercberg S, Scalbert A. Urinary excretion of 13 dietary flavonoids and phenolic acids in free-living healthy subjects - variability and possible use as biomarkers of polyphenol intake. Eur J Clin Nutr 2008;62:519-25.

37. Atkinson C, Skor HE, Fitzgibbons ED et al. Overnight urinary isoflavone excretion in a population of women living in the United States, and its relationship to isoflavone intake. Cancer Epidemiol Biomarkers Prev 2002;11:253-60.

38. Hodgson JM, Chan SY, Puddey IB et al. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. Br J Nutr 2004;91:301-6.

39. Landberg R, Kamal-Eldin A, Andersson A, Vessby B, Aman P. Alkylresorcinols as biomarkers of whole-grain wheat and rye intake: plasma concentration and intake estimated from dietary records. Am J Clin Nutr 2008;87:832-8.

TABLE 1. Baseline characteristics of the 1000 participants examined.

Characteristic	Non wine consumers (*n*=391)	Intermittent wine consumers (*n*=151)	Daily wine consumers (*n*=458)	*P*
Mean (SD) age, *y*	68.1 (6.1)	66.1 (6.2)	65.5 (5.9)	<0.001
Men, *n (%)*	170 (43.8)	72 (47.7)	238 (52.0)	0.014
Mean (SD) BMI, kg/m^2	29.6 (3.5)	29.2 (3.4)	29.1 (3.0)	0.152
Current smokers, *n (%)*	34 (8.7)	22 (14.6)	103 (22.5)	<0.001
Type 2 diabetes mellitus,*n(%)*	214 (54.7)	64 (42.4)	192 (39.7)	<0.001
Hypertension, *n (%)*	318 (81.3)	124 (82.1)	349 (76.2)	0.069
Dyslipidemia, *n (%)*	237 (60.6)	97 (64.2)	280 (61.1)	0.914
Education level, *n (%)*				
Primary school	353 (90.2)	108 (71.5)	308 (67.2)	<0.001
First-degree high school	28 (7.2)	29 (19.2)	90 (19.7)	<0.001
High school or university	10 (2.6)	14 (9.3)	60 (13.1)	<0.001

FIGURE LEGENDS

FIGURE 1. Box plots of urinary resveratrol metabolite concentrations among non-wine consumers (n=391), intermittent wine consumers (n=151), and moderate daily wine consumers (n=458).

FIGURE 2. A. Relation between reported wine consumption and morning urinary resveratrol metabolites concentration (r=0.895; *P*<0.001) in 1000 participants of the PREDIMED Study. **B.** Relation between reported dietary resveratrol and morning urinary resveratrol metabolites concentration (r=0.890; *P*<0.001) in 1000 participants of the PREDIMED Study.

FIGURE 3. ROC curve of urine resveratrol metabolites for discrimination of wine consumers from non wine consumers in the PREDIMED Study.

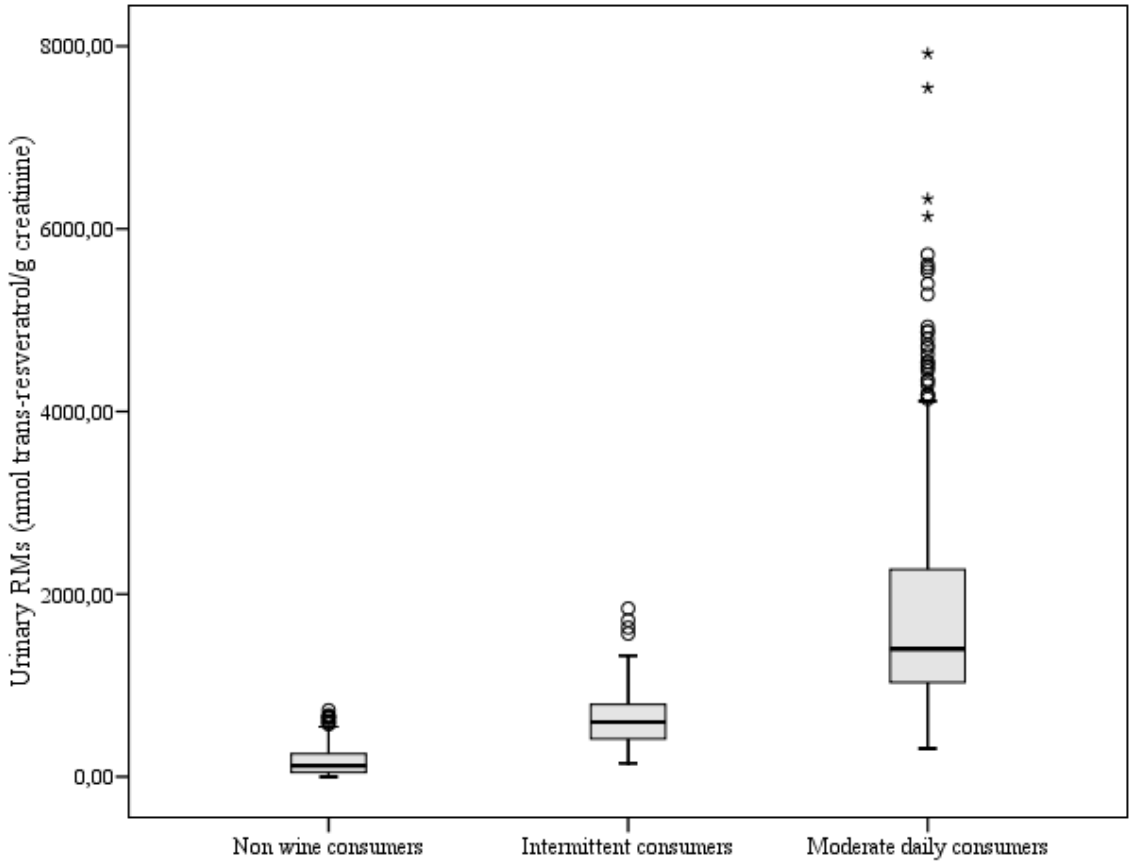

8000,00
6000,00
4000,00
2000,00
0,00
Urinary RMs (nmol trans-resveratrol/g creatinine)
Non wine consumers
Intermittent consumers
Moderate daily consumers

Fig 1

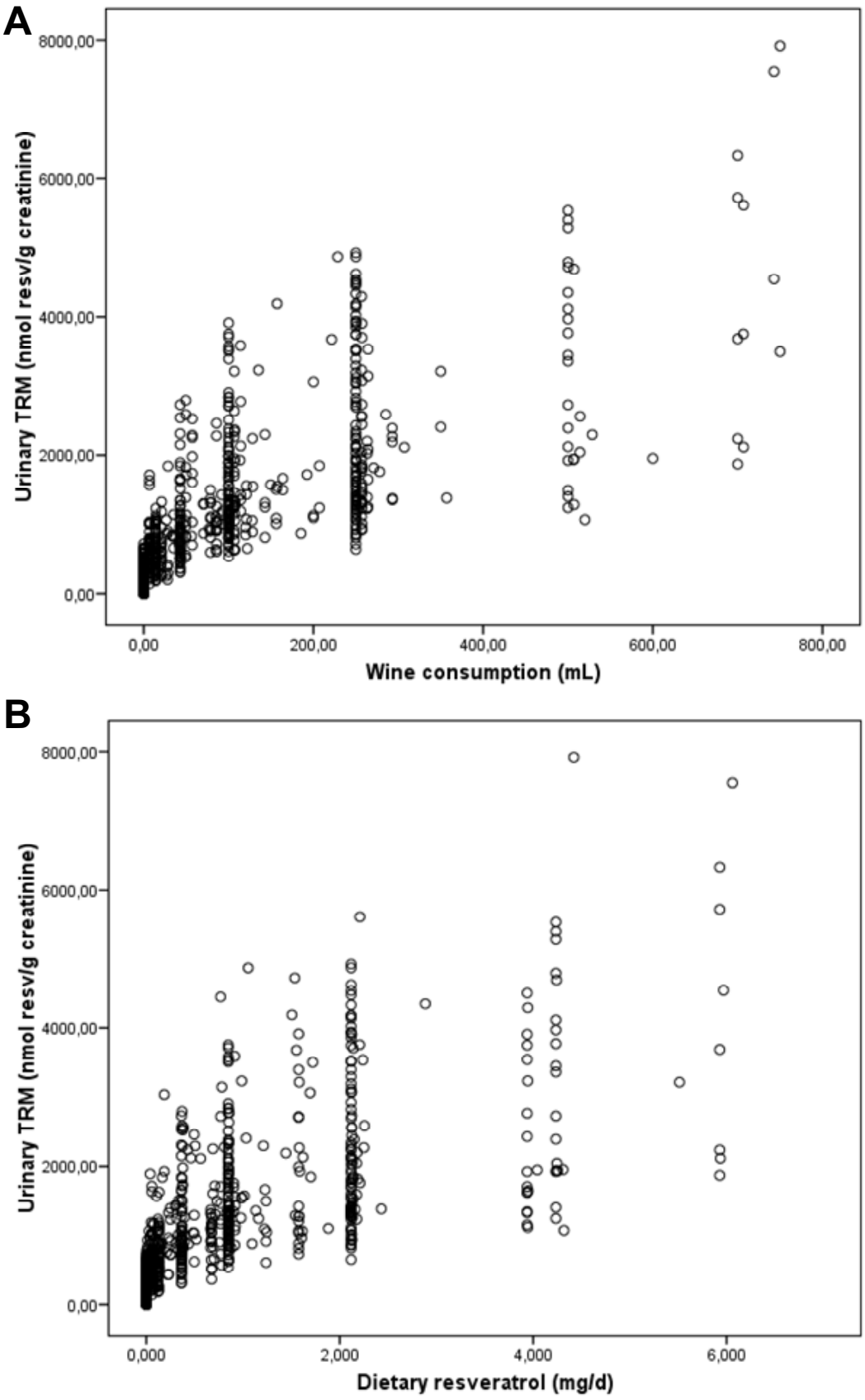

A
Urinary TRM (nmol resv/g creatinine)
8000,00
6000,00
4000,00
2000,00
0,00
0,00
200,00
400,00
600,00
800,00
Wine consumption (mL)
B
Urinary TRM (nmol resv/g creatinine)
8000,00
6000,00
4000,00
2000,00
0,00
0,000
2,000
4,000
6,000
Dietary resveratrol (mg/d)

Fig 2

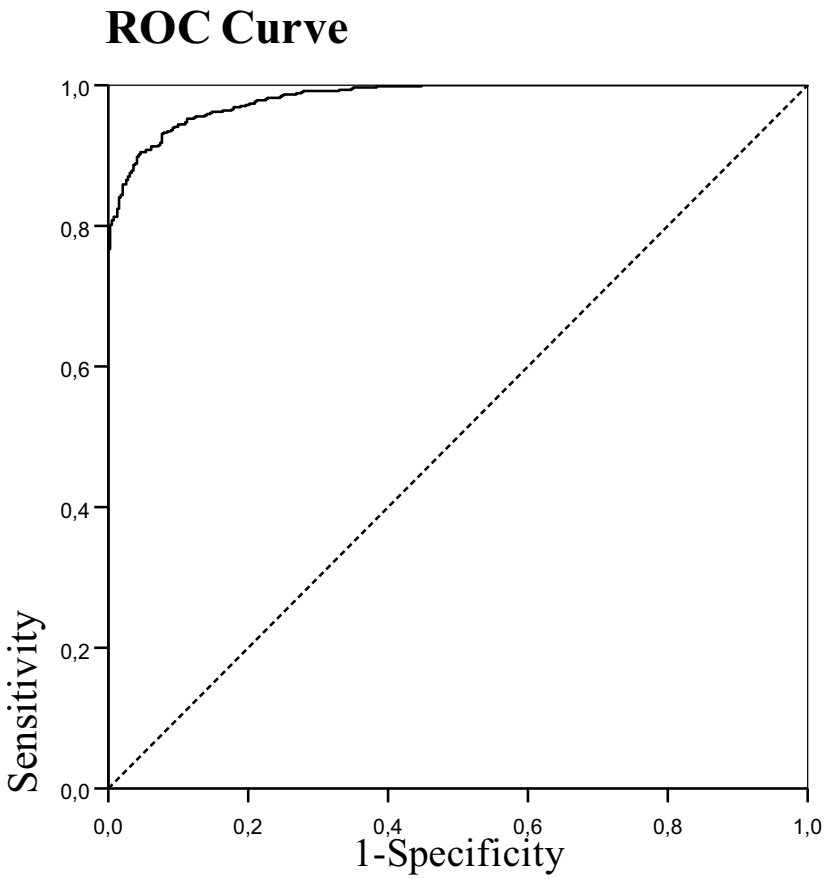

ROC Curve
Sensitivity
1-Specificity
1,0
0,8
0,6
0,4
0,2
0,0
0,0
0,2
0,4
0,6
0,8
1,0

Fig 3

4.2.4. Prevenció de la sobrecàrrega antioxidant per mecanismes d'homeostasi endògens de la xarxa plasmàtica redox. Estudi PREDIMED.

Article 4: Serafini M, Zamora-Ros R, Lamuela-Raventós RM, Estruch E, Miguel Ángel Martínez-González; Covas, MI, Miguel Fiol, José Lapetra y Andres-Lacueva C. Antioxidant overloading is prevented by endogenous tuning mechanism of plasma redox network. **En procés de revisió.**

Resum:

La xarxa antioxidant de l'organisme permet una eficient i completa protecció contra l'atac produït per les especies reactives de l'oxigen i del nitrogen. A pesar de l'elevada complexitat i eficàcia de la homeostasi redox endògena, aquesta ha de ser complementada amb l'aportació d'antioxidants provinents de la dieta per optimitzar la estratègia de defensa. Diversos estudis de consumo agut d'aliments rics en antioxidants han demostrat la capacitat de la dieta de modular la capacitat antioxidant total (TAC). Tot i que diversos meta-anàlisis apareguts recentment han suggerit uns efectes negatius de la suplementació amb antioxidants a dosis farmacològiques en malaltia cardiovascular i mortalitat total. La gran variabilitat en la biodisponibilitat dels antioxidants i l'existència de mecanismes reguladors homeostàtics poden afectar la capacitat dels antioxidants exògens de modular les defenses antioxidants *in vivo*. Així la hipòtesi del present estudi ha estat mostrar si l'estat antioxidant està modulat per mecanismes de regulació endògens dissenyats per evitar la sobrecàrrega redox.

Aquest estudi s'ha portat a terme amb 569 participants seleccionats aleatòriament de l'estudi PREDIMED, els voluntaris van ser admesos des de Octubre del 2003 a Juny del 2005. Els participants seleccionats van ser assignats aleatòriament a les tres intervencions de l'estudi Predimed: dieta mediterrània suplementada amb oli d'oliva verge (n=195), dieta mediterrània suplementada amb fruits secs (n=190) i finalment a la dieta control, baixa en greix (n=184). Als participants se'ls va analitzar la TAC del plasma en el temps inicial i en el seguiment a l'any mitjançant els assaigs del FRAP i del TRAP.

S'han observat increments positius significatius en els nivells plasmàtics de FRAP [72.0μmol/L (95% IC, 34.2-109.2; *P*<0.001) i de TRAP, 45.0 (0.9-89.1; *P*=0.046) després d'un any d'intervenció dietètica amb dieta mediterrània suplementada amb fruits secs. També s'han

observat augments significatius després d'un any de seguiment de dieta mediterrània suplementada amb oli d'oliva verge en els nivells de FRAP 48.9μmol/L (24.3-73.5; *P*<0.001) i quasi significatius en els de TRAP, 36.4 (-4.3-63.0; *P*<0.08). No obstant no s'han observat diferències significatives en el grup control. Els nivells de TRAP i FRAP inicial han estat correlacionats directament amb els deltes d'increment, la diferència entre els nivells de l'any i els bassals, de TRAP i FRAP (r>0.365; *P*<0.001) en els dos grups de dieta mediterrània. En conseqüència els increments en els deltes de TRAP i FRAP són majors en el quartil 1 inicial que en quartils superiors, fins i tot en el quartil 4 els nivells es redueixen després d'un any d'intervenció. A més s'ha observat una associació inversa entre els nivells plasmàtics de FRAP inicials i els de glucosa ajustada (β coefficient=-0.027; *P*=0.012). Alts nivells de glucèmia poden induir l'autooxidació de la glucosa, la glucosidació de proteïnes i l'activació del metabolisme dels poliols. Aquests canvis poden accelerar la generació d'espècies reactives de l'oxigen i del nitrogen augmentant l'estrès oxidatiu.

Les conclusions que es poden extreure del present estudi són que apareixen evidències de la existència de mecanismes moduladors de la xarxa antioxidant plasmàtica que mantenen una homeòstasi de forma dinàmica optimzant la disponibilitat dels antioxidants. Augmentar el coneixement sobre els mecanismes reguladors i les necessitats de l'organisme en antioxidants és necessari per optimitzar les estrategies de prevenció contra l'estrès oxidatiu.

Antioxidant overloading is prevented by endogenous tuning mechanism of plasma redox network.

Mauro Serafini[1], Raul Zamora-Ros[2*], Rosa M. Lamuela-Raventós,[2] Ramon Estruch[3,4], Miguel Ángel Martínez-González[5], Montserrat Fitó[6], Miguel Fiol [4,7], José Lapetra[4,8] and Cristina Andres-Lacueva[2#]*

[1]Antioxidant Research Laboratory at the Unit of Human Nutrition, National Institute for Food and Nutrition Research, Rome, Italy; [2]Nutrition and Food Science Department-XaRTA INSA, Pharmacy School, University of Barcelona, Barcelona, Spain and Ingenio-CONSOLIDER program, FUN-C-FOOD; [3]Internal Medicine Department, Hospital Clínic. Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain; [4]CIBER 03/06 Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Spain; [5]Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona, Spain; [6]Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d´Investigació Mèdica (IMIM), Barcelona, Spain; [7]Departement of Cardiology, Hospital Son Dureta, Palma de Mallorca, Spain; [8]San Pablo Health Center, Sevilla, Spain. *Both authors equally contributed to this research.

[#]**Correspondence to**: Department of Nutrition and Food Science, Pharmacy School, University of Barcelona, Av/Joan XXIII s/n. 08028, Barcelona, Spain; phone 34-93-403-48-40, fax 34-93-403-59-31, e-mail candres@ub.edu

ABSTRACT (70 words)

Scientific literature has recently suggested harmful effect of antioxidant supplementation in human. We show the existence of mechanisms of regulation of antioxidant plasma network aimed to reach a physiological homeostasis. Reduction of antioxidant levels in subjects in the highest quartile of plasma antioxidant capacity, suggest the need of the body to avoid antioxidants overloading. These findings are of practical relevance for optimizing strategies of antioxidant supplementation for oxidative stress prevention.

BRIEF COMMUNICATION (1000 words)

The antioxidant network of the body, due to its wide range of redox potential and localization, allow a complete and efficient protection against attack driven by Reactive Oxygen Nitrogen Species (RONS). However, despite its high grade of complexity and efficiency, there is the need to optimize defense strategies against RONS with dietary antioxidants. Different studies have shown the ability of diet to modulate plasma Total Antioxidant Capacity (TAC) following acute consumption of plant foods in humans[1-3]. In addition, large cohort studies has shown that greater adherence to Mediterranean diet is associated with elevated TAC levels[4,5]. However, conclusion from randomized, placebo-controlled intervention studies, using galenic antioxidants supplements, have been mainly discouraging with some unexpected results where an enhancement of disease risk was observed[6-8]. Moreover recent meta-analyses have suggested a negative effect of galenic antioxidant supplementation on overall mortality[9] as well as on cardiovascular diseases[10] and cancer[11]. Physiological diversity in the absorption and disposal of antioxidants and the existence of homeostatic mechanisms of regulation might affect the ability of exogenous antioxidants to modulate antioxidant defenses *in vivo*[12]. The possibility that, antioxidant status is regulated by endogenous mechanism of control designed to avoid redox overloading, is our working hypothesis. We analyzed the baseline and one year follow-up data of 569 high cardiovascular-risk randomly selected participants (279 men and 290 women, mean (SD) aged 65.1 (9.7), recruited from October 2003 to July 2005 in a substudy of the PREDIMED (PREvención con DIeta MEDiterránea) Trial (www.predimed.org)[13]. This is a large, parallel group, multicenter, controlled, randomized 4-year clinical trial designed to evaluate the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. The study protocol was approved by the Institutional Review Board of all participant

centers, and it has been registered in the Current Controlled Trials, London (ISRCTN 35739639). Participants were assigned to 3 interventions: Mediterranean diet with virgin olive oil, 1 liter per week (n=195); Mediterranean diet with mixed nuts, 30 g/d (n=190); or low-fat diet (n=184).

Plasma TAC was analyzed through the assessment of the Ferric Reducing Antioxidant Potential (FRAP assay)[14] and the Total Radical-trapping Antioxidant Parameter (TRAP assay)[15] indicator of the ability of plasma to reduce iron and to scavenge peroxyl radicals, respectively. Plasma glucose was measured using standard enzymatic automated method (ABX-Horiba Diagnostics, Montpellier, France). Plasma levels of FRAP [mean difference, 72.0μmol/L(95% CI, 34.2-109.9);*P*<0.001;Fig.1a] and TRAP [45.0μmol/L(0.9-89.1);*P*= 0.046;Fig1a]) increased significantly after one year of Mediterranean diet intervention supplemented with the nuts mixture. Mediterranean diet with olive oil supplementation raised significantly FRAP [48.9μmol/L(24.3-73.5);*P*<0.001] and close to significance TRAP [30.36μmol/L(-4.3-63.0);*P*<0.08]) levels. No changes were observed in the control group (Figure 1a). However, the effect of supplementation differed significantly on the basis of starting quartile of plasma TAC levels. People in the lowest quartile showed a significant increase of TRAP level [186.7μmol/L (121.8-251.7); *P*<0.001; Fig. 1b and 152.0μmol/L (71.0-233.0); *P*=0.001; Fig 1c] in the olive oil and nuts groups, respectively, whereas a lack of effect was observed in people at the second and third quartile for both supplements. On the contrary, people in the highest quartile display a decrease of plasma TRAP levels [-139.9μmol/L(-209.6 to -70.2);*P*<0.001;Fig.1b] in the olive oil group and a lack of changes in the nuts group [-77.5μmol/L (-172.6. to 17.5);*P*=0.105;Fig1b]. People at lowest, 2nd and 3rd quartile (nuts only) increased further, but at different degree, FRAP

levels (micromolar increase for first quartile [114.9μmol/L(70.0-159.9);*P*<0.001 and 132.4μmol/L(75.9-189.0);*P*<0.001]; for 2nd quartile[77.2μmol/L(30.5-123.8);*P*=0.002 and 88.2μmol/L(38.5-137.9);*P*=0.001]; for third quartile [30.1μmol/L(-11.5-71.8);*P*=0.152 and 89.6μmol/L(2.3-176.9);*P*=0.045] respectively for olive oil and nuts Fig. 1b and 1c). No increase was observed for people at highest quartile of FRAP levels. Significant Pearson correlations (All r >0.365; All *P*<0.001) have been observed between baseline levels and delta of increase of plasma TRAP and FRAP in both supplemented groups (Figure 2a and 2b).

A significant inverse association (β coefficient=-0.027; *P*=0.012) has been observed between glucose and TAC levels in our subjects by multiple linear regression model adjusted by age, sex, body weight and drugs. Interestingly, stratification in quartiles of plasma FRAP revealed higher levels of glucose concentration for people in the lowest quartile of TAC (Fig. 2c). High glucose level can induce the autoxidation of glucose, glycation of proteins, and the activation of polyol metabolism. These changes accelerate generation of RONS and increases oxidative chemical modification of bio-molecules[16]. This pro-oxidant action of glucose might be at the basis of the inverse association with plasma TAC, despite further evidences are needed to substantiate our finding.

For the first time, we provide evidences on the existence of tuning mechanisms of plasma antioxidant network to maintain a dynamic homeostasis and to optimize antioxidant absorption. The mechanisms through physiological levels are reached is still unclear, and it might involve dietary as well as endogenous antioxidant. Our results suggest an efficiency of antioxidant supplementation based on the specific requirement of the body for antioxidant equivalents. Indirect evidences to our findings came from the SUVIMAX (Supplémentation en Vitamines et en Minéraux Antioxydants) study,

where a cocktail of antioxidants and minerals was used at nutritional dosages for 8 years, supplementation was effective only in men, characterized by lower levels of - carotene and vitamin E respect to women, reducing cancer incidence of about 30%[17]. It is possible that, antioxidant supplementation might be inefficient in subjects with plasma levels of TAC within physiological ranges. The clear reduction of TAC levels in subjects in the highest quartile, suggest the necessity of the body to trigger constraint mechanisms avoiding an antioxidants overcapacity in subjects with plasma levels already within the "physiological ranges". The possibility that, chronic antioxidant supplementation at doses far high respect to the physiological need of the body, can overwhelm the mechanism of antioxidant control is feasible and should be considered in designing intervention studies. A better understanding of the mechanism regulating the requirement of the body for antioxidants is needed in order to manage antioxidant overloading optimizing strategies for oxidative stress prevention.

REFERENCES

1. Serafini,M., Ghiselli,A., & Ferro-Luzzi,A. Red wine, tea, and antioxidants. *Lancet* **344**, 626 (1994).

2. Serafini,M. *et al.* Plasma antioxidants from chocolate. *Nature* **424**, 1013 (2003).

3. Serafini,M., Villano,D., Spera,G., & Pellegrini,N. Redox molecules and cancer prevention: the importance of understanding the role of the antioxidant network. *Nutr. Cancer* **56**, 232-240 (2006).

4. Dragsted,L.O. *et al.* The 6-a-day study: effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers. *Am. J Clin. Nutr.* **79**, 1060-1072 (2004).

5. Pitsavos,C. *et al.* Adherence to the Mediterranean diet is associated with total antioxidant capacity in healthy adults: the ATTICA study. *Am. J Clin. Nutr.* **82**, 694-699 (2005).

6. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N. Engl. J Med.* **330**, 1029-1035 (1994).

7. Lonn,E. *et al.* Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA* **293**, 1338-1347 (2005).

8. Omenn,G.S. *et al.* Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N. Engl. J Med.* **334**, 1150-1155 (1996).

9. Miller,E.R., III *et al.* Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann. Intern. Med.* **142**, 37-46 (2005).

10. Bjelakovic,G., Nikolova,D., Simonetti,R.G., & Gluud,C. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. *Lancet* **364**, 1219-1228 (2004).

11. Vivekananthan,D.P., Penn,M.S., Sapp,S.K., Hsu,A., & Topol,E.J. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet* **361**, 2017-2023 (2003).

12. Serafini,M., Del,R.D., Crozier,A., & Benzie,I.F. Effect of changes in fruit and vegetable intake on plasma antioxidant defenses in humans. *Am. J Clin. Nutr.* **81**, 531-532 (2005).

13. Estruch,R. *et al.* Effects of a Mediterranean-Style Diet on Cardiovascular Risk Factors: A Randomized Trial. *Ann. Intern. Med.* **145**, 1-11 (2006).

14. Serafini,M. *et al.* Effect of acute ingestion of fresh and stored lettuce (Lactuca sativa) on plasma total antioxidant capacity and antioxidant levels in human subjects. *Br. J Nutr.* **88**, 615-623 (2002).

15. Benzie,I.F. & Strain,J.J. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol* **299**, 15-27 (1999).

16. Halliwell,B. & Gutteridge,J.M.C. *Free radicals in Biology and Medicine*(Clarendon Press, Oxford, 2007).

17. Hercberg,S. *et al.* The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch. Intern. Med.* **164**, 2335-2342 (2004).

FIGURE CAPTIONS

Figure 1. Effects on TAC assessed as TRAP and FRAP after one year of Mediterranean dietary treatment supplemented with nuts or virgin olive oil. **A,** Mean changes from baseline in TRAP and FRAP after 1 year of Mediterranean diet supplemented with nuts, Mediterranean diet supplemented with virgin olive oil or low-fat diet. Values are means and 95% CI. Asterisks indicate differences from baseline (t-test): **P*<0.05; ***P*<0.01; ****P*<0.001. **B,** Means of TRAP at base line and after one year of Mediterranean diet supplemented with virgin olive oil by quartile of plasma TRAP levels. Asterisks indicate differences from baseline (t-test): **P*<0.05; ***P*<0.01; ****P*<0.001. **C,** Means of FRAP at base line and after one year of Mediterranean diet supplemented with nuts by quartile of plasma FRAP levels. Asterisks indicate differences from baseline (t-test): **P*<0.05; ***P*<0.01; ****P*<0.001.

Figure 2. A, Relation between μmol/L of TRAP at baseline and μmol/L of ΔTRAP (differences from base line) after 1 year of Mediterranean diet supplemented with virgin olive oil (green marks, r=0.585;*P*<0.001) and Mediterranean diet supplemented with nuts (blue marks, r=0.442;*P*<0.001). **B,** Relation between μmol/L of FRAP at baseline and μmol/L of ΔFRAP (differences from baseline) after 1 year of Mediterranean diet

supplemented with olive oil (green marks, r=0.366;*P*<0.001) and Mediterranean diet supplemented with nuts (blue marks, r=0.396;*P*<0.001).

Means of TAC (TRAP and FRAP) at base line and after one year of Mediterranean diet supplemented with virgin olive oil by quartile of plasma TAC levels. **C,** Mean (SD; *error bars*) of adjusted blood glucose by FRAP quartile at baseline. The values were adjusted by using a multiple linear regression approach. The model was adjusted by age, gender, body weight and drugs. Significant differences: *,*P*=0.022; ***P*=0.001; ******P*<0.001 for difference from values for quartile 1.

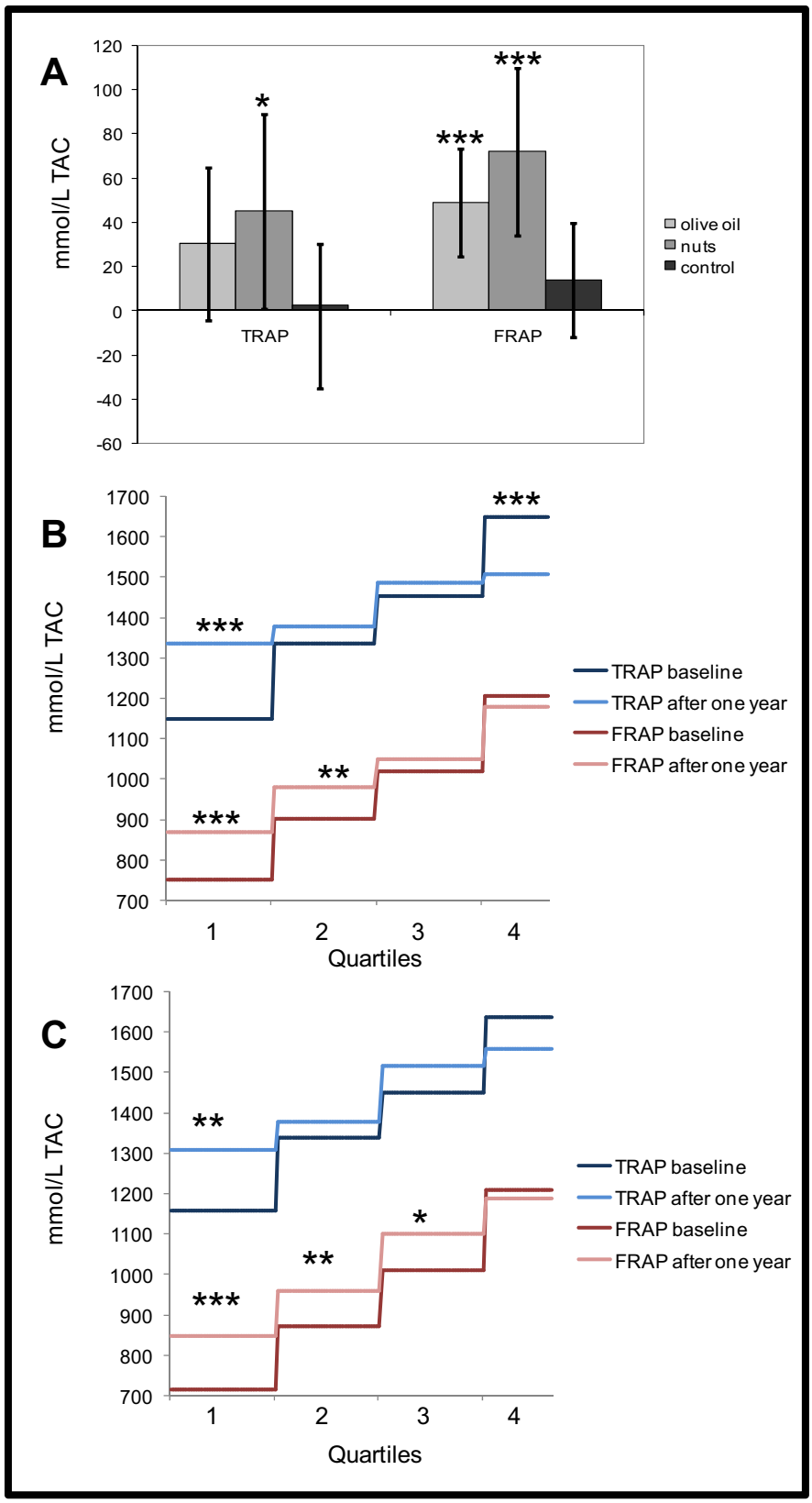

A
mmol/L TAC
120
100
80
60
40
20
0
-20
-40
-60
*
***
***
TRAP
FRAP
olive oil
nuts
control
B
mmol/L TAC
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
***
**
***
***
1
2
3
4
Quartiles
TRAP baseline
TRAP after one year
FRAP baseline
FRAP after one year
C
mmol/L TAC
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
**
*
**
***
1
2
3
4
Quartiles
TRAP baseline
TRAP after one year
FRAP baseline
FRAP after one year

Figure 1

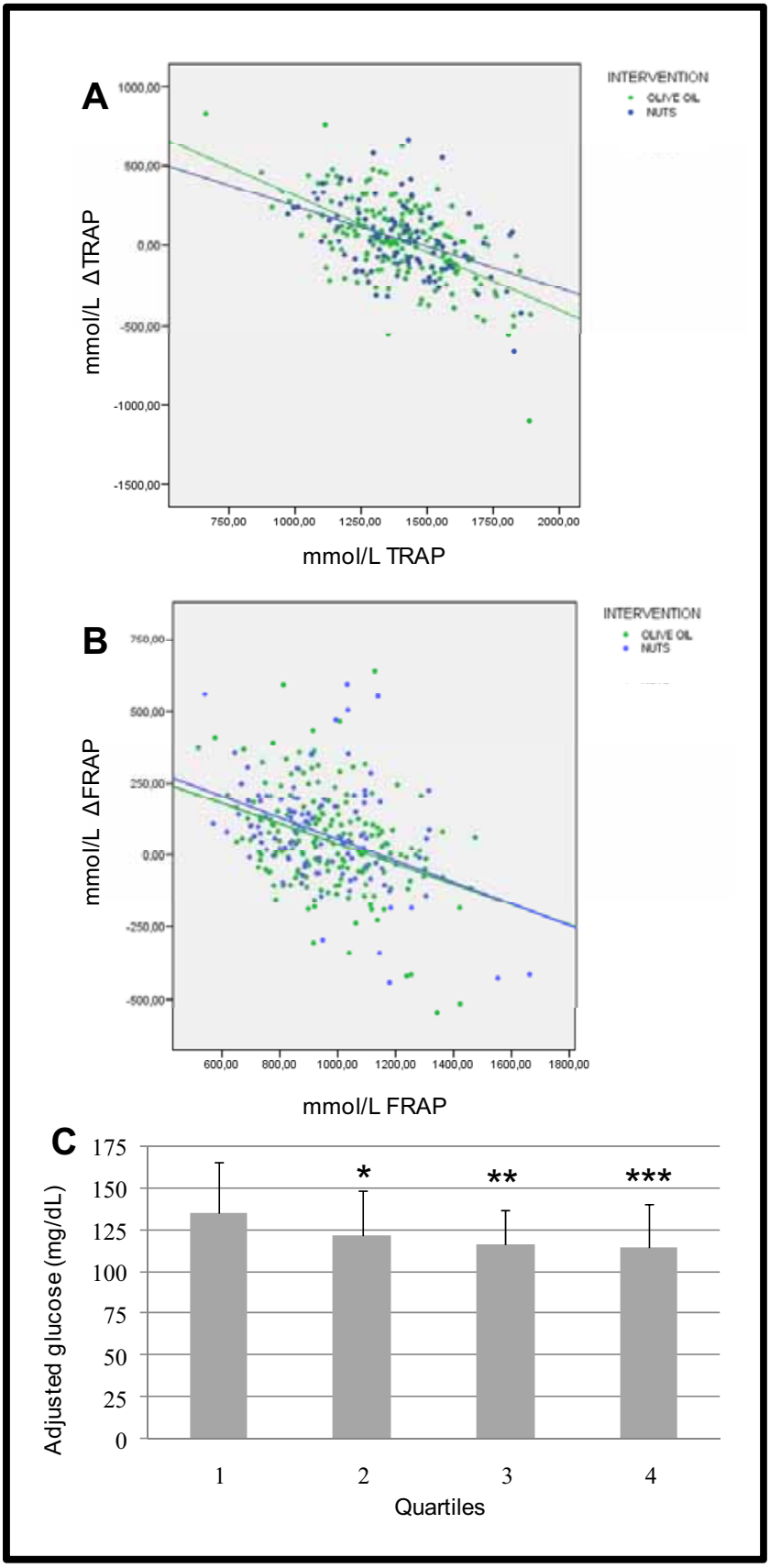

A
INTERVENTION
OLIVE OIL
NUTS
mmol/L ΔTRAP
1000,00
500,00
0,00
-500,00
-1000,00
-1500,00
750,00
1000,00
1250,00
1500,00
1750,00
2000,00
mmol/L TRAP
B
INTERVENTION
OLIVE OIL
NUTS
mmol/L ΔFRAP
750,00
500,00
250,00
0,00
-250,00
-500,00
600,00
800,00
1000,00
1200,00
1400,00
1600,00
1800,00
mmol/L FRAP
C
Adjusted glucose (mg/dL)
175
150
125
100
75
50
25
0
*
**
***
1
2
3
4
Quartiles

Figure 2

RESULTATS GLOBALS

DISCUSSIÓ GENERAL

5. RESULTATS GLOBALS/DISCUSSIÓ GENERAL

El treball realitza en aquesta tesi doctoral ha permès identificar un marcador nutricional del consum moderat de vi tant en estudis clínics controlats com en estudis epidemiològics . Per ésser considerat un bon biomarcador, aquest ha de acomplir els requisits revisats i proposats per Spencer *et al.*(Spencer et al., 2008), l'assegurar-se de l'acompliment d'aquests criteris és part fonamental dintre d'aquesta memòria i estan breument resumits també en la carta d'opinió.

1) Metodologia robusta. La metodologia proposada per a la determinació del resveratrol i dels seus metabòlits en orina consisteix en una extracció en fase sòlida seguida per un anàlisi en cromatografia líquida acoblada a un espectròmetre de masses en tàndem. Aquesta tècnica ha estat validada i publicada (Urpi-Sarda et al., 2005;Urpi-Sarda et al., 2007) com una tècnica molt adequada per l'anàlisi de mostres biològiques, les quals, intrínsecament presenten moltes interferències i baixes concentracions de l'analit d'interès. També s'ha millorat la metodologia per poder ser utilitzada en estudis epidemiològics amb un elevat nombre de mostres.

2) Sensibilitat. El nostre biomarcador s'ha comprovat que reflexa correctament els canvis d'ingesta. En l'article 1 es pot comprovar que en els estudis clínics controlats després de les diferents dosis creixents de resveratrol (cava, vi blanc, vi negre) s'aprecien diferències en la mesura del biomarcador. En l'estudi PREDIMED la mesura per valorar la sensibilitat és la bona correlació dosi-resposta, en l'article 1 amb 52 voluntaris s'obté un coeficient de correlació de 0.654 (P<0.001), en canvio quan s'avaluen 1000 participants el coeficiente augmenta fins el 0.895(P<0.001),possiblement degut a l'augment en la potència de la mostra.

3) Especificitat. Un biomarcador nutricional únicament s'hauria de modificar amb la ingesta d'un sol aliment o grup d'aliments d'interès. No obstant actualment això és pràcticament impossible. En el nostre cas el resveratrol s'ha comprovat, en el article 3, que la font dietètica més important és el vi (98.4%). A més a més les concentracions de resveratrol en altres aliments (cacauets, festucs, fruites del bosc) són molt baixes en comparació amb les descrites al raïm i als productes derivats del raïm.

   La sensibilitat i l'especifitat també han estat valorades en els articles 1 i 4 mitjançant la determinació de les proves diagnòstiques. La corba ROC, ens proporciona el punt de

tall òptim per distingir entre els consumidors i els no consumidors de vi. El punt de tall de 90nmol de resveratrol/g creatinina obtingut en l'artícle 1 és menor que l'obtingut en l'article 4 (411.490nmol de resveratrol/g creatinina), perquè en aquest últim es consideren més metabòlits (els sulfats de resveratrol) respecte el primer article, degut a les millores analítiques introduïdes. Els paràmetres de sensibilitat, especifitat i valors predictius són superiors en l'article 4, ja que s'ha incrementat el nombre d'individus valorats.

4) Biodisponibilitat. El coneixement dels paràmetres farmacocinètics és de gran importància per saber en quina forma i en quina quantitat el compost s'absorbeix, se metabolitza, se distribueix i s'excreta (Boocock et al., 2007). Els temps de vida mitjana són també importants per determinar de quants dies ens aporta informació el biomarcador, consum puntual i actual o un consum més habitual. En els estudis epidemiològics s'observa que el biomarcador és útil fins i tot en els bevedors de vi ocasionals o intermitents (1-2 copes de vi a la setmana).

S'ha comprovat que els metabòlits de resveratrol en orina acompleixen amb tots els requisits exposats anteriorment, per tant podem concloure que la suma de metabòlits de resveratrol en orina de primera hora del matí és un bon biomarcador del consum de vi. Aquest biomarcador ens és molt útil per avaluar el consum de vi en estudis tant clínics com epidemiològics, així com en els estudis clínics comprovar l'acompliment de la intervenció (ingesta de cava) article 2.

CONCLUSIONS

6. CONCLUSIONS

- S'ha determinat els metabòlits del resveratrol en orina després de la ingesta regular de cava, vi blanc o negre en assaigs clínics controlats, així com després del consum de vi diari o ocasional en una població no controlada (*free living*).

- S'ha demostrat la capacitat d'aquests metabòlitos per discriminar entre consumidors i no consumidors de vi, amb uns adequats paràmetres de sensibilitat, especificitat i valors predictius tant positius com negatius calculats mitjançant la corba ROC.

- Ss'ha envidenciat una bona correlació entre la ingesta de vi i la concentració en orina dels metabòlits del resveratrol. Per aquests motius podem afirmar que la suma de metabòlits del resveratrol és un biomarcador útil del consum moderat de vi.

- S'ha compilat una completa taula de composició dels aliments en resveratrol i piceid (formes *cis* i *trans*) amb 160 ítems, agrupats en 18 aliments genèrics.

- S'ha comprobat, per primera vegada, que en una població adulta espanyola (cohort EPIC-Espanya) que l'aport dietètic de resveratrol és de 100 μg/dia (mediana) i de 933 μg/dia (mitjana), els quals provenen majoritàriament de la ingesta de vi (98.4%) i en menor proporció del raïm i most, aproximadament un 1.6%. Finalment el percentatge degut a altres fonts alimentàries com els cacauets, festucs i fruites del bosc és inferior al 0.01%. També cal destacar que dels 4 estilbens avaluats el més abundant en la dieta és el *trans*-piceid (53.6%), seguit pel *trans*-resveratrol (20.9%), *cis*-piceid (19.3%) i *cis*-resveratrol (6.2%).

- La capacitat antioxidant total de l'organismo posterior al consumo d'una dieta suplementada amb antioxidants es troba modulada per l'estat redox inicial de l'individu, amb la finalitat d'evitar una sobrecàrrega d'antioxidantes. La importancia d'aquesta conclusió radica en la seva potencial aplicació pràctica a l'hora d'optimitzar estrategies de suplementació amb antioxidants per la prevenció de l 'estrès oxidatiu.

BIBLIOGRAFIA

7. BIBLIOGRAFIA

Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, and Okada H. 1991. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr 54:1093-1100.

Adrian M, Jeandet P, Douillet-Breuil AC, Tesson L, and Bessis R. 2000. Stilbene content of mature Vitis vinifera berries in response to UV-C elicitation. Journal of Agricultural and Food Chemistry 48:6103-6105.

Afaq F, Adhami VM, and Ahmad N. 2003. Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice. Toxicol Appl Pharmacol 186:28-37.

Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, and Takada Y. 2004. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 24:2783-2840.

Al-Regaiey KA, Masternak MM, Bonkowski M, Sun L, and Bartke A. 2005. Long-lived growth hormone receptor knockout mice: interaction of reduced insulin-like growth factor i/insulin signaling and caloric restriction. Endocrinology 146:851-860.

Alarcón de la Lastra C and Villegas I. 2005. Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. Mol Nutr Food Res 49:405-430.

Álvarez-Sala L, Slowing-Barrillas K, Gómez-Serranillos P, Torres-Segovia F, Valderrama-Rojas M, and Millán-Núñez J. 2000. Variabilidad del contenido de polifenoles de distintos tipos de vino y su potencial aplicación al conocimiento de sus efectos biológicos. Medicina Clínica 114:331-332.

Amos AF, McCarty DJ, and Zimmet P. 1997. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 14 Suppl 5:S1-85.

Andlauer W, Kolb J, Siebert K, and Furst P. 2000. Assessment of resveratrol bioavailability in the perfused small intestine of the rat. Drugs Exp Clin Res 26:47-55.

Andres-Lacueva C, Ibern-Gomez M, Lamuela-Raventos RM, Buxaderas S, and de la Torre-Boronat M. 2002. Cinnamates and resveratrol content for sparkling wine characterization. Am J Enol Vitic 53:147-150.

Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC, and Andriantsitohaina R. 1997. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. Br J Pharmacol 120:1053-1058.

Anjaneyulu ASR, Raghava Reddy AV, Reddy DSK, Ward D, Adhikesavalu D, and Stanley Cameron T. 1984. Pacherin: a new dibenzo (2,3-6,7)oxepin derivative from Bauhinia racemosa LAmk. Tetrahedron 40:4245-4252.

Arija Val V and Fernández Ballart J. 2002. Métodos de valoración del consumo alimentario. In: Salvador-Salas J, Bonada A, Trallero R, and Saló ME, editors. Nutrición y dietética clínica. Barcelona: Masson S.A. p 55-68.

Arts IC and Hollman PC. 2005. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 81:317S-325S.

Asensi M, Medina I, Ortega A, Carretero J, Bano MC, Obrador E, and Estrela JM. 2002. Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med 33:387-398.

Aumont V, Krisa S, Battaglia E, Netter P, Richard T, Merillon JM, Magdalou J, and Sabolovic N. 2001. Regioselective and stereospecific glucuronidation of trans- and cis-resveratrol in human. Arch Biochem Biophys 393:281-289.

Badia E, Sacanella E, Fernandez-Sola J, Nicolas JM, Antunez E, Rotilio D, de Gaetano G, Urbano-Marquez A, and Estruch R. 2004. Decreased tumor necrosis factor-induced adhesion of human monocytes to endothelial cells after moderate alcohol consumption. Am J Clin Nutr 80:225-230.

Bauer JH, Goupil S, Garber GB, and Helfand SL. 2004. An accelerated assay for the identification of lifespan-extending interventions in Drosophila melanogaster. Proc Natl Acad Sci U S A 101:12980-12985.

Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le CD, Shaw RJ, Navas P, Puigserver P, Ingram DK, de CR, and Sinclair DA. 2006b. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444:337-342.

Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le CD, Shaw RJ, Navas P, Puigserver P, Ingram DK, de CR, and Sinclair DA. 2006a. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444:337-342.

Baur JA and Sinclair DA. 2006. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5:493-506.

bd El-Mohsen M, Bayele H, Kuhnle G, Gibson G, Debnam E, Kaila SS, Rice-Evans C, and Spencer JP. 2006. Distribution of [3H]trans-resveratrol in rat tissues following oral administration. Br J Nutr 96:62-70.

Beaton GH, Burema J, and Ritenbaugh C. 1997. Errors in the interpretation of dietary assessments. Am J Clin Nutr 65:1100S-1107S.

Belguendouz L, Fremont L, and Linard A. 1997. Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. Biochem Pharmacol 53:1347-1355.

Benzie IFF and Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of ''antioxidant power'': The FRAP assay. Analytical Biochemistry 239:70-76.

Bergamini E, Cavallini G, Donati A, and Gori Z. 2003. The anti-ageing effects of caloric restriction may involve stimulation of macroautophagy and lysosomal degradation, and can be intensified pharmacologically. Biomed Pharmacother 57:203-208.

Bertelli AA, Giovannini L, Giannessi D, Migliori M, Bernini W, Fregoni M, and Bertelli A. 1995. Antiplatelet activity of synthetic and natural resveratrol in red wine. Int J Tissue React 17:1-3.

Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, and Pezzuto JM. 2001. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. Cancer Res 61:7456-7463.

Bingham SA and Cummings JH. 1985. Urine nitrogen as an independent validatory measure of dietary intake: a study of nitrogen balance in individuals consuming their normal diet. Am J Clin Nutr 42:1276-1289.

Bingham SA, Gill C, Welch A, Cassidy A, Runswick SA, Oakes S, Lubin R, Thurnham DI, Key TJ, Roe L, Khaw KT, and Day NE. 1997. Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers. Int J Epidemiol 26 Suppl 1:S137-S151.

Blache D, Rustan I, Durand P, Lesgards G, and Loreau N. 1997. Gas chromatographic analysis of resveratrol in plasma, lipoproteins and cells after in vitro incubations. J Chromatogr B Biomed Sci Appl 702:103-110.

Bogers RP, Dagnelie PC, Westerterp KR, Kester AD, van Klaveren JD, Bast A, and van den Brandt PA. 2003. Using a correction factor to correct for overreporting in a food-frequency questionnaire does not improve biomarker-assessed validity of estimates for fruit and vegetable consumption. J Nutr 133:1213-1219.

Boocock DJ, Faust GE, Patel KR, Schinas AM, Brown VA, Ducharme MP, Booth TD, Crowell JA, Perloff M, Gescher AJ, Steward WP, and Brenner DE. 2007. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 16:1246-1252.

Bordone L and Guarente L. 2005. Calorie restriction, SIRT1 and metabolism: understanding longevity. Nat Rev Mol Cell Biol 6:298-305.

Bradamante S, Barenghi L, and Villa A. 2004. Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 22:169-188.

Brakenhielm E, Cao R, and Cao Y. 2001. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. FASEB J 15:1798-1800.

Bravo L. 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Reviews 56:317-333.

Brill SS, Furimsky AM, Ho MN, Furniss MJ, Li Y, Green AG, Bradford WW, Green CE, Kapetanovic IM, and Iyer LV. 2006. Glucuronidation of trans-resveratrol by human liver and intestinal microsomes and UGT isoforms. J Pharm Pharmacol 58:469-479.

Brito P, Almeida LM, and Dinis TC. 2002. The interaction of resveratrol with ferrylmyoglobin and peroxynitrite; protection against LDL oxidation. Free Radic Res 36:621-631.

Burkon A and Somoza V. 2008. Quantification of free and protein-bound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides - Two novel resveratrol metabolites in human plasma. Mol Nutr Food Res 52:549-557.

Burns J, Crozier A, and Lean ME. 2001. Alcohol consumption and mortality: is wine different from other alcoholic beverages? Nutr Metab Cardiovasc Dis 11:249-258.

Burns J, Yokota T, Ashihara H, Lean ME, and Crozier A. 2002. Plant foods and herbal sources of resveratrol. J Agric Food Chem 50:3337-3340.

Callemien D, Jerkovic V, Rozenberg R, and Collin S. 2005. Hop as an interesting source of resveratrol for brewers: Optimization of the extraction and quantitative study by liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. Journal of Agricultural and Food Chemistry 53:424-429.

Cantos E, Espín J, and Tomás-Barberán. 2001. Postharvest induction modeling method using UV irradiation pulses for obtaining resveratrol-enriched table grapes: a new 'functional' fruit? Journal of Agricultural & Food Chemistry 49:5052-5058.

Cantos E, Espin JC, and Tomas-Barberan FA. 2002a. Postharvest stilbene-enrichment of red and white table grape varieties using UV-C irradiation pulses. Journal of Agricultural and Food Chemistry 50:6322-6329.

Cantos E, Espin JC, and Tomas-Barberan FA. 2002b. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. Journal of Agricultural and Food Chemistry 50:5691-5696.

Cantos E, Tomas-Barberan FA, Martinez A, and Espin JC. 2003. Differential stilbene induction susceptibility of seven red wine grape varieties upon post-harvest UV-C irradiation. European Food Research and Technology 217:253-258.

Cao Z and Li Y. 2004. Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. Eur J Pharmacol 489:39-48.

Chang TK, Lee WB, and Ko HH. 2000. Trans-resveratrol modulates the catalytic activity and mRNA expression of the procarcinogen-activating human cytochrome P450 1B1. Can J Physiol Pharmacol 78:874-881.

Chen WP, Chi TC, Chuang LM, and Su MJ. 2007. Resveratrol enhances insulin secretion by blocking K(ATP) and K(V) channels of beta cells. Eur J Pharmacol 568:269-277.

Chen Z, Zheng W, Custer LJ, Dai Q, Shu XO, Jin F, and Franke AA. 1999. Usual dietary consumption of soy foods and its correlation with the excretion rate of isoflavonoids in overnight urine samples among Chinese women in Shanghai. Nutr Cancer 33:82-87.

Chi TC, Chen WP, Chi TL, Kuo TF, Lee SS, Cheng JT, and Su MJ. 2007. Phosphatidylinositol-3-kinase is involved in the antihyperglycemic effect induced by resveratrol in streptozotocin-induced diabetic rats. Life Sci 80:1713-1720.

Chukwumah YC, Walker LT, Verghese M, Bokanga M, Ogutu S, and Alphonse K. 2007. Comparison of extraction methods for the quantification of selected phytochemicals in peanuts (Arachis hypogaea). J Agric Food Chem 55:285-290.

Chung HT, Pae HO, Choi BM, Billiar TR, and Kim YM. 2001. Nitric oxide as a bioregulator of apoptosis. Biochem Biophys Res Commun 282:1075-1079.

Chung HY, Kim HJ, Kim KW, Choi JS, and Yu BP. 2002. Molecular inflammation hypothesis of aging based on the anti-aging mechanism of calorie restriction. Microsc Res Tech 59:264-272.

Chung IM, Park MR, Chun JC, and Yun SJ. 2003. Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic stresses and hormones in peanut plants. Plant Science 164:103-109.

Ciolino HP, Daschner PJ, and Yeh GC. 1998. Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. Cancer Res 58:5707-5712.

Ciolino HP and Yeh GC. 1999. Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol. Mol Pharmacol 56:760-767.

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de CR, and Sinclair DA. 2004. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305:390-392.

Collins T and Cybulsky MI. 2001. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest 107:255-264.

Creasy LL and Coffee M. 1988. Phytoalexin production potential of grape berries. J Am Soc Hortic Sci 113:230-234.

Cruz MN, Luksha L, Logman H, Poston L, Agewall S, and Kublickiene K. 2006. Acute responses to phytoestrogens in small arteries from men with coronary heart disease. Am J Physiol Heart Circ Physiol 290:H1969-H1975.

Das S, Alagappan VK, Bagchi D, Sharma HS, Maulik N, and Das DK. 2005. Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. Vascul Pharmacol 42:281-289.

Davies MJ. 1999. The birth, growth, and consequences of the atherosclerotic plaque. Dialogues in Cardiovascular Medicine 4:115-130.

Davies PS, Coward WA, Gregory J, White A, and Mills A. 1994. Total energy expenditure and energy intake in the pre-school child: a comparison. Br J Nutr 72:13-20.

Day AJ and Williamson G. 2001. Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma. Br J Nutr 86 Suppl 1:S105-S110.

de Andres-de PR, Yuste-Rojas M, Sort X, ndres-Lacueva C, Torres M, and Lamuela-Raventos RM. 2007. Effect of soil type on wines produced from Vitis vinifera L. cv. Grenache in commercial vineyards. J Agric Food Chem 55:779-786.

de Santi C, Pietrabissa A, Mosca F, and Pacifici GM. 2000a. Glucuronidation of resveratrol, a natural product present in grape and wine, in the human liver. Xenobiotica 30:1047-1054.

de Santi C, Pietrabissa A, Spisni R, Mosca F, and Pacifici GM. 2000b. Sulphation of resveratrol, a natural product present in grapes and wine, in the human liver and duodenum. Xenobiotica 30:609-617.

de Santi C, Pietrabissa A, Spisni R, Mosca F, and Pacifici GM. 2000c. Sulphation of resveratrol, a natural compound present in wine, and its inhibition by natural flavonoids. Xenobiotica 30:857-866.

Delgado M, Llorca J, and Doménech JM. 2008. Investigación científica: Fundamentos metodológicos y estadísticos. Barcelona: Signo.

Delmas D, Jannin B, and Latruffe N. 2005a. Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res 49:377-395.

Delmas D, Jannin B, and Latruffe N. 2005b. Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res 49:377-395.

Delmas D, Lancon A, Colin D, Jannin B, and Latruffe N. 2006. Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. Curr Drug Targets 7:423-442.

Doménech JM. 2006b. Fundamentos de Diseño y Estadística. UD 3: Teoría y cálculo de probabilidades. Pruebas diagnósticas. Barcelona: Signo.

Doménech JM. 2006a. Fundamentos de Diseño y Estadística. UD 13: Correlación y regresión lineal. Barcelona: Signo.

El-Mowafy AM. 2002. Resveratrol activates membrane-bound guanylyl cyclase in coronary arterial smooth muscle: a novel signaling mechanism in support of coronary protection. Biochem Biophys Res Commun 291:1218-1224.

Elosua R, Marrugat J, Molina L, Pons S, Pujol E, Arquer A, Covas MI, Deflores T, Fornells X, Martinez S, Perea M, Pujol P, Richart JA, Rubies J, and Tomas A. 1994. Validation of the Minnesota Leisure-Time Physical-Activity Questionnaire in Spanish Men. American Journal of Epidemiology 139:1197-1209.

Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutiérrez V, Covas MI, Fiol M, Gómez-Gracia E, López-Sabater MC, Vinyoles E, Arós F, Conde M, Lahoz C, Lapetra J, Sáez G, and Ros E. 2006. Effects of a Mediterranean-Style Diet on Cardiovascular Risk Factors: A Randomized Trial. Ann Intern Med 145:1-11.

Estruch R, Sacanella E, Badia E, Antunez E, Nicolas JM, Fernandez-Sola J, Rotilio D, de Gaetano G, Rubin E, and Urbano-Marquez A. 2004. Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial; Effects of wine on inflammatory markers. Atherosclerosis 175:117-123.

Facchini FS, Hua N, Abbasi F, and Reaven GM. 2001. Insulin resistance as a predictor of age-related diseases. J Clin Endocrinol Metab 86:3574-3578.

Farran Codina A and Zamora-Ros R. 2006. Tablas de composición de alimentos: aplicaciones en salud pública. In: Serra Majem L and Aranceta Bartrina J, editors. Nutrición y Salud Pública: Métodos, Bases Científicas y Aplicaciones. Barcelona: Masson S.A. p 228-238.

Fenn JB, Mann M, Meng CK, Wong SF, and Whitehouse CM. 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science 246:64-71.

Fernandez-Pachon MS, Villano D, Troncoso AM, and Garcia-Parrilla MC. 2006. [Review of the different methods for the evaluation of the in vitro antioxidant activity of wine and study of in vivo effects]. Arch Latinoam Nutr 56:110-122.

Ferrer P, Asensi M, Priego S, Benlloch M, Mena S, Ortega A, Obrador E, Esteve JM, and Estrela JM. 2007. Nitric oxide mediates natural polyphenol-induced Bcl-2 down-regulation and activation of cell death in metastatic B16 melanoma. J Biol Chem 282:2880-2890.

Ferrer P, Asensi M, Segarra R, Ortega A, Benlloch M, Obrador E, Varea MT, Asensio G, Jorda L, and Estrela JM. 2005. Association between pterostilbene and quercetin inhibits metastatic activity of B16 melanoma. Neoplasia 7:37-47.

Flesch M, Schwarz A, and Bohm M. 1998. Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. Am J Physiol 275:H1183-H1190.

Floreani M, Napoli E, Quintieri L, and Palatini P. 2003. Oral administration of trans-resveratrol to guinea pigs increases cardiac DT-diaphorase and catalase activities, and protects isolated atria from menadione toxicity. Life Sci 72:2741-2750.

Fragopoulou E, Nomikos T, Antonopoulou S, Mitsopoulou CA, and Demopoulos CA. 2000. Separation of biologically active lipids from red wine. J Agric Food Chem 48:1234-1238.

Frankel EN, Waterhouse AL, and Kinsella JE. 1993. Inhibition of human LDL oxidation by resveratrol. Lancet 341:1103-1104.

Frankenfeld CL, Patterson RE, Horner NK, Neuhouser ML, Skor HE, Kalhorn TF, Howald WN, and Lampe JW. 2003. Validation of a soy food-frequency questionnaire and evaluation of correlates of plasma isoflavone concentrations in postmenopausal women. Am J Clin Nutr 77:674-680.

Fremont L. 2000. Biological effects of resveratrol. Life Sci 66:663-673.

Fremont L, Belguendouz L, and Delpal S. 1999. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. Life Sci 64:2511-2521.

Fulda S and Debatin KM. 2006. Resveratrol modulation of signal transduction in apoptosis and cell survival: a mini-review. Cancer Detect Prev 30:217-223.

Gao L, Chu Q, and Ye J. 2002. Determination of trans-resveratrol in wines, herbs and health food by capillary electrophoresis with electrochemical detection. Food Chemistry 78:255-260.

Garber K. 2008. A mid-life crisis for aging theory. Nat Biotechnol 26:371-374.

Gehm BD, Levenson AS, Liu H, Lee EJ, Amundsen BM, Cushman M, Jordan VC, and Jameson JL. 2004. Estrogenic effects of resveratrol in breast cancer cells expressing mutant and wild-type estrogen receptors: role of AF-1 and AF-2. J Steroid Biochem Mol Biol 88:223-234.

Gentile C, Tesoriere L, Butera D, Fazzari M, Monastero M, Allegra M, and Livrea MA. 2007. Antioxidant activity of Sicilian pistachio (Pistacia vera L. var. Bronte) nut extract and its bioactive components. J Agric Food Chem 55:643-648.

Ghiselli A, Serafini M, Maiani G, Azzini E, and Ferro-Luzzi A. 1995. A fluorescence-based method for measuring total plasma antioxidant capability. Free Radic Biol Med 18:29-36.

Goldberg DM, Karumanchiri A, Ng E, Yan J, Diamandis EP, and Soleas GJ. 1995. Direct Gas-Chromatographic Mass-Spectrometric Method to Assay Cis-Resveratrol in Wines - Preliminary Survey of Its Concentration in Commercial Wines. Journal of Agricultural and Food Chemistry 43:1245-1250.

Goldberg DM, Yan J, and Soleas GJ. 2003. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 36:79-87.

Goldfinger TM. 2003. Beyond the French paradox: the impact of moderate beverage alcohol and wine consumption in the prevention of cardiovascular disease. Cardiol Clin 21:449-457.

Gonzalez-Barrio R, Beltran D, Cantos E, Gil MI, Espin JC, and Tomas-Barberan FA. 2006. Comparison of ozone and UV-C treatments on the postharvest stilbenoid monomer, dimer, and trimer induction in var. 'Superior' white table grapes. J Agric Food Chem 54:4222-4228.

Gronbaek M. 2002. Alcohol, type of alcohol, and all-cause and coronary heart disease mortality. Ann N Y Acad Sci 957:16-20.

Guarente L and Picard F. 2005. Calorie restriction--the SIR2 connection. Cell 120:473-482.

Haider UG, Sorescu D, Griendling KK, Vollmar AM, and Dirsch VM. 2002. Resveratrol suppresses angiotensin II-induced Akt/protein kinase B and p70 S6 kinase phosphorylation and subsequent hypertrophy in rat aortic smooth muscle cells. Mol Pharmacol 62:772-777.

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stocker RH, and . 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361:153-156.

Hanawa F, Tahara S, and Mizutani J. 1992. Antifungal stress compounds from Veratrum grandiflorum leaves treated with cupric chloride. Phytochemistry 31:3005-3007.

Hansen T, Seidel A, and Borlak J. 2007. The environmental carcinogen 3-nitrobenzanthrone and its main metabolite 3-aminobenzanthrone enhance formation of reactive oxygen intermediates in human A549 lung epithelial cells. Toxicol Appl Pharmacol 221:222-234.

Harborne JB. 1989. Methods in plant biochemistry, I: plant phenolics. London: Academic Press.

Harrington DJ, Booth SL, Card DJ, and Shearer MJ. 2007. Excretion of the urinary 5C- and 7C-aglycone metabolites of vitamin K by young adults responds to changes in dietary phylloquinone and dihydrophylloquinone intakes. J Nutr 137:1763-1768.

Harris MI, Hadden WC, Knowler WC, and Bennett PH. 1987. Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in U.S. population aged 20-74 yr. Diabetes 36:523-534.

Heilbronn LK, Civitarese AE, Bogacka I, Smith SR, Hulver M, and Ravussin E. 2005. Glucose tolerance and skeletal muscle gene expression in response to alternate day fasting. Obes Res 13:574-581.

Henry C, Vitrac X, Decendit A, Ennamany R, Krisa S, and Merillon JM. 2005. Cellular uptake and efflux of trans-piceid and its aglycone trans-resveratrol on the apical membrane of human intestinal Caco-2 cells. J Agric Food Chem 53:798-803.

Henry-Vitrac C, Desmouliere A, Girard D, Merillon JM, and Krisa S. 2006. Transport, deglycosylation, and metabolism of trans-piceid by small intestinal epithelial cells. Eur J Nutr 45:376-382.

Hillis WE, Hart JH, and Yazaki Y. 1974. Polyphenols of Eucaliptus sideroxylon wood. Phytochemistry 13:1591-1595.

Hipskind JD and Paiva NL. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to Phoma medicaginis. Molecular Plant-Microbe Interactions 13:551-562.

Hodgson JM, Chan SY, Puddey IB, Devine A, Wattanapenpaiboon N, Wahlqvist ML, Lukito W, Burke V, Ward NC, Prince RL, and Croft KD. 2004b. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. Br J Nutr 91:301-306.

Hodgson JM, Chan SY, Puddey IB, Devine A, Wattanapenpaiboon N, Wahlqvist ML, Lukito W, Burke V, Ward NC, Prince RL, and Croft KD. 2004a. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. Br J Nutr 91:301-306.

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, and Sinclair DA. 2003. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 425:191-196.

Huang D, Ou B, and Prior RL. 2005. The chemistry behind antioxidant capacity assays. J Agric Food Chem1841-1856.

Ibern-Gomez M, Roig-Pérez S, Lamuela-Raventos RM, and de la Torre-Boronat M. 2000. Resveratrol and piceid levels in natural and blended peanut butters. J Agric Food Chem 48:6352-6354.

Imai S, Armstrong CM, Kaeberlein M, and Guarente L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403:795-800.

Ingram DK, Anson RM, de CR, Mamczarz J, Zhu M, Mattison J, Lane MA, and Roth GS. 2004. Development of calorie restriction mimetics as a prolongevity strategy. Ann N Y Acad Sci 1019:412-423.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, and Pezzuto JM. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275:218-220.

Jarolim S, Millen J, Heeren G, Laun P, Goldfarb DS, and Breitenbach M. 2004. A novel assay for replicative lifespan in Saccharomyces cerevisiae. FEMS Yeast Res 5:169-177.

Jeandet P, Bessis R, Maume BF, Meunier P, Peyron D, and Trollat P. 1995. Effect of Enological Practices on the Resveratrol Isomer Content of Wine. Journal of Agricultural and Food Chemistry 43:316-319.

Jerkovic V, Callemien D, and Collin S. 2005. Determination of stilbenes in hop pellets from different cultivars. Journal of Agricultural and Food Chemistry 53:4202-4206.

Juan ME, Lamuela-Raventos RM, de la Torre-Boronat MC, and Planas JM. 1999. Determination of trans-resveratrol in plasma by HPLC. Anal Chem 71:747-750.

Kaldas MI, Walle UK, and Walle T. 2003. Resveratrol transport and metabolism by human intestinal Caco-2 cells. J Pharm Pharmacol 55:307-312.

Kampa M, Nifli AP, Notas G, and Castanas E. 2007. Polyphenols and cancer cell growth. Rev Physiol Biochem Pharmacol 159:79-113.

Kaneider NC, Mosheimer B, Reinisch N, Patsch JR, and Wiedermann CJ. 2004. Inhibition of thrombin-induced signaling by resveratrol and quercetin: effects on adenosine nucleotide metabolism in endothelial cells and platelet-neutrophil interactions. Thromb Res 114:185-194.

Kaneuchi M, Sasaki M, Tanaka Y, Yamamoto R, Sakuragi N, and Dahiya R. 2003. Resveratrol suppresses growth of Ishikawa cells through down-regulation of EGF. Int J Oncol 23:1167-1172.

Kelemen LE. 2006. Food frequency questionnaires: not irrelevant yet. Cancer Epidemiol Biomarkers Prev 15:1054.

Kim HJ, Jung KJ, Yu BP, Cho CG, Choi JS, and Chung HY. 2002a. Modulation of redox-sensitive transcription factors by calorie restriction during aging. Mech Ageing Dev 123:1589-1595.

Kim HJ, Jung KJ, Yu BP, Cho CG, and Chung HY. 2002b. Influence of aging and calorie restriction on MAPKs activity in rat kidney. Exp Gerontol 37:1041-1053.

Kim HJ, Yu BP, and Chung HY. 2002c. Molecular exploration of age-related NF-kappaB/IKK downregulation by calorie restriction in rat kidney. Free Radic Biol Med 32:991-1005.

Kipnis V, Midthune D, Freedman LS, Bingham S, Schatzkin A, Subar A, and Carroll RJ. 2001. Empirical evidence of correlated biases in dietary assessment instruments and its implications. Am J Epidemiol 153:394-403.

Kobayashi S, Ding CK, Nakamura Y, Nakajima I, and Matsumoto R. 2000. Kiwifruits (Actinidia deliciosa) transformed with a Vitis stilbene synthase gene produce piceid (resveratrol-glucoside). Plant Cell Reports 19:904-910.

Kollar P, Kotolova H, Necas J, Karpisek M, Bartosikova L, and Karesova P. 2000. [Experimental study of resveratrol and flavonoids in red wine with regard to their possible hypolipemic effects]. Vnitr Lek 46:856-860.

Kristal AR, Peters U, and Potter JD. 2005. Is it time to abandon the food frequency questionnaire? Cancer Epidemiol Biomarkers Prev 14:2826-2828.

Kuhnle G, Spencer JPE, Chowrimootoo G, Schroeter H, Debnam ES, Srai SKS, Rice-Evans C, and Hahn U. 2000. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochem Biophys Res Commun 272:212-217.

Kumar RJ, Jyostna D, Krupadanam GL, and Srimannarayana G. 1988. Phenanthrene and stilbenes from Pterolobium hexapetallum. Phytochemistry 27:3625-3626.

Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, and Auwerx J. 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 127:1109-1122.

Lampe JW, Gustafson DR, Hutchins AM, Martini MC, Li S, Wahala K, Grandits GA, Potter JD, and Slavin JL. 1999. Urinary isoflavonoid and lignan excretion on a Western diet: relation to soy, vegetable, and fruit intake. Cancer Epidemiol Biomarkers Prev 8:699-707.

Lamuela-Raventós R, Romero-Pérez A, Waterhouse A, and de la Torre-Boronat M. 1995. Direct HPLC analysis of cis- and trans-Resveratrol and piceic isomers in spanish red vitis vinifera wines. J Agric Food Chem 43:281-283.

Landberg R, Kamal-Eldin A, Andersson A, Vessby B, and Aman P. 2008. Alkylresorcinols as biomarkers of whole-grain wheat and rye intake: plasma concentration and intake estimated from dietary records. Am J Clin Nutr 87:832-838.

Leckband G and Lorz H. 1998. Transformation and expression of a stilbene synthase gene of Vitis vinifera L. in barley and wheat for increased fungal resistance. Theoretical and Applied Genetics 96:1004-1012.

Lee S, Lee S, Kim M, Chun J, Cheong Y, and Lee J. 2004. Analysis of trans-resveratrol in peanuts and peanut butters consumed in Korea. Food Research International 37:247-251.

Levi F, Pasche C, Lucchini F, Ghidoni R, Ferraroni M, and La VC. 2005. Resveratrol and breast cancer risk. Eur J Cancer Prev 14:139-142.

Li HF, Chen SA, and Wu SN. 2000. Evidence for the stimulatory effect of resveratrol on Ca(2+)-activated K+ current in vascular endothelial cells. Cardiovasc Res 45:1035-1045.

Li Z, Bowerman S, and Heber D. 2005. Health ramifications of the obesity epidemic. Surg Clin North Am 85:681-701, v.

Liu DQ, Xia YQ, and Bakhtiar R. 2002. Use of a liquid chromatography/ion trap mass spectrometry/triple quadrupole mass spectrometry system for metabolite identification. Rapid Commun Mass Spectrom 16:1330-1336.

Liu JC, Chen JJ, Chan P, Cheng CF, and Cheng TH. 2003. Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. Hypertension 42:1198-1205.

Liu S, Hu Y, Wang X, Zhong J, and Lin Z. 2006. High content of resveratrol in lettuce transformed with a stilbene synthase gene of Parthenocissus henryana. J Agric Food Chem 54:8082-8085.

Lyons MM, Yu C, Toma RB, Cho SY, Reiboldt W, Lee J, and van Breemen RB. 2003. Resveratrol in raw and baked blueberries and bilberries. J Agric Food Chem 51:5867-5870.

Maier-Salamon A, Hagenauer B, Wirth M, Gabor F, Szekeres T, and Jager W. 2006. Increased transport of resveratrol across monolayers of the human intestinal Caco-2 cells is mediated by inhibition and saturation of metabolites. Pharm Res 23:2107-2115.

Manach C, Scalbert A, Morand C, Remesy C, and Jimenez L. 2004. Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727-747.

Marier JF, Vachon P, Gritsas A, Zhang J, Moreau JP, and Ducharme MP. 2002. Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. J Pharmacol Exp Ther 302:369-373.

Marshall JR. 2003. Methodologic and statistical considerations regarding use of biomarkers of nutritional exposure in epidemiology. J Nutr 133 Suppl 3:881S-887S.

Martinez-Ortega MV, Carcia-Parrilla MC, and Troncoso AM. 2000. Resveratrol content in wines and musts from the south of Spain. Nahrung-Food 44:253-256.

Maskarinec G, Singh S, Meng L, and Franke AA. 1998. Dietary soy intake and urinary isoflavone excretion among women from a multiethnic population. Cancer Epidemiol Biomarkers Prev 7:613-619.

Masternak MM, Al-Regaiey KA, Del Rosario Lim MM, Jimenez-Ortega V, Panici JA, Bonkowski MS, Kopchick JJ, Wang Z, and Bartke A. 2006. Caloric restriction and growth hormone receptor knockout: effects on expression of genes involved in insulin action in the heart. Exp Gerontol 41:417-429.

Matsuda H, Tomohiro N, Hiraba K, Harima S, Ko S, Matsuo K, Yoshikawa M, and Kubo M. 2001. Study on anti-Oketsu activity of rhubarb II. Anti-allergic effects of stilbene components from Rhei undulati Rhizoma (dried rhizome of Rheum undulatum cultivated in Korea). Biol Pharm Bull 24:264-267.

Mattivi F, Reniero F, and Korhammer S. 1995. Isolation, Characterization, and Evolution in Red Wine Vinification of Resveratrol Monomers. J Agric Food Chem 43:1820-1823.

Meng X, Maliakal P, Lu H, Lee MJ, and Yang CS. 2004. Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. J Agric Food Chem 52:935-942.

Mennen LI, Sapinho D, Ito H, Bertrais S, Galan P, Hercberg S, and Scalbert A. 2006. Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods. Br J Nutr 96:191-198.

Mietus-Snyder M, Gowri MS, and Pitas RE. 2000. Class A scavenger receptor up-regulation in smooth muscle cells by oxidized low density lipoprotein. Enhancement by calcium flux and concurrent cyclooxygenase-2 up-regulation. J Biol Chem 275:17661-17670.

Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA, Olefsky JM, Jirousek MR, Elliott PJ, and Westphal CH. 2007. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature 450:712-716.

Ministerio de Sanidad y Consumo. Mortalidad por causa de muerte 1981 - 2004. España y comunidades autónomas. 2004.
Ref Type: Internet Communication

Miura D, Miura Y, and Yagasaki K. 2003. Hypolipidemic action of dietary resveratrol, a phytoalexin in grapes and red wine, in hepatoma-bearing rats. Life Sci 73:1393-1400.

Miyazaki R, Ichiki T, Hashimoto T, Inanaga K, Imayama I, Sadoshima J, and Sunagawa K. 2008. SIRT1, a Longevity Gene, Downregulates Angiotensin II Type 1 Receptor Expression in Vascular Smooth Muscle Cells. Arterioscler Thromb Vasc Biol.

Mnjoyan ZH and Fujise K. 2003. Profound negative regulatory effects by resveratrol on vascular smooth muscle cells: a role of p53-p21(WAF1/CIP1) pathway. Biochem Biophys Res Commun 311:546-552.

Moreno-Labanda J, Mallavia R, Pérez-Fons L, Lizama V, Saura D, and Micol V. 2004. Determination of piceid and resveratrol in Spanish wines deriving from Monastrell (Vitis vinifera L.) grape variety. Journal of Agricultural & Food Chemistry 52:5396-5403.

Mukamal KJ, Conigrave KM, Mittleman MA, Camargo CA, Jr., Stampfer MJ, Willett WC, and Rimm EB. 2003. Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men. N Engl J Med 348:109-118.

Murias M, Handler N, Erker T, Pleban K, Ecker G, Saiko P, Szekeres T, and Jager W. 2004. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. Bioorg Med Chem 12:5571-5578.

Nicoletti I, De Rossi A, Giovinazzo G, and Corradini D. 2007. Identification and quantification of stilbenes in fruits of transgenic tomato plants (Lycopersicon esculentum Mill.) by reversed phase HPLC with photodiode array and mass spectrometry detection. Journal of Agricultural and Food Chemistry 55:3304-3311.

Nielsen SE, Freese R, Kleemola P, and Mutanen M. 2002. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. Cancer Epidemiol Biomarkers Prev 11:459-466.

Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, and Carruba MO. 2005. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310:314-317.

Noroozi M, Burns J, Crozier A, Kelly IE, and Lean MEJ. 2000. Prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion. European Journal of Clinical Nutrition 54:143-149.

Olas B and Wachowicz B. 2005. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. Platelets 16:251-260.

Olas B, Wachowicz B, Stochmal A, and Oleszek W. 2002. Anti-platelet effects of different phenolic compounds from Yucca schidigera Roezl. bark. Platelets 13:167-173.

Oleszek W, Sitek M, Stochmal A, Piacente S, Pizza C, and Cheeke P. 2001. Resveratrol and other phenolics from the bark of Yucca schidigera Roezl. Journal of Agricultural & Food Chemistry 49:747-752.

Olson ER, Naugle JE, Zhang X, Bomser JA, and Meszaros JG. 2005. Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol. Am J Physiol Heart Circ Physiol 288:H1131-H1138.

Pal S, Ho N, Santos C, Dubois P, Mamo J, Croft K, and Allister E. 2003. Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. J Nutr 133:700-706.

Patlak M. 2002. New weapons to combat an ancient disease: treating diabetes. FASEB J 16:1853.

Pinto MC, Garcia-Barrado JA, and Macias P. 1999. Resveratrol is a potent inhibitor of the dioxygenase activity of lipoxygenase. J Agric Food Chem 47:4842-4846.

Plakke T, Berkel J, Beynen AC, Hermus RJ, and Katan MB. 1983. Relationship between the fatty acid composition of the diet and that of the subcutaneous adipose tissue in individual human subjects. Hum Nutr Appl Nutr 37:365-372.

Potischman N. 2003. Biologic and methodologic issues for nutritional biomarkers. J Nutr 133 Suppl 3:875S-880S.

Pozo-Bayon MA, Hernandez MT, Martin-Alvarez PJ, and Polo MC. 2003. Study of low molecular weight phenolic compounds during the aging of sparkling wines manufactured with red and white grape varieties. J Agric Food Chem 51:2089-2095.

Ragab AS, Van FJ, Jankowski B, Park JH, and Bobzin SC. 2006. Detection and quantitation of resveratrol in tomato fruit (Lycopersicon esculentum Mill.). J Agric Food Chem 54:7175-7179.

Rajala MW and Scherer PE. 2003. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 144:3765-3773.

Ratna WN and Simonelli JA. 2002. The action of dietary phytochemicals quercetin, catechin, resveratrol and naringenin on estrogen-mediated gene expression. Life Sci 70:1577-1589.

Renaud S and de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339:1523-1526.

Rimando AM, Kalt W, Magee JB, Dewey J, and Ballington JR. 2004. Resveratrol, Pterostilbene, and Piceatannol in Vaccinium Berries. J Agric Food Chem 52:4713-4719.

Rodriguez-Delgado M, González G, Pérez-Trujillo J, and García-Montelongo F. 2002. Trans-resveratrol in wines from the Canary Islands (Spain). Analysis by high performance liquid chromatography. Food Chemistry 76:371-375.

Romero Perez AI, Lamuela Raventos RM, Waterhouse AL, and de laTorre Boronat MC. 1996. Levels of cis- and trans-resveratrol and their glucosides in white and rose Vitis vinifera wines from Spain. J Agric Food Chem 44:2124-2128.

Romero-Perez AI, Ibern-Gomez M, Lamuela-Raventos RM, and Torre-Boronat MC. 1999. Piceid, the major resveratrol derivative in grape juices. J Agric Food Chem 47:1533-1536.

Romero-Perez AI, Lamuela-Raventos RM, Andres-Lacueva C, and Torre-Boronat MC. 2001. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. J Agric Food Chem 49:210-215.

Ross JA and Kasum CM. 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annual Reviews of Nutrition19-34.

Roth GS, Ingram DK, Black A, and Lane MA. 2000. Effects of reduced energy intake on the biology of aging: the primate model. Eur J Clin Nutr 54 Suppl 3:S15-S20.

Roth GS, Lane MA, and Ingram DK. 2005. Caloric restriction mimetics: the next phase. Ann N Y Acad Sci 1057:365-371.

Roupe KA, Remsberg CM, Yáñez JA, and Davies NM. 2006. Pharmacometrics of stilbenes: Seguing towards the clinic. Curr Clin Pharmacol 1:81-101.

Rudolf JL, Resurreccion AVA, Saalia FK, and Phillips RD. 2005. Development of a reverse-phase high-performance liquid chromatography method for analyzing trans-resveratrol in peanut kernels. Food Chemistry 89:623-638.

Ruhmann S, Treutter D, Fritsche S, Briviba K, and Szankowski I. 2006. Piceid (resveratrol glucoside) synthesis in stilbene synthase transgenic apple fruit. Journal of Agricultural and Food Chemistry 54:4633-4640.

Sacanella E, Vazquez-Agell M, Mena MP, Antunez E, Fernandez-Sola J, Nicolas JM, Lamuela-Raventos RM, Ros E, and Estruch R. 2007. Down-regulation of adhesion molecules and other inflammatory biomarkers after moderate wine consumption in healthy women: a randomized trial. Am J Clin Nutr 86:1463-1469.

Sanders TH, McMichael RW, and Hendrix KW. 2000. Occurrence of resveratrol in edible peanuts. J Agric Food Chem 48:1243-1246.

Scalbert A and Williamson G. 2000. Dietary intake and bioavailability of polyphenols. J Nutr 130:2073S-2085S.

Schneider Y, Vincent F, Duranton B, Badolo L, Gosse F, Bergmann C, Seiler N, and Raul F. 2000. Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. Cancer Lett 158:85-91.

Sengottuvelan M, Viswanathan P, and Nalini N. 2006. Chemopreventive effect of trans-resveratrol--a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis. Carcinogenesis 27:1038-1046.

Serafini M and Del Rio D. 2004. Understanding the association between dietary antioxidants, redox status and disease: is the Total Antioxidant Capacity the right tool? Redox Rep 9:145-152.

Serafini M, Villano D, Spera G, and Pellegrini N. 2006. Redox molecules and cancer prevention: the importance of understanding the role of the antioxidant network. Nutr Cancer 56:232-240.

Sgambato A, Ardito R, Faraglia B, Boninsegna A, Wolf FI, and Cittadini A. 2001. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat Res 496:171-180.

Shahidi F and Naczk M. 2004. Biosynthesis, Classification, and Nomenclature of Phenolics in Food and Nutraceuticals. In: Shahidi F and Naczk M, editors. Phenolics in Food and Nutraceuticals. CRC Press. p 1-16.

Shigematsu S, Ishida S, Hara M, Takahashi N, Yoshimatsu H, Sakata T, and Korthuis RJ. 2003. Resveratrol, a red wine constituent polyphenol, prevents superoxide-dependent inflammatory responses induced by ischemia/reperfusion, platelet-activating factor, or oxidants. Free Radic Biol Med 34:810-817.

Shu N, Zhou H, and Hu C. 2006. Simultaneous determination of the contents of three stilbene oligomers in Caragana sinica collected in different seasons using an improved HPLC method. Biol Pharm Bull 29:608-612.

Siemann EH and Creasy LL. 1992. Concentration of the Phytoalexin Resveratrol in Wine. American Journal of Enology and Viticulture 43:49-52.

Sinclair DA. 2005. Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev 126:987-1002.

Sinha K, Chaudhary G, and Gupta YK. 2002. Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. Life Sci 71:655-665.

Sobolev VS and Cole RJ. 1999. trans-resveratrol content in commercial peanuts and peanut products. J Agric Food Chem 47:1435-1439.

Soleas GJ, Yan J, and Goldberg DM. 2001. Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. J Chromatogr B Biomed Sci Appl 757:161-172.

Spencer JP, bd El Mohsen MM, Minihane AM, and Mathers JC. 2008. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. Br J Nutr 99:12-22.

Stark-Lorenzen P, Nelke B, Hänßler G, Mülbach HP, and Thomzik JE. 1997. Transfer of a grapevine stilbene synthase gene to rice (Oryza sativa L.). Plant Cell Reports 16:668-673.

Stewart JR, Ward NE, Ioannides CG, and O'Brian CA. 1999. Resveratrol preferentially inhibits protein kinase C-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism. Biochemistry 38:13244-13251.

Su HC, Hung LM, and Chen JK. 2006. Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab 290:E1339-E1346.

Szankowski I, Briviba K, Fleschhut J, Schonherr J, Jacobsen HJ, and Kiesecker H. 2003. Transformation of apple (Malus domestica Borkh.) with the stilbene synthase gene from grapevine (Vitis vinifera L.) and a PGIP gene from kiwi (Actinidia deliciosa). Plant Cell Rep 22:141-149.

Szkudelski T. 2007. Resveratrol-induced inhibition of insulin secretion from rat pancreatic islets: evidence for pivotal role of metabolic disturbances. Am J Physiol Endocrinol Metab 293:E901-E907.

Szkudelski T. 2006. Resveratrol inhibits insulin secretion from rat pancreatic islets. Eur J Pharmacol 552:176-181.

Thurberg BL and Collins T. 1998. The nuclear factor-kappa B/inhibitor of kappa B autoregulatory system and atherosclerosis. Curr Opin Lipidol 9:387-396.

Tokusoglu O, Unal MK, and Yemis F. 2005. Determination of the Phytoalexin Resveratrol (3,5,4'-Trihydroxystilbene) in Peanuts and Pistachios by High-Performance Liquid Chromatographic Diode Array (HPLC-DAD) and Gas Chromatography-Mass Spectrometry (GC-MS). J Agric Food Chem 53:5003-5009.

Tomás-Barberán FA. 2003. Los polifenoles de los alimentos y la salud. Alimentación, Nutrición y Salud 10:41-53.

Tsai SH, Lin-Shiau SY, and Lin JK. 1999. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. Br J Pharmacol 126:673-680.

Tseng SH, Lin SM, Chen JC, Su YH, Huang HY, Chen CK, Lin PY, and Chen Y. 2004. Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. Clin Cancer Res 10:2190-2202.

Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas M, and Andres-Lacueva C. 2005. Uptake of diet resveratrol into the human low density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. Anal Chem 77:3149-3155.

Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos RM, Cherubini A, Jauregui O, de la Torre R, Covas M, Estruch R, Jaeger W, and Andres-Lacueva C. 2007. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. Clinical Chemistry 53:292-299.

Urquiaga I and Leighton F. 2005. Wine and health: evidence and mechanisms. World Rev Nutr Diet 95:122-139.

Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, and Cellerino A. 2006. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr Biol 16:296-300.

Vazquez-Agell M, Sacanella E, Tobias E, Monagas M, Antunez E, Zamora-Ros R, ndres-Lacueva C, Lamuela-Raventos RM, Fernandez-Sola J, Nicolas JM, and Estruch R. 2007. Inflammatory markers of atherosclerosis are decreased after moderate consumption of cava (sparkling wine) in men with low cardiovascular risk. J Nutr 137:2279-2284.

Vinas P, Lopez-Erroz C, Marin-Hernandez JJ, and Hernandez-Cordoba M. 2000. Determination of phenols in wines by liquid chromatography with photodiode array and fluorescence detection. Journal of Chromatography A 871:85-93.

Viswanathan M, Kim SK, Berdichevsky A, and Guarente L. 2005. A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span. Dev Cell 9:605-615.

Vitaglione P, Sforza S, Galaverna G, Ghidini C, Caporaso N, Vescovi PP, Fogliano V, and Marchelli R. 2005. Bioavailability of trans-resveratrol from red wine in humans. Mol Nutr Food Res 49:495-504.

Vitrac X, Desmouliere A, Brouillaud B, Krisa S, Deffieux G, Barthe N, Rosenbaum J, and Merillon JM. 2003. Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. Life Sci 72:2219-2233.

Walle T, Hsieh F, DeLegge MH, Oatis JE, and Walle UK. 2004. High Absorption but Very Low Bioavailability of Oral Resveratrol in Humans. Drug Metab Dispos 32:1377-1382.

Wallerath T, Deckert G, Ternes T, Anderson H, Li H, Witte K, and Forstermann U. 2002. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 106:1652-1658.

Wang Q, Xu J, Rottinghaus GE, Simonyi A, Lubahn D, Sun GY, and Sun AY. 2002b. Resveratrol protects against global cerebral ischemic injury in gerbils. Brain Res 958:439-447.

Wang Q, Xu J, Rottinghaus GE, Simonyi A, Lubahn D, Sun GY, and Sun AY. 2002a. Resveratrol protects against global cerebral ischemic injury in gerbils. Brain Res 958:439-447.

Wang Y, Catana F, Yang Y, Roderick R, and van Breemen RB. 2002c. An LC-MS method for analyzing total resveratrol in grape juice, cranberry juice, and in wine. J Agric Food Chem 50:431-435.

Wang YJ, He F, and Li XL. 2003. The neuroprotection of resveratrol in the experimental cerebral ischemia. Zhonghua Yi Xue Za Zhi 83:534-536.

Wang Z, Huang Y, Zou J, Cao K, Xu Y, and Wu JM. 2002d. Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. Int J Mol Med 9:77-79.

Wang Z, Zou J, Huang Y, Cao K, Xu Y, and Wu JM. 2002e. Effect of resveratrol on platelet aggregation in vivo and in vitro. Chin Med J (Engl) 115:378-380.

Wenzel E, Soldo T, Erbersdobler H, and Somoza V. 2005. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res 49:482-494.

Wild S, Roglic G, Green A, Sicree R, and King H. 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27:1047-1053.

Willcox BJ, Willcox DC, Todoriki H, Fujiyoshi A, Yano K, He Q, Curb JD, and Suzuki M. 2007a. Caloric restriction, the traditional Okinawan diet, and healthy aging: the diet of the world's longest-lived people and its potential impact on morbidity and life span. Ann N Y Acad Sci 1114:434-455.

Willcox DC, Willcox BJ, Shimajiri S, Kurechi S, and Suzuki M. 2007b. Aging gracefully: a retrospective analysis of functional status in Okinawan centenarians. Am J Geriatr Psychiatry 15:252-256.

Willcox DC, Willcox BJ, Sokolovsky J, and Sakihara S. 2007c. The cultural context of "successful aging" among older women weavers in a northern Okinawan village: the role of productive activity. J Cross Cult Gerontol 22:137-165.

Willcox DC, Willcox BJ, Todoriki H, Curb JD, and Suzuki M. 2006. Caloric restriction and human longevity: what can we learn from the Okinawans? Biogerontology 7:173-177.

Wolk A, Vessby B, Ljung H, and Barrefors P. 1998. Evaluation of a biological marker of dairy fat intake. Am J Clin Nutr 68:291-295.

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, and Sinclair D. 2004. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 430:686-689.

Yu BP and Chung HY. 2001. Stress resistance by caloric restriction for longevity. Ann N Y Acad Sci 928:39-47.

Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, Hirschelman WH, Pezzuto JM, Mehta RG, and van Breemen RB. 2002. Human, rat, and mouse metabolism of resveratrol. Pharm Res 19:1907-1914.

Yu C, Shin YG, Kosmeder JW, Pezzuto JM, and van Breemen RB. 2003a. Liquid chromatography/tandem mass spectrometric determination of inhibition of human cytochrome P450 isozymes by resveratrol and resveratrol-3-sulfate. Rapid Commun Mass Spectrom 17:307-313.

Yu L, Sun ZJ, Wu SL, and Pan CE. 2003b. Effect of resveratrol on cell cycle proteins in murine transplantable liver cancer. World J Gastroenterol 9:2341-2343.

Zamora-Ros R, ndres-Lacueva C, Lamuela-Raventos RM, Berenguer T, Jakszyn P, Martinez C, Sanchez MJ, Navarro C, Chirlaque MD, Tormo MJ, Quiros JR, Amiano P, Dorronsoro M, Larranaga N, Barricarte A, Ardanaz E, and Gonzalez CA. 2007. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. Br J Nutr1-9.

Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Vazquez-Agell M, Serrano-Martinez M, Jaeger W, and ndres-Lacueva C. 2006. Diagnostic Performance of Urinary Resveratrol Metabolites as a Biomarker of Moderate Wine Consumption. Clin Chem 52:1373-1380.

Zern TL, West KL, and Fernandez ML. 2003. Grape polyphenols decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized guinea pigs. J Nutr 133:2268-2272.

Zhu J, Yong W, Wu X, Yu Y, Lv J, Liu C, Mao X, Zhu Y, Xu K, Han X, and Liu C. 2008. Anti-inflammatory effect of resveratrol on TNF-alpha-induced MCP-1 expression in adipocytes. Biochem Biophys Res Commun.

Zhu Z, Klironomos G, Vachereau A, Neirinck L, and Goodman DW. 1999. Determination of trans-resveratrol in human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 724:389-392.

ANNEX

8. ANNEX

En aquest annex s'inclou una publicació, no inclosa com a treball de tesi doctoral, però en la que també s'ha col·laborat.

8.1. Mètode de LC-MS/MS per caracteritzar el metabolisme del resveratrol en humans.

Artícle 5: Urpí-Sardà M, Zamora-Ros R, Lamuela-Raventós RM, Cherubini A, Jauregui O, De la Torre R, Covas MI, Estruch R, Jaeger W, Andres-Lacueva C. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. *Clinical Chemistry.* **2007**; 53 (2):292-9.

Clinical Chemistry 53:2
292–299 (2007)

Automation and
Analytical Techniques

HPLC–Tandem Mass Spectrometric Method to Characterize Resveratrol Metabolism in Humans

Mireia Urpi-Sarda,[1] Raul Zamora-Ros,[1] Rosa Lamuela-Raventos,[1]
Antonio Cherubini,[2] Olga Jauregui,[3] Rafael de la Torre,[4] Maria Isabel Covas,[5]
Ramon Estruch,[6] Walter Jaeger,[7] and Cristina Andres-Lacueva[1*]

Background: **Nutritional biomarkers are alternatives to traditional dietary assessment tools. We sought to develop a method for nutritional analysis of resveratrol, a phenolic compound with purported health-promoting properties, and to determine all resveratrol metabolites.**
Methods: **We obtained LDL and urine samples from 11 healthy male volunteers who had consumed 250 mL of Merlot red wine. We measured resveratrol and its metabolites with 96-well solid-phase extraction plates coupled with HPLC-tandem mass spectrometry. Hexestrol was used as the internal standard. Gradient chromatography in multiple reaction monitoring mode was performed on a Luna C_{18} column, maintained at 40 °C; *m/z* transitions were as follows: resveratrol, 227/185; resveratrol glucosides, 389/227; resveratrol glucuronides, 403/227; resveratrol sulfates, 307/227; taxifolin, 303/285; and hexestrol, 269/134.**
Results: **Standard calibration curves were linear at 4.4–3289.5 nmol/L. Residual analyses were 100% (3.2) for *trans*-resveratrol and 100% (11.1) for *trans*-piceid. In both matrices, imprecision (CV) was <10.8% at all concentrations. Detection limits for resveratrol were 0.2 nmol/L (LDL), 0.3 nmol/L (synthetic urine), and 4.0 nmol/L (blank urine). Resveratrol and metabolites were checked for stability, and no degradation was observed.**
Conclusions: **The HPLC–tandem mass spectrometry method enabled us to identify resveratrol sulfates in human LDL and to characterize the complete profile of resveratrol metabolism in human LDL and urine. This method provides an accurate index of exposure to resveratrol and its metabolites, which can be used as nutritional biomarkers for evaluating the biological effects of moderate wine intake on human health.**

Resveratrol is a phenolic compound that has been linked to the beneficial effects of red wine *(1)* (Fig. 1), which have been proposed to be mimetic of caloric restriction in mammals *(2)*. In red wine, resveratrol occurs predominantly as its glucose derivative, piceid. Several in vitro studies have demonstrated that resveratrol acts as an antioxidant *(3)*, reduces the synthesis of proatherosclerotic substances *(4)*, is a potential cancer preventative *(5)*, and acts as a neuroprotector *(6)*. Few authors, however, have studied resveratrol metabolism in humans. As with many polyphenols, resveratrol is reasonably well absorbed but has low bioavailability *(7)*. Therefore, the health benefits attributed to the ingestion of resveratrol are most likely related to biologically active metabolites. In vivo characterization of resveratrol's metabolic profile may reveal which metabolites act as signaling molecules within tissues *(6)* or reach target organs and account for the health benefits of resveratrol *(8)*.

Nutritional biomarkers of nutrient exposure may be useful alternatives to traditional dietary assessment tools but require a clear understanding of the metabolism of the specific phytochemical. The metabolism of resveratrol has been partially characterized *(9–13)*. After resveratrol ingestion, the main metabolites found in biological fluids are glucuronide and sulfate conjugates *(9–12)*. Resveratrol glucuronide was reported to be a nutritional biomarker of wine consumption *(13)*, but underestimation of

[1] Nutrition and Food Science Department, CeRTA, Pharmacy School, University of Barcelona, Barcelona, Spain.
[2] Institute of Gerontology and Geriatrics, Department of Clinical and Experimental Medicine, University of Perugia Medical School, Perugia, Italy.
[3] Scientific and Technical Services and [6] Department of Internal Medicine, Hospital Clínic, Institut d'Investigació, Biomèdica August Pi i Sunyer, University of Barcelona, Barcelona, Spain.
[4] Pharmacology Research Unit and [5] Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d'Investigació Mèdica, Barcelona, Spain.
[7] Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna, Austria.
[*] Address correspondence to this author at: Nutrition and Food Science Department, CeRTA, Pharmacy School, University of Barcelona, 08028 Barcelona, Spain. Fax 34-93-4035931; e-mail candres@ub.edu.
Received April 18, 2006; accepted November 8, 2006.
Previously published online at DOI: 10.1373/clinchem.2006.071936

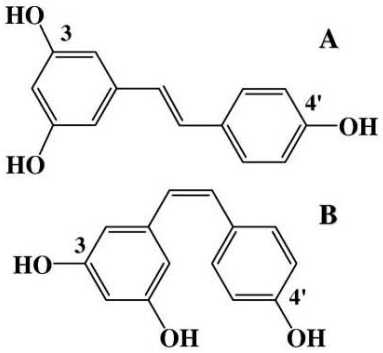


Fig. 1. Structure of *trans*-resveratrol (*A*) and *cis*-resveratrol (*B*).

sulfate conjugates due to poor chromatographic behavior has limited the analytical methods used for the analysis of resveratrol metabolites *(9–13)*. Other drawbacks included rather laborious sample preparation *(14–16)*, long total analysis time *(9–20)*, and the use of enzymatic hydrolysis that precluded direct detection of conjugates *(14, 19, 20)*.

We describe an HPLC–tandem mass spectrometry (HPLC-MS/MS)[1] method to characterize the metabolic profile of resveratrol in human urine and LDL after sample clean-up with solid-phase extraction (SPE).

Materials and Methods

STANDARDS AND REAGENTS

All samples and standards were handled with no exposure to light. Standards of *trans*-resveratrol (99% purity), *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity), diethylstilbestrol (≥99% purity), diethylstilbestrol dipropionate, dienestrol, hexestrol (≥98% purity), and human blank LDL were purchased from Sigma-Aldrich. Trismethoxy resveratrol (≥98% purity) was purchased from Cayman Chemical, diethylstilbestrol-d6 from RIVM, taxifolin (>90% purity) from Extrasynthese, and creatinine from Fluka.

Methanol, acetone, and acetonitrile of HPLC grade were purchased from SDS. Glacial acetic acid, ethyl acetate, and *o*-phosphoric acid were purchased from Panreac. Ultrapure water (MilliQ) was obtained from Millipore. Synthetic urine was prepared as previously described *(21)*.

We purified standard resveratrol metabolites from the livers of male Wistar rats raised at the Institut für Versuchstierzucht und-haltung (University of Vienna). Ethics Review Board approval was obtained for the animal studies. The animals were humanely treated. The livers were perfused with 20 μmol/L of *trans*-resveratrol in a recirculating system as previously described *(22)*. We purified resveratrol metabolites from multiple bile samples collected over a time period of 60 min. After collection the samples were pooled and lyophilized. Chemical structures were confirmed by nuclear magnetic resonance *(10)*.

STUDY DESIGN AND SAMPLES

We obtained human LDL samples from 11 healthy male volunteers (ages 18–50) before and 24 h after the consumption of 250 mL of Merlot red wine *(10)*. All volunteers were considered healthy based on the results of physical examination and standard biochemical and hematological tests. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1996. The Ethics Committee of our institution (Comité Ético de Investigación Clínica–Institut Municipal d'Investigació Mèdica) approved the protocol, and all the participants provided signed informed consent. Exercise was monitored with the Minnesota Leisure Time Physical Activity Questionnaire *(23)*.

Before administration, the volunteers followed a 10-day washout period in which they consumed a controlled diet from days 1 to 7, avoiding excess intake of antioxidants. During the immediate 3 days before and on the intervention day, the volunteers consumed a standardized low phenolic compound diet. On the intervention day they drank a single dose of 250 mL of red wine. We collected EDTA blood at baseline and at 24 h after wine consumption. LDL was isolated by sequential flotation ultracentrifugation *(24)*. We immediately froze all LDL samples at −80 °C, with thawing immediately before analysis. Protein content was determined with the red pirogalol method (Sigma-Aldrich).

We obtained urine samples from 5 healthy male volunteers (ages 25–28 years). The study design and conditions were similar to those of Meng et al. *(9)*, with the exception that urine was collected at baseline and during the 4 h after wine consumption. Urine creatinine was measured by a colorimetric assay using picric acid *(25)*.

We used the same red wine in both studies and analyzed resveratrol by HPLC *(26)*. The mean (SD) amount of total resveratrol consumed was 5.4 (0.4) mg, corresponding to 2.6 (0.0) mg of *trans*-piceid, 2.0 (0.2) mg of *cis*-piceid, 0.4 (0.1) mg of *trans*-resveratrol, and 0.4 (0.1) mg of *cis*-resveratrol.

SAMPLE EXTRACTION

LDL (1 mL) was treated with 20 μL of *o*-phosphoric acid (850 mL/L) and vortex-mixed. Urine was centrifuged at 10 000*g* at 4 °C for 3 min and then vortex-mix mixed after addition of 20 μL of the hexestrol as internal standard (92.6 μmol/L) to 1 mL of sample. Samples were then loaded onto a Waters Oasis® HLB 96-well SPE plate (30 mg) that had been preconditioned with 1 mL of methanol and equilibrated with 1 mL of 2 mol/L acetic acid in

[1] Nonstandard abbreviations: MS/MS, tandem mass spectrometry; SPE, solid-phase extraction; LC, liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring; DP, declustering potential.

water. The plate was 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 1mol/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. We reconstituted the residue with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase as an additional the external standard.

HPLC-MS/MS ANALYSES

We performed liquid chromatography (LC) analyses using a Perkin-Elmer series 200 system equipped with a quaternary pump and a refrigerated plate autosampler. An Applied Biosystems API 3000 triple quadrupole mass spectrometer, equipped with a Turbo IonSpray source ionizing in the negative mode, was used to obtain the mass spectrometry (MS) and MS/MS data. A Phenomenex Luna C_{18} column, 50 × 2.0 mm i.d., 3 μm, maintained at 40 °C, was used for chromatographic separation. The injection volume was 15 μL, and the flow rate was 550 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. We applied a linear gradient profile with the following proportions (v/v) of phase B [*t*(min), %B]: (0, 15), (1, 15), (1.5, 40), (2.5, 100), (4.5, 100), (4.8, 15), (10, 15). The column was reequilibrated for 6 min. The MS and MS/MS parameters were as previously described *(10)*.

The identification of metabolites in biological samples was based on 3 indicators *(10, 27)*: (*a*) comparison of retention time of available standard, (*b*) multiple reaction monitoring (MRM) of metabolite and resveratrol transitions [with higher declustering potential (DP) in collision-induced dissociation MS/MS conditions], or (*c*) product ion spectra. For MS/MS, a product ion scan was used at a cycle time of 2 s. The product ion spectra of metabolites showed the deprotonated molecule (*m/z* 403 or *m/z* 307, respectively) and the ion corresponding to resveratrol (*m/z* 227) through the neutral loss of the glucuronide or sulfate unit (−176 u or −80 u, respectively) from the glucuronide or sulfate. MRM mode was used with a dwell time of 200 ms, monitoring 6 transitions for each analysis: resveratrol (227/185), resveratrol glucosides (389/227), resveratrol glucuronides (403/227), resveratrol sulfates (307/227), taxifolin (303/285), and hexestrol (269/134). The concentrations of resveratrol metabolites were expressed as *trans*-resveratrol equivalents *(10, 20)*.

EVALUATION OF INTERNAL STANDARDS

Several compounds, structurally similar to resveratrol, were evaluated as possible internal standards. MRM transitions were 267/237 for diethylstilbestrol, 273/254 for diethylstilbestrol-d6, 269/134 for hexestrol, and 265/93 for dienestrol. Trismethoxy resveratrol and diethylstilbestrol dipropionate were not ionizable in negative mode.

ASSAY VALIDATION

We assessed endogenous interference by analyzing blank human LDL, synthetic urine, and blank urine samples (n = 5) collected from volunteers after the washout period. Recovery and linearity were investigated by adding *trans*-resveratrol and *trans*-piceid, at 10 concentrations, to blank urine (Table 1). The limit of detection was defined as the concentration of analyte that produced a signal-to-noise ratio of 3. The lowest standard on the calibration curve was accepted as the limit of quantification *(28)*. Within- and between-day imprecision and recovery were evaluated with use of 10 different concentrations of resveratrol and piceid (n = 3) over a 10-day period. We evaluated stability during the analytical process, after freeze and thaw cycles, and after short-term and long-term storage. Control materials with resveratrol concentrations of 219.3 nmol/L and 2193.0 nmol/L, and piceid concentrations of 140.8 nmol/L and 1145.6 nmol/L, in the proper matrices, were stored under the same conditions (−80 °C) as biological samples. We assessed the stability of metabolites with urine from volunteers who had consumed red wine.

After we had validated the analytical method for routine use, we used resveratrol at concentrations of 21.9, 219.3, and 2193.0 nmol/L and piceid at concentrations of 12.8, 128.2, and 1282.0 nmol/L in duplicate as QC samples *(28)*.

STATISTICAL ANALYSIS

SPSS statistical software, Windows version 11.5.1, was used. Kolmogorov–Levene and a paired Student *t*-test were employed. A weighted least-squares regression analysis was used to obtain correlation coefficients and slopes. Statistical significance was defined as $P < 0.05$. Data are shown as the mean (SD).

Results

SELECTIVITY

Under the chromatographic and MS/MS conditions used for the assay, metabolites and standards were well resolved (Fig. 2, Table 2). Endogenous peaks at the retention time of the analytes of interest were not observed in blank human LDL or in synthetic urine. Blank urine from volunteers showed some endogenous peaks, but none at the same retention time of the analytes.

EXTRACTION RECOVERY AND LINEARITY

The mean (SD) recoveries of known amounts of *trans*-resveratrol and *trans*-piceid added to blank matrices were 92 (11.5)% and 89 (6.3)%, respectively. The 9-point calibrator concentrations showed a linear and reproducible curve for standards. Weighted ($1/x^2$) least-square regression analysis yielded equation regression lines and residual analysis [mean range (SD)] as follows: $y = 35.2x - 0.07$ ($r^2 = 0.996$) and 100% (3.2) for *trans*-resveratrol and $y = 19.3x + 1.3$ ($r^2 = 0.967$) and 100% (11.1) for *trans*-piceid.

Table 1. Within- and between-day precision and recovery data obtained from the LC-MS/MS of *trans*-resveratrol and *trans*-piceid in blank human urine.

	trans-Resveratrol				*trans*-Piceid			
Imprecision	Added, nmol/L	Mean, nmol/L	Precision (RSD), %	Recovery (error), %	Added, nmol/L	Mean, nmol/L	Precision (RSD), %	Recovery (error), %
Within-day (n = 3)								
	4.4	4.4	4.5	99.8				
	21.9	22.8	2.1	104.0	12.8	13.0	8.7	101.6
	43.9	40.9	3.2	93.2	25.6	27.0	2.8	105.3
	87.7	90.6	2.8	103.3	51.3	52.6	8.7	102.6
	219.3	226.1	3.7	103.1	128.2	133.6	0.7	104.2
	329.0	318.7	10.5	96.9	192.3	209.4	9.4	108.9
	438.6	475.6	6.0	108.4	256.4	264.1	6.0	103.0
	1096.5	1106.4	8.1	100.9	641.0	658.3	5.5	102.7
	2193.0	2022.6	6.8	92.2	1282.0	1287.2	4.6	100.4
	3289.5	3441.1	3.1	104.6	1923.1	1857.8	8.5	96.6
Between-day (n = 10)								
	4.4	4.7	10.8	106.6				
	21.9	24.2	10.5	110.3	12.8	12.5	9.8	97.3
	43.9	39.4	8.1	89.9	25.6	27.7	10.5	108.0
	87.7	95.5	10.1	108.9	51.3	52.8	4.4	102.9
	219.3	227.2	4.4	103.6	128.2	137.8	8.2	107.5
	328.9	295.7	10.2	89.9	192.3	211.2	10.4	109.8
	438.6	475.7	9.2	108.5	256.4	247.0	9.5	96.3
	1096.5	1076.5	8.4	98.2	641.0	705.1	10.5	110.0
	2193.0	1994.1	8.5	90.9	1282.0	1193.3	9.6	93.1
	3289.5	3605.9	10.4	109.6	1923.1	1727.0	8.8	89.8

PRECISION, RECOVERY, AND DETECTION LIMIT

Precision and recovery (Table 1) met acceptance criteria (*28*) at all concentrations. According to these criteria, the lowest standards of *trans*-resveratrol and *trans*-piceid, 4.4 and 12.8 nmol/L, respectively, were accepted as the limit of quantification in human blank urine, and 0.4 and 1.9 nmol/L, respectively, in the LDL matrix (*10*). Limits of detection for *trans*-resveratrol and *trans*-piceid were 0.2 and 1.2 nmol/L, respectively, in LDL matrix, 4.0 and 8.4 nmol/L, respectively, in human blank urine, and 0.3 and 1.9 nmol/L, respectively, in synthetic urine.

STABILITY

To evaluate short-term temperature stability, 3 aliquots of each concentration were thawed at room temperature, maintained at this temperature for 3 h, and then analyzed. This time represents the average sample preparation time for 96-well plates. The aliquots were then put in a refrigerated autosampler and analyzed at 10 and 25 h, the average time required to analyze 96 samples. Under these conditions, and after freeze and thaw cycles, we observed differences <5% for *trans*-resveratrol and *trans*-piceid. Evaluation of the long-term stability of resveratrol glucuronide stored at −80 °C for 5 years yielded an observed CV of 10.8% (n = 5). After testing the stability of human urine after moderate consumption of red wine, we observed no statistically significant differences in glucuronided and sulfated metabolites at freeze and thaw

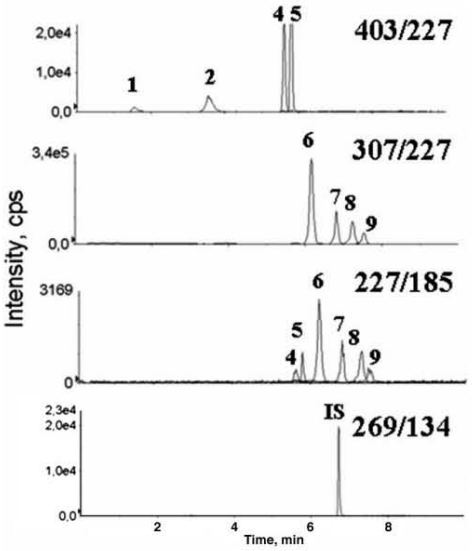


Fig. 2. MRM trace chromatogram of sulfated and glucuronidated standards of resveratrol and MRM of 227/185 (DP = −80) in LC-MS/MS conditions as described in the text.

Numbered peaks refer to Table 2.

Table 2. Description of relative molecular mass, retention times, negative mode multiple reaction monitoring transitions, mean concentrations (SD) of 24-h LDL and 4-h urine after moderate consumption of red wine, and percentage of volunteers who demonstrated each metabolite.

					LDL samples		Urine samples	
Compound	Peak no.	M_r	R_t, min	MS/MS Ions, *m/z*	pmol resveratrol/mg LDL protein, mean (SD)	Volunteers, %	nmol resveratrol/g creatinine, mean (SD)	Volunteers, %
trans-Resveratrol-4′-*O*-glucuronide	1	404	1.6	403/227	37.8 (43.6)	27	59.6 (88.7)	80
Taxifolin	ES[a]	304	2.4	303/285	ES		ES	
trans-Resveratrol-3-*O*-glucuronide	2	404	3.3	403/227	111.7 (126.0)	36	179.2 (276.0)	80
trans-Resveratrol	3	228	5.5	227/185	3.5 (4.6)	73	ND	ND
cis-Resveratrol-4′-*O*-glucuronide	4	404	5.6	403/227	ND	ND	355.8 (567.4)	80
cis-Resveratrol-3-*O*-glucuronide	5	404	5.8	403/227	7.1 (5.8)	27	893.5 (894.6)	100
trans-Resveratrol-4′-sulfate	6	308	6.2	307/227	2.0 (1.9)	36	2.4 (14.8)	40
Hexestrol	IS	270	6.7	269/134	IS		IS	
trans-Resveratrol-3-sulfate	7	308	6.8	307/227	4.0 (5.4)	36	74.7 (339.0)	40
cis-Resveratrol-4′-sulfate	8	308	7.3	307/227	7.1 (5.2)	64	9294.2 (8219.2)	100
cis-Resveratrol-3-sulfate	9	308	7.5	307/227	5.4 (2.9)	36	221.2 (1010.1)	40

[a] ES, additional external standard; IS, internal standard; ND, not detected.

cycles and after short- and long-term stability. We concluded that the metabolites were stable under the storage and sample handling conditions used for this assay.

INTERNAL STANDARD EVALUATION

Diethylstilbestrol and diethylstilbestrol-d6 showed 2 unstable peaks over time. Hexestrol and dienestrol, both veterinary synthetic products, were absent in human nutritional and body fluids. Their mean recoveries (n = 11) at the concentrations used in the assay procedure (1851.8 and 1879.7 nmol/L, respectively) were 96% and 89%, respectively. Although the mean recoveries were acceptable for both, dienestrol showed a higher variability (CV >15%) than hexestrol (CV = 11.2%). Hexestrol was selected as the internal standard.

QUALITY CONTROL RESULTS

trans-Resveratrol showed that 83% of QC were within 15% of their nominal value. *trans*-Piceid showed that 67% of QC were within 15% of their nominal value.

APPLICATION TO LDL SAMPLES

To identify sulfated metabolites of resveratrol and to complete its metabolic profile (*10*), we analyzed LDL samples with this LC-MS/MS method. Three different profiles of 24-h LDL glucuronide and sulfate conjugates of resveratrol after a single dose of red wine are shown in Fig. 3. Six metabolites were identified in volunteer A, 5 in volunteer B, and 4 in volunteer C. Volunteer B showed several peaks with 403/227 transition, but only 2 of them were positively identified as resveratrol glucuronides. Mean (SD) concentrations are shown in Table 2.

In addition to the well-described phase II metabolites of resveratrol, we also screened phase I metabolites, such as methylated (241/227) and hydroxylated (243/159) resveratrol, and their respective phase II metabolites, such as hydroxyresveratrol-glucuronide (419/243) and hydroxyresveratrol-sulfate (323/243). We also screened microflora metabolites, such as dihydroresveratrol-glucuronide (405/229) and dihydroresveratrol-sulfate (309/229) (*12*). After checking for these

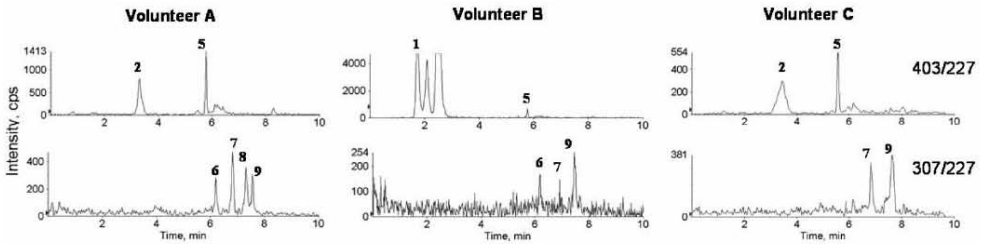


Fig. 3. MRM trace chromatogram of LDL after the intake of 250 mL of red wine (volunteers A, B, and, C).
Numbered peaks refer to Table 2.

transitions, some peaks were observed but were below the limit of detection.

APPLICATION TO URINE SAMPLES

Glucuronided and sulfated metabolites were characterized in human urine by LC-MS/MS. MRM chromatograms of sulfates (307/227) and glucuronides (403/227) in urine from 4 volunteers are shown in Fig. 4. As can be seen in Fig. 4, the application of a higher DP (−80) in the collision-induced dissociation MS/MS experiment allowed the confirmation of all the metabolites through the characteristic 227/185 transition for resveratrol. The means (SD) of the metabolites for these volunteers are presented in Table 2 as nmol resveratrol/g creatinine.

Discussion

We have developed a new method to evaluate resveratrol metabolism in human samples. With this HPLC-MS/MS method, we determined the resveratrol metabolic profile in 10 min in different types of matrices. We emphasize that because of the observed differences in limits of detection, human blank urine is a better tool than synthetic urine because it shows the real matrix effect (*29*).

Investigations on human resveratrol metabolism have only recently been performed. In 2003, Goldberg et al. (*30*) were the first to administer resveratrol to humans. Subsequent published studies have shown glucuronides and sulfates to be the main metabolites of resveratrol. Only the glucuronide metabolites have been well characterized because of the poor chromatographic behavior of resveratrol sulfates (*12*).

We have circumvented the drawbacks of previous methods. To improve the resolution of the sulfates (*10*, *12*), acetone was incorporated into mobile phase B. Acetone allows better resolution of sulfates by improving the peak shape and reducing the relative retention time. The incorporation of a shorter chromatographic column also reduced the chromatographic time to 10 min (*9–20*). The use of a 96-well SPE plate helped avoid laborious sample preparation (*14–16*), requiring ~3 h of preparation per plate. The use of LC-MS/MS avoids the need to perform enzymatic hydrolysis (*14*, *19*, *20*), thus simplify-

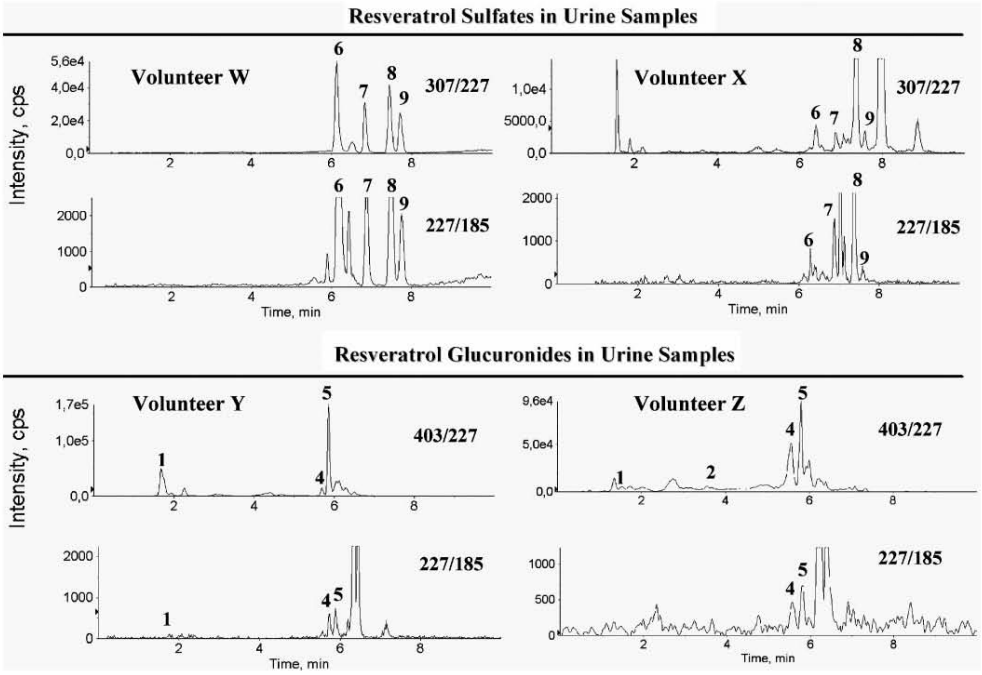


Fig. 4. MRM trace chromatogram of resveratrol sulfates (307/227), resveratrol glucuronides (403/227), and resveratrol (227/185; DP −80) in urine samples of representative volunteers after the consumption of 250 mL of red wine.

Numbered peaks refer to Table 2.

ing the quantitative and qualitative profiling of the resveratrol metabolites.

Another highlight of the present method is the ability to differentiate between the *trans* and *cis* isomers of resveratrol-4′-*O*-glucuronide, resveratrol-3-*O*-glucuronide, resveratrol-4′-sulfate, and resveratrol-3-sulfate. This method is the first to identify the entire profile of resveratrol sulfates in human LDL and urine (Figs. 3 and 4).

There was variability between volunteers (Table 2), but all sulfates were found in similar concentrations in LDL. The main sulfate in LDL was the *cis*-resveratrol-4′-sulfate, and the main glucuronide was *trans*-resveratrol-3-*O*-glucuronide. The *trans*-resveratrol-*O*-glucuronides were in greater concentrations than sulfates. Resveratrol can be glucuronidated at 2 positions on the molecule. Although the 3 position seemed to be the preferential glucuronidation site in vitro in human liver microsomes, the 4′ position is also a possible site of metabolism in humans in vivo *(11)*. Considering activity, the presence of the 4′-OH is a requisite for inhibition of cell proliferation *(31)*. Our results show major glucuronidation of resveratrol in 3-position at 24 h maintaining the 4′-OH free. Although the glucuronide metabolites of resveratrol have previously been described in LDL *(10)*, this new method is able to determine resveratrol sulfates without reducing the resolution of glucuronides.

After successful characterization of the resveratrol metabolites profile in LDL, we applied the method to urine samples. Urine is a more adequate sample to be used in large-scale population studies to establish nutritional biomarkers *(32)*. Meng et al. *(9)* described the rapid excretion of resveratrol in urine (after 2–3 h) when low amounts are consumed. In this study, the urine was collected during the 4 h after moderate red wine intake. When absorbed, resveratrol is rapidly cleared through the glucuronidation and sulfation pathways, and metabolites are principally excreted in urine *(9, 12)*. All the resveratrol metabolites previously described were found in these urine samples. Concerning the stereoselectivity of glucuronidation, *cis*-isomers were glucuronidated faster than *trans*-isomers *(15)*. This observation is in accordance with our results of our study, in which greater amounts of *cis*-*O*-glucuronide are obtained. Because this is the first time that sulfates of resveratrol have been well characterized, there are no published data about sulfate stereoselectivity. Taking into account the concentration results (Table 2), however, the behavior of sulfates seems similar to that of glucuronides, showing higher amounts for *cis* isomers. The variability shown in these results has been seen previously in LDL *(10)* and is attributable to polymorphisms of intestinal enzymes *(33)* or to interactions with other compounds *(34)*. Further investigations on resveratrol variability with more volunteers are needed.

This method can be used in future epidemiological and clinical intervention trials. In studies aimed at evaluating the biological effects of resveratrol intake via moderate wine consumption, knowledge of the resveratrol profile may facilitate better estimation of resveratrol consumption than dietary data obtained by food frequency questionnaires.

We are grateful to the volunteers for their valuable cooperation in the study. We are also grateful for the financial support of the following Spanish Departments: Agriculture (INIA project VIN00-027-C3-2), Education and Science (MEC) (AGL2004-08378-C02-01/02), and Health: Instituto de Salud Carlos III, Red de Grupo G03/140 (PREDIMED study). M.U.-S. and R.Z.-R. thank the Formación de Personal Investigator fellowship program from MEC and Departament d'Universitats, Recerca i Societat de la Informació, respectively. We are grateful to Dr. Isidre Casals from Scientific and Technical Services, to Marta Burrull and Xavier Rodriguez from Waters, and to Dr. Bénédicte Duretz from Applied Biosystems for technical assistance.

References

1. Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. Lancet 1993;341:1103–4.
2. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 2006;5:493–506.
3. Olas B, Wachowicz B. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. Platelets 2005;16:251–60.
4. Corder R, Douthwaite JA, Lees DM, Khan NQ, Viseu Dos Santos AC, Wood EG, et al. Endothelin-1 synthesis reduced by red wine. Nature 2001;414:863–4.
5. Rodrigue CM, Porteu F, Navarro N, Bruyneel E, Bracke M, Romeo PH, et al. The cancer chemopreventive agent resveratrol induces tensin, a cell-matrix adhesion protein with signaling and antitumor activities. Oncogene 2005;24:3274–84.
6. Dore S. Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. Neurosignals 2005;14:61–70.
7. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.
8. Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, et al. How should we assess the effects of exposure to dietary polyphenols in vitro? Am J Clin Nutr 2004;80:15–21.
9. Meng X, Maliakal P, Lu H, Lee MJ, Yang CS. Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. J Agric Food Chem 2004;52:935–42.
10. Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas MI, et al. Uptake of diet resveratrol into the human low-density lipoprotein: identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. Anal Chem 2005;77:3149–55.
11. Vitaglione P, Sforza S, Galaverna G, Ghidini C, Caporaso N, Vescovi PP, et al. Bioavailability of trans-resveratrol from red wine in humans. Mol Nutr Food Res 2005;49:495–504.
12. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 2004;32:1377–82.
13. Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Vazquez-Agell M, Serrano-Martinez M, et al. Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. Clin Chem 2006;52:1373–80.

14. Asensi M, Medina I, Ortega A, Carretero J, Bano MC, Obrador E, et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med 2002;33:387–98.

15. Aumont V, Krisa S, Battaglia E, Netter P, Richard T, Merillon JM, et al. Regioselective and stereospecific glucuronidation of trans- and *cis*-resveratrol in human. Arch Biochem Biophys 2001;393: 281–9.

16. Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res 2005;49:482–94.

17. Andlauer W, Kolb J, Siebert K, Furst P. Assessment of resveratrol bioavailability in the perfused small intestine of the rat. Drugs Exp Clin Res 2000;26:47–55.

18. Kuhnle G, Spencer JP, Chowrimootoo G, Schroeter H, Debnam ES, Srai SK et al. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochem Biophys Res Commun 2000; 272:212–7.

19. Marier JF, Vachon P, Gritsas A, Zhang J, Moreau JP, Ducharme MP. Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. J Pharmacol Exp Ther 2002;302: 369–73.

20. Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS et al. Human, rat, and mouse metabolism of resveratrol. Pharm Res 2002;19: 1907–14.

21. Miro-Casas E, Farre AM, Covas MI, Rodriguez JO, Menoyo CE, Lamuela Raventos RM, et al. Capillary gas chromatography-mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake. Anal Biochem 2001;294:63–72.

22. Jager W, Zembsch B, Wolschann P, Pittenauer E, Senderowicz AM, Sausville EA, et al. Metabolism of the anticancer drug flavopiridol, a new inhibitor of cyclin dependent kinases, in rat liver. Life Sci 1998;62:1861–73.

23. Elosua R, Marrugat J, Molina L, Pons S, Pujol E. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators. Am J Epidemiol 1994; 139:1197–209.

24. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–53.

25. Jaffé M. Über den niederschlag welchen pikrinsäure in normalen harn erzeugt und uber eine neue reaction des kreatinins. Z Physiol Chem 1886;10:391–400.

26. Lamuela-Raventos RM, Romero-Perez AI, Waterhouse AL, de la Torre-Boronat MC. Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceid isomers in Spanish red *Vitis vinifera* wines. J Agric Food Chem 1995;43:281–3.

27. Hernandez F, Ibanez M, Sancho JV, Pozo OJ. Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental water based on the use of identification points. Anal Chem 2004;76:4349–57.

28. US Department of Health and Human Services, Food and Drug Administration. Guidance for Industry Bioanalytical Method Validation. 2001. http://www.fda.gov/cder/guidance/4252fnl.htm (accessed December 2006).

29. Annesley TM, Clayton LT. Quantification of mycophenolic acid and glucuronide metabolite in human serum by HPLC-tandem mass spectrometry. Clin Chem 2005;51:872–7.

30. Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 2003;36:79–87.

31. Stivala LA, Savio M, Carafoli F, Perucca P, Bianchi L, Maga G, et al. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. J Biol Chem 2001;276:22586–94.

32. Potischman N. Biologic and methodologic issues for nutritional biomarkers. J Nutr 2003;133(Suppl 3):875–80.

33. Manach C, Donovan JL. Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Radic Res 2004;38:771–85.

34. Pacifici GM. Inhibition of human liver and duodenum sulfotransferases by drugs and dietary chemicals: a review of the literature. Int J Clin Pharmacol Ther 2004;42:488–95.

8.2. Pósters y comunicación oral

Comunicación 1: Poster

Zamora-Ros,R.;Andrés-Lacueva,C.;Izquierdo-Pulido,M.;Lamuela-Raventós,R.M.

Consumo de flavonoides en la población infantil y adulta española.

V Congreso Internacional de Barcelona sobre Dieta Mediterránea. Barcelona (SPAIN) 2004

CONSUMO DE FLAVONOIDES EN LA POBLACIÓN INFANTIL Y ADULTA ESPAÑOLA

Raül Zamora-Ros[1] ; Cristina Andrés-Lacueva[1]; María Izquierdo-Pulido[1,2] ; Rosa María Lamuela-Raventós[1].

[1]Departamento de Nutrición y Bromatología. Facultad de Farmacia. Universidad de Barcelona. Av. Joan XXIII s/n, 08028, Barcelona
E-mail: lamuela@ub.edu
[2] Nutrexpa

Introducción:

Los flavonoides son sustancias polifenólicas, con estructura química de difenilpropanos (C6-C3-C6), ampliamente distribuidas en el mundo vegetal, en frutas, verduras, cacao, té y vino. Los flavonoides se pueden desglosar en 6 subgrupos: antocianos, flavonoles, flavan-3-oles, flavanonas, flavonas e isoflavonas.

En los últimos años, los flavonoides, debido a su elevada actividad antioxidante, están adquiriendo un especial interés, ya que su ingesta puede tener efectos beneficiosos en la prevención de enfermedades cardiovasculares, neurodegenerativas y cáncer.

Sin embargo, y a pesar de su interés, existen muy pocos datos disponibles a nivel internacional sobre su consumo y, concretamente en España, no existe ningún dato publicado En la tabla 1 se presentan los estudios más importantes sobre consumo de flavonoides.

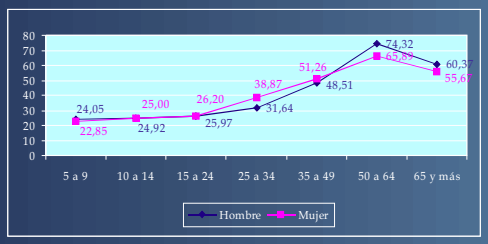
Objetivo:

Determinar la ingesta de flavonoides en la población infantil y adulta española, y qué alimentos son los responsables mayoritarios de este aporte.

Tabla 1. Estudios más representativos sobre consumo de flavonoides.

Estudio	Individuos	Flavonoides estudiados	mg/día	Alimentos	Efecto protector
Grecia (2003)	2368 mujeres (50-60años)	Flavonoids	110,9		Cáncer de pecho
Zuphten (Holanda) (1993)	805 hombres (65-84años)	Flavan-3-oles, flavonoles y flavonas	97,9	81% te 9% manzana 3,5% cebolla	Enfermedad vascular y cáncer de piel
Morgen (Holanda) (2001)	13.651 individuos (20-59años)	Flavan-3-oles, flavonoles y flavonas	58	65% te 13% manzana 5% cebolla	Enfermedad pulmonar obstructiva
3 estudios en USA (2002)	30.000 individuos (40-75años)	Flavan-3-oles, flavonoles y flavonas	45,5	46% te 15% manzana 10% cebolla	Enfermedad vascular y cáncer de colon
Reino Unido (1997)	1.900 hombres (45-59anys)	Flavonoles	26	82% te 10% cebolla	Enfermedad vascular

Figura 3. Ingesta de flavonoides (mg/día) por intervalos de edad y por género en la población española.



Material y métodos:

Se han utilizado como base de datos de composición de flavonoides en alimentos la publicada por el USDA (U.S Department of Agriculture, 2003) formada por 242 alimentos y 26 flavonoides (11 flavan-3-oles, 6 antocianos, 4 flavonoles, 2 flavonas y 3 flavanonas). Además se incluyeron valores propios del grupo para el cava y el cacao soluble debido a la ausencia de los mismos en la base de datos de composición.

El consumo de alimentos se ha estimado a partir del estudio "Family Food Panel", cuestionario de frecuencia de consumo cualitativo de alimentos realizado a 15.288 individuos residentes en España (13.098 adultos y 2.190 niños) elaborado por Taylor Nelson Sofres, 2002. Las cantidades de alimentos ingeridos se han ajustado mediante los datos de consumo del Ministerio de Agricultura, Pesca y Alimentación del 2002.

Resultados:

Se ha estimado un consumo medio de flavonoides de 45,9mg/día en la población española. La población adulta (mayores de 15 años) ingiere 49,5mg/día mientras que la infantil (de 5 a 14 años) es de 24,2mg/día. Los alimentos que más flavonoides aportan a la dieta de los adultos son: té (32%), vino (31%) y frutas y verduras (27%) figura 1 . En los niños los más representativos son: cacao (54%), frutas y verduras (29%) y zumos de fruta (14%) figura 2.

Figura 1. Distribución porcentual de alimentos por aporte de flavonoides en la población adulta española.

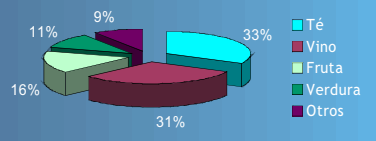
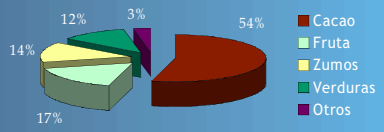


Figura 2. Distribución porcentual de alimentos por aporte de flavonoides en la población infantil española.



Conclusiones:

- Por primera vez en nuestro país, se obtienen datos sobre la ingesta media de flavonoides en población española, adulta e infantil.
- Nuestra alimentación, fundamentalmente mediterránea, provoca que las frutas, las verduras y especialmente el vino (en adultos) sean los alimentos con un peso específico más importante.
- En niños, destaca el cacao (alimento habitual en el desayuno) ya que aportaría más del 50% de la ingesta total de estas sustancias.

Bibliografía:

- USDA Database for the flavonoid content for selected foods. United State Departament of Agricultura l. (2003).
- Food Panell Questionary. Taylor Nelson Sofres. (2002).
- La Alimentación en España. MAPA. (2002).
- Ross JA, Kassum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr. (2002); 22:19-34.
- Peterson J, Lagiou P, Samoli E, Lagiou A, Katsouyanni K, La Vecchia C, Dwyer J, Trichopoulos D. Flavonoid intake and breast cancer risk: a case-control study in Greece. British Journal of Cancer; (2003). 89: 1255-1259.
- Hertog M, Feskens E, Hollman P, Katan M, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. The Lancet; (1993). 342: 1007-1011.
- Arts ICW, Hollman PCH, Feskens EJM, Bas Bueno de Mesquita H, Kromhout D. Catechin intake might expl ain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly St udy. American Journal of Clinical Nutrition; (2001). 74: 227-232.
- Tabak C, Arts IWC, Smit HA, Heederik D, Kromhout D. Chronic obstructive pulmonary disease and intake of catechins, flavonols and flavones. Am. J. Crit. Care Med.; (2001). 164: 61-64.
- Sampson L, Rimm E, Hollman P, de Vries J, Katan M. Flavonol and flavone intake in US health professionals. Journal of the american dietetic association; (2002). 102 (10): 1414-1420
- Hertog M, Sweetnam P, Fehily A, Elwood P, Kromhout D. Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study. American Journal of Clinical Nutrition; (1997); 65: 1489-1494.
- Rimm EB, Katan MB, Ascherio A, Stampfer MJ, Willet WC. Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. Annals of Internal Medicine; (1996). 125: 384-389.

Comunicación 2: Poster

Zamora-Ros, R.; Urpí-Sardà, M.; Jauregui, O.; Lamuela-Raventós, R.M.; Ibern-Gómez, M.; Estruch, R.; Vázquez, M.; Andrés-Lacueva, C.

Identification of resveratrol glucuronide in urine after moderate sparkling wine consumption by LC-ESI-MS/MS

Poster

II Reunión Nacional de Espectrometría de Masas. Barcelona (SPAIN) 2004

IDENTIFICATION OF RESVERATROL GLUCURONIDE IN URINE AFTER MODERATE SPARKLING WINE CONSUMPTION BY LC-ESI-MS/MS

R. Zamora-Ros[1], M. Urpí-Sardà[1], O. Jáuregui[2], R. M. Lamuela-Raventós[1], M. Ibern-Gómez[1], R. Estruch[3], M. Vázquez[3], C. Andrés-Lacueva[1]

UNIVERSITAT DE BARCELONA

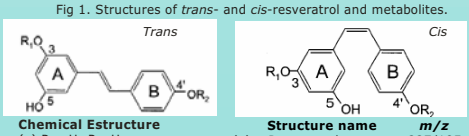
[1]Departament de Nutrició i Bromatologia: Facultat de Farmàcia, Universitat de Barcelona
[2]Serveis de Suport a la Recerca: Universitat de Barcelona
[3]Departament de Medicina Interna, Hospital Clínic, IDIBAPS, UB.
E-mail: rzamora@ub.edu / candres@ub.edu

Introduction

Resveratrol (3, 5, 4'-trihydroxystilbene) and piceid (resveratrol 3*O-β* -glucoside) are stilbenes found in only low quantities in the human diet. Cava is a grape-derived beverage with a low content of stilbenes, ranged from 0.5 to 1.5mg/L, and mainly in piceid form [1].
Regular and moderate consumption of resveratrol may achieve beneficial effects [2].
Bioavailability of resveratrol has been scarcely studied and it was considered in three ways: depending the experimental model, the resveratrol source, and the administrated dose. i) Extensively in rodents, resveratrol standard, single dose between 20-50mg/Kg [3,4]. ii) Humans, food supplemented with resveratrol standard, single dose ranged from 0.3 to 1mg/Kg [5-7]. iii) Humans, food, single dose between 0.005-0.027mg/Kg total stilbenes mainly in piceid form. Below 0.014mg/Kg administration dose no metabolites were detected neither in urine nor in plasma [7]. In all studies the mainly metabolite of resveratrol was the glucuronidated form [3-7].
In these conditions, high sensitivity and sensibility were required and LC-ESI/MS/MS using a triple-quadrupole mass spectrometer is recommended. MRM (multiple reaction monitoring) assay is the method of choice to search for potential metabolites present at trace levels.

Aim of this study
To establish a biomarker of moderate wine intake
Determinate resveratrol metabolites after regular and moderate sparkling wine consumption versus gin as a beverage without resveratrol content.

Fig 1. Structures of *trans*- and *cis*-resveratrol and metabolites.



Chemical Estructure	Structure name	*m/z*
(a) R_1= H; R_2=H	(a) Resveratrol	227/185
(b) R_1= glucose; R_2=H	(b) Piceid	389/227
(c) R_1= glucuronic acid; R_2= H	(c) **3-*O*-glucuronide**	**403/227**
(d) R_1= H; R_2= glucuronic acid	(d) 4'-*O*-glucuronide	403/227
(e) R_1= SO_3H; R_2= H	(e) 3-*O*-sulfate	307/227
(f) R_1= H; R_2= SO_3H	(f) 4'-*O*-sulfate	307/227

Material and methods

Subjects a study design
Protocol was approved by the Institutional Review Board of the Hospital Clinic (Barcelona).
Five healthy men consumed 30 g ethanol/day during 28 days:
- Cava (300mL of beverage with 0.35mg of total stilbenes, mainly in piceid form)
- Gin (100mL of beverage without stilbenes)
- Washout period of 3 weeks previous every diet.

Samples
Morning urine and serum after last sparkling wine intake and gin period were collected.
Urine and serum were acidified until 200mM with HCl and were stored at -80ºC.

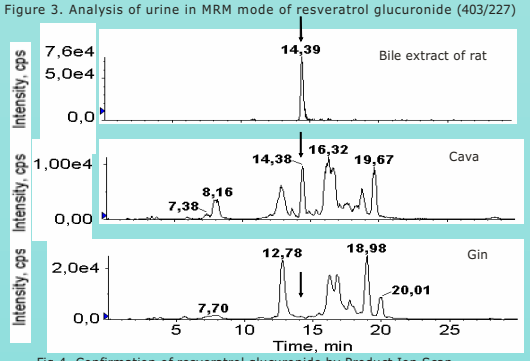
Sample preparation
Polyphenols were extracted with a SPE cartridge (Oasis HLB, Waters). Resveratrol metabolites were eluted with 1mL acidic methanol solution and 2mL of ethyl acetate. The organic solution was evaporated under N_2 avoiding dryness. Taxifolin was used as internal standard. The samples were reconstituted with mobile phase A until 100µL. Finally samples were filtration with 4mm PTFE filter 0.45µm (Waters).

Analysis sample
Analysis of resveratrol metabolites in urine was carried out by LC-MS/MS as described by Urpí [11] with slight modifications.

LC conditions
Perkin Elmer series 200 (Norwalk, CT, USA) quaternary pump, autosampler.
Column: Luna C_{18} (150 x 2,0 mm i.d., 5mm) (Phenomenex, Torrance, CA, USA).
Solvents: A: 0,05% acetic acid in water. B: acetonitrile
Gradient (t(min), %B): (0,15); (2,15); (10,40); (20,70); (25,100); (30,100).
Flow-rate: 400µL/min Volume injected: 15µL

MS/MS conditions
Triple quadrupole mass spectrometer (API 3000, PE Sciex, Concord, ON, Canada).
TURBO ION SPRAY
Ionization Mode: Negative
Acquisition Mode:
- MRM (Multiple Reaction Monitoring)
- Product Ion Scan of 403
- Precursor Ion Scan of 227
- Neutral Loss Scan

Figure 3. Analysis of urine in MRM mode of resveratrol glucuronide (403/227)

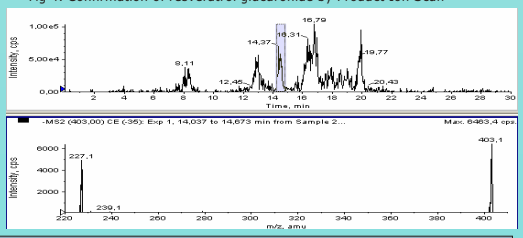


Results and conclusions

- The positive identification of resveratrol glucuronide in urine samples from cava ingestion was based on its retention time, and ions fragmentation in different MS/MS modes (product ion scan and precursor ion scan of *m/z* 227), compared with those of purified standard when available.
- Following 28 days of dietary supplementation with cava, only resveratrol glucuronide was found in the urine of all volunteers, while no resveratrol metabolites were detected in the urine of control diet volunteers.
- However, any resveratrol metabolites were detected in serum samples.
- To our knowledge, this is the first time that resveratrol glucuronide has been identified in urine following food (cava) not supplemented intake and with low dose of total resveratrol (0,005mg/Kg).
- Advances in analytical techniques let propose resveratrol glucuronide in urine as biomarker of sparkling wine consumption.
- In the future, this study could be the base for the application of this biomarker in epidemiological studies.

Fig 2: Amounts of resveratrol glucuronide excreted in urine.

Fig 4. Confirmation of resveratrol glucuronide by Product Ion Scan



References

1. C. Andres-Lacueva; et al. American Journal of Enology and Viticulture 53:147-150 (2002).
2. J.F. Marier; et al. J.Pharmacol.Exp.Ther. 302:369-373 (2002)
4. M. Asensi; et al. Estrela, Free Radic.Biol.Med. 33:387-398 (2002).
5. G.J. Soleas; J. Yan; D.M. Goldberg. J.Chromatogr.B Biomed.Sci.Appl. 757:161-172 (2001).
6. D.M. Goldberg; J. Yan; G.J. Soleas. Clin.Biochem. 36:79-87 (2003)
7. X. Meng; et al. J.Agric.Food Chem. 52:935-942 (2004).
8. M. Urpí-Sardà;C. Andrés-Lacueva; O. Jáuregui; M Ibern-Gómez; M. Covas; R.M. Lamuela-Raventós. Determination of diet resveratrol and its metabolites in human LDL by LC-ESI MS/MS. 1st International Conference on Polyphenols and Health. Vichy (FRANCIA) 2003. pp: 290.

Acknoledgement

The *"Consejo Regulador del Cava"*, Vilafranca del Penedés (Barcelona) and the *Instituto de Salud Carlos III* (Red de Grupo G03/140), Madrid, Spain supported this study. RZR was supported by Departament d'Universitats, Recerca i Societat de la Informació. CAL thanks the Ramón y Cajal program by the Ministry of Science and Technology from Spain and the ESF (European Social Found).

Comunicación 3: Poster

Andrés-Lacueva, C.; Zamora-Ros, R.; Urpí-Sardà, M.; Estruch, R.; Vázquez-Agell, M.; Jaeger, W.; Lamuela-Raventós, R.M.

Phenolic metabolites as nutritional biomarkers in humans. Two randomized controlled clinical trials

II International Conferences on Polyphenols and Health. Davis, CA (UNITED STATES) 2005

PHENOLIC METABOLITES AS NUTRITIONAL BIOMARKERS IN HUMANS. TWO RANDOMIZED CROSSOVER CLINICAL TRIALS

C. Andrés-Lacueva[1], R. Zamora-Ros[1], M. Urpí-Sardà[1], R. Estruch[2], M. Vázquez[2], W. Jaeger[3], R. M. Lamuela-Raventós[1]

[1]Nutrition and Food Science Department-CeRTA, Faculty of Pharmacy, University of Barcelona, Spain.
[2]Departament de Medicina Interna, Hospital Clínic, IDIBAPS, University of Barcelona, Spain.
[3]Institute of Pharmaceutical Chemistry. University of Vienna, Vienna, Austria.
E-mail: candres@ub.edu

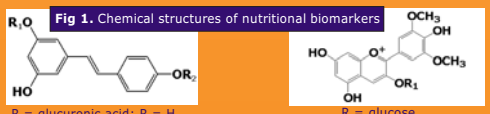
Introduction

Several studies have reported an association between moderate wine consumption and a lower risk CHD[1]. In nutritional studies, accurate quantification of diet is critical. For this reason, nutritional biomarkers are used to measure exposure because that have less error than dietary data[2].
Resveratrol (3, 5, 4'-trihydroxystilbene) and piceid (resveratrol-3*O*-*β* -glucoside) are stilbenes present mainly in grapes and wine. Bioavailability of resveratrol in humans has been scarcely studied. After single dose intake (0.014mg/Kg) glucuronidate form was the only metabolite detected in urine [3]. In plasma it's needed a high dose (0.3mg/Kg) to detect resveratrol metabolites [4]. To our knowledge, this is the first time it has been measured resveratrol metabolites after a regular wine intake.
Malvidin-3-glucoside (M-3-G) is an anthocyanin caracteristic of red grape and red wine responsable of the color. This compound is not present in white wines, so it could be a good biomarker when white *versus* red wine intake is tested. Previous studies of bioavailability of anthocyanins in humans in single dose showed poor absorption of M-3-G (1-5% of the ingested amount), and M-3-G not detected after 6 hours of last intake [5].
Biomarkers of nutrient intake are useful in epidemiological and clinical assays and are preferred over purely dietary data. Considering the limitations of the food composition data, direct nutritional markers are more precise and provide a more proximal measure of specific nutrient intake as an integrated measure of the metabolism of the component.

Aim of this study

- To purpose a biomarker of moderate wine intake
- To determine resveratrol metabolites after regular and moderate wine consumption.
- To determine M-3-G after regular and moderate red wine intake.

Fig 1. Chemical structures of nutritional biomarkers



R_1= glucuronic acid; R_2= H
***trans*-Resveratrol-3-*O*-glucuronide**

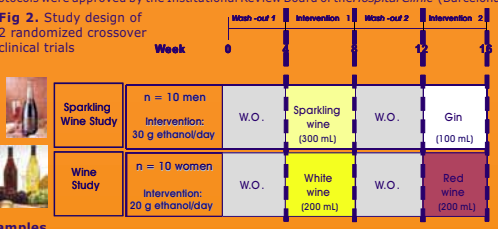
R_1= glucose
Malvidin-3-glucoside

Material and methods

Subjects a study design

Protocols were approved by the Institutional Review Board of the *Hospital Clinic* (Barcelona).

Fig 2. Study design of 2 randomized crossover clinical trials



Samples

After overnight fasting of last day of intervention or wash-out period, morning urine and serum were collected.
Urine and serum were acidified until 200mM with HCl and were stored at -80ºC.

Analysis sample

		Resveratrol (Urpi-Sarda et al. 2005)	Anthocyans (Andres-Lacueva et al. 2005)
LC-MS/MS		Perkin Elmer series 200 (Norwalk, CT, USA), API 3000 triple quadrupole mass spectometer (Applied Biosystems) (PE Sciex, Concord, Ontario, Canada)	
Mobile Phases		A/ 0.05% acetic acid B/ Acetonitrile	A/ 5% formic acid B/ Acetonitrile
Column		Luna Stable Bond C_{18} (150 x 2.0 mm, 5µm)	Zorbax Stable Bond C_{18} (150 x 2.1 mm, 5µm)
Flow-rate, injection volume		400 µL/min; 15 µL	
Source		Turbo Ion Spray	
Ionization Mode		Negative	Positive
Acquisition Mode		Multiple Reaction Monitoring (MRM)	
Metabolites	Compound	Resveratrol-3-*O*-glucuronide	Malvidin-3-GAL or GLU
	MW	404	492
	MRM	403/227	493/331

Results and conclusions

- Following 28 days of dietary supplementation with sparkling wine, white wine and red wine, *trans*-resveratrol-3-*O*-glucuronide was found in the urine of all volunteers.
- Only baseline levels of resveratrol metabolites were detected in the urine of control diet volunteers.
- No resveratrol metabolites were detected in serum samples in both studies.
- Following 4 weeks of red wine consumption, M-3-G was observed in the urine of all volunteers, while traces levels were found after white wine or wash-out periods.
- Advances in analytical techniques let propose resveratrol glucuronide in urine as biomarker of wine consumption. M-3-G may be used as biomarker only when red wine is compared*versus* white wine intake.
- In the future, this study could be the base for the application of these biomarkers in epidemiological or intervention studies.

Fig 2. Amounts of resveratrol glucuronide excreted in urine (P>0.05)

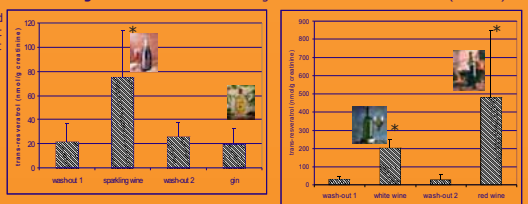


Figure 4. Amounts of malvidin-3-glucoside excreted in urine (P=0.005)

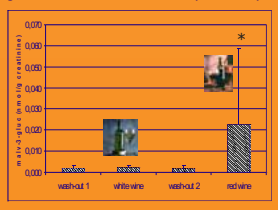


Figure 5. Cromatogram of malvidin-3-glucoside (*m/z* 493/331)

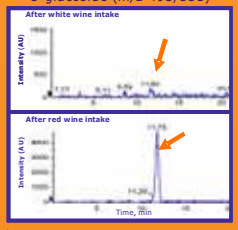


Table 1. Phenolic characteritzation of wines

	SPARKLING WINE	WHITE WINE	RED WINE
Grape variety	Chardonnay	Xarel·lo	Tempranillo
Alcohol strength (%)	12,5	12,5	12,5
Total phenolic content (mg gallic acid/L)	202	308	1945
Total resveratrol content (mg/L)	1,21	1,26	12,79
Total anthocyanin (mg/L)	ND	ND	164,85
ANTHOCYANINS by HPLC (mg/L)			
Malvidin-3-glucoside			92,8
Delphinidin-3-glucoside			21,14
Peonidin-3-glucoside			4,24
Petunidin-3-glucoside			24,22
Malvidin-6-acetil-3-glucoside			11,78
Malvidin-6-coumaroil-3-glucoside			10,67
			164,85
RESVERATROL by HPLC (mg/L)			
trans-resvetratrol	0,137	0,327	1,677
cis-resveratrol	0,126	0,159	0,616
trans-piceid	ND	0,801	2,782
cis-piceid	0,922	0,696	7,716

References

1. Renaud, SC; et al. Am J Clin Nutr. 80:621-625 (2004).
2. Potischman, N. J Nutr. 133 Suppl 3:875S-880S (2003).
3. Meng,X; et al. J.Agric.Food Chem. 52:935-942 (2004).
4. Walle, T; et al. Drug Metab Dispos. 32:1377-1382 (2004).
5. Bub, A; et al. Eur J Nutr. 40:113-120 (2001).
7. Urpi-Sarda,M; et al. Anal Chem. 77: 3149-3155 (2005).
8. Andres-Lacueva,C; et al. Nutr Neurosci. 8:111-120 (2005).

Acknoledgement

The authors express their gratitude to the financial support of *Red de Grupo* G03/140 from the *Instituto de Salud Carlos* III and of AGL2004-08378-C02-01 from Education and Science Ministry (MEC). R. Z.-R. was supported by *Departament d'Universitats, Recerca i Societat de la Informació* and M. U-S. and C. A.-L. thank the FPI fellowship and Ramon y Cajal Programs, respectively, from MEC and European Social Fund. We thank Dr. Maite Ibern-Gomez the project management. We are grateful to Dr. Olga Jauregui from Scientific and Technical Services (University of Barcelona) and to Dr. Bénédicte Duretz of Applied Biosystems for technical assistance and advice in the LC-MS/MS analyses.

II International Conference on Polyphenols and Health, October 4-7, 2005, Davis, California

Comunicación 4: Poster

Zamora-Ros, R.; Lamuela-Raventós, R.M; Andrés-Lacueva, C.

Development of a food composition database for the estimation of dietary intake of resveratrol.

I World Congress International of Public Health Nutrition. Barcelona (SPAIN) 2006

DEVELOPMENT OF A FOOD COMPOSITION DATABASE FOR THE ESTIMATION OF DIETARY INTAKE OF RESVERATROL

Zamora-Ros R, Lamuela-Raventós RM[a], Urpí-Sardà M, Andrés-Lacueva C.

Nutrition and Food Science Department-CeRTA, Faculty of Pharmacy, University of Barcelona, Spain.
E-mail: candres@ub.edu

Introduction

Resveratrol (3,5,4'-trihydroxystilbene) is the parent compound of a family of molecules, including glycosides (piceid), existing in *cis* and *trans* configurations, and polymers (viniferins) classified as stilbens.

Resveratrol is of great interest in nutrition and medicine due to its potential health benefits, sch as anti-carcinogenic properties (1, 2), neuroprotective effects (3), antioxidant capacity, modulation of lipid and lipoprotein metabolism, antiplatelet aggregation (4), and estrogenic activity. Caloric restriction is probably the only other treatment for which such a broad array of protective effects is observed in mammals (5). Indeed, resveratrol has been hypothesized to use the same pathways activated by caloric restriction (5, 6).

To our knowledge, only one epidemiologic study exist concerning the daily consumption of the *trans*-resveratrol, tertil 2 ranged from 0.0731 to 0.1607 mg/day, in adult women of Swiss Canton of Vaud (7). However, only white and red wines and grapes were taking intoaccount as resveratrol sources. In this study was concluded an inverse association between resveratrol from grapes, but not from wine, and breast cancer risk.

Aim of this study

The aim of the present study was to develop a new food composition database on resveratrol content from foods and beverages based on the literature survey.

Material and methods

We performed a literature search on the food content of these compounds using the Medline and Food Science and Technology Abstracts databases. The relevant papers containing analytical data were selected. The data were inserted in the database using Access software, which is specially developed for data acquisition, compilation, and retrieval. Each composition value was attached to the description of the food or beverage, the literature source, the sampling plan and procedures, the analytical method, and the analytical quality control of the laboratory. The individual food items were compiled in food groups with noun "non specified". The relative quality and the reliability of the data were measured with a quality index, which was evaluated following the key points originally developed in the EU-AIR NETTOX Project (8):

- Is the food correctly identified?
- Is the food acceptable market quality?
- Has the chemical identity of the bioactive compound been correctly established?
- Are the extraction and analytical procedures reliable?
- Have appropriate sampling procedures been applied?

It was agreed that the grading of papers dealt with by evaluators should be:

- A: data that was thoroughly reliable.
- B: data that may have been sound but did not provide enough information to meet all the required criteria for an A grade.
- C: data that was not of the best quality that might be expected by current standards.
- U: data for which there was insufficient information available to allow assignment to other categories.

Table 1. Content of resveratrol and piceid in group foods (ppm)

Food Name	Nº references	Nº samples	*trans*-resveratrol	*cis*-resveratrol	*trans*-picoid	*cis*-picoid	sume	Quality Index
Red wine	36	1881	2,10	2,81	4,03	1,22	10,16	A
White wine	9	259	0,14	0,12	0,27	0,16	0,69	A
Rose wine	3	36	0,53	0,52	0,51	0,79	2,35	B
Sparkling wine	3	199	0,08	0,14	0,11	0,31	0,65	A
Fortified wines	4	62	0,37	0,20	1,09	0,39	2,05	C
White grape juice	4	133	0,05	0,00	0,16	0,43	0,64	B
Red Grape Juice	5	43	0,11	0,01	1,13	0,26	1,50	B
White Grape raw	6	23	0,49	0,00	1,43	nd	1,92	B
Red Grape raw	8	66	2,88	0,00	0,91	0,00	3,80	B
Sultanas or raisins	1	1	nd	nd			nd	C
Peanut, raw	9	208	0,37				0,37	A
Peanut, roasted	3	24	0,06				0,06	B
Peanut, boiled	1	11	3,06				3,06	C
Peanut butter	3	35	0,43				0,43	B
Pistachio, raw	1	7	1,17				1,17	C
Cranberry, raw	1	2	0,12				0,12	C
Cranberry, juice	2	2	tr				tr	C
Blueberry, raw	2	71	0,07				0,07	B
Blueberry, baked	1	20	0,01				0,01	C
Sparkleberry, raw	1	3	0,04				0,04	C
Bilberry raw	2	7	0,06				0,06	C
Bilberry baked	1	5	0,02				0,02	C
Deerberry, raw	1	17	0,03				0,03	C
Lingonberry, raw	1	2	0,76				0,76	C
Partridgeberry, raw	1	2	0,12				0,12	C
Hop pellets	2	10	5,35	nd	3,55	0,52	9,42	B
Itadori tea (infusion)	1	1	0,68	nd	9,06	nd	9,74	C
Rhubarb, raw	2	2	detected				detected	U

nd. not detected; tr. Traces.

Results

Adequate data for the resveratrol content were available from 85 published studies providing 160 individual food items classified in 27 food groups. The highest values found for total resveratrol is for red wine and itadori tea; moderate levels are observed in grape and grape products; and low levels are observed in berries, peanuts, and pistachios (Table 1). Table 2 shows the food group "red wine non specified" divided in specific red wines. The figure 1 shows the number of papers which contained food composition data classified by food group. The figure 2 shows the quality of total Food Composition DataBase by means of the % of quality index grade of food groups.

Table 2. Content of resveratrol and piceid in specific red wines

Food Name	Nº references	Nº samples	*trans*-resveratrol	*cis*-resveratrol	*trans*-picoid	*cis*-picoid	sume	Quality Index
Pinot noir	11	243	3,57	1,59	2,47	0,68	8,31	A
Merlot	11	41	2,80	2,09	5,32	1,17	11,38	B
Cabernet Sauvignon	14	257	1,70	1,22	0,77	0,46	4,14	A
Tempranillo	4	21	1,24	0,41	0,97	0,59	3,21	B
Grenache	2	17	1,91	0,88	2,63	0,89	6,30	B
Gamay	5	61	1,86	1,09	na	na		C
Beaujolais nouveaux	1	6	3,60	na	na	na		C
Zinfandel	2	20	2,13	1,80	na	na		C
Shirah	2	18	2,23	na	na	na		C
Nebbiolo	1	19	1,60	na	na	na		C
Sangiovese	2	17	2,41	na	na	na		C
Cabernet franc	5	22	1,19	1,86	na	na		C
Muscadine	2	16	7,58	9,20	na	na		C
Cencibel	1	1	0,16	0	0	0	0,16	U
Moristel	1	2	1,60	0,92	na	na		C
Monastrell	1	58	1,23	0,00	8,72	2,26	12,21	B
Marechal Foch	1	1	1,04	1,22	na	na		C
DeChaunac	1	1	0,04	0,10	na	na		C
Baco noir	1	1	0,03	0,90	na	na		C
Concord	2	7	1,33	1,84	na	na		C
Lemberger	1	9					11,53	U
Xinomauro	1	4	0,83	na	na	na		C
Liatiko	1	3	1,05	na	na	na		C
Mandilaria	1	2	1,62	na	na	na		C
Agiorgitiko	2	8	0,55	na	na	na		C
Blended wines	26	1013	1,67	3,48	1,87	0,86	7,89	B

Na, not analyzed; nd, not detected.

Figure 1. Number of references by food group.

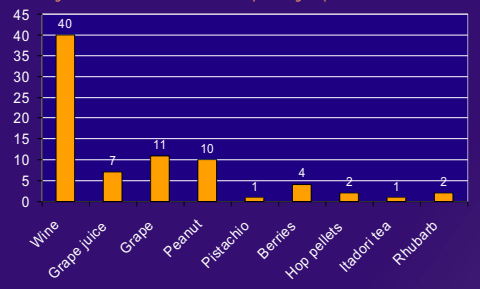
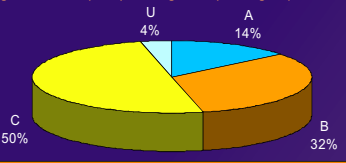


Figure 2. % of quality index grade by food groups.



Conclusions

In the future, this resveratrol food database will be used to calculate the dietary exposure of the resveratrol and to study the associations of resveratrol intake with the risk of cardiovascular disease and cancer, in epidemiological, intervention and clinical studies to predict the health protective effect of resveratrol in food and beverages (eg. a moderate consumption of wine) in a context of the Mediterranean diet.

References

1. Delmas, D; et al. Curr Drug Targets. 7:423-442 (2006).
2. Signorelli, P & Ghidoni, R. J Nutr Biochem. 16:449-466 (2005).
3. Dore,S. Neurosignals. 14:61-70 (2005).
4. Olas, B & Wachowicz, B. Platelets. 16:251-260 (2005).
5. Baur, JA & Sinclair, DA. Nat Rev Drug Discov. 5:493-506 (2006).
6. Ingram, DK; et al. Aging Cell. 5:97-108 (2006).
7. Levi, F; et al. Eur J Cancer Prev. 14:139-142 (2005).
8. Moller, A; et al. http://www. foodcomp. dk/basis/ . (1998).

Acknoledgement

R. Z.-R. was supported by *Departament d'Universitats, Recerca i Societat de la Informació* and M. U-S. thank the FPI fellowship from MEC. The authors express their gratitude to the financial support of *Red de Grupo* G03/140 from the *Instituto de Salud Carlos* III and of AGL2004-08378-C02-01 from Education and Science Ministry (MEC).

I World Congress of Public Health Nutrition, September 28-30, 2006, Barcelona, Spain

Comunicación 5: Comunicació oral invitada.

Zamora-Ros, R.; Urpí-Sardà, M.; Lamuela-Raventós, R.M.; Estruch, R.; Vázquez-Agell, M.; Serrano-Martínez, M.; Jaeger, W.; Andres-Lacueva, C.

Urinary resveratrol metabolites as a biomarker of moderate wine consumption.

11th Congress of the European Society for Biomedical Research on Alcoholism.

Berlin (GERMANY) 2007

ISSN 0735-0414

Volume 42 Supplement 1 August/September 2007

ALCOHOL AND ALCOHOLISM

International Journal of the
Medical Council on Alcohol

Journal of the European Society for
Biomedical Research on Alcoholism

ABSTRACTS ESBRA 2007

www.alcalc.oxfordjournals.org

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

a similar magnitude as abstinence. Low-dose consumption on a controlled drinking situation should be considered on those chronic alcohol abusers who are not able to achieve complete abstinence.

Presentation S28-3

SCIENTIFIC EVIDENCE OF THE BENEFICIAL EFFECTS OF MODERATE ALCOHOL CONSUMPTION ON HEALTH

Estruch R, Sacanella E, Vazquez-Agell M, Mena MP, Monagas M, Fernandez-Solà J (Spain)

Aims. Numerous epidemiologic studies have found an association between moderate alcohol consumption and a reduced risk of coronary heart disease and ischemic stroke.
On the other hand, other types of evidence are related to the biologic plausibility of this hypothesis. Since atherosclerosis seems to be an inflammatory disease, the aims of the clinical trials performed were to evaluate the effects of red and white wines compared to those of an alcoholic beverage with low polyphenol content, on inflammatory biomarkers related to atherosclerosis.
Methods. Two randomized, crossover trials studies were performed. In study 1, 20 men consumed 30 g ethanol/day as cava (sparkling wine) or gin over 28 days, after a 15-day washout period. In study 2, 35 women consumed 20 g ethanol/day as white or red wine for 28 days. Serum and urine samples were collected after each 28-day period in both studies. Adhesion molecules involved in lymphocyte and monocyte—endothelium interactions were determined on the cell surface, and adhesions of human monocytes to endothelial cells were also measured in basal and stimulated conditions.
Results. Serum levels of C-reactive protein, intercellular adhesion molecule-1, CD40L, and interleUkin-6 decreased after either alcoholic beverage ($P < 0.01$; all). However, red wine and cava showed higher anti-inflammatory properties probably due to their polyphenolic content.
Conclusions. Although there is increasing evidence on the beneficial effects of moderate alcohol consumption on the health, without data from large randomized clinical trials, it is unclear how a physician can be in a position to advise his or her patients.

Presentation S28-4

URINARY RESVERATROL METABOLITES AS A BIOMARKER OF MODERATE WINE CONSUMPTION

Zamora-Ros R, Urpi M, Sarda Rosa M, Lamuela-Raventos Estruch, R, Vazquez-Agell M, Serrano-Martinez M, Jaeger W, Andres-Lacueva C (Spain and USA)

Aims. Background: Nutritional biomarkers may be better measures of dietary exposure than self-reported dietary data. We evaluated resveratrol metabolites, potential biomarkers of wine consumption. in humans after moderate consumption of sparkling, white, or red wines.
Methods. We performed 2 randomized, crossover trials and a cohort study. In the first study, 10 healthy men consumed 30 g of ethanol/day as sparkling wine or gin for 28 days. In the second trial, 10 healthy women consumed 20 g of ethanol/day as white or red wine for 28 days. We also evaluated 52 participants in a study on the effects of a Mediterranean diet on primary prevention of cardiovascular disease (the PREDIMED Study). We used liquid chromatography-tandem mass spectrometry to analyze urinary total resveratrol metabolites (TRMs) and predictive values and ROC curve analyses to assess the diagnostic accuracy.
Results. We observed significant increases in TRMs [72.4 (95% confidence interval, 48.5–96.2; $P = 0.005$), 211.5 (166.6–256.3; $P = 0.005$), and 560.5 nmol/g creatinine (244.9–876.1; $P = 0.005$)] after consumption of sparkling, white, or red wine, respectively, but no changes after the washout or gin periods. In the cohort study, the reported daily dose of wine consumption correlated directly with TRMs ($r = 0.654$; $P < 0.001$). Using a cutoff of 90 nmol/g, we were able to use TRMs to differentiate wine consumers from abstainers with a sensitivity of 72% (60%–84%); and a specificity of 94% (87%–100%).
Conclusions. Resveratrol metabolites in urine may be useful biomarkers of wine intake in epidemiologic and intervention studies.

SYMPOSIUM 29 WEDNESDAY SEPT. 26TH 9.00 AM–10.30 AM; ROOM: LECTURE HALL 1

GABA-B receptor: a new target for treating alcohol dependence?

Chairpersons: Leite-Morris KA (USA), Colombo G (Italy)

Presentation S29-1

ACTIVATION OF THE GABA(B) RECEPTOR IN ANIMAL MODELS OF ALCOHOL DEPENDENCE

Colombo G, Maccioni P, Orrù A, Lobina C, Agabio R, Addolorato G, Gessa GL, Carai MAM (Italy)

Aims. The present paper summarizes the different lines of experimental evidence featuring the suppressing effect of the GABA(B) receptor full agonists, baclofen and CGP44532, and positive allosteric modulators, CGP7930 and GS39783, on different alcohol-motivated behaviors.
Methods. These studies have been conducted testing different procedures of alcohol intake and alcohol self-administration in Sardinian alcohol-preferring (sP) rats, one of the few rat lines selectively bred worldwide for high alcohol preference and consumption.
Results. Administration of non-sedative doses of baclofen, CGP44532, CGP7930, and GS39783 to sP rats have been found to suppress: (a) acquisition and maintenance of alcohol drinking behavior under the standard 2-bottle 'alcohol vs water' choice regimen; (b) relapse-like drinking after a period of alcohol abstinence (the so-called 'alcohol deprivation effect'); (c) the increase in alcohol intake induced by the acute administration of opioids and cannabinoids; (d) oral self-administration of alcohol in rats trained to lever-press for alcohol on an FR4 schedule of reinforcement; (e) the motivational properties of alcohol, measured by the progressive ratio schedule of reinforcement and the extinction responding procedure in rats previously trained to self-administer alcohol on an FR4 schedule; (f) reinstatement (induced by the presentation of non-contigent, alcohol-associated stimuli) of alcohol-seeking behavior in rats trained to self-administer alcohol (another experimental model of alcohol relapse). Finally, acute administration of baclofen has been found to suppress the severity of different signs of alcohol withdrawal syndrome, including tremors and seizures, in Wistar rats made physically dependent on alcohol.
Conclusions. Taken together, these data suggest the involvement of the GABA(B) receptor in the neural substrate(s) controlling alcohol intake and relapse-like drinking, mediating alcohol's reinforcing and motivational properties, and underlying alcohol withdrawal syndrome. Of interest, subsequent preliminary clinical studies with baclofen have extended to human alcohol-dependent patients the majority of the above observations (Addolorato *et al*., this meeting).

Presentation S29-2

GABA(B) RECEPTOR ACTIVATION MODULATES NEUROTRANSMITTER LEVELS DURING ALCOHOL SEEKING

Leite-Morris KA (USA)

Aims. Dopamine neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and other forebrain regions are implicated in the motivational behaviors related to ethanol seeking and intake. This presentation will discuss the findings that direct activation of GABA (B) receptors in the VTA modulate mesoaccumbens circuits and alter dopamine responses in the NAc that underlie the motivation of appetitive behaviors.
Methods. An animal model combining in vivo microdialysis with alcohol self-administration was designed to collect brain dialysate concurrently while subjects were lever pressing (extinction sessions). Long Evans rats were trained to perform a fixed number of lever presses (RR20) for a 20 minute presentation of 2% sucrose or 10% ethanol. Subjects were surgically implanted with guide cannula into the VTA for microinjections and NAc for in vivo microdialysis.
Following administration of intra-VTA artificial cerebral spinal fluid (aCSF), or the GABA (B) receptor agonist baclofen (0.25, 0.5, 1.0 ug) and/or the antagonist CGP 35348 (10 ug), single extinction sessions were performed each week. Brain dialysate was collected at 5 minute intervals prior to and during extinction trials.
Results. Intra-VTA administration of baclofen (versus aCSF) resulted in a significant dose-dependent inhibition of ethanol seeking (lever pressing)

Comunicación 5: Poster

Lamuela-Raventós, R.M.; Zamora-Ros, R.; Urpí-Sardà, M.; Estruch, R.; Vázquez-Agell, M.; Jaeger, W.; Andrés-Lacueva, C.

Phenolic metabolites as nutritional biomarkers in humans. Two randomized controlled clinical trials.

III International Congress on wine and health. Bordeaux (FRANCE) 2007

PHENOLIC METABOLITES AS NUTRITIONAL BIOMARKERS IN HUMANS. TWO RANDOMIZED CONTROLLED CLINICAL TRIALS

R. M. Lamuela-Raventós[1*], R. Zamora-Ros[1], M. Urpí-Sardà[1], R. Estruch[2], M. Vázquez-Agell[2], W. Jaeger[3], C. Andrés-Lacueva[1]

[1]Nutrition and Food Science Department-XaRTA,INSA, Pharmacy School, University of Barcelona, Spain.
[2]Departament de Medicina Interna, Hospital Clínic, IDIBAPS, University of Barcelona, Spain.
[3]Institute of Pharmaceutical Chemistry. University of Vienna, Vienna, Austria.
*E-mail: lamuela@ub.edu

Introduction

Several studies have reported an association between moderate wine consumption and a lower risk CHD[1]. In nutritional studies, accurate quantification of diet is critical. For this reason, nutritional biomarkers are used to measure exposure because that have less error than dietary data[2].

Resveratrol (3, 5, 4'-trihydroxystilbene) and piceid (resveratrol-3*O*-*β* -glucoside) are stilbenes present mainly in grapes and wine. Bioavailability of resveratrol in humans has been scarcely studied. After single dose intake (0.014mg/Kg) glucuronidate form was the only metabolite detected in urine [3]. In plasma it's needed a high dose (0.3mg/Kg) to detect resveratrol metabolites [4]. To our knowledge, this is the first time it has been measured resveratrol metabolites after a regular wine intake.

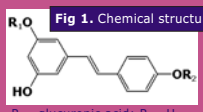
Malvidin-3-glucoside (M-3-G) is an anthocyanin caracteristic of red grape and red wine responsable of the color. This compound is not present in white wines, so it could be a good biomarker when white *versus* red wine intake is tested. Previous studies of bioavailability of anthocyanins in humans in single dose showed poor absorption of M-3-G (1-5% of the ingested amount), and M-3-G not detected after 6 hours of last intake [5].

Biomarkers of nutrient intake are useful in epidemiological and clinical assays and are preferred over purely dietary data. Considering the limitations of the food composition data, direct nutritional markers are more precise and provide a more proximal measure of specific nutrient intake as an integrated measure of the metabolism of the component.

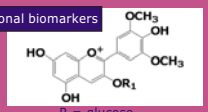
Aim of this study

- To purpose a biomarker of moderate wine intake
- To determine resveratrol metabolites after regular and moderate wine consumption.
- To determine M-3-G after regular and moderate red wine intake.

Fig 1. Chemical structures of nutritional biomarkers



R_1= glucuronic acid; R_2= H
***trans*-Resveratrol-3-*O*-glucuronide**



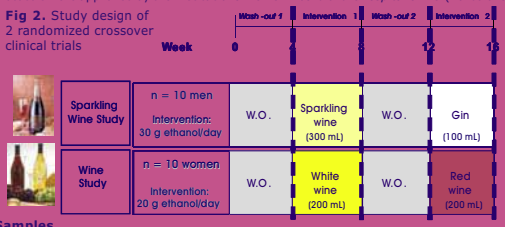
R_1= glucose
Malvidin-3-glucoside

Material and methods

Subjects a study design

Protocols were approved by the Institutional Review Board of the*Hospital Clinic* (Barcelona).

Fig 2. Study design of 2 randomized crossover clinical trials



Samples

After overnight fasting of last day of intervention or wash-out period, morning urine and serum were collected.

Urine and serum were acidified until 200mM with HCl and were stored at -80ºC.

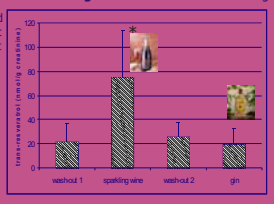
Analysis sample

		Resveratrol (Urpi-Sarda et al. 2005)	Anthocyans (Andres-Lacueva et al. 2005)
LC-MS/MS		Perkin Elmer series 200 (Norwalk, CT, USA), API 3000 triple quadrupole mass spectometer (Applied Biosystems) (PE Sciex, Concord, Ontario, Canada)	
Mobile Phases		A/ 0.05% acetic acid B/ Acetonitrile	A/ 5% formic acid B/ Acetonitrile
Column		Luna Stable Bond C_{18} (150 x 2.0 mm, 5µm)	Zorbax Stable Bond C_{18} (150 x 2.1 mm, 5µm)
Flow-rate, injection volume		400 µL/min; 15 µL	
Source		Turbo Ion Spray	
Ionization Mode		Negative	Positive
Acquisition Mode		Multiple Reaction Monitoring (MRM)	
Metabolites	Compound	Resveratrol-3-*O*-glucuronide	Malvidin-3-GAL or GLU
	MW	404	492
	MRM	403/227	493/331

Results and conclusions

- Following 28 days of dietary supplementation with sparkling wine, white wine and red wine, *trans*-resveratrol-3-*O*-glucuronide was found in the urine of all volunteers.
- Only baseline levels of resveratrol metabolites were detected in the urine of control diet volunteers.
- No resveratrol metabolites were detected in serum samples in both studies.
- Following 4 weeks of red wine consumption, M-3-G was observed in the urine of all volunteers, while traces levels were found after white wine or wash-out periods.
- Advances in analytical techniques let propose resveratrol glucuronide in urine as biomarker of wine consumption. M-3-G may be used as biomarker only when red wine is compared*versus* white wine intake.
- In the future, this study could be the base for the application of these biomarkers in epidemiological or intervention studies.

Fig 2. Amounts of resveratrol glucuronide excreted in urine (P>0.05)



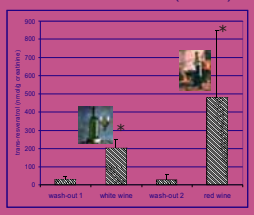


Figure 4. Amounts of malvidin-3-glucoside excreted in urine (P=0.005)

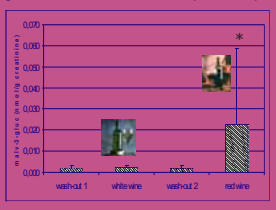


Figure 5. Cromatogram of malvidin-3-glucoside (*m/z* 493/331)

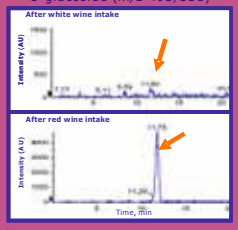


Table 1. Phenolic characteritzation of wines

	SPARKLING WINE	WHITE WINE	RED WINE
Grape variety	Chardonnay	Xarel·lo	Tempranillo
Alcohol strength (%)	12,5	12,5	12,5
Total phenolic content (mg gallic acid/L)	202	308	1945
Total resveratrol content (mg/L)	1,21	1,26	12,79
Total anthocyanin (mg/L)	ND	ND	164,85
ANTHOCYANINS by HPLC (mg/L)			
Malvidin-3-glucoside			92,8
Delphinidin-3-glucoside			21,14
Peonidin-3-glucoside			4,24
Petunidin-3-glucoside			24,22
Malvidin-6-acetil-3-glucoside			11,78
Malvidin-6-coumaroil-3-glucoside			10,67
			164,85
RESVERATROL by HPLC (mg/L)			
trans-resvetratrol	0,137	0,327	1,677
cis-resveratrol	0,126	0,159	0,616
trans-piceid	ND	0,801	2,782
cis-piceid	0,922	0,696	7,716

References

1. Renaud, SC; et al. Am J Clin Nutr. 80:621-625 (2004).
2. Potischman, N. J Nutr. 133 Suppl 3:875S-880S (2003).
3. Meng,X; et al. J.Agric.Food Chem. 52:935-942 (2004).
4. Walle, T; et al. Drug Metab Dispos. 32:1377-1382 (2004).
5. Bub, A; et al. Eur J Nutr. 40:113-120 (2001).
7. Urpi-Sarda,M; et al. Anal Chem. 77: 3149-3155 (2005).
8. Andres-Lacueva,C; et al. Nutr Neurosci. 8:111-120 (2005).

Acknowledgements

The authors express their gratitude to the financial support of Red de Grupo G03/140 and RETICS RD06/0045/0003 from the Instituto de Salud Carlos III and of CICYT´s (AGL2005-002823 and AGL2006-14228-C03-02) and CONSOLIDER CSD2007-063 from Education and Science Ministry (MEC). R. Z-R. was supported by Departament d'Universitats, Recerca i Societat de la Informació and M. U-S. thank the FPI fellowship from MEC. We thank Dr. Maite Iberni-Gomez the project management. We are grateful to Dr. Olga Jauregui from Scientific and Technical Services (University of Barcelona).

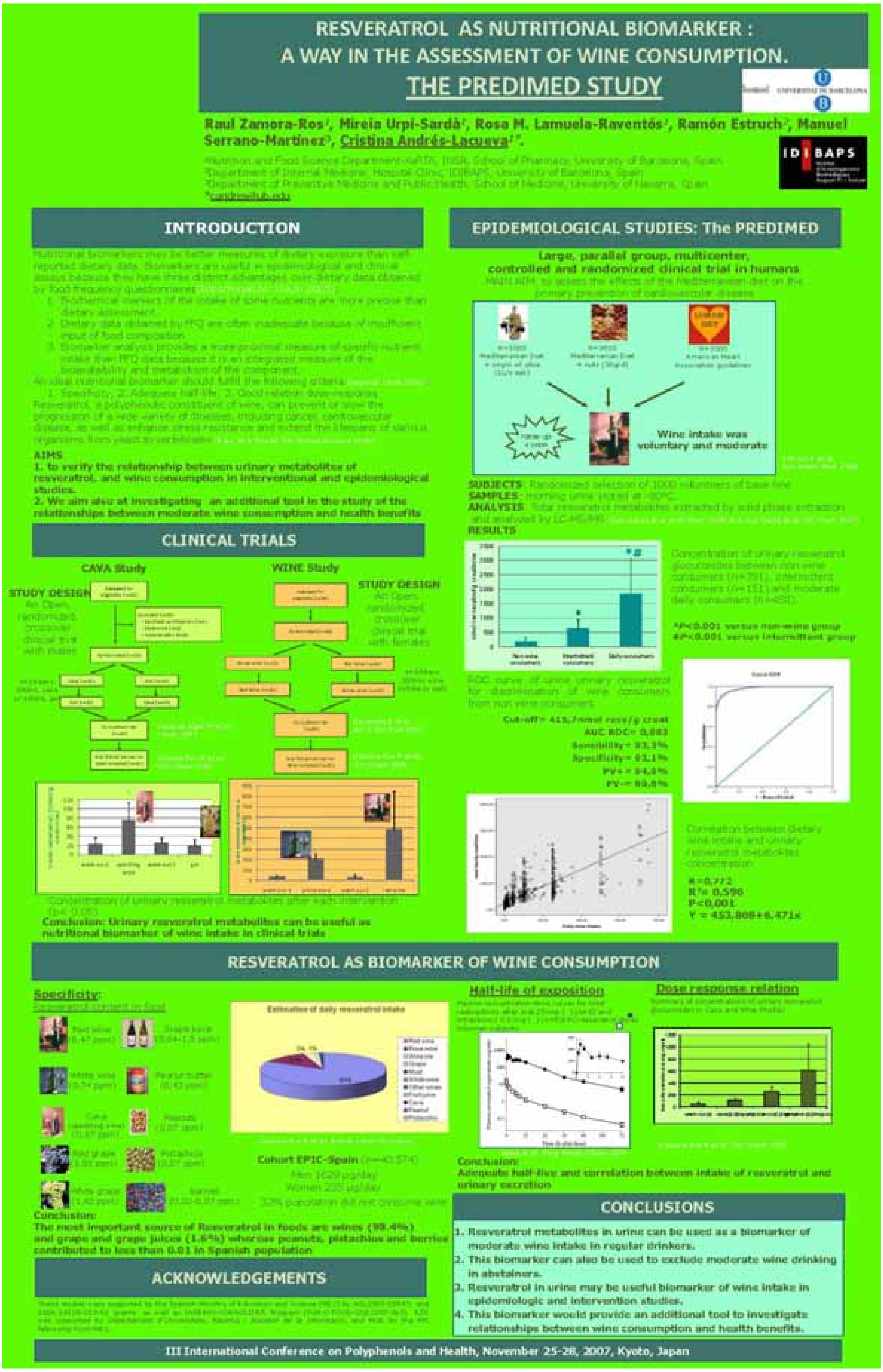
III International Congress on wine and health, September 20-22, 2007, Bordeaux, France

Comunicación 6: Poster

Zamora-Ros, R.; Urpí-Sardà, M.; Lamuela-Raventós, R.M.; Estruch, R.; Serrano-Martínez, M.; Andrés-Lacueva, C.

Resveratrol as nutritional biomarker: a way in the assessment of wine consumption. The PREDIMED Study.

III International Conference on Polyphenols and Health. Kyoto (JAPAN) 2007

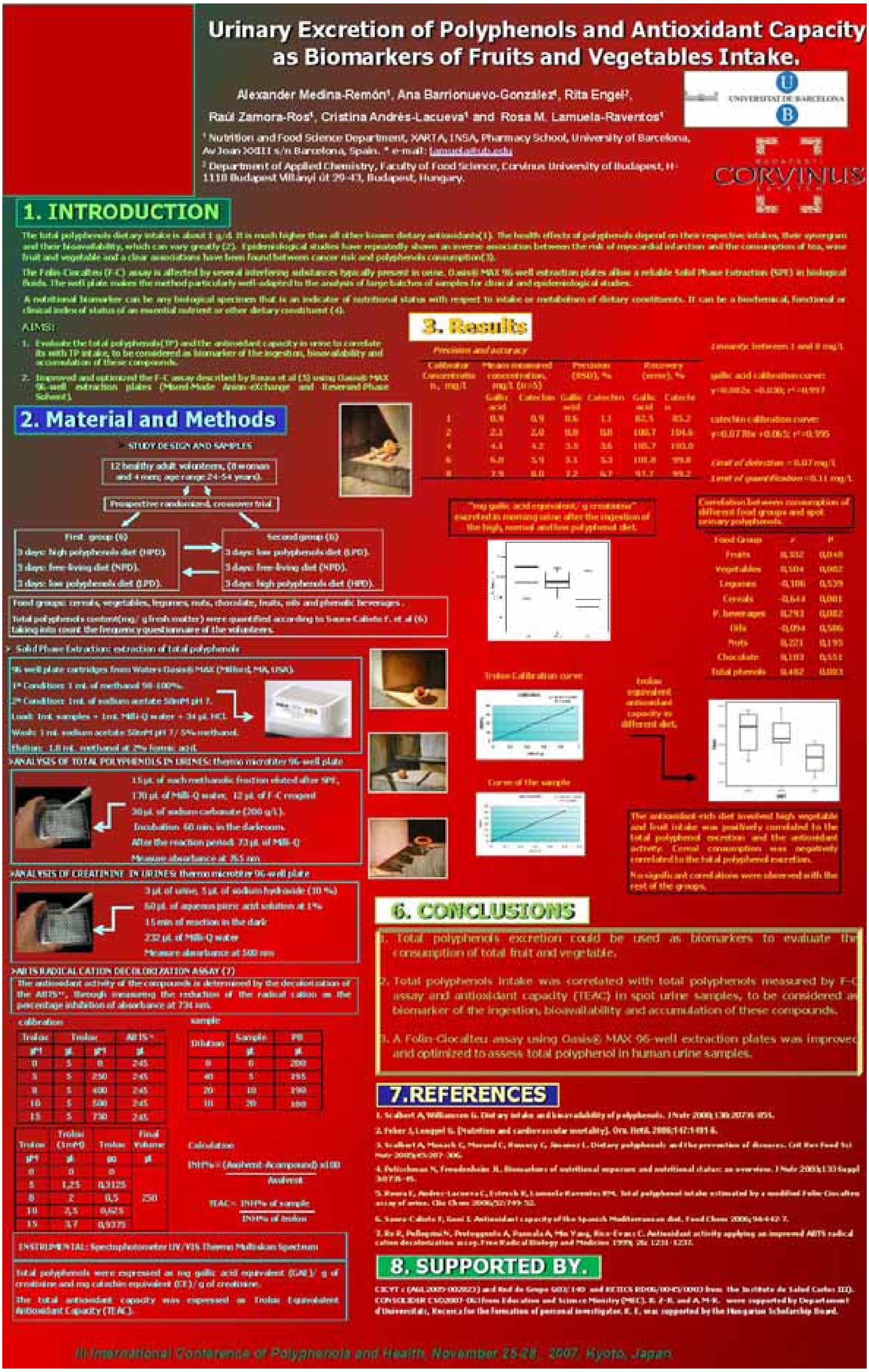

RESVERATROL AS NUTRITIONAL BIOMARKER :
A WAY IN THE ASSESSMENT OF WINE CONSUMPTION.
THE PREDIMED STUDY
Raul Zamora-Ros[1], Mireia Urpí-Sardà[1], Rosa M. Lamuela-Raventós[1], Ramón Estruch[2], Manuel Serrano-Martínez[3], Cristina Andrés-Lacueva[1*].
INTRODUCTION
AIMS
1. to verify the relationship between urinary metabolites of resveratrol, and wine consumption in interventional and epidemiological studies.
2. We aim also at investigating an additional tool in the study of the relationships between moderate wine consumption and health benefits
EPIDEMIOLOGICAL STUDIES: The PREDIMED
Large, parallel group, multicenter, controlled and randomized clinical trial in humans
Wine intake was voluntary and moderate
SUBJECTS
SAMPLES
ANALYSIS
RESULTS
*P<0.001 versus non-wine group
#P<0.001 versus intermittent group
Cut-off= 411,7nmol resv/g creat
AUC ROC= 0,883
Sensibility= 83,3%
Specificity= 82,1%
PV+= 84,0%
PV-= 80,8%
R=0,772
R²= 0,596
P<0,001
Y = 453,808+6,471x
CLINICAL TRIALS
CAVA Study
WINE Study
STUDY DESIGN
STUDY DESIGN
Conclusion: Urinary resveratrol metabolites can be useful as nutritional biomarker of wine intake in clinical trials
RESVERATROL AS BIOMARKER OF WINE CONSUMPTION
Specificity:
Estimation of daily resveratrol intake
Cohort EPIC-Spain
Half-life of exposition
Dose response relation
Conclusion:
The most important source of Resveratrol in foods are wines (98.4%) and grape and grape juices (1.6%) whereas peanuts, pistachios and berries contributed to less than 0.01 in Spanish population
Conclusion:
Adequate half-live and correlation between intake of resveratrol and urinary excretion
ACKNOWLEDGEMENTS
CONCLUSIONS
1. Resveratrol metabolites in urine can be used as a biomarker of moderate wine intake in regular drinkers.
2. This biomarker can also be used to exclude moderate wine drinking in abstainers.
3. Resveratrol in urine may be useful biomarker of wine intake in epidemiologic and intervention studies.
4. This biomarker would provide an additional tool to investigate relationships between wine consumption and health benefits.
III International Conference on Polyphenols and Health, November 25-28, 2007, Kyoto, Japan

Comunicación 7: Poster

Medina-Remón, A.; Barrionuevo-González, A.; Engel, R.; Zamora-Ros, R.; Andres-Lacueva, C.; Lamuela-Raventos, RM.

Urinary Excretion of Polyphenols and Antioxidant Capacity as Biomarkers of Fruits and Vegetables Intake.

III International Conference on Polyphenols and Health. Kyoto (JAPAN) 2007



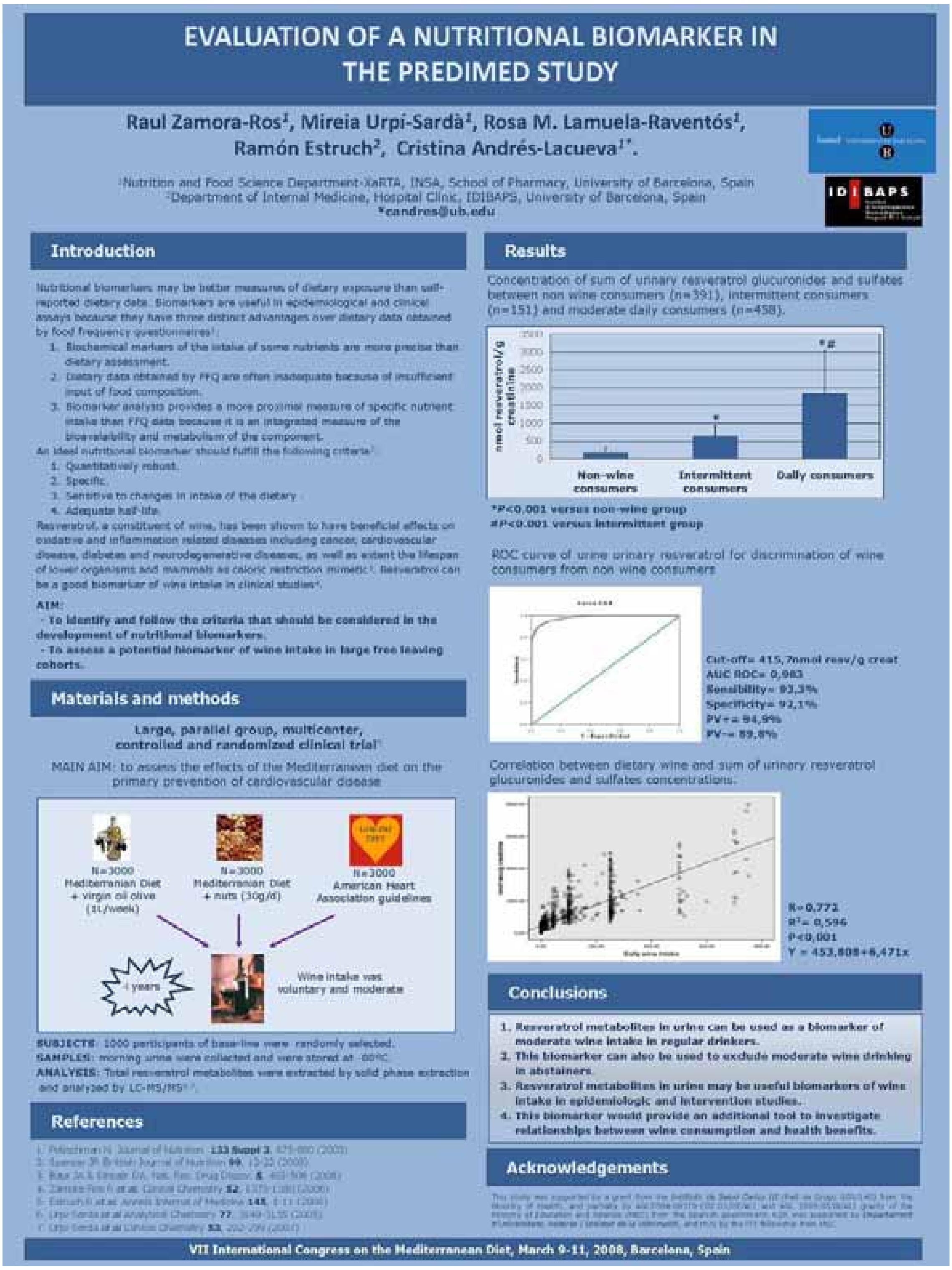
Urinary Excretion of Polyphenols and Antioxidant Capacity as Biomarkers of Fruits and Vegetables Intake.
Alexander Medina-Remón[1], Ana Barrionuevo-González[1], Rita Engel[2],
Raúl Zamora-Ros[1], Cristina Andrés-Lacueva[1] and Rosa M. Lamuela-Raventos[1]
[1] Nutrition and Food Science Department, XARTA, INSA, Pharmacy School, University of Barcelona, Av Joan XXIII s/n Barcelona, Spain.
[2] Department of Applied Chemistry, Faculty of Food Science, Corvinus University of Budapest, H-1118 Budapest Villányi út 29-43, Budapest, Hungary.
1. INTRODUCTION
AIMS:
2. Material and Methods
3. Results
6. CONCLUSIONS
1. Total polyphenols excretion could be used as biomarkers to evaluate the consumption of total fruit and vegetable.
2. Total polyphenols intake was correlated with total polyphenols measured by F-C assay and antioxidant capacity (TEAC) in spot urine samples, to be considered as biomarker of the ingestion, bioavailability and accumulation of these compounds.
3. A Folin-Ciocalteu assay using Oasis® MAX 96-well extraction plates was improved and optimized to assess total polyphenol in human urine samples.
7.REFERENCES
8. SUPPORTED BY.
III International Conference of Polyphenols and Health, November 25-28, 2007, Kyoto, Japan

Comunicación 8: Poster

Zamora-Ros, R.; Urpí-Sardà, M.; Lamuela-Raventós, R.M.; Estruch, R.; Andrés-Lacueva, C.

Evaluation of a nutritional biomarker in the PREDIMED Study.

VII International Congress on the Mediterranean Diet. Barcelona (SPAIN) 2008


EVALUATION OF A NUTRITIONAL BIOMARKER IN THE PREDIMED STUDY
Raul Zamora-Ros1, Mireia Urpí-Sardà1, Rosa M. Lamuela-Raventós1, Ramón Estruch2, Cristina Andrés-Lacueva1*.
1Nutrition and Food Science Department-XaRTA, INSA, School of Pharmacy, University of Barcelona, Spain
2Department of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona, Spain
*candres@ub.edu
IDIBAPS
Introduction
Nutritional biomarkers may be better measures of dietary exposure than self-reported dietary data. Biomarkers are useful in epidemiological and clinical assays because they have three distinct advantages over dietary data obtained by food frequency questionnaires1:
1. Biochemical markers of the intake of some nutrients are more precise than dietary assessment.
2. Dietary data obtained by FFQ are often inadequate because of insufficient input of food composition.
3. Biomarker analysis provides a more proximal measure of specific nutrient intake than FFQ data because it is an integrated measure of the bioavailability and metabolism of the component.
An ideal nutritional biomarker should fulfil the following criteria2:
1. Quantitatively robust.
2. Specific.
3. Sensitive to changes in intake of the dietary.
4. Adequate half-life.
Resveratrol, a constituent of wine, has been shown to have beneficial effects on oxidative and inflammation related diseases including cancer, cardiovascular disease, diabetes and neurodegenerative diseases, as well as extent the lifespan of lower organisms and mammals as caloric restriction mimetic3. Resveratrol can be a good biomarker of wine intake in clinical studies4.
AIM:
- To identify and follow the criteria that should be considered in the development of nutritional biomarkers.
- To assess a potential biomarker of wine intake in large free leaving cohorts.
Materials and methods
Large, parallel group, multicenter, controlled and randomized clinical trial5
MAIN AIM: to assess the effects of the Mediterranean diet on the primary prevention of cardiovascular disease
N=3000 Mediterranian Diet + virgin oil olive (1L/week)
N=3000 Mediterranian Diet + nuts (30g/d)
N=3000 American Heart Association guidelines
4 years
Wine intake was voluntary and moderate
SUBJECTS: 1000 participants of baseline were randomly selected.
SAMPLES: morning urine were collected and were stored at -80ºC.
ANALYSIS: Total resveratrol metabolites were extracted by solid phase extraction and analyzed by LC-MS/MS6,7.
References
Results
Concentration of sum of urinary resveratrol glucuronides and sulfates between non wine consumers (n=391), intermittent consumers (n=151) and moderate daily consumers (n=458).
nmol resveratrol/g creatinine
3500
3000
2500
2000
1500
1000
500
0
Non-wine consumers
Intermittent consumers
Daily consumers
*#
*
*P<0.001 versus non-wine group
#P<0.001 versus intermittent group
ROC curve of urine urinary resveratrol for discrimination of wine consumers from non wine consumers
Cut-off= 415,7nmol resv/g creat
AUC ROC= 0,983
Sensibility= 93,3%
Specificity= 92,1%
PV+= 94,9%
PV-= 89,8%
Correlation between dietary wine and sum of urinary resveratrol glucuronides and sulfates concentrations.
Daily wine intake
R=0,772
R2= 0,596
P<0,001
Y = 453,808+6,471x
Conclusions
1. Resveratrol metabolites in urine can be used as a biomarker of moderate wine intake in regular drinkers.
2. This biomarker can also be used to exclude moderate wine drinking in abstainers.
3. Resveratrol metabolites in urine may be useful biomarkers of wine intake in epidemiologic and intervention studies.
4. This biomarker would provide an additional tool to investigate relationships between wine consumption and health benefits.
Acknowledgements
VII International Congress on the Mediterranean Diet, March 9-11, 2008, Barcelona, Spain