Estudi del benefici de la ingesta de polifenols en una població d'edat avançada i amb risc cardiovascular

Study of the benefits of polyphenol intake in an elderly population at high cardiovascular risk

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Memòria presentada per Anna Tresserra i Rimbau per optar al títol de doctor per la Universitat de Barcelona, dirigida per:

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Abreviatures i acrònims

ACE	*Angiotensin-converting enzyme* (Enzim convertidor de l'angiotensina)
AHA	*American Health Association*
ATPIII	*Adult Treatment Panel III*
C_{max}	Concentració màxima
col.	Col·laboradors
CV	Cardiovascular
DM	Dieta Mediterrània
DMOO	Dieta Mediterrània complementada amb oli d'oliva verge extra
DMFS	Dieta Mediterrània complementada amb fruits secs
DBG	Dieta baixa en greixos
DE	Desviació estàndard
EAG	Equivalents d'Àcid Gàl·lic
EDHF	*Endothelium-Derived Hyperpolarizing Factor* (Factor hiperpolaritzant derivat de l'endoteli)
EFSA	*European Food Safety Administration* (Agència europea de seguretat alimentària)
EGCG	*Epigallocatechin gallate* (gal·lat d'epigal·locatequina)
F-C	Folin-Ciocalteu
FC	Freqüència cardíaca
FEE	Fracció etiològica en exposats
FMD	*Flow Mediated endothelium-dependent Dilation* (dilatació mediada per l'endoteli)
HDL	*High Density Lipoproteins* (Lipoproteïnes d'alta densitat)
HPLC	*High Performance Liquid Cromatography* (Cromatografia de líquids d'alta eficàcia)
HR	*Hazard Ratio*
I	Taxa d'incidència
IA	Taxa d'incidència acumulada
IDF	*International Diabetes Federation*
IC	Interval de confiança
ICAM-1	*Soluble Inter-Cellular Adhesion Molecule-1* (Molècules d'adhesió intercel·lulars solubles)

IGFBP	*Insulin-like Growth Factor Binding Protein*
IMC	Índex de Massa Corporal
INRA	*Institut Nacional de la recherche agronomique*
LDL	*Low Density Lipoproteins* (Lipoproteïnes de baixa densitat)
NHLBI	*National Heart, Lung, and Blood Institute*
NNT	Nombre necessari a tractar
NO	Òxid nítric
OMS	Organització Mundial de la Salut
OR	Odds Ratio
P	Prevalença
PA	Pressió arterial
PAD	Pressió arterial diastòlica
PAS	Pressió arterial sistòlica
PREDIMED	*PREvención con DIeta MEDiterránea*
QFC	Qüestionari de freqüència de consum
RAe	Risc Atribuïble als exposats
ROS	*Reactive Oxygen Species* (Espècies reactives de l'oxigen)
RR	Risc relatiu
RTI	Raó de taxa d'incidència
SM	Síndrome metabòlica
SPE	*Solid Phase Extraction* (Extracció en fase sòlida)
TLGS	*Tehran Lipid and Glucose Study*
UBE	Unitat de Beguda Estàndard
USDA	*United States Department of Agriculture* (Departament d'agricultura dels Estats Units)
UHPLC	*Ultra High Performance Liquid Cromatography* (Cromatografia de líquids d'ultra alta eficàcia)
VCAM-1	*Vascular Cell Adhesion Molecule-1* (Molècules d'adhesió a cèl·lules vasculars)

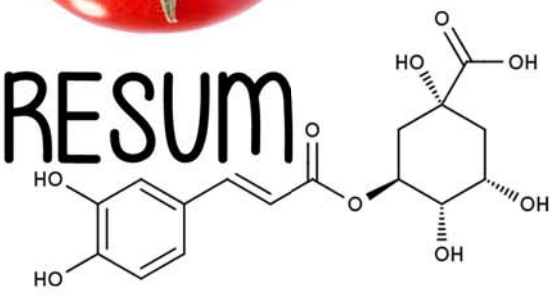
Abbreviations and acronyms

ACE	Angiotensin-converting enzyme
AHA	American Health Association
ARe	Attributable Risk among the exposed
ATPIII	Adult Treatment Panel III
BMI	Body Mass Index
BP	Blood Pressure
C_{max}	Maximum concentration
CI	Cumulative Incidence
CI	Confidence Interval
CV	Cardiovascular
DBP	Dyastolic Blood Pressure
EDHF	Endothelium-Derived Hyperpolarizing Factor
EFSA	European Food Safety Administration
EFE	Etiological Fraction among the Exposed
EGCG	Epigallocatechin gallate
F-C	Folin-Ciocalteu
FMD	Flow Mediated endothelium-dependent Dilation
FFQ	Food Frequency Questionnaire
GAE	Gallic Acid Equivalents
HDL	High Density Lipoproteins
HPLC	High Performance Liquid Cromatography
HR	Hazard Ratio
I	Incidence
ICAM-1	Soluble Inter-Cellular Adhesion Molecule-1
IDF	International Diabetes Federation
IGFBP	Insulin-like Growth Factor Binding Protein
INRA	*Institut Nacional de la recherche agronomique*
IRR	Incidence Rate Ratio
LDL	Low Density Lipoproteins
LFD	Low-fat Diet

MD	Mediterranean Diet
MD-EVOO	Mediterranean Diet supplemented with extra virgin olive oil
MD-nuts	Mediterranean Diet supplemented with nuts
MS	Metabolic Syndrome
NHLBI	National Heart, Lung, and Blood Institute
NNT	Number Needed to Treat
NO	Nitric Oxide
OR	Odds Ratio
P	Prevalence
PREDIMED	*PREvención con DIeta MEDiterránea*
ROS	Reactive Oxygen Species
RR	Relative Risk
SBP	Systolic Blood Pressure
SBU	Standard Beverage Units
SPE	Solid Phase Extraction
SD	Standard Deviation
TLGS	Tehran Lipid and Glucose Study
USDA	United States Department of Agriculture
UHPLC	Ultra High Performance Liquid Cromatography
VCAM-1	Vascular Cell Adhesion Molecule-1
WHO	World Health Organization

Índex

RESUM



I. Resum

Segons la Organització Mundial de la Salut (OMS), més d'un quart de la població mundial pateix algun tipus de malaltia relacionada amb el sistema circulatori[1]. Actualment, les malalties cardiovasculars (CV) són la principal causa de mort i discapacitat en els països desenvolupats. Només a Espanya, l'any 2012, les malalties del sistema circulatori van ser responsables de 122.097 morts, que representen un 33.3% del total de defuncions, seguides dels càncers (27.5%) i de les malalties del sistema respiratori (11.7%)[2]. Però no només són un problema de salut, sinó també econòmic ja que suposen una despesa de 3000 milions d'euros anuals, un 17.7% del cost de tota l'atenció hospitalària del Sistema Nacional de Salut espanyol[3].

La prevenció, més que el tractament, és una prioritat màxima per les agències de salut pública, que s'esforcen per sensibilitzar a la població amb l'objectiu de trencar aquesta tendència. El nostre paper com a investigadors en el camp de la salut és estudiar els factors de risc d'aquestes malalties i buscar la manera de reduir-los, per exemple, amb un canvi d'estil de vida i de dieta.

La dieta mediterrània (DM) i altres dietes riques en fruites i hortalisses s'han considerat una bona manera de reduir la majoria de factors de risc de malalties CV ja que són riques en components bioactius, vitamines i minerals, fibra, i àcids grassos mono i poliinsaturats. En aquest estudi ens hem focalitzat en els polifenols, un grup de compostos que ens aporten la major part d'antioxidant de la dieta i que s'ha demostrat que tenen efectes beneficiosos per a la salut ja que milloren alguns factors de risc com ara l'aterosclerosi, la resistència a la insulina, els biomarcadors d'inflamació, o la pressió arterial, entre d'altres.

Els polifenols són un grup de compostos molt heterogeni i nombrós: hi ha centenars de molècules descrites en diferents aliments i begudes. Aquesta varietat és un problema a l'hora d'estudiar-ne l'efecte, la biodisponibilitat o els mecanismes que expliquen els seus beneficis i, sovint, s'opta per escollir només un polifenol o un grup reduït de polifenols i estudiar-lo amb detall. No obstant, això impedeix tenir una visió global i els grups de polifenols més minoritaris han quedat sistemàticament exclosos dels estudis. Així doncs, aquest treball pretén, en primer lloc, estimar el contingut de polifenols totals ingerits a través de la dieta en la població espanyola de l'estudi PREDIMED (PRevención con DIeta MEDiterránea) i valorar l'associació d'aquests amb el risc de patir una malaltia CV i el risc de mortalitat.

Els qüestionaris de freqüència de consum (QFC) són una eina àmpliament utilitzada en estudis nutricionals. No obstant, els biomarcadors nutricionals són un bon complement ja que cobreixen les limitacions pròpies dels qüestionaris. Aquesta tesi fa una revisió dels últims avenços en biomarcadors de consum de polifenols i s'explica un mètode colorimètric ràpid i senzill, adaptat del mètode de Folin-Ciocalteu (F-C), per determinar polifenols en orina.

La hipertensió és un dels factors de risc CV més freqüents i estudiats. Es coneix que la ingesta d'aliments rics en polifenols pot ajudar a disminuir la pressió arterial (PA) i per tant, evitar un d'aquests factors de risc. En aquesta tesi es revisen les últimes evidències sobre la influència dels polifenols sobre la PA i els mecanismes d'acció que explicarien aquest efecte. Aquests coneixements s'han aplicat en la població PREDIMED per demostrar que, efectivament, la disminució de la PA està mediada per l'increment dels nivells d'òxid nítric (NO) en plasma.

Les begudes alcohòliques han estat tradicionalment una font de conflicte quan es tracta d'establir recomanacions nutricionals o dietètiques, ja que la línia entre el benefici i el risc és molt fina i els efectes dels excessos d'alcohol han suposat una mala publicitat per al sector. Volíem doncs, aportar més informació sobre el consum, sempre moderat, de vi i la síndrome metabòlica, un desordre metabòlic que resulta de la combinació dels principals factors de risc CV: obesitat, hipertensió, dislipèmia i hiperglucèmia.

I. Abstract

According to the World Health Organization (WHO), more than a quarter of the world's population suffers from a circulatory system-related disease[1]. Nowadays, cardiovascular (CV) diseases are the leading cause of mortality and disability in developed countries. In Spain, in 2012, CV diseases were responsible for 122,097 deaths, which represent 33.3% of total deaths, followed by cancer (27.5%) and respiratory-related diseases (11.7%)[2]. Moreover, CV diseases are not only a health issue, but also an economic problem, requiring an annual expenditure of 3,000 million euros by the Spanish state, or 17.7% of the total hospital care under the Spanish National Health System[3].

Prevention, even more than treatment, of CV diseases is a priority for the public health agencies, which make huge efforts to raise awareness in the population with the aim of reversing this trend. Our role as researchers is to study the risk factors of these diseases and how to reduce them, for instance, by changing lifestyles and dietary habits.

The Mediterranean Diet (MD) and other diets rich in fruits and vegetables have been proposed as an effective way to reduce CV risk factors because they are rich in antioxidant components, vitamins, minerals, fiber, and mono and polyunsaturated fatty acids. In this study, we focused on polyphenols, a group of compounds that are the main source of bioactive compounds in our diet and have proven beneficial effects on our health because they improve certain risk factors such as atherosclerosis, insulin resistance, inflammation biomarkers, or blood pressure, among others.

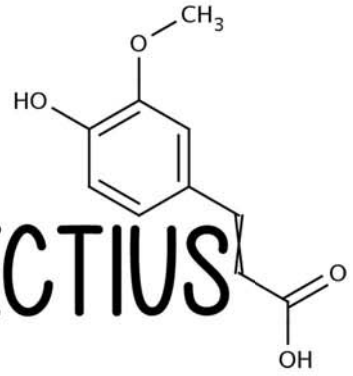
Polyphenols are a large and heterogeneous group of compounds: there are hundreds of molecules described in many foods and beverages. Such variety becomes a problem when studying their effects, bioavailability or the mechanisms of action that explain their benefits. This is the reason why usually only one polyphenol or a group of polyphenols is chosen to study in detail. However, this does not allow a global view and minor polyphenol groups have been systematically excluded from the studies. Therefore, this thesis aims, firstly, to estimate the total intake of polyphenols in the Spanish PREDIMED (PRevención con DIeta MEDiterránea) population and to evaluate the association of dietary polyphenols with a lower risk of CV disease or mortality.

Food Frequency Questionnaires (FFQ) are widely used in nutritional studies. However, nutritional biomarkers are good complements because they cover the limitations of FFQ. This thesis reviews recent evidences on biomarkers of polyphenol intake. Moreover, a new, fast and simple method to determine polyphenols in urine is explained. This method was an adaptation of the colorimetric Folin-Ciocalteu (F-C) method.

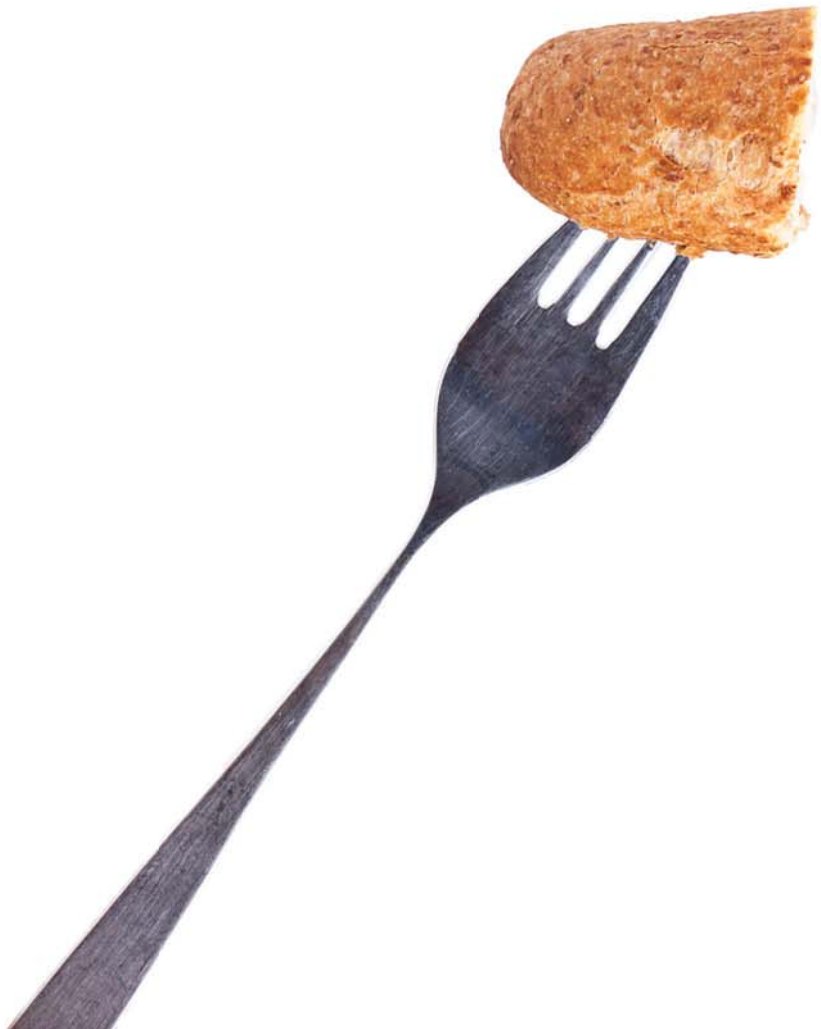
Hypertension is a very frequent and much studied CV risk factor. It is known that consumption of polyphenol-rich foods helps to decrease blood pressure (BP) and, therefore, one of the CV diseases risk factors. In this thesis we reviewed the last evidences about the influence of polyphenols on BP and the mechanisms of action that explain this effect. This knowledge has been applied to the PREDIMED population to demonstrate that, indeed, the BP decrease was mediated by the increase in plasma nitric oxide (NO).

Alcoholic beverages have traditionally been a source of conflict when it comes to establishing dietary recommendations, since the consequences of excessive alcohol intake create a bad image for the products. Therefore, our aim was to provide more information about moderate

wine consumption and metabolic syndrome, a disorder arising from a combination of the main CV risk factors: obesity, hypertension, dyslipidaemia and hyperglycaemia.



HIPÒTESI I OBJECTIUS



II. Hipòtesi i objectius

Hipòtesi

La hipòtesis conceptual general és la següent:

Els polifenols són components de la dieta amb beneficis demostrats per a la salut. La dieta mediterrània, rica en aquests compostos, ha estat proposada com a model exemplar d'alimentació i d'estil de vida. La nostra hipòtesi era que els polifenols de la dieta tindrien un efecte beneficiós en la prevenció primària de malalties cròniques i, en especial, de malalties cardiovasculars, en una població espanyola d'edat avançada i amb un alt risc de patir una malaltia coronària (cohort de l'estudi PREDIMED).

Objectius

L'objectiu principal d'aquesta tesi era estudiar el paper dels polifenols de la dieta en la prevenció primària de malalties cròniques.

Objectius específics:

- ✓ Estimar de forma molt detallada la ingesta de polifenols en una població mediterrània d'avançada edat i risc cardiovascular (PREDIMED) utilitzant la nova base de dades Phenol-explorer i identificar els aliments que més contribueixen a aquesta ingesta.
- ✓ Estudiar l'associació entre el consum de polifenols (totals i per grups) i els esdeveniments cardiovasculars (infart, ictus o mort cardiovascular) en la població del PREDIMED.
- ✓ Estudiar l'associació entre el consum de polifenols (totals i per grups) i la mortalitat per qualsevol causa en la població del PREDIMED.
- ✓ Revisar els últims avenços sobre biomarcadors de consum de polifenols
- ✓ Revisar les últimes evidències sobre la influència dels polifenols sobre la PA i els mecanismes d'acció que explicarien aquest efecte.
- ✓ Avaluar si el consum de polifenols disminueix la PA a través de l'increment de la producció d'òxid nítric (NO) en plasma: estudi de la població PREDIMED després d'un any d'intervenció.
- ✓ Estudiar la relació entre el consum de vi i la síndrome metabòlica en la població del PREDIMED.

II. Hypothesis and aims

Hypothesis

The general hypothesis is the following:

Polyphenols are dietary components with proven benefits for our health. The Mediterranean diet, highly rich in polyphenols, has been proposed as a good pattern of nutrition and lifestyle. In this study we hypothesized that dietary polyphenols would help to prevent chronic diseases, especially cardiovascular diseases, in an elderly Spanish population at high cardiovascular risk (cohort from the PREDIMED study).

Aims

The main objective of this thesis was to study the role of dietary polyphenols in the primary prevention of chronic diseases.

Specific aims:

- ✓ To estimate in detail the polyphenol intake of an elderly Mediterranean population at high cardiovascular risk (PREDIMED cohort), using the recently launched Phenol-explorer database, and to identify the foods that contribute to this intake.
- ✓ To study the association between polyphenol intake (total and by groups) and cardiovascular events (myocardial infarction, stroke or cardiovascular death) within the PREDIMED population.
- ✓ To study the association between polyphenol intake (total and by groups) and all-cause mortality within the PREDIMED population.
- ✓ To review recent evidences on biomarkers of polyphenol intake
- ✓ To review the last evidences about the influence of polyphenols on BP and the mechanisms of action that explain this effect.
- ✓ To evaluate whether polyphenol intake decreases BP due to the increase in plasma nitric oxide (NO) production: study of the PREDIMED population after one year of intervention.
- ✓ To evaluate the relationship between wine consumption and the metabolic syndrome within the PREDIMED cohort.

INTRODUCCIÓ

III. Introducció

1. Els polifenols

1.1. Estructura i classificació

Els polifenols són un ampli grup de compostos que provenen del metabolisme secundari de les plantes. Aquest grup, format per centenars de compostos descrits, és la principal font d'antioxidants de la dieta humana. La seva estructura consta, com a mínim, d'un anell aromàtic amb un o més grups hidroxil[4].

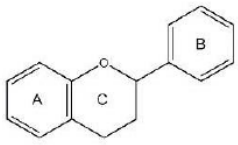
En general es classifiquen segons el nombre d'anells fenòlics i les estructures que en pengen. Una de les classificacions més usada és la que divideix els polifenols en dos grups: flavonoids i no flavonoids. Els primers, representats a la **Taula 1.1**, tenen una estructura C6-C3-C6 i hi podem trobar flavones, flavonols, flavan-3-ols o flavanols (i els seus polímers, les proantocianidines), flavanones, antocianidines, i isoflavones, i en menor proporció, xalcones, dihidroxalcones, dihidroflavonols, flavan-3,4-diols, cumarins i aurones. Els no flavonoids, les estructures dels quals es poden veure a la **Taula 1.2**, es classifiquen segons el nombre de carbonis. Dins d'aquest grup hi trobem els àcids fenòlics, els estilbens, els lignans i altres polifenols com els fenols simples[4,5].

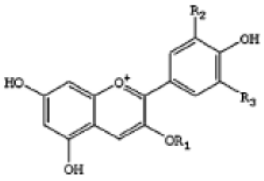
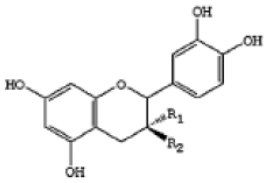
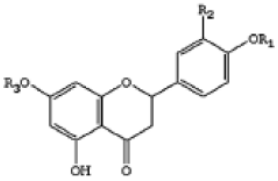
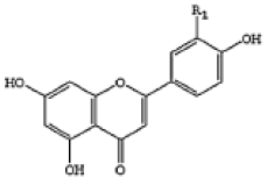
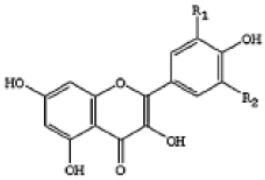
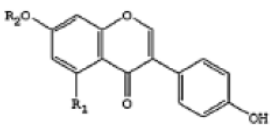
1.2. Distribució i anàlisi dels polifenols en els aliments

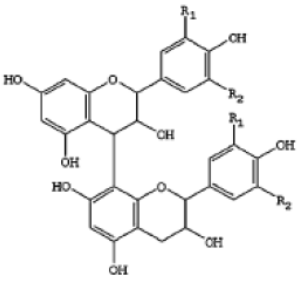
Per fer una estimació acurada de la ingesta de polifenols és imprescindible disposar d'informació fiable sobre el contingut fenòlic dels aliments. Les matrius alimentàries poden ser molt complexes. En general, hi ha dos aspectes claus a tenir en compte: aspectes físics o estructurals, i aspectes biològics. Així doncs, és d'esperar que el contingut fenòlic de la pell d'una fruita no sigui el mateix que el de la polpa o les llavors. També influiran el grau de maduració, el clima, la manipulació posterior, etc.[6–8]. Les plantes sintetitzen polifenols com a mecanisme de defensa en front dels agents externs. Donada l'escassa o nul·la utilització de pesticides en l'agricultura ecològica, les fruites i hortalisses provinents d'aquest mètode de conreu tenen un contingut de polifenols més elevat que els seus equivalents de l'agricultura tradicional[9].

Els flavonols, un subgrup dels flavonoids, són els polifenols més abundants en els aliments. Els podem trobar al cacau, al te, al vi, als fruits vermells, a les cebes, als espàrrecs i en moltes espècies, entre d'altres. Les flavones com l'apigenina o la luteolina es troben principalment a les carxofes, al pebrot, a l'api, al raïm o a les taronges. Les flavononones són característiques dels cítrics mentre que les isoflavones les trobem a les lleguminoses com la soja i els seus derivats. Els préssecs, els fruits vermells, les pomes, i el raïm negre són rics en flavanols o flavan-3-ols, en proantocianidines (també conegudes com tanins condensats) i antocianidines[6]. Tot i que el grup dels flavonoids ha estat el més estudiat, els no flavonoids també tenen una gran contribució en la dieta i en alguns casos són els responsables de característiques nutricionals úniques[10]. Aquest és el cas de l'oli d'oliva, que conté un gran nombre de fenols simples com ara l'hidroxitirosol i el tirosol[11]. El grup dels àcids fenòlics, que inclouen els àcids hidroxicinnàmics i hidroxibenzòics, el trobem principalment als fruits vermells, a les

FLAVONOIDS

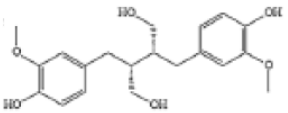
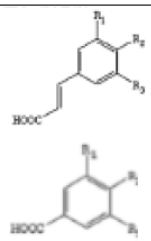
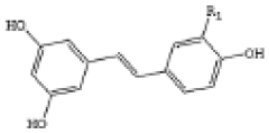
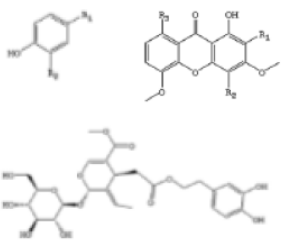


Grup	Estructura	Exemples	Exemple de fonts alimentàries
Antocianidines		Delfinifina Cianidina Malvidina	
Flavan-3-ols o flavanols		Epicatequina Catequina Epigal·locatequina	
Flavanones		Hesperitina Naringenina Eriodictiol	
Flavones		Luteolina Apigenina Tangeretina	
Flavonols		Quercetina Campferol Miricetina	
Isoflavonoids		Genisteïna Daidzeïna Gliciteïna	

Proantocianidines		Polímers de flavanols	

Taula 1.1. Característiques, classificació i fonts alimentàries dels flavonoids.

NO FLAVONOIDS

Grup	Estructura	Exemples	Fonts alimentàries
Lignans		Lariciresinol Pinoresinol Sesamina	
Àcids fenòlics		Àcid gàl·lic Àcid cafèic Àcid vaníl·lic	
Estilbens		Resveratrol Pal·lidol Piceatanol	
Altres		Tirosol Oleuropeïna Xantotoxina Ferulaldehid Eugenol Curcumina	

Taula 1.2. Característiques, classificació i fonts alimentàries dels no flavonoids.

olives, a les nous, i al te. Els estilbens i els lignans, encara que en baixa concentració, són característics del vi negre i els cereals, respectivament. L'estilbè més conegut i estudiat és el *trans*-resveratrol, al qual se li han atribuït múltiples beneficis per a la salut[12,13].

A l'hora d'analitzar la concentració de polifenols en un aliment és important recordar la seva sensibilitat a la llum i a la temperatura. Els processos mecànics de pelar, tallar i triturar, que són necessaris per dur a terme les anàlisis, exposen els polifenols al medi exterior i donen lloc a reaccions enzimàtiques d'enfosquiment que transformen els polifenols. El fred, l'ús de dissolvents orgànics, la liofilització i treballar sense llum ultraviolada són els mètodes més usats per prevenir l'oxidació dels compostos fenòlics. A més, també es poden donar reaccions d'isomerització i hidròlisi[14].

En la fase d'extracció s'han de poder extreure el màxim nombre de compostos però evitant-ne la degradació. Cal tenir en compte, no només la complexitat de la matriu alimentària, sinó també la presència de substàncies interferents, la solubilitat dels diferents compostos fenòlics, la temperatura, el temps d'extracció, etc. A l'hora d'analitzar-los, una de les dificultats rau en els diferents nivells de concentració, que van des de les traces fins al mil·ligrams. Per a la separació de polifenols la tècnica més utilitzada és la cromatografia de líquids (HPLC i UHPLC), tot i que, en alguns casos, es pot utilitzar la cromatografia de gasos o l'electroforesi capil·lar[15].

Pel que fa a la identificació i quantificació, l'espectrometria de masses és la metodologia més habitual avui en dia ja que és una tècnica molt versàtil gràcies a les múltiples combinacions de fonts de ionització (ionització per electroesprai, ionització química, per bombardeig atòmic, ionització per desorció làser assistida per una matriu...) i detectors (triple quadrupol, detectors de temps de vol, ressonància ciclotrònica, detectors "diode array"...). Si l'objectiu no és l'obtenció d'un perfil fenòlic detallat sinó la quantificació de tots els polifenols o d'un grup determinat, els mètodes d'elecció són els espectrofotomètrics. El mètode de Folin-Ciocalteu (F-C) és àmpliament utilitzat per determinar el contingut de polifenols total ja que el reactiu de F-C no és específic. Hi ha reactius específics per a determinar proantocianidines, tanins hidrolitzables, antocianidines, i flavan-3-ols[5,15,16].

Podem concloure, doncs, que l'anàlisi de polifenols en un aliment és un procés complex que requereix tenir en compte múltiples factors i que variarà segons l'aliment i el tipus de polifenol d'interès.

1.3. La base de dades Phenol-explorer

Tradicionalment, la USDA (United States Department of Agriculture) Flavonoid Database ha estat la base de dades de referència pel que fa al contingut de polifenols en aliments. No obstant, aquesta base de dades no té en compte el grup dels no flavonoids que, com s'ha mencionat anteriorment, tenen una gran importància, no tant en termes quantitatius, sinó qualitatius.

L'agost del 2009 es va publicar la base de dades Phenol-explorer (www.phenol-explorer.eu), un projecte liderat per l'*Institut Nacional de la recherche agronomique* (INRA), França, on s'hi pot consultar el contingut de polifenols (uns 500) de més de 400 aliments. Aquests valors s'extreuen de més de 1300 publicacions científiques. El 2011 va sortir la versió 2.0, que incloïa, a més, informació sobre metabòlits dels polifenols. Actualment, la versió 3.0 ha introduït dades sobre els efectes que tenen el processat i el cuinat sobre el contingut fenòlic dels aliments[17].

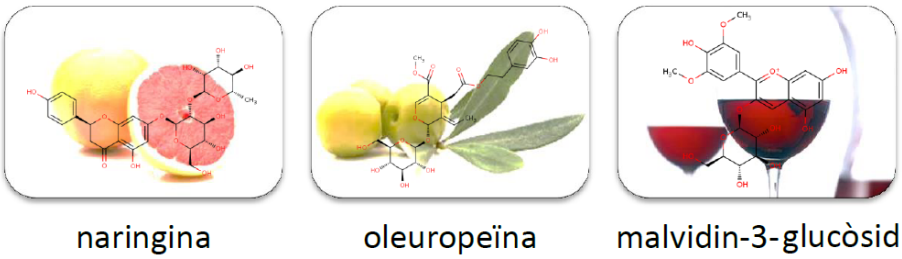


Figura 1.1. Exemples de polifenols que aporten amargor a alguns aliments.

1.4. Característiques organolèptiques dels polifenols

Els polifenols són, en part, els responsables de les propietats organolèptiques d'alguns aliments d'origen vegetal (**Figura 1.1**). Per exemple, l'amargor dels pomelos i de les olives és causada per la naringina i l'oleuropeïna, respectivament. Els tanins condensats o proantocianidines i els tanins hidrolitzables confereixen astringència, especialment a les llavors i a la pell de certes fruites com el raïm i el seu principal derivat, el vi. L'eugenol, en canvi, és el responsable de l'aroma característic del plàtan[18,19]. Altres polifenols confereixen el color característic d'alguns fruits i vegetals: les antocianines donen coloracions vermelles, liles i blaves (cireres, col lombarda, vi negre, rabes, etc.) mentre que els flavonols són de color groguenc (vi blanc, poma, te, etc.). A vegades, els polifenols majoritaris com els àcids hidroxicinàmics no tenen un impacte directe sobre les característiques organolèptiques de l'aliment però afecten negativament si s'oxiden, ja que donen lloc a polímers de color marró[19].

1.5. Els polifenols i la salut

1.5.1. Biodisponibilitat dels compostos fenòlics

De forma general, la biodisponibilitat és la proporció en què un ingredient és absorbit i es torna disponible en el lloc de l'acció. Això inclou l'alliberament i la digestió en el sistema digestiu, el transport a través de la membrana intestinal cap al torrent sanguini, la distribució cap als diferents teixits, la metabolització dels compostos i, per acabar, l'eliminació[8].

L'àmplia varietat existent de polifenols fa que la seva biodisponibilitat sigui també molt variable. Els polifenols més abundants de la dieta no són, necessàriament, els més biodisponibles i, per contra, alguns polifenols que es consumeixen a nivells de traces poden tenir una gran activitat biològica[20]. L'absorció dels polifenols depèn de la ingesta de greix, la matriu alimentària, la dosi, i el trànsit intestinal[8]. Per exemple, l'àcid gàl·lic, les isoflavones, les catequines, els flavonols, les flavanones i els glucòsids de la quercetina són els polifenols que millor s'absorbeixen, mentre que les proantocianidines, les gal·locatequines i les antocianines són els menys absorbits[8,20].

En alguns casos, l'organisme necessita metabolitzar els polifenols per tal de poder-los absorbir, així, mentre que les aglicones i les antocianines s'absorbeixen sense metabolitzar a l'estómac i a l'intestí prim, els èsters, els glicòsids i els polímers necessiten ser hidrolitzats pels enzims intestinals o per la microbiota del colon abans de ser absorbits. Durant el procés d'absorció, als enteròcits de l'intestí prim i al fetge, els polifenols són conjugats, principalment mitjançant reaccions de metilació, sulfatació i/o glucurunidació[4,21]. Aquestes conjugacions varien en funció de la naturalesa del substrat i de la dosi ingerida. A continuació, els metabòlits dels polifenols viatgen pel torrent sanguini units a transportadors com l'albúmina. Les concentracions plasmàtiques de polifenols van de 0 a 4 μmol/L prenent com a referència una ingesta de 50 mg d'equivalents d'aglicones. Pel que fa a les cinètiques, la concentració plasmàtica

màxima (C_{max}) s'assoleix després de 1.5-5.5 hores depenent del lloc d'absorció[8,20].

L'eliminació dels polifenols es pot fer a través de dues vies depenent del pes molecular. Els més pesats, que també són els més conjugats, se solen eliminar per via biliar, mentre que els de menor pes molecular presenten una major probabilitat de ser excretats per la via renal a través de la orina[20].

A part de la naturalesa estructural dels polifenols, hi ha altres factors que afecten a la seva biodisponibilitat i farmacocinètica. En primer lloc, els polifenols es consumeixen com a components dels aliments que els contenen, i es troben units a macronutrients (proteïnes, hidrats de carboni i lípids) i altres micronutrients que n'afecten l'absorció. El sexe i l'edat de l'individu també afecten el procés d'absorció[22], així com el processat dels aliments. La cocció dels aliments afecta a l'aborció dels polifenols en direccions oposades. Per una part, la calor trenca la matriu alimentària i ajuda a alliberar els compostos fent-los més biodisponibles. D'altra banda, les altes temperatures degraden els antioxidants. En alguns casos, els compostos passen d'un medi a l'altre, per exemple, de l'aliment a l'aigua o de l'oli a l'aliment que s'està fregint[23].

1.5.2. Efectes beneficiosos del consum de polifenols

Els polifenols constitueixen la principal font d'antioxidants de la nostra dieta. La seva capacitat de captar radicals lliures els fa bons candidats per a la prevenció de malalties relacionades amb l'estrès oxidatiu. Nombrosos estudis clínics i epidemiològics han demostrat que el seu consum pot protegir contra les malalties cardiovasculars, les malalties neurodegeneratives, alguns càncers, la resistència a la insulina, i la obesitat, entre d'altres.

a) L'estrès oxidatiu i envelliment

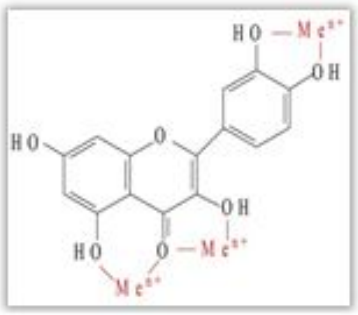


Figura 1.2. Posicions favorables per la quelació de metalls.

Els processos fisiològics normals, com la respiració i les reaccions metabòliques que tenen lloc al nostre organisme, donen com a resultat espècies reactives de l'oxigen o ROS (de l'anglès reactive oxygen species). El peròxid d'hidrogen (H_2O_2), el superòxid (O_2^-) i el radical hidroxil ($OH\bullet^-$) són exemples d'aquestes ROS[24]. El cos té uns mecanismes de defensa que són antioxidants endògens com ara el superòxid dismutasa, el catalasa o el glutatió reductasa que permeten eliminar aquestes ROS que es van produint contínuament. Aquests i altres mecanismes no enzimàtics actuen evitant la formació de les ROS, reduint-les, reparant el dany oxidatiu, eliminant les molècules ja afectades i prevenint les mutacions. L'estrès, la contaminació ambiental i l'envelliment trenquen l'equilibri entre la producció i l'eliminació d'aquestes ROS donant lloc al que es coneix com a estrès oxidatiu[25].

Les ROS són radicals lliures i molècules oxidants que es poden unir a l'ADN, als lípids i a les proteïnes i alterar-ne l'estabilitat, donant lloc a diverses patologies. Aquest estrès oxidatiu contribueix al desenvolupament de malalties com la diabetis, l'Alzheimer, el Parkinson, el càncer, malalties del sistema cardiovascular i del sistema respiratori.

Els polifenols juguen un paper important en la disminució de l'estrès oxidatiu gràcies a la seva capacitat antioxidant. Les estructures d'aquests compostos, amb anells fenòlics molt estables, permeten que els hidroxils cedeixin un protó amb molta facilitat i s'oxidin. L'activitat

antioxidant dels polifenols depèn del nombre de grups hidroxil i de la seva posició relativa, essent la posició *orto* la més favorable. La combinació de la cetona amb el doble enllaç també ajuda a la formació de formes ressonants i, per tant, afavoreix la pèrdua d'electrons. Per últim, els polifenols també tenen diferents zones que permeten la quelació amb metalls, com els hidroxils en orto o les cetones amb hidroxils contigus (**Figura 1.2**).

Alguns dels polifenols que s'han proposat per retardar l'envelliment són el gal·lat d'epigallocatequina, la quercetina o el resveratrol[25]. Aquests i altres polifenols de característiques similars, així com les seves fonts principals es troben resumits a la **Taula 1.3**.

b) Malalties cardiovasculars

Les malalties CV són les responsables de més de 16 milions de morts anuals arreu del món, el que representa un 30% del total de defuncions. En els països desenvolupats, les malalties coronàries són la principal causa de morbiditat i mortalitat, fet que ha posat en alerta els organismes de salut pública. Aquestes malalties engloben totes les malalties coronàries, la hipertensió, els infarts aguts de miocardi i les embòlies[25,26].

Hi ha alguns factors de risc, com l'edat, el sexe o la predisposició genètica que no són modificables, però la gran majoria depenen de l'estil de vida i, a més, estan fortament relacionats entre ells (**Figura 3**). Així doncs, dur una vida tranquil·la, dormir bé, fer exercici, menjar equilibradament i no fumar ajuden a mantenir un pes saludable, i disminueixen el risc de tenir hipertensió, dislipèmia, o diabetis que, al seu temps, també són factors de risc CV[27]. Actualment, se sap que prevenir o tractar aquests factors de risc és molt més efectiu que tractar les malalties CV i és on cal, per tant, centrar més els esforços.

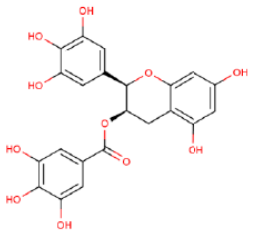
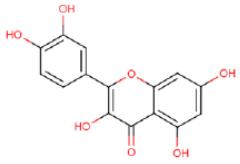
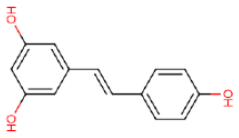
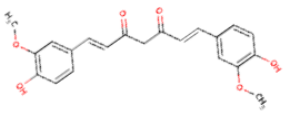
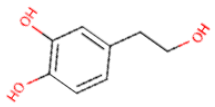
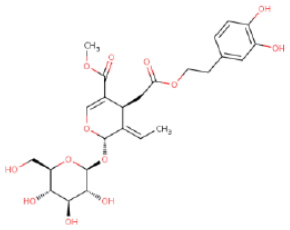
Un gran nombre d'estudis epidemiològics han associat el consum de polifenols amb una disminució del risc de malaltia CV o coronària. En un meta-anàlisi sobre el te i aquestes malalties es va concloure que el consum de te tenia un efecte cardioprotector[28]. Una revisió bibliogràfica sobre consum moderat de vi posava de manifest resultats molt similars[29]. Més recentment, un meta-anàlisi d'estudis d'intervenció aleatoritzats relacionava els flavan-3-ols amb una disminució de biomarcadors de risc cardiovascular[30].

Altres estudis s'han centrat en el consum d'un o més tipus de polifenols: dos estudis prospectius van associar la ingesta de flavanones i antocianines amb un menor risc de malaltia CV i mortalitat total[31,32]. Hertog i col. van trobar una associació dèbil però positiva entre el consum de flavonols i la mortalitat per qualsevol causa però no van trobar cap associació significativa per malaltia coronària ni per càncer[33].

L'efecte protector dels polifenols es pot explicar per les millores que exerceixen sobre diferents factors de risc. Així, diferents estudis clínics, amb models animals i amb humans, han demostrat que els polifenols milloren la funció endotelial mitjançant la millora de paràmetres com el colesterol LDL (low-density lipoprotein), l'agregació plaquetària, la invasió i la proliferació de les cèl·lules musculars llises en la paret arterial, l'òxid nítric (NO) i alguns marcadors d'inflamació[34].

La disfunció endotelial

L'endoteli és la capa més interna de la paret dels vasos sanguinis. Les cèl·lules endotelials, en resposta a diversos estímuls, alliberen factors vasodilatadors, substàncies vasoconstrictores, factors promotors o inhibidors del creixement, moduladors de la inflamació i factors hemostàtics i trombolítics. Aquests factors són els responsables de mantenir el to vascular, controlar el creixement del múscul llis vascular i modular la coagulació, la fibrinòlisi i l'adhesió de cèl·lules sanguínies a la paret endotelial.

Polifenol	Estructura	Fonts
Gal·lat d'epigal·locatequina		Te verd
Quercetina		Ceba, pomes, bròquil, te, tàperes, cacau, prunes...
Resveratrol		Vi negre, raïm negre, fruits vermells
Curcumina		Cúrcuma
Hidroxitirosol		Oli d'oliva i olives
Oleuropeïna		Oli d'oliva i olives

Taula 1.3. Polifenols relacionats amb la prevenció de l'estrès oxidatiu i l'envelliment.

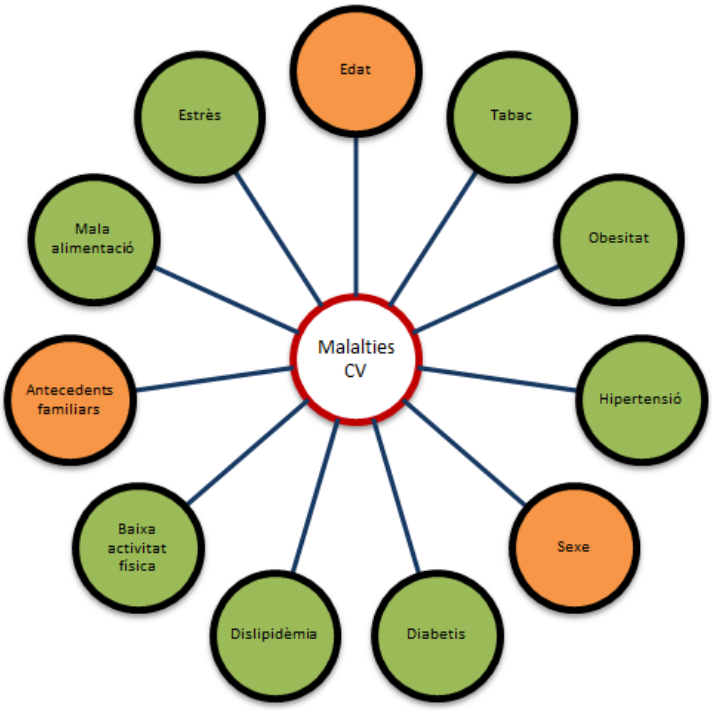


Figura 1.3. Factors de risc de les malalties cardiovasculars. En taronja, factors de risc no modificables. En verd, factors de risc modificables.

Parlem de disfunció endotelial quan es trenca l'equilibri homeostàtic i les funcions de l'endoteli es veuen alterades. La disfunció endotelial és el primer pas en el progrés de l'aterosclerosi i, per tant, del desenvolupament de malalties cardiovasculars.

L'òxid nítric, per exemple, és un vasodilatador i disminueix la pressió arterial (PA). L'estrès oxidatiu disminueix la biodisponibilitat dels radicals de NO i afavoreix, per tant, els processos d'inflamació. Una hipòtesi és que els aliments rics en polifenols disminueixen la PA mitjançant l'activació del NO sintasa. En experiments *in vitro* amb arteries aïllades es va observar que els polifenols augmentaven la formació de NO endotelial i causaven relaxacions dependents de l'endoteli i mediades pel NO [35].

L'aterosclerosi

L'aterosclerosi és un procés inflamatori crònic de les parets de les grans arteries com a conseqüència de la disfunció endotelial. La diabetis, la hipertensió, el tabac, nivells de colesterol LDL elevats o nivells de colesterol HDL (High Density Lipoproteins) baixos són factors de risc d'aterosclerosi.

El colesterol LDL és una peça clau en el procés de formació de la placa d'ateroma (**Figura 1.4**). Les lipoproteïnes LDL s'acumulen a l'endoteli vascular i travessen les cèl·lules endotelials. Aquest procés es veu facilitat quan augmenta la pressió arterial. Allà, les LDL s'oxiden i causen inflamació, posant en alerta el sistema immunitari que respon enviant monòcits. Aquests, entren a la íntima mitjançant les molècules d'adhesió com la ICAM-1 (Soluble Inter-Cellular Adhesion Molecule-1) i la VCAM-1 (Vascular Cell Adhesion Molecule-1) i es transformen en macròfags que fagociten les molècules de LDL, convertint-se llavors en cèl·lules espumoses (foam cells) que no poden sortir de la íntima. Els macròfags, juntament amb les cèl·lules T, estimulen la proliferació de cèl·lules musculars llises que formen una placa fibrosa juntament amb les cèl·lules espumoses. La placa d'ateroma provoca una disminució

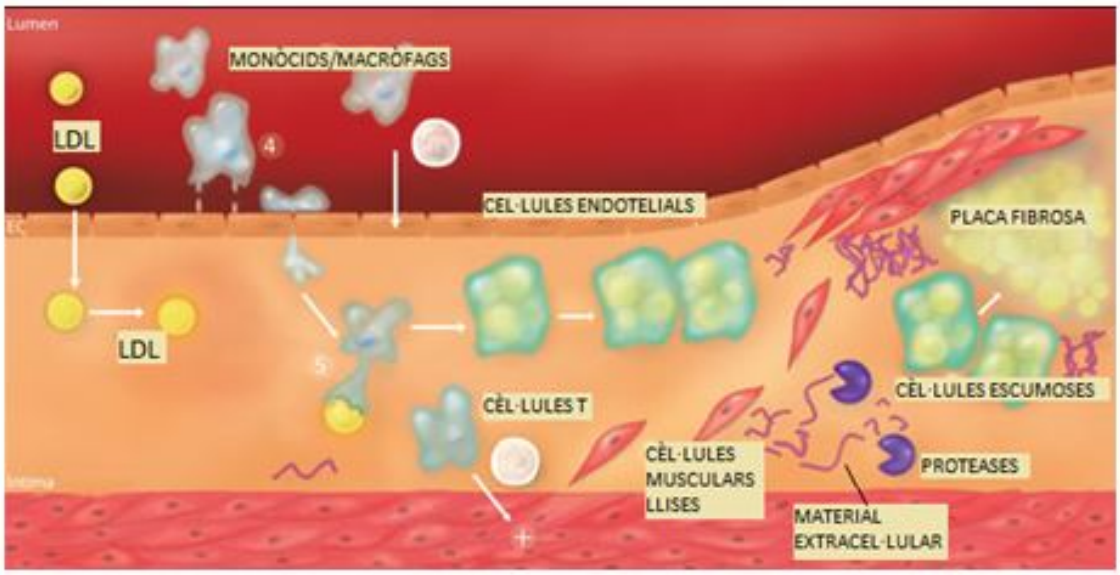


Figura 1.4. Desenvolupament de la placa d'ateroma.

de la llum arterial i, per tant, del flux sanguini. L'ateroma en estat avançat pot desprendre's formant coàguls (trombosi) que viatgen pel sistema circulatori fins que s'encallen provocant, per exemple, un infart agut de miocardi o una embòlia[36,37].

La hipertensió

La hipertensió és un problema de salut pública que afecta a més de 1 bilió de persones al món i causa 7.6 milions de morts anuals[38]. A Espanya, s'estima que entre un 30% i un 45% de la població major d'edat és hipertensa, la qual cosa suposa que hi ha uns 8 milions d'individus amb aquesta condició. Una persona es considera hipertensa si té, de forma mantinguda, una pressió arterial sistòlica (PAS) superior a 140 mm Hg i/o una pressió arterial diastòlica (PAD) superior a 90 mm Hg. Tot i que el diagnòstic d'hipertensió és senzill, una gran part de la població no sap que és hipertensa i, per tant, no es medica ni es controla.

La hipertensió és, per si sola, un dels principals factors de risc de malalties de l'aparell circulatori, especialment de malalties coronàries, malalties cerebrovasculars i d'insuficiència cardíaca. La hipertensió, a més, també està relacionada amb altres factors de risc com la obesitat, la manca d'exercici físic, una mala alimentació i un consum d'alcohol elevat.

Diferents estudis observacionals i d'intervenció han demostrat que el consum d'aliments rics en polifenols està associat amb una disminució de la PA. Per exemple, en un estudi d'intervenció creuat i doble cec dut a terme amb pacients hipertensos, es va concloure que un consum de 100 g al dia de xocolata negra rica en flavonoids disminuïa la PA de forma significativa, i augmentava la dilatació mediada per l'endoteli (Flow Mediated endothelium-dependent Dilation o FMD), mentre que no s'observà cap millora amb la xocolata blanca (sense flavonoids)[39].

A la **Taula 1.4** es resumeixen els resultats d'algunes publicacions sobre l'efecte dels polifenols en la PA obtinguts a partir d'estudis d'intervenció en humans[40–61].

c) Càncer

Els efectes anticancerígens dels polifenols han estat àmpliament demostrats en models animals. L'administració de polifenols en rates o ratolins amb tumors o sota l'efecte d'agents carcinògens protegeix i sovint redueix el nombre de tumors o el seu creixement[62].

Referència	Tipus d'estudi	Nombre d'individus ǂ	Característiques	Edat	Substància administrada	Polifenols ǂ	Dosi/dia	Durada	Biomarcador	Canvis en la PA ǂ
43	Crònic, controlat i paral·lel	12 (8h, 4d)	Hipertensos	42-62	Híbrid d'aranja i pomelo	Flavonoids	0.5 L (889 mg/L)	5 setmanes	PAS PAD	? ?
44	Crònic, controlat i paral·lel	45 (40h,5d)	Pacients amb malaltia coronària isquèmica i miocàrdia.	58-80	Suc d'aranja	Tanins, antocianines	240 mL	90 dies	PA	↔
45	Crònic, un braç, sense control	21 (homes)	Sans	30-46	Suc d'aranyons americans	Flavonoids, àcids fenòlics	7 mL/Kg pes	14 dies	PAS	↓ 2% (no estadísticament significatiu)
46	Crònic, controlat i paral·lel	57	Hiperlipidèmics després de cirurgia de by-pass.	39-72	Suc d'aranja o pomelo	Flavonoids, antocianines	Una fruita (20 mg/100 g pes fresc)	30 dies	PAS PAD	↔ ↔
47	Crònic, controlat i paral·lel	44 (33h,11d)	Supervivents d'infarts de miocardi amb estatines durant 6 mesos.	57-75	Extracte de flavonoids d'*Aronia melanocarpa*	Antocianines, procianidines	3 x 85 mg	42 dies	PAD PAS sèrum ACE	↓ 7.2 mmHg ↓11 mmHg ↓33.3%
48	Crònic, controlat i paral·lel	44 (20h,24d)	Hipertensos	56-73	Xocolata negra	Flavonoids	6.3 g (30 mg)	18 setmanes	PAS PAD	↓2.9 mmHg ↓1.9 mmHg
49	Crònic, un braç, sense control	60 (49h,11d)	Sans	32-73	Extracte de te verd	Catequines	1 paquet (544 mg)	2 mesos	PAD	↓4 mmHg
50	Crònic, controlat i paral·lel	187 (38h,149d)	Sans	19-20	Extracte d'aranyons americans	Antocianines, àcids fenòlics	480 mL	Postprandial	PA FC	↔ ↔
51	Crònic, un braç, sense control	10	Sans	Adults	Suc de marula	Tanins hidrolitzables, catequines	200 mL (56 mg/dL)	21 dies	PA	↔
52	Crònic, controlat i transversal	71 (25h,46d)	Alt risc cardiovascular	51-64	Puré de nabius negres i vermells, grosella negra, maduixa i suc de gerds	Antocianines	150 g (837 mg)	56 dies	PAS	↓ 7·3mmHg En els voluntaris amb PA alta a l'inici
53	Crònic, controlat i transversal	42 (19h,23d)	Sans	58-81	Cacau en pols (amb llet desnatada)	Flavonoids	40 g (495.2 mg)	28 dies	PAS PAD FC	↔ ↔ ↔
54	Crònic, controlat i transversal	35 (homes)	Sans	18-45	Extractes de raïm i llavors (càpsules)	Antocianines, àcids fenòlics	6 càpsules (800 mg)	14 dies	PAS PAD FC	↔ ↔ ↔

55	Creuat, aleatoritzat i controlat	24 (homes)	Sans i amb sobrepès	50-65	Suc de taronja o beguda amb hesperidina	Hesperidina	500 mL (292 mg)	28 dies	PAD	↓(en els dos grups d'intervenció)
56	Creuat, aleatoritzat i controlat	24 (homes)	Amb síndrome metabòlica	30-70	Extracte de raïm (càpsules)	Flavanols, antocianines	46 g/dia	30 dies	PAS FMD NO	↓ ↑ ↔
57	Paral·lel, aleatoritzat, controlat	97(38h,69d)	Amb sobrepès	19-55	Extracte d'algues marines (*Ecklonia cava*)	Fluorotanins	Llaunes (246 mL i 72 mg extracte)	12 setmanes	PAS	↓ (amb la dosi alta)
58	Creuat, aleatoritzat, controlat	10 (homes)	Sans	45-50	Vi, vi desalcoholitzat i ginebra	Flavanols, antocianines	272 mL de vi (733-798 mg EAG/dia) o 100 mL de ginebra	20 dies	PAD PAS	↓(amb vi) ↓(amb vi i vi desalcoholitzat)
59	Paral·lel, aleatoritzat i controlat	51(16h, 35d)	Sans	30-50	Suc de magrana	Tanins hidrolitzables i antocianines	330 mL/d	4 setmanes	PAS PAD PA	↓(-3.14 mmHg) ↓(-2.33 mmHg) ↓(-2.60 mmHg)
60	Creuat, aleatoritzat, controlat	67 (homes)	Amb alt risc cardiovascular	55-75	Vi, vi desalcoholitzat i ginebra	Flavanols, antocianines	272 mL de vi (733-798 mg EAG/dia) o 100 mL de ginebra	4 setmanes	PAS i PAD NO en plasma	↓ ↑ (amb vi desalcoholitzat)
61	Paral·lel, aleatoritzat, controlat	84 (31h, 53d)	Sans o amb hipertensió lleu	35-75	Te negre	Catequines	3 tasses de te/dia (429 mg)	4 setmanes	PAS i PAD FC	↔ ↓
62	Creuat, aleatoritzat, controlat	49 (homes)	Sans	48-68	Quercetina	Quercetina	Càpsules (150 mg/dia)	8 setmanes	PAS postprandial	↓
63	Paral·lel, aleatoritzat, controlat.	70 (38h, 32d)	Hipertensió de grau 1 o menor	35-75	Extracte de llavors de raïm	Catequina, dímers de procianidines	Càpsules (300 mg/dia)	8 setmanes	PAS i PAD	↓ (no estadísticament significatiu)
64	Estudi pilot	6 (4h, 2d)	Sans	34-68	Suc de "boysenberries"	Dímers de procianidines, epicatequina	180 mL/dia (351 mg)	4 setmanes	FMD PAS	↑ ↓

PAS, pressió arterial sistòlica; PAD, pressió arterial diastòlica; PA, pressió arterial; sèrum ACE, enzim convertidor de l'angiotensina; FC: freqüència cardíaca; EAG: equivalents d'àcid gàl·lic.
* La taula està ordenada cronològicament, en ordre ascendent.
† h: homes, d: dones
‡ Només els dos polifenols principals s'han afegit a la taula.
¥ ↑augment; ↓ disminució; ↔ sense canvis; si no s'especifica el contrari, % es refereix als canvis respecte a l'inici de l'estudi.

Taula 1.4. Efecte dels polifenols sobre la pressió arterial en estudis d'intervenció en humans (continuat).

Atribuir aquest efecte a les propietats antioxidants dels polifenols és fer una simplificació. Així doncs, s'han proposat diversos mecanismes que explicarien aquesta protecció contra el càncer. En primer lloc, els polifenols poden actuar bloquejant les fases inicials de la malaltia modulant l'expressió dels enzims del citocrom P-450 involucrats en l'activació de carcinògens i limitant la formació de cèl·lules iniciades estimulant la reparació de l'ADN. D'altra banda, els polifenols alenteixen o aturen el creixement de tumors mitjançant la inhibició de l'expressió dels gens involucrats en la proliferació d'aquests o bé induint l'apoptosi de les cèl·lules malignes. Per últim, es creu que també inhibeixen l'angiogènesi i limiten la invasió tumoral. Així doncs, els polifenols actuen, tant en la fase d'iniciació com en les de promoció i progressió[34,63].

Els principals problemes plantejats fins ara són la traducció dels estudis animals als humans i la dosi de polifenols administrada, que sempre és molt superior a la dosi habitual ingerida a través de la dieta. En alguns casos, s'han observat efectes oposats segons la dosi administrada. Per exemple, l'àcid cafeic en dosis altes (0.5-2% de la dieta) va induir hiperplàsia i tumors a l'estómac i als ronyons de rates i ratolins, mentre que administrat en dosis inferiors (0.05-0.15%) tenia propietats anticarcinogèniques[64]. Les conclusions finals, doncs, caldria extreure-les d'estudis clínics amb humans administrant dosis reals o bé mitjançant estudis epidemiològics.

Malgrat l'escàs nombre d'estudis clínics i epidemiològics, comparats amb les investigacions *in vitro* o amb animals, hi ha suficient evidència científica per afirmar que el gal·lat d'epigallocatequina (EGCG), un flavonoid present en el te, és un agent quimioprotector. El consum de te verd s'ha relacionat amb un menor risc de càncer de mama[65], càncer de boca[63,66], i càncer de pròstata[67]. En canvi, els resultats per al te negre o pels càncers de colon i bufeta no són concloents[65,68,69]. Diferents estudis *in vitro*, *in vivo* i en humans assenyalen que els polifenols del raïm, de les baies, de l'oli d'oliva i del cacau també tenen propietats anticancerígenes[66,70–72].

d) Malalties neurodegeneratives

Degut a l'envelliment de la població, la prevalença de malalties neurodegeneratives, estretament lligades a l'edat, ha anat en augment. La demència és una síndrome clínica caracteritzada per un conjunt de símptomes tals com pèrdua de memòria, canvis de conducta i altres afectacions que impedeixen o dificulten les tasques diàries. Actualment, més de 25 milions de persones al món tenen algun tipus de demència, un 75% de les quals pateixen la malaltia d'Alzheimer. A Europa, el 6.4% de la població major de 65 anys pateix alguna malaltia neurodegenerativa[73]. Algunes demències són un efecte secundari de les malalties isquèmiques com l'embòlia cerebral. Amb el temps, les malalties neurodegeneratives comporten pèrdua de memòria o una pèrdua motora que resulta en diferents graus de dependència.

El cervell és un òrgan amb un elevat consum d'oxigen i els radicals lliures són productes normals del seu metabolisme. Hi ha suficient evidència científica que relaciona la producció de radicals lliures, la inducció de necrosi, la inflamació i la patogènesi de les malalties neurodegeneratives[74,75]. Així doncs, aquestes malalties estan estretament lligades a l'estrès oxidatiu i és per aquest motiu que es creu que els antioxidants poden ajudar en la seva prevenció[34]. Nombrosos estudis s'han focalitzat en l'efecte de la vitamina C, la vitamina E i el β-carotè però encara hi ha moltes incògnites referents als polifenols[76].

Molts dels estudis s'han dut a terme amb models animals, especialment ratolins i rates, tot i que també s'han utilitzat cèl·lules neuronals en experiments *in vitro*. Per exemple, l'administració d'una combinació de polifenols provinents del raïm va disminuir els pèptids β-amiloides en ratolins[77]. Aquests pèptids estan involucrats en la patogènesi de la malaltia d'Alzheimer. L'ús d'altres polifenols aïllats com el resveratrol, les proantocianidines, la epicatequina, la catequina i l'àcid ferúlic van donar resultats similars[78–82], així com els polifenols procedents

de les maduixes, els espinacs, els mirtils o nabius, el te, el pebrot vermell i l'all[76].

Altres estudis també han pogut demostrar l'efecte dels polifenols sobre les malalties neurodegeneratives en humans. Els polifenols del vi negre i el raïm van tenir efectes beneficiosos millorant la memòria en persones grans amb problemes cognitius lleus[83]. En altres estudis amb els mateixos polifenols es demostrà que interferien en la generació i l'agregació de pèptids β-amiloides[84–86]. Nurk i col. van analitzar l'efecte del consum de vi negre, xocolata i te (per separat o conjuntament) en un estudi transversal realitzat en 2000 persones grans, d'entre 70 i 74 anys. Aquells que consumien aquests aliments mostraren millors resultats en diferents testos cognitius de forma dependent de la dosi[87]. El consum de flavonoids es va associar amb una millor capacitat cognitiva a l'inici i una millor evolució en una cohort de 1640 persones majors de 65 anys que es van seguir durant 10 anys[88].

Els polifenols estan relacionats amb una millora de les malalties neurodegeneratives gràcies al seu efecte antioxidant ben demostrat en models *in vitro* però sembla que aquest no seria l'únic mecanisme d'acció i, a més, no està clar si interaccionen directament amb els sistemes neuronals o hi actuen de forma indirecta[76] ja que no se sap si tots els polifenols són capaços d'arribar al cervell. En aquesta direcció, només un grup de recerca japonès i, més recentment, un de la Universitat de Barcelona van demostrar que alguns polifenols són capaços de travessar la barrera hematoencefàlica de models animals. Per exemple, després de la ingesta d'un extracte de te i de EGCG es van trobar aquests polifenols i alguns metabòlits en diferents òrgans de ratolí, inclòs el cervell[89]. D'altra banda, el consum d'un suplement de nabius millorava els resultats obtinguts per unes rates en el test del laberint aquàtic de Morris. En aquest estudi, els metabòlits de les antocianines es van poder identificar al cerebel, al còrtex, a l'hipocamp i al nucli estriat del cervell[90]. Calen però, més estudis epidemiològics i, sobretot, clínics, que esclareixin en quin grau els polifenols poden alentir la progressió de les malalties neurodegeneratives i mitjançant quins mecanismes.

e) Síndrome metabòlica, obesitat i diabetis

La síndrome metabòlica (SM) és un desordre metabòlic que consisteix en una combinació de múltiples factors de risc cardiovascular: obesitat, hipertensió, dislipèmia i hiperglucèmia. No existeix un criteri universal per al diagnòstic d'aquest trastorn, fet que dificulta el coneixement de la prevalença real d'aquesta malaltia i fa difícil la comparació dels diferents estudis científics. Un dels criteris més estesos per al seu diagnòstic és el proposat per l'Adult Treatment Panel III (ATPIII) l'any 2001, el qual considera que un individu té SM si compleix, com a mínim, 3 dels següents criteris: 1) obesitat abdominal $\geq$102 en homes i $\geq$88 en dones, 2) nivells de triglicèrids en sang $\geq$150 mg/dL (o medicació hipotrigliceridemiant), 3) nivells de colesterol HDL $<$40 mg/dL en homes i $<$50 mg/dL en dones, 4) pressió arterial $\geq$130/85 mmHg (medicació per a la hipertensió), i 5) glucosa plasmàtica en dejú $\geq$100 mg/dL (o medicació per a la diabetis)[91,92].

Aquesta síndrome és el resultat de la interacció de múltiples causes entre les quals hi ha factors genètics, menys importants, i ambientals: falta d'activitat física, tabac i hàbits alimentaris, sobretot el consum de sucres simples i greixos saturats. Si no es controla, pot derivar en accidents cardio i cerebrovasculars i en diabetis tipus-2. El consum d'aliments rics en polifenols pot prevenir la SM a través de l'efecte protector sobre la inflamació crònica, lligada a la obesitat, la resistència a la insulina, la dislipèmia, i la hipertensió[92]. Alguns dels polifenols que s'han relacionat amb el tractament i la prevenció de la SM són el resveratrol, la quercetina, l'epigalocatequina-3-galat i la curcumina[93].

El cacau, un aliment ric en epicatequina, catequina i proantocianidines és un aliment amb activitat antioxidant, antihipertensiva, antiinflamatòria, antiaterogènica, millora la resitència a la insulina, la funció endotelial i els nivells de NO. Aquests efectes, confirmats en múltiples

revisions bibliogràfiques i meta-anàlisis demostren que podria ser un bon aliat en el tractament i la prevenció de la SM[94]. S'han observat resultats similars amb el consum de te verd, una beguda molt extesa i també rica en catequines[95] i amb l'oli d'oliva[96].

Soham i col. van dur a terme un estudi amb 2618 participants (19-84 anys) de l'estudi TLGS (Tehran Lipid and Glucose Study). Mitjançant dades extretes d'un qüestionari de freqüència de consum es va relacionar la ingesta de polifenols totals, flavonoids, àcids fenòlics, estilbens i lignans amb la prevalença de SM i els seus components. Van concloure que aquells que consumien més flavonoids tenien menys probabilitats de patir SM i 4 dels 5 components (excepte la hipertensió). En canvi, els lignans semblaven afavorir el risc d'hipertrigliceridèmia i hiperglicèmia, i els estilbens, la hipertensió[97].

En un assaig controlat, creuat, aleatori i doble-cec amb 45 participants de mitjana edat i índex de massa corporal (IMC) de 28±2 kg/m^2, es va donar càpsules amb un extracte de fulla d'olivera durant 12 setmanes (riques en oleuropeïna i hidroxitirosol, entre d'altres polifenols). La sensibilitat a la insulina va millorar en un 15% amb el suplement comparat amb el placebo. També es va notar una millora en la resposta d'un 28% de cèl·lules beta pancreàtiques, va augmentar la interleuquina-6 en dejú i les concentracions de IGFBP-1 (Insulin-like growth factor I binding protein) i IGFBP-2[98]. En un estudi similar amb dones amb SM i administrant suc d'aranyons americans durant 8 setmanes es va observar una disminució significativa del colesterol LDL i un augmenta de la capacitat antioxidant del plasma[99].

Pel que fa a la relació dels polifenols amb la diabetis tipus 2 es proposen diferents mecanismes d'acció. Per un costat, els polifenols podrien inhibir l'absorció de glucosa a l'intestí prim i la seva reabsorció en el fetge. D'altra banda, exerceixen diferents accions en teixits perifèrics, entre les quals hi ha la inhibició de la gluconeogènesi, la estimulació adrenèrgica del consum de glucosa, o la estimulació de l'alliberament d'insulina per part de les cèl·lules beta pancreàtiques[34]. Per exemple, els polifenols de la canyella, el resveratrol, les isoflavones, i els polifenols del te, el cacau i de les llavors de raïm milloren la sensibilitat a la insulina, hormona que regula els nivells de glucosa en sang[100]. Els compostos fenòlics també podrien estar lligats al control de la obesitat. Diferents estudis *in vitro*, en animals i en humans demostren que els polifenols poden disminuir l'absorció de greixos en el tracte intestinal, activar la termogènesi i modular la resposta hormonal que regula la ingesta d'aliments i la sacietat[101].

2. Epidemiologia

L'OMS defineix l'epidemiologia com una disciplina científica que estudia la distribució, la freqüència, les causes i el control dels factors relacionats amb la salut i l'aplicació d'aquests estudis al control de malalties i altres problemes de salut de la població[1]. L'epidemiologia és una ciència bàsica de la medicina preventiva i una eina fonamental per a les polítiques dels organismes de salut pública. Els estudis epidemiològics ajuden a donar resposta a preguntes del tipus: els telèfons mòbils poden augmentar el risc de patir càncer? Quin és el mínim d'exercici físic que cal fer per disminuir el risc de malaltia cardiovascular o diabetis? Ens hem de preocupar pel mercuri que conté el peix que consumim?

Les branques de l'epidemiologia són la descriptiva, en la qual es mesura la freqüència i la distribució d'una malaltia, i l'analítica, que busca, mitjançant l'observació o l'experimentació, la mesura d'associació (o la relació causa-efecte) entre una malaltia i una exposició (**Figura 2.1**). Una branca complementa a l'altra ja que l'epidemiologia descriptiva serveix per plantejar hipòtesis que l'epidemiologia analítica ha de respondre[102].

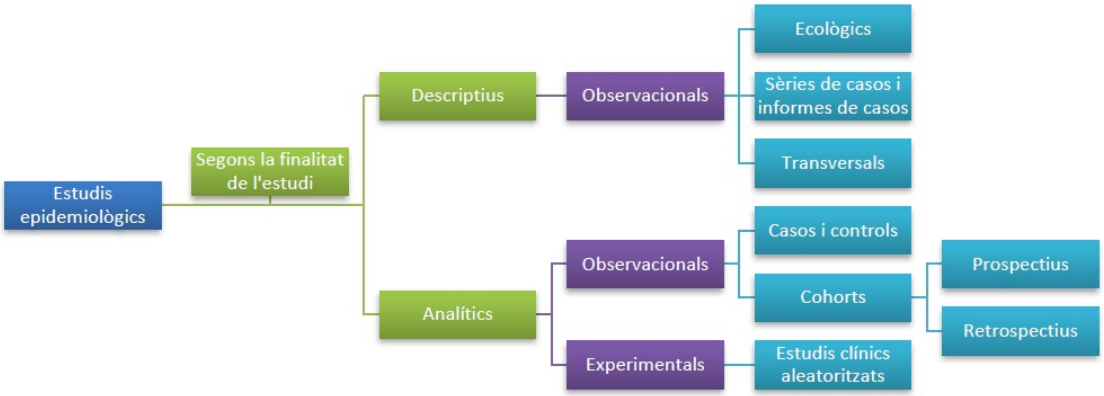


Figura 2.1. Classificació d'estudis epidemiològics.

Segons la seqüència temporal, els estudis també es poden classificar en transversals o longitudinals. Considerem que un estudi és transversal quan les dades dels individus es prenen en un moment concret de temps. Com que les variables es mesuren de manera simultània, no es poden establir relacions causa-efecte. Els estudis transversals són, per definició, descriptius. Quan existeix un interval de temps entre les diferents variables que s'avaluen parlem d'estudis longitudinals. En aquests casos, cal tenir en compte la direccionalitat temporal, que pot anar de la causa al desenllaç (estudis experimentals i de cohorts) o del desenllaç a la causa (estudis de casos i controls)[103,104].

Els estudis ecològics o correlacionals serveixen per comparar freqüències de malalties entre diferents grups de població dins un període de temps determinat, o dins d'una mateixa població però en diferents períodes de temps. Al comparar poblacions no podem assumir que l'associació serà la mateixa a nivell individual. Altres limitacions són la confusió per altres variables, i que els valors d'exposició utilitzats són les mitjanes de la població, no els valors reals. Els estudis de sèries de casos, a diferència dels estudis ecològics, utilitzen informació sobre pacients de forma individualitzada, amb dades detallades sobre factors relacionats amb la malaltia que es vol estudiar. La principal limitació és l'absència d'un grup control, fet que impedeix dimensionar l'efecte[105].

Els estudis descriptius transversals mesuren la prevalença d'una malaltia en un moment determinat. La informació sobre l'exposició i el resultat es prenen al mateix temps i per a tots els individus. Són estudis ràpids i barats però no es pot establir cap relació temporal, excepte en el cas d'exposicions invariables en el temps, com ara la raça, factors genètics, el sexe, etc. Així doncs, els estudis descriptius serveixen per descriure patrons d'incidència de malalties en relació a característiques personals, llocs i temps. La informació sol ser fàcil i ràpida d'obtenir ja que sovint forma part de processos rutinaris (informes mèdics, enquestes de població, etc.). Les dades que se n'obtenen són utilitzades pels organismes de salut pública per localitzar problemes i poder focalitzar els recursos i els programes de prevenció i educació[103].

Els estudis analítics solucionen totes les limitacions dels descriptius: estudien individus en comptes de poblacions, hi ha grup control i seqüència de temps i, a més, permeten ajustar per variables de confusió. N'hi ha de dos tipus: observacionals o d'intervenció. En els primers, l'exposició és aleatòria o deguda a l'ambient i l'investigador és un observador passiu. En canvi, en els estudis d'intervenció, l'exposició l'assigna l'investigador (**Figura 2.2**)[103].

Els estudis observacionals poden ser de cohorts o de casos i controls. La diferència està en el punt de vista de l'investigador o, dit d'una altra manera, en quines dades s'utilitzen

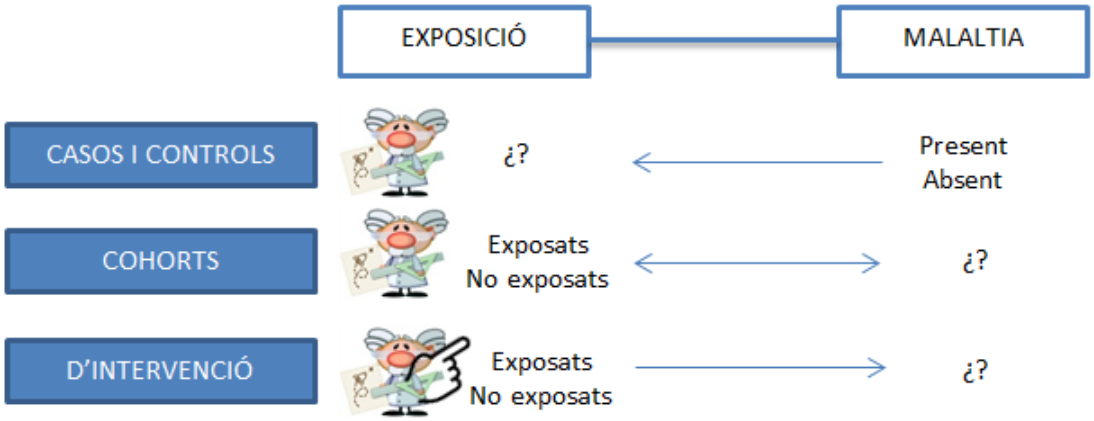


Figura 2.2. Esquema de funcionament dels estudis epidemiològics analítics.

per seleccionar els grups. En els estudis de casos i controls, els individus es classifiquen segons si pateixen o no una malaltia i s'investiga l'exposició. Per contra, en els estudis de cohorts la població es divideix segons si està exposada o no al factor que s'estudia. Es parla d'estudis de cohorts prospectius quan s'espera un temps determinat a l'aparició de la malaltia i retrospectius quan es mira si la malaltia ja existeix o havia existit en el passat. En algunes ocasions es poden donar les dues situacions alhora. Els estudis de cohorts no es poden utilitzar per estudiar malalties rares, mentre que els de casos i controls estan limitats a exposicions freqüents[106,107].

En els estudis experimentals o d'intervenció l'equip investigador assigna el factor de l'estudi, de forma aleatòria o no, i el controla segons un pla establert. Poden ser controlats o no depenent de si existeix un grup control per comparar. Si existeix un control, els estudis clínics poden ser paral·lels o creuats. Ben dissenyats, permeten provar causalitat però són cars, es limiten a resoldre problemes molt concrets i plantegen problemes ètics[102,108].

L'elecció del mètode dependrà de la pregunta que es vulgui respondre, del temps i dels recursos disponibles. És important conèixer les avantatges i les limitacions de cada estudi a l'hora d'interpretar els resultats: es poden generalitzar els resultats?, es pot establir relació causal?, hi ha algun mecanisme biològic plausible que expliqui els resultats? Finalment, la recerca hauria d'acabar amb un missatge clar i entenedor que pugui ser comunicat a altres científics, als organismes de salut pública o a la població.

2.1. L'epidemiologia nutricional

L'epidemiologia nutricional és una branca de l'epidemiologia té com a objectiu l'avaluació dels efectes de la dieta sobre el risc de patir alguna malaltia. Aquest coneixement és utilitzat pels organismes de salut pública en les seves tasques de prevenció. Per a conèixer la influència de la dieta sobre la salut i poder planificar programes d'intervenció cal determinar amb exactitud la ingesta d'aliments o de nutrients (**Figura 2.3**). La valoració de la dieta es pot dur a terme mitjançant enquestes alimentàries i/o biomarcadors nutricionals. Els dos sistemes tenen avantatges i inconvenients que es resumeixen a la **Taula 2.1** i que cal tenir en compte a l'hora d'escollir-los i d'extreure conclusions. Si es disposa de recursos, la combinació dels dos mètodes és la millor solució[109].

Figura 2.3. Relació entre els diferents camps involucrats en l'epidemiologia nutricional.

Figura 2.4. Tipus d'enquestes alimentàries.

2.1.1. Estimació de la ingesta de nutrients

2.1.1.1. Enquestes alimentàries

Hi ha diferents mètodes per obtenir informació sobre els hàbits alimentaris d'una població (**Figura 2.4**). Es diferencien en la manera de recollir la informació i en el període que comprenen, i cadascun té avantatges i inconvenients. L'elecció d'un mètode concret dependrà de la població que es vol estudiar, dels aliments o nutrients d'interès, dels recursos disponibles i del disseny de l'estudi. En el cas de l'estudi PREDIMED, es va optar pels qüestionaris anuals de freqüència de consum (QFC) d'aliments, recollits a nivell individual i mitjançant entrevistes personals amb una dietista. De forma complementària, es va dissenyar un qüestionari curt, de 14 preguntes, per valorar l'adhesió de l'individu a la DM tradicional. Un QFC és un mètode retrospectiu que utilitza una llista tancada (i classificada) d'aliments i begudes i es pregunta sobre la freqüència de consum (mai, ocasionalment, setmanalment, diàriament, etc.) durant un període de temps determinat [109–111].

2.1.1.2. Biomarcadors nutricionals

Un biomarcador nutricional és un compost extern a l'organisme humà, per exemple components d'aliments o metabòlits d'aquests. El seu anàlisi en mostres biològiques, ja siguin

	Qüestionaris de freqüència de consum d'aliments	Biomarcadors nutricionals
Avantatges	Econòmics. Ràpid i senzill d'administrar. Capacitat de classificar individus per categories de consum.	Objectius. Precisos. Tenen en compte la biodisponibilitat i el metabolisme. Permeten valorar l'acompliment d'una intervenció nutricional.
Inconvenients	Depenen de la memòria i la fiabilitat de l'entrevistat. No té en compte la biodisponibilitat. No sempre es disposa de taules de composició d'aliments. Poca precisió en l'estimació i la quantificació de les porcions d'aliments. Requereix temps per part de l'enquestat.	Cost elevat. No sempre existeix un biomarcador. Complexa interpretació del resultat ja que depèn de la biodisponibilitat i el metabolisme. Varietat interindividual. Requereix extreure, conservar i treballar amb mostres biològiques.

Taula 2.1. Comparació entre les enquestes de consum i els biomarcadors nutricionals.

sang, orina, teixits, etc. permet estimar la ingesta de certs aliments o compostos. Un bon biomarcador ha de tenir les següents característiques[112,113]:

1. Disposar d'un mètode analític exacte, reproduïble, fiable, vàlid i robust per a poder-lo quantificar.
2. Les concentracions del biomarcador en la mostra biològica han de ser sensibles als canvis en la ingesta del compost o l'aliment estudiat. Sobretot és imprescindible que permeti distingir els consumidors dels no consumidors.
3. El biomarcador ha de ser específic de l'aliment estudiat. Aquest criteri és el més difícil tot i que en alguns casos es compleix, com en el cas del tirosol i l'hidroxitirosol com a biomarcadors de l'oli d'oliva[114].

En l'estudi PREDIMED es van utilitzar biomarcadors nutricionals per verificar el correcte compliment de la intervenció. L'hidroxitirosol i el tirosol en orina, així com l'àcid oleic en plasma demostraven el correcte seguiment de la DM suplementada amb oli d'oliva verge extra. Pel grup de fruits secs es va analitzar l'àcid α-linolènic en plasma ja que es característic de les nous. D'altra banda, el consum de fruites, verdures i begudes riques en polifenols es va relacionar amb una excreció superior de polifenols totals en orina[115].

2.1.2. Quantificació de polifenols totals en orina mitjançant el mètode de Folin-Ciocalteu

L'anàlisi colorimètric amb el reactiu de F-C ha estat àmpliament utilitzat per a la quantificació de polifenols totals en mostres d'aliments[116–118]. La primera vegada que es va fer aplicar

a mostres biològiques va ser per utilitzar-lo com a biomarcador de consum de vi[119,120]. El reactiu de F-C és una solució de color groc brillant que conté una mescla de complexos de fosfomolibdat i fosfotungstat amb l'estructura següent:

$3\ H_2O \bullet P_2O_5 \bullet 13\ WO_3 \bullet 5\ MoO_3 \bullet 10\ H_2O$

$3\ H_2O\ x\ P_2O_5 \bullet 14\ WO_3 \bullet 4\ MoO_3 \bullet 10\ H_2O$

En medi bàsic, la transferència d'electrons del reactiu als fenols redueix els complexos a òxids de tungstè (W_8O_{23}) i molibdè (Mo_8O_{23}), que són cròmofors de color blau intens. Aquesta coloració es pot mesurar espectrofotomètricament a 765 nm i és proporcional al número de grups hidroxil de la molècula[121]. Així doncs, no tots els polifenols reaccionaran amb la mateixa intensitat i això pot donar lloc a errors de quantificació.

El reactiu de F-C no reacciona de forma específica amb els polifenols sinó que també ho fa amb sucres, amines aromàtiques, diòxid de sofre, àcid ascòrbic, àcids orgànics, Fe(II) i altres substàncies orgàniques no fenòliques però oxidables que es troben habitualment a l'orina[122]. Així doncs, és imprescindible eliminar les interferències en les mostres d'orina fent-les passar per un cartutx d'extracció en fase sòlida (SPE) abans del seu anàlisi[115].

3. Bioestadística

La bioestadística és una branca de l'estadística aplicada que utilitza els mètodes estadístics per resoldre problemes mèdics i biològics. Es divideix en dues branques: descriptiva, per sintetitzar i presentar la informació continguda en unes dades, i l'analítica, que permet demostrar associacions i relacions entre les característiques observades mitjançant contrastos d'hipòtesis i intervals de confiança. Així doncs, l'epidemiologia s'encarrega de dissenyar un pla d'investigació i una estratègia òptima de recollida de dades i la bioestadística tracta matemàticament aquestes dades per obtenir-ne informació. Un cop obtinguts els resultats, és necessari tornar al camp de l'epidemiologia per interpretar-los amb sentit crític ja que uns resultats estadísticament significatius no són necessàriament vàlids si no tenen plausibilitat biològica[123,124].

3.1. Correlació i regressió

La correlació té com a finalitat examinar la magnitud i la direcció de l'associació entre dues variables quantitatives. La mesura del grau d'associació ens la donen els coeficients de correlació, que poden ser de dos tipus: el de Pearson (r), per a dades paramètriques i que estima l'adaptació a un model lineal, i el de Spearman (rho), per a dades no paramètriques i que mesura qualsevol tipus d'associació, lineal o no[124].

El coeficient de Pearson pot prendre valors de -1 a 1, essent 0 el valor nul, és a dir, sense correlació. Quan r pren valors positius parlarem d'associació directa: al augmentar una variable també augmenta l'altra. En canvi, si r és negativa, l'associació serà indirecta. Valors per sobre de 0.7 (en valor absolut) representen associacions fortes. Si les variables mesurades no compleixen els criteris de normalitat o no són ordinals farem servir el coeficient de Spearman[124]. A diferència de la regressió, la correlació no distingeix entre variables dependents i independents ja que no hi ha relació causa-efecte i, per tant, són intercanviables. A més, el coeficient de correlació no està influït per les unitats de mesura[124].

La regressió descriu d'una manera més detallada la relació entre dues variables de manera

que pot tenir finalitats predictives: acceptant un marge d'error, es pot predir el valor d'una variable si sabem el valor de l'altra. Sempre suposarem que hi ha una variable independent o predictora, controlada per l'investigador, i una variable dependent o resposta[125].

Hi ha dos tipus de regressió lineal, la simple i la múltiple. La primera analitza la relació entre dues variables quantitatives per determinar en quin grau s'ajusta a la linealitat. Per obtenir estimacions més precises s'utilitza la regressió lineal múltiple, que té més d'una variable explicativa. Permet saber, entre un conjunt de variables, quines tenen major influència sobre la variable dependent.

Un cas particular de regressió seria la logística, que s'empra quan la variable dependent és dicotòmica (malalt/sa, mort/no mort, etc.). La mesura entre variables es fa mitjançant el coeficient de probabilitats o odds ratio (OR). Quan es té en compte el temps de seguiment fins que es produeix el fenomen d'interès s'utilitza la regressió de Cox, que mesura raons de taxes o Hazard ratios (HR). Així doncs, la OR té un sentit estàtic mentre que el HR és dinàmic. El model de Cox fa una mitjana ponderada de les HR de tots els moments en els que es produeix una mort o un esdeveniment, és com fer moltes regressions logístiques[124,125].

3.2. Mesures de freqüència i associació

En epidemiologia, les mesures de freqüència s'usen per descriure i comparar la magnitud d'una malaltia o un determinat estat de salut en diferents poblacions. N'hi ha de dos tipus, les que descriuen la proporció de casos existents en un moment determinat (prevalença, P) i les que descriuen l'aparició de nous casos en un període de temps en forma de proporció (incidència acumulada, IA) o de taxa (taxa d'incidència, I)[126].

$$P = \frac{N°\text{de casos en un moment determinat}}{\text{Total de la població}}$$

$$IA = \frac{N°\text{de casos nous durant un període de temps}}{\text{Total de la població sense la malaltia i en risc}}$$

$$I = \frac{N°\text{de casos nous durant un període de temps}}{\text{Total de persones} - \text{temps d'observació}}$$

Les mesures d'associació o mesures relatives de risc (**Taula 3.1**) són indicadors epidemiològics que avaluen amb quina força una malaltia o un indicador de malaltia es relaciona a un determinat factor que es pensa que pot ser una causa. Les mesures més sòlides són les que es calculen utilitzant incidències ja que existeix una relació temporal entre la causa i l'efecte[127].

$$\text{Raó de riscos} = \frac{\text{Risc de malaltia dels exposats}}{\text{Risc de malaltia dels no exposats}}$$

- No té unitats
- Pren valors de 0 a infinit
- Significat dels valors:
  - <1 – el factor d'estudi és protector
  - =0 – no existeix associació (valor nul)
  - >1 – el factor d'estudi és factor de risc

Les mesures d'impacte potencial o mesures absolutes del risc (**Taula 3.1**) indiquen la contribució d'un determinat factor en el desenvolupament d'una malaltia entre els individus exposats així com el grau de benefici de les accions preventives. Totes ens donen informació similar però la manera de calcular-les depèn del disseny de l'estudi (cohorts, casos i controls, etc.)[127].

3.3. Associació versus causalitat

Idealment, un estudi hauria d'avaluar si una relació és causal, és a dir, si una alteració en l'exposició modifica el risc de malaltia. No obstant, les relacions causa-efecte són difícils de demostrar per diversos motius. Els períodes de latència de les malalties cròniques són molt llargs, solen durar anys, i és difícil mantenir els estudis durant tant de temps. Abans de considerar que una relació és causal cal considerar tres explicacions alternatives: l'efecte dels factors de confusió, el biaix (d'observació i de selecció) i la casualitat[126,128].

Un cop controlades les explicacions alternatives que desestimarien una relació causal hi ha altres criteris per concloure aquesta relació. Una força d'associació gran minimitza l'impacte de factors de confusió no controlats, ja sigui per desconeixement o per la impossibilitat de controlar-los. La consistència de resultats entre estudis fets per diferents investigadors i tipus de poblacions també recolza la teoria d'una relació causa-efecte. Per últim, hi hauria d'haver un mecanisme o una credibilitat biològica que expliqués els resultats i, si és possible, una relació dosi-resposta[126,128].

3.4. Anàlisi de supervivència

En els anàlisis de supervivència, la variable d'interès no és quantitativa ni qualitativa sinó temporal. Combinen dos elements, un de dicotòmic (aparició o no d'un esdeveniment), i un de quantitatiu (quan de temps transcorre fins a l'esdeveniment). El desenllaç no necessàriament ha de ser la mort de l'individu però només es pot produir una vegada i impliquen l'existència d'informació truncada o censurada, amb temps d'observació incomplets[124,129].

El mètode de Kaplan-Meier és la forma més comuna d'estimar la distribució de la supervivència. És un mètode no paramètric mitjançant el qual es pot calcular la proporció de supervivència i el temps de supervivència. Se sol representar gràficament, amb el temps a l'eix d'abscisses i el percentatge de supervivència al d'ordenades. Per comparar dues o més corbes de supervivència s'utilitza el test del Log-rank, que té en compte les diferències de su-

	Mesura	Fórmula	Tipus d'estudi	Interpretació
Raó de mesures (escala relativa)	Risc relatiu, raó d'incidències acumulades, o raó entre dos riscos.	$RR = \frac{IA_e}{IA_o}$	Estudis de cohorts amb pacients amb el mateix període de seguiment.	Els exposats tenen X vegades més risc de desenvolupar una malaltia que els no exposats.
	Raó de taxa d'incidència, o raó de densitats d'incidència.	$RTI = \frac{I_e}{I_o}$	Estudis de cohorts amb pacients amb diferent període de seguiment.	Els exposats tenen una taxa de la malaltia X vegades més alta que els no exposats.
	Odds ratio	$OR = \frac{\text{odds d'exposició en els casos}}{\text{odds d'exposició en els controls}}$	Estudis de casos i controls	Els exposats tenen X vegades més odds (probabilitats) de tenir la malaltia que els no exposats.
Diferències entre riscos (escala absoluta)	Risc atribuïble als exposats	$RAE = IA_e - IA_o$	Estudis de cohorts amb pacients amb el mateix període de seguiment.*	La freqüència de malaltia atribuïble a l'exposició és X.
	Fracció etiològica en exposats	$FEE = \frac{IA_e - IA_o}{IA_e} = \frac{RAE}{IA_e}$	Estudis de cohorts amb pacients amb el mateix període de seguiment.*	Assumint causalitat, un X% de la malaltia entre els exposats està causada per l'exposició.
	Nombre necessari a tractar	$NNT = \frac{1}{RAE}$	Estudis de cohorts amb pacients amb el mateix període de seguiment.*	Caldria tractar (o evitar l'exposició) a X persones per evitar 1 malalt.

*Si el període de seguiment no és el mateix entre tots els pacients, es parla de raó de risc atribuïble i s'utilitza la incidència.
IA: Incidència acumulada, I: Incidència, e: exposats, o: no exposat

Taula 3.1. Estimació i interpretació de les principals mesures d'associació i d'impacte potencial.

pervivència entre grups en tots els punts del temps que dura el seguiment. De forma similar, el mètode de Nelson-Aalen genera una funció dels hazard rates acumulats[124,129].

4. L'estudi PREDIMED

L'estudi PREDIMED (PREvención amb DIeta MEDiterrània, ISRCTN35739639) ha estat un assaig d'intervenció prospectiu, aleatoritzat, multicèntric i controlat. El seu objectiu va ser determinar els efectes beneficiosos de la dieta mediterrània en la prevenció primària de malalties cardiovasculars[130,131]. L'assaig va tenir una durada de 9 anys, els primers voluntaris es van reclutar l'any 2004 i va ser finançat per l'*Instituto de Salud Carlos III* (G03/140).

Per a l'estudi es van reclutar pacients a través de centres d'atenció primària de 8 comunitats autònomes i se'ls va assignar de forma aleatòria un dels tres grups d'intervenció nutricional:

a) Dieta Mediterrània complementada amb oli d'oliva verge extra (DMOO)

b) Dieta Mediterrània complementada amb fruits secs (DMFS)

c) Grup control: dieta baixa en greixos (DBG) segons les recomanacions de la American Heart Association, AHA[132].

A continuació es resumeixen els criteris d'inclusió que es van escollir[131]. Finalment, 7447 participants complien amb els criteris i van participar en l'estudi.

- Edat: 55-80 anys (homes) i 60-80 anys (dones).
- Lliures de malalties cardiovasculars.
- Diagnosticats de Diabetis Mellitus tipus 2 o que compleixin tres o més dels factors de risc següents:
  - Fumadors (>1 cigarreta/dia durant l'últim mes)
  - Hipertensió arterial (PAS≥140 mm Hg i/o PAD≥90 mm Hg, o medicació antihipertensiva)
  - Hipercolesterolèmia (Colesterol LDL≥160 mg/dL, colesterol HDL≤40 mg/dL en homes o ≤50 mg/dL en dones, o medicació pel colesterol)
  - Sobrepès o obesitat (IMC≥25 kg/m^2)
  - Història familiar de cardiopatia isquèmica precoç.
- Tenir capacitat i voluntat de canviar d'hàbits alimentaris[133].
- No patir cap malaltia greu que impedeixi la participació en un estudi d'intervenció dietètica.
- No tenir o haver tingut alcoholisme o drogoaddicció.

Un cop firmat el consentiment informat, als voluntaris se'ls agafaven dades mèdiques, com la PA (per triplicat) i antropomètriques, com l'altura, el pes i el perímetre de cintura, i se'ls feia omplir el següents qüestionaris:

a) Qüestionari d'inclusió.

b) Qüestionari general: dades demogràfiques i sociològiques.

c) Qüestionari de seguiment

d) Qüestionari de freqüència de consum alimentari[134,135]

e) Qüestionari d'adherència a la DM[134]

f) Qüestionari d'activitat física[136]

A més, s'han fet diferents determinacions bioquímiques a partir de mostres biològiques (sang, orina i ungles), se'ls va mesurar la PA i se'ls va realitzar un electrocardiograma. Els pacients es visitaven un cop l'any i es repetien els qüestionaris i la presa de mostres biològiques.

Els participants van rebre assessorament personalitzat sobre dieta mediterrània o dieta baixa en greixos i cada tres mesos, assistien a unes xerrades sobre alimentació per a aconseguir canvis en els seus hàbits alimentaris. En aquestes sessions, els del grup de DMOO rebien oli d'oliva verge extra (1L per setmana), als de DMFS se'ls proporcionaven bosses amb 30 g de fruits secs per dia (50% nous, 25% ametlles, 25% avellanes), i als del grup control se'ls premiava amb altres tipus de regals (vaixella, davantal, etc.). A més, rebien informació per escrit sobre aliments, receptes i ajudes per a fer la llista de la compra[130].

Després d'una mitjana de 4.8 anys de seguiment, i un any i mig abans que finalitzés el període de seguiment, un comitè científic extern encarregat d'avaluar el projecte va advertir que les diferències entre els grups mediterranis i el grup que seguia la dieta baixa en greix eren prou significatives com per aturar l'estudi. Els resultats revelaren que ambdós grups de dieta mediterània, suplementada amb oli d'oliva verge extra o amb fruits secs, tenien una incidència 30% menor de malalties cardiovasculars que el grup control (dieta baixa en greix). En concret, el grup de DMOO, en el qual es van registrar 96 esdeveniments cardiovasculars, el valor de HR ajustat va ser de 0.70 (IC 95%=0.54-0.92) i en el grup de DMFS, amb 83 casos, va ser lleugerament superior (HR=0.72, IC 95%=0.54-0.96). Amb aquests resultats, es comunicà als voluntaris del grup control que havien de modificar la dieta per ajustar-la a un patró més mediterrani, incloent oli d'oliva i fruits secs[137].

Dins l'estudi PREDIMED, s'han fet nombrosos sub-estudis que demostraren, per exemple, que la DMOO i la DMFS reduïen el colesterol LDL, la glucosa, la PA i els biomarcadors d'inflamació després de només 3 mesos d'intervenció[130,138]. També es va fer un subestudi amb 1224 participants comparant els dos grups de DM amb el control després d'un any per demostrar que la DM revertia la síndrome metabòlica de forma significativa (OR=1,3 per la DMOO i OR=1,7 per la DMFS, en comparació amb el control)[139]. Altres articles s'han focalitzat en els beneficis de la DM sobre la obesitat[140,141], el deteriorament cognitiu[142] o la diabetis tipus 2[143], entre d'altres.

Per tant, aquests resultats apunten que s'hauria d'incloure el patró de DM complementada amb fruits secs i oli d'oliva verge a les recomanacions nutricionals, especialment les dirigides a persones grans amb risc elevat de malaltia cardiovascular.

III. Introduction

1. Polyphenols

1.1. Structure and classification

Polyphenols are naturally occurring compounds mainly found in fruits, vegetables, cereals and beverages, since they are secondary metabolites of plants. This group of compounds, hundreds of which have been described, is the main source of antioxidants in our diet. Their structure consists of at least one aromatic ring carrying one or more hydroxyl moiety[4].

Polyphenols are classified according to the number of phenol rings they bear and the structures that bind these rings. One generally accepted classification divides polyphenols into two groups: flavonoids and nonflavonoids. The former, shown in **Table 1.1**, have a C6-C3-C6 structure and comprise the following polyphenol subgroups: flavones, flavonols, flavan-3-ols or flavanols (and their polymeric forms, proanthocyanidins), flavanones, anthocyanidins, and isoflavones, and in smaller amounts, chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins and aurones. The nonflavonoid group, whose structures are depicted in **Table 1.2**, is classified according to the number of carbons they possess, and include phenolic acids, stilbenes, lignans and other polyphenols such as simple phenols[4,5].

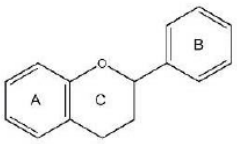
1.2. Distribution and analysis of polyphenols in food

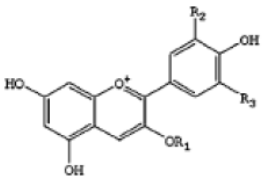
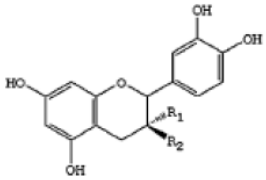
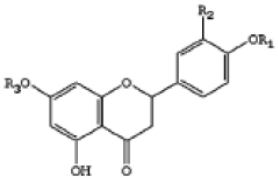
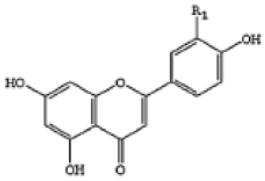
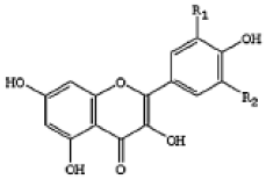
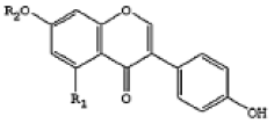
It is essential to have reliable information about the phenolic content in foods in order to estimate polyphenol intake accurately. Food matrices can be highly complex and influence the analysis and bioavailability of their components. In general, there are two key aspects to take into account: physical/structural and biological. Indeed, it is expected that the phenolic content of the skin of a given fruit will not be the same as that of the pulp or seeds. Maturity, climate, or manipulation will also influence the concentration of polyphenols[6–8]. Plants synthesize polyphenols as a mechanism of defense against external agents. Given the scarce or null use of pesticides in organic agriculture, it produces fruit and vegetables with a higher polyphenol content than traditional agriculture[9].

Flavonols, one of the flavonoid subgroups, are the most abundant polyphenols in food. They are found in cocoa, tea, wine, berries, onions, asparagus, and in most of the spices, among other sources. Flavones like apigenin and luteolin are mainly found in artichokes, pepper, celery, grapes or oranges. Flavanones are typical polyphenols of citrus fruits, whereas isoflavones are found in legumes such as soy and its products. Peaches, berries, apples and black grapes are rich in flavanols or flavan-3-ols, procyanidins (also called condensed tannins) and anthocyanidins[6].

Although flavonoids have traditionally been the most broadly studied group, nonflavonoids also contribute significantly to our polyphenol dietary intake and can be responsible for the nutritional characteristics of food[10]. This is the case of olive oil, which contains several simple phenols such as hydroxytyrosol and tyrosol[11]. The phenolic acids, including hydroxycinnamic acids and hydroxybenzoic acids, are mainly found in berries, olives, walnuts, and tea. Stilbenes and lignans, albeit in low concentrations, are characteristic of red wine

FLAVONOIDS



Group	Chemical structure	Examples	Example of food source
Anthocyanidins		Delphinidin Cyanidin Malvidin	
Flavan-3-ols or flavanols		Epicatechin Catechin Epigallocatechin	
Flavanones		Hesperitin Naringenin Eriodictyol	
Flavones		Luteolin Apigenin Tangeretin	
Flavonols		Quercetin Kaempferol Myricetin	
Isoflavonoids		Genistein Daidzein Glycitein	

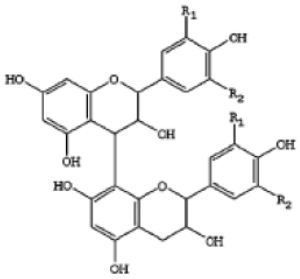
Proanthocyanidins		Polymers of flavanols	

Table 1.1. Characteristics, classification and examples of flavonoids.

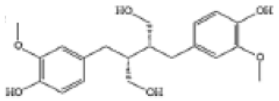
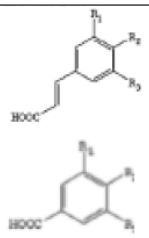
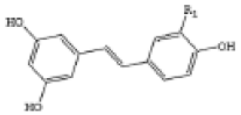
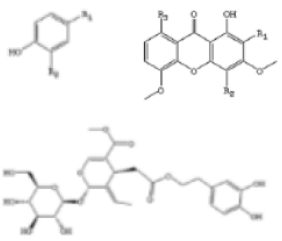
NONFLAVONOIDS			
Group	Chemical structure	Examples	Example of food source
Lignans		Lariciresinol Pinoresinol Sesamin	
Phenolic acids		Gallic acid Caffeic acid Vanillic acid	
Stilbenes		Resveratrol Pallidol Piceatannol	
Others		Tyrosol Oleuropein Xanthotoxin Ferulaldehyde Eugenol Curcumin	

Table 1.2. Characteristics, classification and examples of nonflavonoids.

and cereals, respectively. The most known and studied stilbene is trans-resveratrol, which is reported to have multiple health effects[12,13].

When analyzing polyphenol content in food, it is important to remember they are sensitive to UV light and temperature. Polyphenols are exposed to the external environment by the peeling, cutting and grinding usually required for food analysis and are transformed by the resulting enzymatic browning reactions. Low temperatures, organic solvents, lyophilization and working under UV-free light conditions are extensively used methods to prevent the oxidation of polyphenolic compounds. Moreover, polyphenols also commonly undergo isomerization and hydrolysis during food analysis[14].

The extraction step aims to extract the maximum number of compounds while avoiding degradation. Not only should the complexity of the food matrix be born in mind, but also possible interferences, the variable solubility of phenolic compounds, temperature, extraction time, etc. When it comes to the analysis, one of the main difficulties lies in the varying concentration levels, ranging from traces to milligrams. To separate polyphenols, the most frequent method is liquid chromatography (HPLC and UHPLC), although in some cases gas chromatography and capillary electrophoresis can also be used[15].

The most common technique for identification and quantification is mass spectrometry, due to its versatility in allowing numerous combinations of ionization sources (e.g. electrospray ionization, chemical ionization, fast atom bombardment, matrix-assisted laser desorption/ionization) and detectors (e.g. triple quadrupole, time of flight detector, cyclotonic resonance, diode-array). When the main objective is not a detailed phenolic profile but the quantification of total polyphenols or those of a given group, the election method should be spectrophotometric. The Folin-Ciocalteu (F-C) method has been extensively used to determine total polyphenol content. Other more specific reactives have been used to determine proanthocyanidins, hydrolyzable tannins, anthocyanidins, and flavan-3-ols[5,15,16].

Therefore, we can conclude that analysis of polyphenols in food is a highly complex process that requires multiple factors to be considered and varies according to the food and the studied polyphenol.

1.3. The Phenol-explorer database

Traditionally, the USDA (United States Department of Agriculture) Flavonoid Database has been the reference database for polyphenol content in foods. However, this database does not include nonflavonoids, which, as mentioned above, are qualitatively rather than quantitatively relevant.

In August 2009, the Phenol-explorer database (www.phenol-explorer.eu) was launched. It was a project led by the National Institute of Agricultural Research (*Institut Nacional de la recherche agronomique*, INRA), in France, and provides data on more than 500 different polyphenols in more than 400 foods. Values were extracted from over 1300 original papers. In 2011, the 2.0 version was released, with information about phenolic metabolites. The most recent Phenol-explorer 3.0 includes information about the effect of food processing and cooking[17].

1.4. Organoleptic characteristics of polyphenols

Polyphenols are partly responsible for some organoleptic characteristics of several plant-origin foods (**Figure 1.1**). For example, the bitterness of grapefruit and olives is caused by narin-

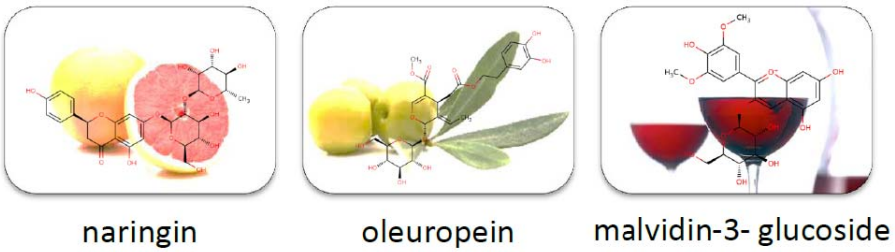


Figure 1.1. Examples of polyphenols that confer bitterness.

gine and oleuropein, respectively. Condensed tannins or proanthocyanidins and hydrolyzable tannins confer astringency, especially to the seeds and skin of fruits such as grapes and their product, wine. Moreover, eugenol is responsible for the banana flavor[18,19]. Other polyphenols are responsible for fruit and vegetable colors: anthocyanins are red, purple and blue (plums, red cabbage, red wine, radishes, etc.), while flavonols are yellowish (white wine, apples, tea, etc.). Sometimes, major polyphenols like hydroxycinnamic acids have no direct impact on the organoleptic characteristics of food but have a negative effect when they oxidize since they produce brown polymers[19].

1.5. Polyphenols and health

1.5.1. Bioavailability of polyphenol compounds

Generally, bioavailability is the proportion in which an ingredient is absorbed and becomes available at the site of action. This includes the release and digestion in the digestive system, transport across the intestinal membrane into the bloodstream, distribution to different tissues, metabolism of compounds and, finally, elimination[8]

The wide variety of existing polyphenols is matched by their variable bioavailability. The most abundant dietary polyphenols are not necessarily the most bioavailable, while on the contrary, some polyphenols consumed in trace levels can have a high biological activity[20]. Absorption of polyphenols depends on fat intake, the food matrix, dose, and intestinal transit[8]. For example, gallic acid, isoflavones, catechins, flavonols, flavanones and quercetin glycosides are the most easily absorbed polyphenols, while proanthocyanidins, gallocatechines and anthocyanins are less absorbed[8,20].

In some cases, the organism needs to metabolize polyphenols in order to improve their absorbtion, while aglycones and anthocyanins do not need to be metabolized and are directly absorbed in the stomach and small intestine. In contrast, esters, glycosides and polymers need to be hydrolyzed by intestinal enzymes or by colonic microbiota before being absorbed. During the absorption process, polyphenols are conjugated mainly by methylation reactions, sulfation and/or glucuronidation in the enterocytes of the small intestine and liver[4,21]. These conjugations vary depending on the nature of the substrate and dose. The polyphenol metabolites then travel through the bloodstream attached to carriers such as albumin. Plasma concentrations of polyphenols range from 0 to 4 mol/L with an intake of 50 mg aglycone equivalents. Regarding kinetics, maximum plasma concentration (C_{max}) is reached after 1.5-5.5 hours, depending on the site of absorption[8,20].

Polyphenol excretion can occur in two different ways, depending on the molecular weight. The heavier compounds, which are also the most conjugated, are usually eliminated by the

biliary tract, while polyphenols with lower molecular weights have a higher probability of being excreted through the urine via the kidney[20].

Besides the different polyphenol structures, there are other factors that affect bioavailability and pharmacokinetics. Firstly, since polyphenols are consumed as food they are linked to macronutrients (proteins, carbohydrates and lipids) and micronutrients that affect their absorption. The sex and age of the consumer also affects the absorption process[22], as does food processing. The cooking method affects the polyphenol absorption in contrasting ways. On the one hand, heat helps to break down the food matrix and release compounds, thus increasing their bioavailability, but on the other hand, high temperatures degrade antioxidants. In some cases, the compounds pass from one medium to another, for example, from food to water when boiling or from oil to food when frying[23].

1.5.2. Beneficial effects of polyphenol intake

Polyphenols are the main source of antioxidants in our diet. Their ability to capture free radicals makes them good candidates for the prevention of diseases associated with oxidative stress. Numerous clinical and epidemiological studies have shown that polyphenol consumption may protect against cardiovascular disease, neurodegenerative diseases, some cancers, insulin resistance, and obesity, among others.

a) Oxidative stress and ageing

Normal physiological processes such as respiration and metabolic reactions that take place in our body produce reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($OH\bullet^-$) are examples of ROS[24]. As defence mechanisms, our body has endogenous antioxidants, such as superoxide dismutase, catalase or the glutathione reductase, to eliminate the ROS that are continuously being produced. These and other non-enzymatic mechanisms act by preventing the formation of ROS, reducing them, repairing oxidative damage by eliminating the molecules involved and preventing mutations. Stress, pollution and ageing break the balance between ROS production and elimination, resulting in what is known as oxidative stress[25].

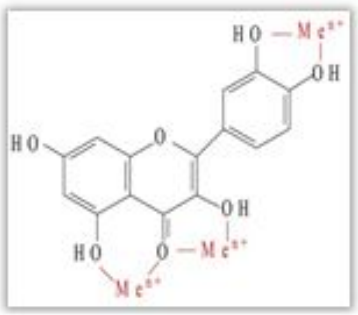


Figure 1.2. Favorable position for metal chelation.

ROS are free radicals and oxidizing molecules that can bind to DNA, lipids and proteins, altering their stability and leading to various diseases, such as diabetes, Alzheimer's, Parkinson's, cancer, CV related diseases and respiratory diseases.

Polyphenols play an important role in reducing oxidative stress through their antioxidant capacity. The structures of these compounds, bearing very stable phenolic rings, allow the hydroxyl groups to easily lose a proton and therefore oxidize. The antioxidant activity of polyphenols depends on the number of hydroxyl groups and their relative position, the ortho position being the most favorable. The combination of the ketone with the double bond also facilitates the formation of resonant forms and therefore promotes the loss of electrons. Finally, polyphenols also have different areas that allow chelation with metals, such as hydroxyl groups in ortho or ketones with adjacent hydroxyl groups (**Figure 1.2**).

Polyphenols proposed to delay ageing include epigallocatechin gallate, chercetin, or resvera-

trol[25]. These and other similar polyphenols, as well as their main food sources, are summarized in **Table 1.3**.

b) Cardiovascular diseases

CV diseases are responsible for over 16 million deaths worldwide, representing 30% of all deaths. In developed countries, heart disease is the leading cause of morbidity and mortality, which has alerted the public health agencies. These diseases include coronary heart diseases, hypertension, myocardial infarction and stroke[25,26].

Some risk factors, such as age, sex and genetic predisposition, are not modifiable, but others depend on lifestyle and are also strongly related to each other (**Figure 3**). Thus, leading a quiet life, sleeping adequately, doing physical exercise, not smoking and eating a balanced diet help to maintain a healthy weight and reduce the risk of hypertension, dyslipidemia, and diabetes, which, in turn, are also CV risk factors[27]. Nowadays, it is known that preventing or treating these risk factors is much more effective than treating the CV disease itself. Therefore, it is necessary to focus more efforts on prevention.

A large number of epidemiological studies have associated the consumption of polyphenols with a decreased risk of CV or coronary heart disease. In a meta-analysis of the relation of tea with these diseases, the authors concluded that consumption of tea had a cardioprotective effect[28]. A literature review on moderate wine consumption showed similar results[29]. More recently, a meta-analysis of randomized intervention studies related flavan-3-ols with a reduction in biomarkers of cardiovascular risk[30].

Other studies have focused on the use of one or more groups of polyphenols: two prospective studies associated intake of anthocyanins and flavanones with a lower risk of CV disease and total mortality[31,32]. Hertog and colleagues found a low but positive association between the consumption of flavonols and all-cause mortality but found no significant association with heart disease or cancer[33].

The protective effect of polyphenols may be explained by the improvements they confer on various risk factors. Thus, clinical studies, using animal models and humans, have shown that polyphenols improve endothelial function by improving parameters such as LDL (low-density lipoproteins) cholesterol, platelet aggregation, invasion and proliferation of smooth muscle cells in the arterial wall, nitric oxide (NO) and some markers of inflammation[34].

Endothelial disfunction

The endothelium is the innermost layer of the blood vessel walls. Endothelial cells, in response to various stimuli, release vasodilator factors, vasoconstrictor substances, growth factor promoters or inhibitors, modulators of inflammation and hemostatic and thrombolytic factors. These factors are responsible for maintaining vascular tone, controling the growth of vascular smooth muscle and modulate coagulation, fibrinolysis and adhesion of blood cells to the endothelial wall.

Endothelial dysfunction occurs when the balance is disrupted and the homeostatic functions of the endothelium are altered. Endothelial dysfunction is the first step in the progress of atherosclerosis and, therefore, the development of cardiovascular disease.

Nitric oxide (NO), for example, is a vasodilator and reduces blood pressure (BP). Oxidative stress reduces the bioavailability of NO radicals and therefore promotes the inflammation process. One hypothesis is that polyphenol-rich foods decrease blood pressure by activation of NO synthase. *In vitro* experiments with isolated arteries showed that polyphenols increased endothelial NO formation and caused NO-mediated endothelium-dependent relaxations[35].

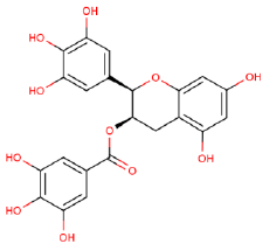
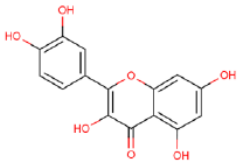
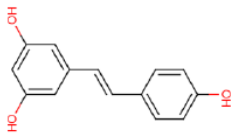
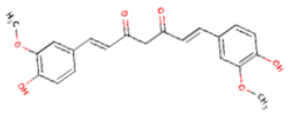
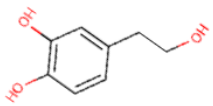
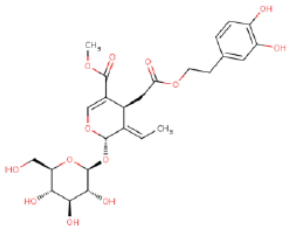
Poyphenol	Structure	Sources
Epigallocatechin gallate		Green tea
Quercetin		Onion, apple, broccoli, tea, cappers, cocoa, plums
Resveratrol		Red wine, grapes, berries
Curcumin		Curcumin
Hydroxytyrosol		Olive oil and olives
Oleuropein		Olive oil and olives

Table 1.3. Polyphenols related with oxidative stress and ageing prevention.

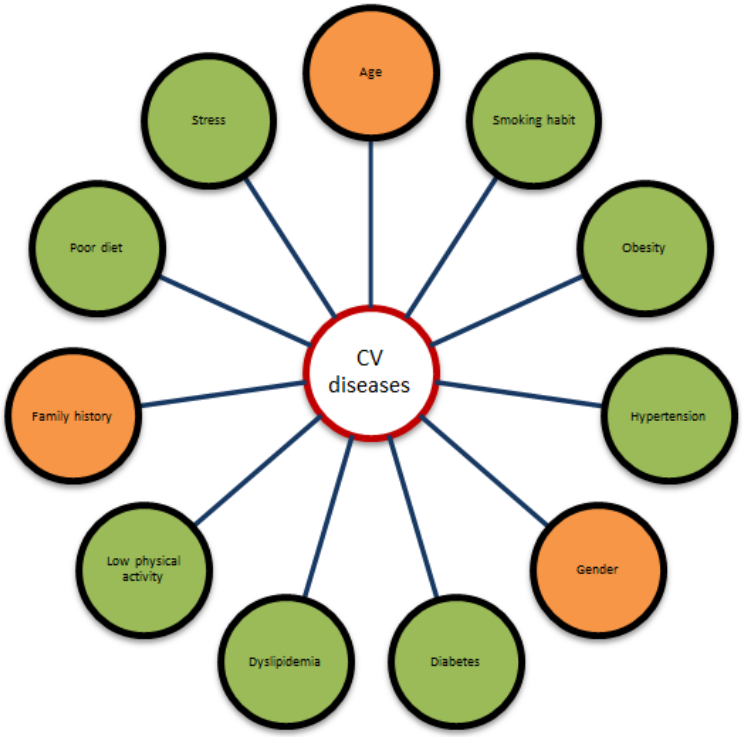


Figure 1.3. Cardiovascular risk factors. In orange, non-modifiable risk factors. In green, modifiable risk factors.

Atherosclerosis

Atherosclerosis is a chronic inflammation of large artery walls as a result of endothelial dysfunction. Diabetes, hypertension, smoking, high LDL cholesterol levels or low HDL (High Density Lipoprotein) cholesterol levels are risk factors for atherosclerosis.

LDL cholesterol is crucial in the process of formation of atherosclerotic plaque (**Figure 1.4**). Low density lipoproteins accumulate in the vascular endothelium and cross the endothelial cells. This process is facilitated when blood pressure is high. Once LDL molecules have crossed endothelial cells, they oxidize and cause inflammation, alerting the immune system, which responds by sending monocytes. These enter inside the intima through adhesion molecules such as ICAM-1 (Inter-Cellular soluble adhesion molecule-1) and VCAM-1 (Vascular Cell adhesion molecule-1) and are transformed into macrophages, which absorb LDL molecules, becoming foam cells trapped within the intima. Macrophages, together with T cells, stimulate the proliferation of smooth muscle cells that form a fibrous plaque with foam cells. The atherosclerotic plaque causes a decrease of arterial lumen and blood flow. Advanced atheroma can become detached, forming clots (thrombosis) that travel through the circulatory system until they become stuck, causing, for example, a heart attack or stroke[36,37].

Hypertension

Hypertension is a public health issue that affects more than 1 billion people worldwide, causing 7.6 million deaths annually[38]. In Spain, it is estimated that 30-45% of the adult population is hypertensive, which means that there are about 8 million individuals with this condition. A person is considered hypertensive if they permanently have systolic blood pressure (SBP) greater than 140 mm Hg and/or diastolic blood pressure (DBP) greater than 90 mm Hg. Although the diagnosis of hypertension is simple, a lot of people are unaware they are hypertensive and, therefore, they are not medicated or controlled.

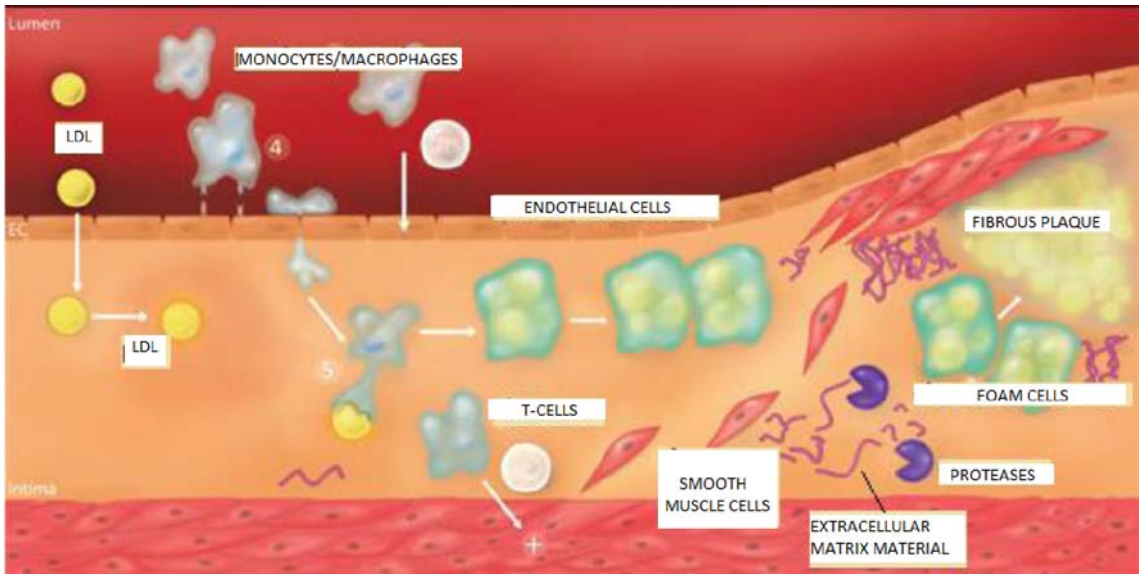


Figure 1.4. Atherosclerotic plaque development

Hypertension itself is a major risk factor for circulatory diseases, especially heart disease, cerebrovascular disease and heart failure. But hypertension is also related to other risk factors such as obesity, lack of physical exercise, a poor diet and high alcohol consumption.

Observational and intervention studies have shown that consumption of polyphenol-rich foods is associated with a decrease in BP. For example, in a double-blind crossover intervention study conducted with hypertensive patients, it was concluded that a daily consumption of 100g of dark chocolate rich in flavonoids significantly decreased BP and increased endothelium-dependent flow mediated dilation (FMD), whereas no improvement was observed with white chocolate (without flavonoids)[39].

Table 1.4 summarizes the results of several publications on the effect of polyphenols in BP obtained from human intervention studies[40–61].

c) Cancer

Anticancerogenic effects of polyphenols have been extensively demonstrated in animal models. The administration of polyphenols to rats or mice with tumors or under the effect of carcinogens protects against cancer and often reduces the number of tumors or their growth[62].

However, to attribute this effect to the antioxidant properties of polyphenols is a simplification and several explanatory mechanisms have been proposed. First, polyphenols may act by blocking the initial stages of the disease by modulating the expression of cytochrome P-450 enzymes involved in the activation of carcinogens and limiting the formation of initiated cells by stimulating DNA repair. Moreover, polyphenols slow or stop tumor growth by inhibiting the expression of genes involved in tumor proliferation or inducing apoptosis of malignant cells. Finally, it is also thought they can inhibit angiogenesis and limit tumor invasion. Thus, polyphenols act at initiation, promotion and progression stages[34,63].

The main issues raised in the research on cancer and polyphenols concern the translation from studies on animals to humans, and the dose of polyphenols administered, which is always much higher than the usual dose ingested through the diet. In some cases, opposite effects have been observed depending on the dose. For example, caffeic acid in high doses (0.5-2% of diet) induced hyperplasia and tumors in the stomach and kidneys of rats and mice, while lower doses (0.05-0.15%) had anticarcinogenic properties[64]. Therefore, conclusions should

Table 4. Effect of polyphenols on blood pressure: human intervention studies.

Reference	Type of study	Number of individuals ǂ	Characteristics	Age	Administrated substance	Polyphenols ǂ	Dose/day	Length of the study	Biomarker	Changes on BP¥
43	Chronic, controlled and parallel	12 (8m, 4w)	Hypertensives	42-62	Sweetie fruit (hybrid between grapefruit and pummelo)	Flavonoids	0.5 L (889 mg/L)	5 weeks	SBP DBP	? ?
44	Chronic, controlled and parallel	45 (40m,5w)	Patients with ischaemic coronary disease and myocardial infarction	58-80	Pomegranate juice	Tannins, anthocyanins	240 mL	90 days	BP	↔
45	Chronic, single arm, no control	21 (men)	Healthy	30-46	Cranberry juice	Flavonoids, phenolic acids	7 mL/Kg weight	14 days	SBP	↓ 2% (not statistically significant)
46	Chronic, controlled and parallel	57	Hyperlipidaemics after coronary bypass surgery	39-72	Blond or red grapefruit	Flavonoids, anthocyanins	One fruit (20 mg/100 g fresh weight)	30 days	SBP DBP	↔ ↔
47	Chronic, controlled and parallel	44 (33m,11w)	Myocardial infarction survivals on statins for 6 months	57-75	Flavonoid extract from *Aronia melanocarpa*	Anthocyanins, procyanidins	3 x 85 mg	42 days	DBP SBP ACE serum	↓ 7.2 mmHg ↓11 mmHg ↓33.3%
48	Chronic, controlled and parallel	44 (20m,24w)	Hypertensives	56-73	Dark chocolate	Flavonoids	6.3 g (30 mg)	18 weeks	SBP DBP	↓2.9 mmHg ↓1.9 mmHg
49	Chronic, single arm, no control	60 (49m,11w)	Healthy	32-73	Green tea extract	Catechins	1 packet (544 mg)	2 months	DBP	↓4 mmHg
50	Chronic, controlled and parallel	187 (38m,149w)	Healthy	19-20	Cranberry juice	Anthocyanins, Phenolic acids	480 mL	Postprandial	BP HR	↔ ↔
51	Chronic, single arm, no control	10	Healthy	Adults	Marula juice	Hydrolizable tannins, catechins	200 mL (56 mg/dL)	21 days	BP	↔
52	Chronic, controlled and cross-sectional	71 (25m,46w)	High cardiovascular risk	51-64	Bilberries, lingonberries, blackcurrant, strawberry puree and raspberry juice	Anthocyanins	150 g (837 mg)	56 days	SBP	↓ 7·3mmHg In subjects with high baseline BP
53	Chronic, controlled and cross-sectional	42 (19m,23w)	Healthy	58-81	Cocoa powder (with skim milk)	Flavonoids	40 g (495.2 mg)	28 days	SBP DBP HR	↔ ↔ ↔
54	Chronic, controlled and cross-sectional	35 (men)	Healthy	18-45	Wine grape or grape seed extract (capsules)	Anthocyanins, phenolic acids	6 capsules (800 mg)	14 days	SBP DBP HR	↔ ↔ ↔

55	Crossover, randomized and controlled	24 (men)	Healthy and overweight	50-65	Orange juice or hesperidin-enriched drink	Hesperidin	500 mL (292 mg)	28 days	DBP	↓(in both intervention groups)
56	Crossover, randomized and controlled	24 (men)	With metabolic syndrome	30-70	Grape extract (capsules)	Flavanols, anthocyanins	46 g/day	30 days	SBP FMD NO	↓ ↑ ↔
57	Parallel, randomized and controlled	97(38m,69w)	Overweight	19-55	Algae extract (*Ecklonia cava*)	Fluorotannins	Cans (246 mL and 72 mg extract)	12 weeks	SBP	↓ (with the highest dose)
58	Crossover, randomized and controlled	10 (men)	Healthy	45-50	Wine, dealcoholized wine and gin	Flavanols, anthocyanins	272 mL wine (733-798 mg EAG/day) or 100 mL gin	20 days	DBP SBP	↓(with wine) ↓(with both wines)
59	Crossover, randomized and controlled	51(16m, 35w)	Healthy	30-50	Pomegranate juice	Hydrolizable tannins, anthocyanins	330 mL/d	4 weeks	SBP DBP BP	↓(-3.14 mmHg) ↓(-2.33 mmHg) ↓(-2.60 mmHg)
60	Crossover, randomized and controlled	67 (men)	High cardiovascular risk	55-75	Wine, dealcoholized wine and gin	Flavanols, anthocyanins	272 mL wine (733-798 mg EAG/day) or 100 mL gin	4 weeks	SBP and DBP Plasma NO	↓ ↑ (with dealcoholized wine)
61	Parallel, randomized and controlled	84 (31m, 53w)	Healthy or mild hypertensives	35-75	Black tea	Catechins	3 cups/day (429 mg)	4 weeks	SBP and DBP HR	↔ ↓
62	Crossover, randomized and controlled	49 (men)	Healthy	48-68	Quercetin	Quercetin	Capsules (150 mg/day)	8 weeks	SBP postprandial	↓
63	Parallel, randomized and controlled.	70 (38m, 32w)	Hypertensives (stage 1 or less)	35-75	Grape seed extract	Catechin, proanthocyanidin dimers	Capsules (300 mg/day)	8 weeks	SBP and DBP	↓ (not statistically significant)
64	Pilot study	6 (4m, 2w)	Healthy	34-68	Boysenberry juice	Proanthocyanidin dimers, epicatechin	180 mL/day (351 mg)	4 weeks	FMD SBP	↑ ↓

SBP, systolic blood pressure; DBP, diastolic blood pressure; BP, blood pressure; ACE serum, Angiotensin Converter Enzyme; HR: heart rate; AGE: gallic acid equivalents; FMD: flow-mediated dilation.
* Table is organized chronologically.
† m: men, w: women
‡ Only the top two polyphenols with the highest concentration are listed
¥ ↑increase; ↓ decrease; ↔ no change; unless otherwise stated, % refers to changes from baseline when test substance is given.

Table 1.4. Effect of polyphenols on blood pressure: human intervention studies (continued)

be extracted from human clinical studies with real doses or from epidemiological studies.

Despite the small number of clinical and epidemiological studies compared with *in vitro* or animal studies, there is enough scientific evidence to describe epigallocatechin gallate (EGCG), a flavonoid found in tea, as a chemopreventive agent. Consumption of green tea has been linked to a lower risk of breast cancer[65], mouth cancer[63,66], and prostate cancer[67]. However, results for black tea or colon and bladder cancers are inconclusive[65,68,69].

Studies *in vitro* and *in vivo* and with humans indicate that grape polyphenols, berries, olive oil and cocoa also have anticarcinogenic properties[66,70–72].

d) Neurodegenerative diseases

Due to population ageing, the prevalence of neurodegenerative diseases, which are closely linked to age, has increased. Dementia is a clinical syndrome characterized by memory loss, behavioral changes and other effects that impede or hinder daily tasks. Nowadays, over 25 million people worldwide have some type of dementia, 75% of whom suffer from Alzheimer's disease. In Europe, 6.4% of the population over 65 suffers from a neurodegenerative disease[73]. Some dementias are a side effect of ischemic diseases like stroke. Over time, neurodegenerative diseases involve memory impairment or motor loss resulting in different degrees of dependency.

The brain is an organ with a high consumption of oxygen and free radicals are products of its normal metabolism. There is sufficient scientific evidence to link the production of free radicals with the induction of necrosis, inflammation and the pathogenesis of neurodegenerative diseases[74,75]. Thus, due to the close relationship between these diseases and oxidative stress, it is believed that antioxidants can help to prevent them[34]. Numerous studies have focused on the effects of vitamin C, vitamin E and β-carotene, but polyphenols remain under-explored in this respect[76].

Many studies on polyphenols and neurodegenerative diseases have been carried out with animal models, particularly mice and rats, but neuronal cells have also been used in experiments *in vitro*. For example, the administration of a combination of polyphenols from grapes reduced β-amyloid peptides in mice[77]. These peptides are involved in the pathogenesis of Alzheimer's disease. The use of isolated polyphenols like resveratrol, proanthocyanidins, epicatechin, catechin and ferulic acid gave similar results[78–82], as well as polyphenols from strawberries, spinach, blueberries or cranberries, tea, red pepper and garlic[76].

Other studies have also demonstrated the effect of polyphenols on neurodegenerative diseases in humans. Polyphenols from red wine and grapes have beneficial effects, improving memory in old people with mild cognitive problems[83]. Other studies with the same polyphenols demonstrated that they interfered in the generation and aggregation of β-amyloid peptides[84–86]. Nurk and colleagues examined the effect of red wine, chocolate and tea consumption (separately or together) in a cross-sectional study conducted in 2,000 elderly people aged between 70 and 74 years. Those who consumed these foods showed the best results in different cognitive tests in a dose-dependent manneri[87]. Flavonoid intake was also associated with better cognitive ability at the beginning and a better evolution in a cohort of 1,640 people over 65 who were followed for 10 years[88].

The association of the polyphenol antioxidant effect with an improvement in symptoms of neurodegenerative diseases has been well demonstrated in *in vitro* models. However, it seems that this is not the only mechanism of action and, moreover, it is unclear whether polyphenols directly interact with neural systems or act indirectly, because it is unknown if all polyphenols are able to reach the brain[76]. Only one Japanese research group and, more recently, a group from the University of Barcelona have shown that some polyphenols are able to cross

the blood-brain barrier in animal models. For example, after intake of a tea extract and gallocatechin gallate, some polyphenols and their metabolites were found in different organs of the mouse, including the brain[89]. Moreover, a cranberry supplement improved the results obtained by rats in the Morris water maze test. In this study, anthocyanin metabolites were identified in the following parts of the brain: cerebellum, cortex, hippocampus and striatum[90].

However, more epidemiological studies and especially clinical trials are needed to clarify the extent to which polyphenols can slow the progression of neurodegenerative diseases and through what mechanisms.

e) Metabolic syndrome, obesity and diabetes

Metabolic syndrome (MS) is a metabolic disorder that consists of a combination of multiple cardiovascular risk factors: obesity, hypertension, dyslipidemia and hyperglycemia. There is no universal criterion for the diagnosis of this disorder, which makes it difficult to know its true prevalence or to compare between scientific studies. One of the most common criteria for diagnosis is that proposed by the Adult Treatment Panel III (ATPIII) in 2001, which considered that an individual had MS when at least 3 of the following were fulfilled: 1) waist circumference $\geq$102 in men and $\geq$ 88 in women, 2) levels of plasma triglycerides $\geq$150 mg / dL (or medication to treat hypertriglyceridemia), 3) HDL cholesterol <40 mg / dL in men and <50 mg / dL in women, 4) blood pressure $\geq$130/85 mmHg (or medication for hypertension), and 5) fasting plasma glucose $\geq$100 mg / dL (or medication for diabetes)[91,92].

This syndrome is the result of the interaction of multiple causes, including genetic factors and environmental factors: lack of physical activity, smoking and dietary habits, especially saturated fat and simple sugar consumption. If this syndrome is not controlled it can lead to cardiovascular and cerebrovascular accidents and type-2 diabetes. The consumption of polyphenol-rich foods can prevent MS through their protective effect on chronic inflammation linked to obesity, insulin resistance, dyslipidemia, and hypertension[92]. Polyphenols that have been linked to the treatment and prevention of MS include resveratrol, quercetin, epigallocatechin-3-gallate's and curcumin[93].

Cocoa, rich in epicatechin, catechin and proanthocyanidins, is an antioxidant food with antioxidant, antihypertensive, anti-inflammatory, and antiatherogenic activities. It also improves insulin resistance, endothelial function and levels of NO. These effects, confirmed in multiple literature reviews and meta-analyzes, show that cocoa could help in the treatment and prevention of MS[94]. Similar results were observed with the consumption of green tea, a widespread drink also rich in catechins[95], and olive oil[96].

Soham *et al.* conducted a study with 2,618 participants (19-84 years) within the TLGS study (Tehran Lipid and Glucose Study). Using data from a food frequency questionnaire, intake of total polyphenols, flavonoids, phenolic acids, lignans and stilbenes was related to the prevalence of MS and its components. They concluded that those who consumed more flavonoids were less likely to suffer from MS and 4 of its 5 components (hypertension was not affected). In contrast, lignans seem to favor the risk of hyperglycemia and hypertriglyceridemia, and stilbenes, hypertension[97].

In a controlled, crossover, randomized, double-blind trial, 45 middle-aged participants with a body mass index (BMI) of 28$\pm$2 kg/m^2 took capsules of olive leaf extract (rich in oleuropein and hydroxytyrosol, among other polyphenols) for 12 weeks. Insulin sensitivity improved by 15% compared with the placebo group. Other results were a 28% improvement in the response of pancreatic beta cells, an increase in interleukin-6 concentration and an increase in fasting IGFBP-1 (Insulin-like Growth Factor 1 Binding Protein) and IGFBP-2 concentrations[98].

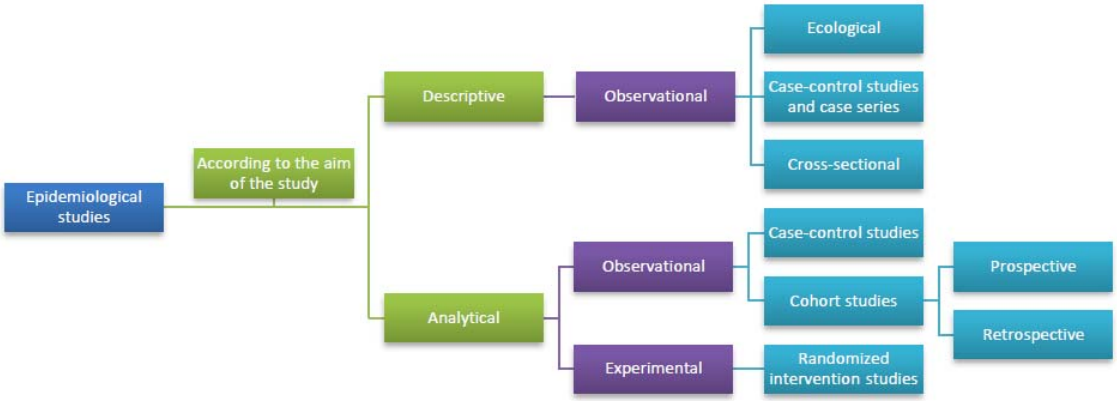


Figure 2.1. Classification of epidemiological studies.

Results from a similar study with women with MS who were administered cranberry juice for 8 weeks showed a significant decrease in LDL cholesterol and a significant increase of plasma antioxidant capacity[99].

Different mechanisms of action have been proposed to explain the effect of polyphenols on type-2 diabetes. On one hand, polyphenols may inhibit glucose absorption in the small intestine and its reabsorption in the liver. On the other hand, polyphenols exert different actions on peripheral tissues, including inhibition of gluconeogenesis, adrenergic stimulation of glucose consumption, or stimulation of insulin release by pancreatic beta cells[34]. For example, polyphenols from cinnamon, resveratrol, isoflavones, and polyphenols from tea, cocoa and grape seeds improve insulin sensitivity, the hormone that regulates plasmatic glucose levels[100].

Phenolic compounds may also be linked to obesity control. Several *in vitro*, animal and human studies have shown that polyphenols can reduce fat absorption in the intestinal tract, activate thermogenesis and modulate the hormonal response that regulates food intake and satiety[101].

2. Epidemiology

The World Health Organization (WHO) defines epidemiology as a scientific discipline that studies the distribution, frequency, causes and control of health-related factors and the application of these studies to control diseases and other health problems of the population[1]. Epidemiology is the basic science of preventive medicine and an essential tool for public health agencies policies. Epidemiological studies help to answer questions such as: can mobile phones increase the risk of cancer? What is the minimum physical exercise you need to do to reduce the risk of cardiovascular disease or diabetes? Should we worry about mercury in the fish we eat?

There are two branches in epidemiology: the descriptive, in which the frequency and distribution of disease is measured, and the analytical, which seeks, through observation and experimentation, to measure association (or cause-effect relationships) between exposure and disease (**Figure 2.1**). One branch complements the other since descriptive epidemiology is used to propose hypotheses that analytical epidemiology clarifies[102].

According to the temporal sequence, studies can also be classified as transversal or longitu-

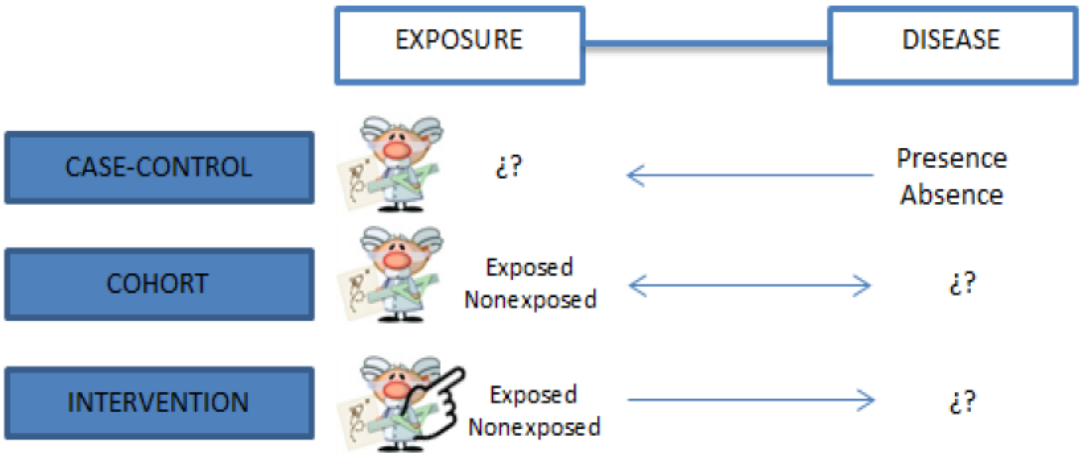


Figure 2.2. Scheme of different analytical epidemiology studies.

dinal. A study is considered cross-sectional when data is taken at a specific point in time. As variables are measured simultaneously, cause-effect relationships cannot be established. Cross-sectional studies are, by definition, descriptive. When there is a time delay between the different evaluated variables, the studies are longitudinal. In these cases, we must take into account temporal directionality, which can go from cause to outcome (cohort and experimental studies) or from outcome to cause (case-control studies)[103,104].

Ecological or correlational studies are used to compare frequencies of disease among different population groups within a given period of time or within a population but in different time periods. When comparing populations we cannot assume that the association will be the same for individuals. Moreover, confusion with other variables may exist. Another limitation is that the values used are the average exposure of the population, not real values. Case series studies, unlike ecological studies, use information about patients individually, with detailed data on factors related to the disease under study. The main limitation is the absence of a control group, which impedes knowing the real dimension of the effect[105].

Cross-sectional descriptive studies measure the prevalence of a disease at a given time. Information about exposure and outcome is taken at the same time and for all individuals. These studies are fast and cheap, but cannot establish any temporal relationship, except for exposures that are invariable over time, such as race, genetic factors, sex, etc. Thus, descriptive studies are used to describe patterns of disease incidence in relation to personal characteristics, places and time. Information is usually quick and easy to obtain, as it is often part of routine processes (medical reports, population surveys, etc.). Data obtained from them are used by public health agencies to locate problems and to focus resources and programs for prevention and education[103].

Analytical studies can solve the limitations of descriptive studies: they focus on populations rather than individuals, there is a control group and time sequence and it is also possible to adjust for confounding variables. There are two types: observational and intervention. In the former, the exposure is random or due to the environment, and the researcher is a passive observer. However, in intervention studies, the researcher assigns the exposure (**Figure 2.2**)[103].

Observational studies can be cohort or case-control studies. The difference is in the point of view of the researcher or, in other words, in the data used to select groups. In case-control studies, individuals are classified according to the presence or absence of a given illness and

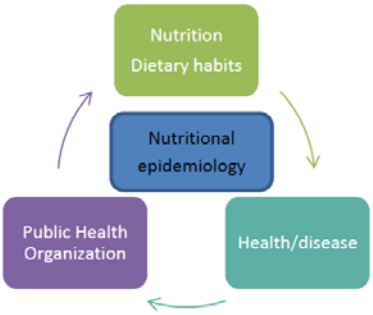


Figure 2.3. Relationship between different fields involved in nutritional epidemiology.

the exposure is investigated. On the contrary, in cohort studies the population is divided according to whether or not individuals are exposed to the studied factor. In prospective cohort studies the researcher waits until the disease appears and in retrospective cohort studies wants to know if the disease already exists or existed in the past. Sometimes the two situations can happen simultaneously. Cohort studies cannot be used to study rare or infrequent diseases, and case-control studies are limited to frequent exposures[106,107].

In experimental or intervention studies the research team assigns the studied factor, randomly or not, and controls it according to a set plan. Studies can be controlled or not depending on whether or not there is a control group for comparison. If there is a control, clinical studies can be parallel or crossover. If studies are correctly designed, they can prove causality but these studies are expensive, limited to solving very specific problems, and they pose ethical problems[102,108].

The method of choice depends on the question that needs answering, the time and available resources. It is important to know the advantages and limitations of each study when interpreting the results: can the results be generalized? Can a causal relationship be established? Is there a plausible biological mechanism to explain the results? Finally, the obtained results should end with a clear and understandable message for other scientists, public health agencies or the general population.

2.1. Nutritional epidemiology

The nutritional branch of epidemiology aims to evaluate the effects of diet on the risk of illness. This knowledge is used by public health agencies in their prevention efforts. To know the influence of diet on health and to plan intervention programs, it is necessary to accurately determine nutrient or food intakes (**Figure 2.3**). Diets can be evaluated using dietary surveys and/or nutritional biomarkers. Both systems have advantages and disadvantages, summarized in **Table 2.1**, which should be taken into account when choosing them and drawing conclusions. If resources are available, the combination of the two methods is the best solution[109].

2.1.1. Nutrient intake estimation

2.1.1.1. Food questionnaires

There are different methods to obtain information about the dietary habits of a population (**Figure 2.4**). They differ in the way information is obtained and the period covered, and each has its advantages and disadvantages. The choice of a particular method depends on

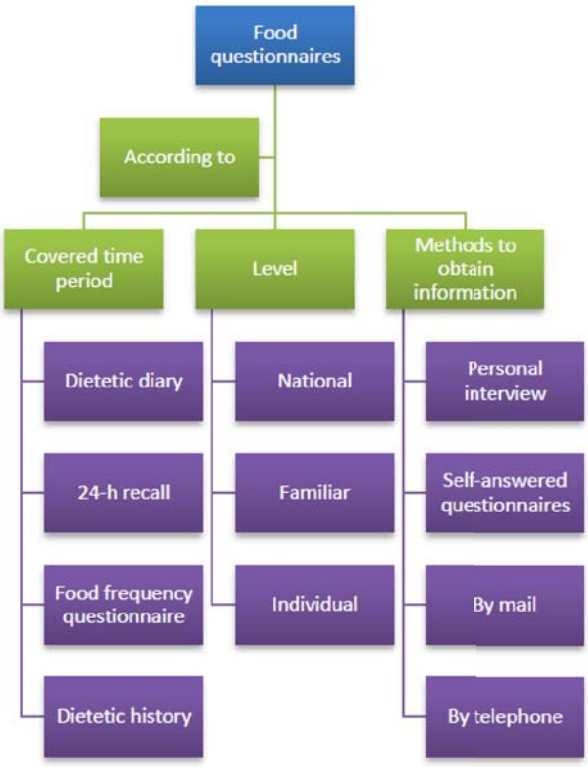


Figure 2.4. Types of food questionnaires.

the studied population, the food or nutrient of interest, available resources and the design of the study. In PREDIMED, annual food frequency questionnaires (FFQ) were chosen. They were collected individually using personal interviews with a dietitian. Complementarily, a short 14-item questionnaire was designed to assess adhesion to the traditional Mediterranean Diet (MD). A retrospective FFQ is a method that uses a closed and classified list of food and drink and asks about the frequency of consumption (never, occasionally, weekly, daily, etc.) during a given period of time[109–111].

2.1.1.2. Nutritional biomarkers

A nutritional biomarker is a compound external to the human body, such as food components or their metabolites. By analyzing them in biological samples (blood, urine, tissues, etc.) it is possible to estimate the intake of certain foods or compounds. A good biomarker should have the following characteristics[112,113]:

1. Availability of an accurate reproducible, reliable, valid and robust analytical method to quantify it.

2. Concentrations of the biomarker in biological samples should be sensitive to changes in the intake of the studied food or compound. Above all, it is essential to distinguish between consumers and non-consumers.

3. The biomarker should be specific to the studied food. This criterion is the most difficult to achieve, but it is possible in some cases. For example, tyrosol and hydroxytyrosol can act as biomarkers of olive oil[114].

	Food frequency questionnaires	Nutritional biomarkers
Advantages	Cheaper Fast and easy to use Ability to classify individuals into consumption categories	Objective Accurate Bioavailability and metabolism are considered Able to track a nutritional intervention
Disadvantages	Depends on interviewee memory and reliability. Bioavailability and metabolism are not considered Food composition tables not always available Low accuracy in estimating and quantifying food portions Long time required by the interviewee.	Expensive Biomarkers are not always available Complex interpretation of the result as it depends on bioavailability and metabolism. Interindividual differences Require extracting, preserving and working with biological samples.

Table 2.1. Comparison between food frequency questionnaires and nutritional biomarkers.

In PREDIMED nutritional biomarkers were used to verify the correct implementation of the intervention. Hydroxytyrosol and tyrosol in urine and oleic acid in plasma demonstrated a correct following of the MD supplemented with extra virgin olive oil. For the MD supplemented with nuts, α-linolenic acid in plasma was analyzed, as this is characteristic of walnuts. Moreover, consumption of fruits, vegetables and polyphenol-rich beverages were associated with a higher excretion of total polyphenols in urine[115].

2.1.2. Quantification of total polyphenols in urine by the Folin-Ciocalteu method

The F-C colorimetric method has been widely used to quantify total polyphenol content in food samples[116–118]. The first time it was applied to biological samples was to use it as a biomarker of wine consumption[119,120]. The F-C reagent is a bright yellow solution that contains a mixture of hexavalent phosphomolybdic/phosphotungstic acid complexes with the following structures:

$3\ H_2O \bullet P_2O_5 \bullet 13\ WO_3 \bullet 5\ MoO_3 \bullet 10\ H_2O$

$3\ H_2O\ x\ P_2O_5 \bullet 14\ WO_3 \bullet 4\ MoO_3 \bullet 10\ H_2O$

In alkaline medium, electron transfer from the reagent to phenols reduces the complexes to tungstic and molybdic oxides (W_8O_{23}) i molibdè (Mo_8O_{23}, respectively), giving a blue coloration that can be measured at 765 nm and it is proportional to the concentration of hydroxiles[121]. Therefore, not all polyphenols will react with the F-C reagent with the same intensity, which can lead to measurement errors.

The F-C method can be hampered by the presence of several water-soluble substance in urine, including sugars, sulfure dioxide, aromatic amines, ascorbic and organic acids, Fe(II) and other non-phenolic but oxidable substances that can be found in urine[122]. Therefore, a solid phase extraction (SPE) clean-up procedure with cartridges is needed before the analysis to avoid interferences[115].

3. Biostatistics

Biostatistics is a branch of applied statistics that uses statistical methods to solve biological and medical problems. It is divided into two branches: descriptive, to synthesize and present information; and analytical, to demonstrate associations and relationships between observed characteristics by contrasting hypotheses and using confidence intervals. Thus, epidemiology is used to design research plans and optimal strategies for data collection and biostatistics analyze these data to obtain information. Once the results are obtained, it is necessary to return to the epidemiology field to interpret them critically, since some statistically significant results are not necessarily valid if there is no biological plausibility[123,124].

3.1. Correlation and regression

Correlation aims to examine the magnitude and direction of the association between two quantitative variables. Correlation coefficients give us a measure of the degree of association and they can be of two types: the Pearson coefficient (r) for parametric data, which estimates adaptation to a linear model, and the Spearman coefficient (rho) for nonparametric data, which measures any type of association, linear or not[124].

The Pearson coefficient can take values from -1 to 1, 0 being the null value, which means that two variables are uncorrelated. When r takes positive values, it means there is a direct association: an increase of one variable makes the other variable increase. However, if r is negative, the association is indirect. Values above 0.7 (in the absolute value) represent strong associations. The Spearman coefficient has to be used when measured variables do not meet normal criteria or they are not ordinal[124].

Unlike regression, correlation does not distinguish between dependent and independent variables, since there is no cause-effect relationship, so they are interchangeable. In addition, the correlation coefficient is not influenced by measurement units[124].

Regression describes the relationship between two variables in more detail, so it may have predictive purposes: to accept a margin of error, we can predict the value of a variable if we know the value of the other. It is always assumed that there is an independent or predictor variable, controlled by the researcher, and a dependent variable or response[125].

There are two types of linear regression: simple and multiple. The former analyzes the relationship between two quantitative variables to determine the extent to which it fits a straight line. For more accurate estimations, multiple linear regression is used, which has more than one explanatory variable. Among a set of variables, it provides information about which have a greater influence on the dependent variable.

A particular case is logistic regression, which is used when the dependent variable is dichotomous (sick/healthy, death/not death, etc.). Measurement of these variables is performed using odds ratios (OR). Cox regression is the used method when considering the follow-up time until the phenomenon of interest. It measures rate ratios or hazard ratios (HR). Thus, OR has a static sense while HR is dynamic. The Cox model calculates a weighted average of HR from all moments when there is a death or an event. It is like performing several logistic regressions[124,125].

3.2. Measures of frequency and association

In epidemiology, measures of frequency are used to describe and compare the magnitude of an illness or a health condition in different populations. There are two different types of measures of frequency of morbidity and mortality, those that describe the proportion of cases at a given point in time (prevalence, P) and those that describe new cases of disease over a given time period (cumulative incidence, CI), which is a proportion, or incidence (I), which is a rate[126]

$$P = \frac{\text{Number of cases at a point in time}}{\text{Total population}}$$

$$IA = \frac{\text{Number of new cases during a period of time}}{\text{Total population at risk}}$$

$$I = \frac{\text{Number of new cases during a period of time}}{\text{Total person} - \text{time of observation}}$$

Measures of association and relative risk (**Table 6**) are epidemiological indicators that assess how strongly a disease or an indicator of disease is related to a specific factor thought to be a cause. Relative risk is a general term to indicate the strength of the association between an exposure and a disease. It can be calculated differently depending on the study design. Hence, risk ratio, rate ratio and odds ratio are similar in meaning but they are calculated differently. Stronger associations are measured using incidence, as there is a temporal cause-effect relationship[127].

$$\text{Relative risk} = \frac{\text{Disease risk among exposed}}{\text{Disease risk among non exposed}}$$

- No units
- Values from 0 to infinite
- Meaning:
  - <1 – the variable is protective
  - =0 – no association (null value)
  - >1 – the variable is a risk factor

Measures of impact or measures of absolute risk (**Table 6**) indicate the contribution of a determined factor to the development of a disease among the individuals who are exposed, as well as the beneficial effect of the preventive actions. All of them provide similar information but the way to calculate them depends on the study design (cohorts, case-control studies, etc.)[127].

	Measure	Equation	Type of study	Interpretation
Ratios (relative scale)	Risk ratio, cumulative incidence ratio, or risk rate.	$RR = \frac{IA_e}{IA_o}$	Cohort studies with patients with different follow-up period.*	Those who are exposed have X times the risk of developing the outcome than those who are non-exposed.
	Incidence rate ratio	$IRR = \frac{I_e}{I_o}$	Cohort studies with patients with different follow-up period.*	Those who are exposed have a disease rate X times higher than the non-exposed.
	Odds ratio	$OR = \frac{\text{odds of exposure among cases}}{\text{odds of exposure among controls}}$	Case-control studies	Those who are exposed have X times the odds of having the disease than the non-exposed.
Risk differences (absolute scale)	Attributable risk	$ARe = IA_e - IA_o$	Cohort studies with patients with the same follow-up period.*	The frequency of the disease attributable to the exposure is X.
	Etiological fraction among the exposed	$EFE = \frac{IA_e - IA_o}{IA_e} = \frac{ARe}{IA_e}$	Cohort studies with patients with the same follow-up period.*	Assuming causality, X% of the illness among the exposed is caused by the exposure.
	Number needed to treat	$NNT = \frac{1}{ARe}$	Cohort studies with patients with the same follow-up period.*	X patients would need to be treated (or avoid exposure) in order to prevent 1 outcome.

*When the follow-up period is not the same for all the patients, an attributable risk ratio and incidence are used.
CI: cumulative incidence, I: Incidence rate, e: exposed, o: non-exposed

Table 3.1. Estimation and interpretation of the main measures of association and impact.

3.3. Association versus causality

Ideally, a study should evaluate whether causality exists among variables, in other words, whether an alteration in the exposure modifies the disease risk. However, cause-effect relationships are difficult to demonstrate for several reasons. Chronic diseases have long periods of latency, usually lasting for years, and it is difficult to maintain studies for so long. Before considering a causal relationship, it is necessary to consider three alternative explanations: the effect of confounding, bias (observation and selection) and causality[126,128].

Once all the alternative explanations other than a causal relationship have been discounted, other criteria can be examined. A strong association minimizes the impact of uncontrolled confounding (unknown or impossible to control). Consistency of results is obtained but different researchers and populations also support cause-effect relationships. Lastly, it is necessary to have a mechanism or biological credibility to explain the results and, if possible, a dose-response relationship[126,128].

3.4. Survival analyses

In survival analysis, the variable of interest is not quantitative or qualitative but temporal. It combines two elements: a dichotomous variable (the occurrence of an event or not) and a quantitative variable (time to event). The outcome is not necessarily the death of the person but it can only happen once and implies the existence of censored or trunked information, with incomplete observation times[124,129].

The Kaplan-Meier method is the most common way to estimate the survival distribution. It is a non-parametric method by which the survival ratio and the survival time are calculated. It is usually plotted with time on the x-axis and the percentage of survival on the y-axis. To compare two or more survival curves, the log-rank test is used, taking into account the differences in survival between groups at all follow-up points. Similarly, the Nelson-Aalen method generates a plot with the cumulative hazard rates[124,129].

4. The PREDIMED study

The PREDIMED study (*PREvención con DIeta MEDiterránea*, ISRCTN35739639) has been a prospective, randomized, multicentric and controlled trial. Its objective was to determine the health benefits of a traditional Mediterranean diet in the primary prevention of cardiovascular diseases[130,131]. The study lasted 9 years, the first volunteers being recruited in 2004, and was funded by the *Instituto de Salud Carlos III* (G03/140).

Volunteers were recruited through primary health care centers from 8 different Spanish regions and they were randomized to one of the following nutritional intervention groups:

Per a l'estudi es van reclutar pacients a través de centres d'atenció primària de 8 comunitats autònomes i se'ls va assignar de forma aleatòria un dels tres grups d'intervenció nutricional:

a) Mediterranean Diet supplemented with extra virgin olive oil (MD-EVOO)

b) Mediterranean Diet supplemented with nuts (MD-nuts)

c) Control group: low-fat diet (LFD) according to the recommendations of the American Heart Association, AHA[132].

In the box below there is a summary of the inclusion criteria that were chosen[131]. Finally, 7447 participants fulfilled the criteria and participated in the study.

- Age: 55-80 years (man) and 60-80 years (women).
- Free of cardiovascular disease.
- Diagnosed with Type-2 Diabetes Mellitus or having three or more of the following CV risk factors:
  - Smokers (>1 cigarette/day during the last month)
  - Arterial hypertension (SBP≥140 mm Hg and/or DBP≥90 mm Hg, or antihypertensive medication)
  - Hypercholesterolemia (LDL cholesterol ≥160 mg/dL, HDL cholesterol≤40 mg/dL for men or ≤50 mg/dL for women, or anticholesterolemic medication)
  - Overweight or obese (BMI≥25 kg/m^2)
  - Family history of early ischemic cardiopathy
- Ability and willingness to change eating habits.
- Not suffering any serious illness that impedes participation in a dietary intervention study.
- Not having or having had alcohol or drug addiction.

Once the informed consent was signed, several personal, anthropometric and health-related data were taken: BP (triplicate), height, weight, waist circumference, etc. and the participants were asked to fill out the following questionnaires:

a) Inclusion questionnaire

b) General questionnaire: demographic and sociological data

c) Follow-up questionnaire

d) Food frequency questionnaire (FFQ)[134,135]

e) Questionnaire of adherence to MD[134]

f) Physical activity questionnaire[136]

Moreover, biochemical determinations were performed with biological samples (blood, urine and toenails). BP and electrocardiograms were also performed. Patients were visited once a year to repeat the questionnaires and take biological samples.

Participants received personal assessment about the MD or low-food diet, depending on their intervention group. Four times a year, every three months, they assisted group meetings to talk about nutrition and receive help in changing dietary habits. After these sessions, the MD-EVOO group received extra virgin olive oil (1L per week for them and their family), the nuts group received 30 g of nuts per day (50% walnuts, 25% almonds and 25% hazelnuts), and the control group was presented with other gifts (silverware, aprons, etc.). Moreover, they were informed about foods, recipes, and shopping lists[130].

After a median of 4.8 years of follow-up, and one year and a half before the follow-up period was over, an external scientific committee warned that the differences between MD groups and the control group were too significant to continue with the study. Results revealed that both MD groups, supplemented with extra virgin olive oil or nuts, had 30% less incidence of CV events than the control group (low-fat diet). Specifically, the MD-EVOO group, in which 96 CV events were confirmed, the adjusted HR was 0.70 (IC 95%=0.54-0.92) and the HR of the MD-nuts group, with 83 confirmed events, was slightly higher (HR=0.72, IC 95%=0.54-0.96). According to these results, volunteers from the control group were given new advice, changing their diet to a more Mediterranean pattern and including olive oil and nuts[137].

Within the PREDIMED study, numerous substudies have demonstrated, for example, that both types of MD, with olive oil or nuts, reduced LDL cholesterol, glucose, BP and biomarkers of inflammation after only 3 months of intervention[130,138]. A substudy was also performed with 1224 participants, comparing both MD groups with the control group after one year to demonstrate that the MD could significantly revert the metabolic syndrome (OR=1.3 for MD-EVOO and OR=1.7 for MD-nuts, compared to the control group)[139]. Other research papers were focused on the beneficial effects of the MD on obesity[140,141], cognitive impairment[142] or type-2 diabetes[143], among others.

Therefore, these results indicate that a MD pattern supplemented with nuts and olive oil should be included in nutritional recommendations, especially when addressed to elderly people at high CV risk.

RESULTATS

IV. Resultats/Results

1. Publicacions en revistes/Research articles

En aquesta secció s'exposen els resultats obtinguts dels treballs experimentals realitzats en aquesta tesi doctoral. Aquests resultats estan recollits en 5 publicacions en revistes del *Science Citation Index*. Abans de cada publicació hi ha un resum on es detallen els objectius, la metodologia, els resultats i les conclusions de cada estudi.

1.1. Publicació 1. Estudi de la ingesta diària i les principals fonts de polifenols en una població espanyola d'alt risc cardiovascular: l'estudi PREDIMED

Article 1. Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk: the PREDIMED study

Anna Tresserra-Rimbau, Alexander Medina-Remón, Jara Pérez-Jiménez, Miguel A. Martínez-González, M. Isabel Covas, Dolores Corella, Jordi Salas-Salvadó, Enrique Gómez-Gracia, José Lapetra, Fernando Arós, Miquel Fiol, Emilio Ros, Lluis Serra-Majem, Xavier Pintó, Miguel Ángel Muñoz, Guillermo T. Saez, Valentina Ruiz-Gutiérrez, Julia Warnberg, Ramón Estruch, Rosa M. Lamuela-Raventós. Nutrition, Metabolism and Cardiovascular Diseases. **2013**, 23(10):953-9.

Resum:

Estudis epidemiològics han demostrat que el consum d'aliments rics en polifenols té un efecte beneficiós per a la salut i pot protegir contra algunes malalties cròniques. L'objectiu d'aquest estudi va ser estimar de forma molt detallada la ingesta de polifenols per part d'una població espanyola d'avançada edat i alt risc CV (estudi PREDIMED), així com determinar els aliments que més van contribuir a aquesta ingesta.

Per a dur-ho a terme, es van utilitzar els QFC basals de 7200 participants de l'estudi PREDIMED i es va calcular la contribució de cada aliment a la ingesta de polifenols total. El contingut de polifenols dels aliments es va treure de la base de dades més completa del moment: la Phenol-explorer database. Fins el moment, el més corrent era utilitzar la base de dades de flavonoids del USDA, fet que portava a subestimar el contingut de polifenols ja que no disposava d'informació sobre àcids fenòlics, estilbens, lignans i altres grups de polifenols més minoritaris. En els càlculs també es van tenir en compte les receptes, que es van separar en ingredients, i el guany o la pèrdua de pes dels aliments durant la cocció. Es va utilitzar el programari Stata versió 10.1 (Stata Corp., TX, USA), tant per als càlculs de les ingestes de polifenols, com per a les anàlisis estadístiques.

D'acord amb la base de dades del Phenol-explorer, 93 dels 137 ítems del QFC d'aliments contenien un total de 290 tipus diferents de polifenols. La mitjana de la ingesta de polifenols totals va ser de 820±323 mg/dia. D'aquests, 443±218 mg/dia eren flavonoids, 304±156 eren àcids fenòlics, i la resta corresponien a altres grups de polifenols. Les fruites són el grup d'aliments que més contribuïren a la ingesta, sobretot les taronges i les pomes. Les begudes no alcohòliques, principalment el cafè, el grup de les hortalisses i el vi negre aportaren un 23%, un 13% i un 8%, respectivament. Més de la meitat dels àcids fenòlics els aportava el cafè, que també és l'aliment que, individualment, contribuí més en la ingesta total, seguit per les taronges, les pomes, les olives i l'oli d'oliva i el vi negre.

Pel que fa als diferents grups de polifenols, el àcids hidroxicinnàmics van ser els més consumits (33%). El segueixen les flavanones, les proanticianidines, els flavonols, les flavones i les antocianines. Dels 290 polifenols estudiats, 86 van ser consumits en quantitats majors de 1 mg/dia. De major a menor, els cinc primers foren: àcid 5-cafeoilquínic, hesperidina, àcid 3-cafeoilquínic, àcid 4-cafeoilquínic i la quercetina 3,4'-O-diglucòsid.

L'oli d'oliva, el principal greix de la dieta mediterrània té, a més d'àcids grassos monoinsaturats, un perfil fenòlic únic i característic. Les olives i l'oli d'oliva proveïren, diàriament, 21.9±10.9 i 68.5±104.0 mg de polifenols totals, respectivament, que representaren un 11% del total de polifenols. Aquests aliments marcaren la diferència en el perfil fenòlic respecte d'altres països no mediterranis.

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Nutrition, Metabolism & Cardiovascular Diseases

Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk: The PREDIMED study

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KEYWORDS
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Mediterranean diet;
Consumption;
Olive oil

Summary *Background and aims:* Epidemiological data have shown an inverse association between the consumption of polyphenol-rich foods and the risk of cardiovascular disease or overall mortality. A comprehensive estimation of individual polyphenol intake in nutritional cohorts is needed to gain a better understanding of this association. The aim of this study was to estimate the quantitative intake of polyphenols and the major dietary sources in the PREDIMED (PREvención con DIeta MEDiterránea) cohort using individual food consumption records.
Methods and results: The PREDIMED study is a large, parallel-group, multicentre, randomised, controlled 5-year feeding trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. A total of 7200 participants, aged 55—80 years, completed a validated 1-year food frequency questionnaire (FFQ) at baseline. Polyphenol consumption was calculated by matching food consumption data from the FFQ with the recently developed Phenol-Explorer database on polyphenol content in foods.
The mean total polyphenol intake was 820 $\pm$ 323 mg day^{-1} (443 $\pm$ 218 mg day^{-1} of flavonoids and 304 $\pm$ 156 mg day^{-1} of phenolic acids). Hydroxycinnamic acids were the phenolic group with the highest consumption and 5-caffeoylquinic acid was the most abundantly ingested individual polyphenol. The consumption of olives and olive oil was a differentiating factor in the phenolic profile of this Spanish population compared with other countries.
Conclusion: In Mediterranean countries, such as Spain, the main dietary source of polyphenols is coffee and fruits, but the most important differentiating factor with respect to other countries is the consumption of polyphenols from olives and olive oil.
Clinical trial registry: International Standard Randomised Controlled Trial Number (ISRCTN of London, England) 35739639.

A high consumption of polyphenols, which are bioactive compounds present mainly in plant foods and beverages, has been suggested to have beneficial effects on human health and provide protection against many chronic illnesses [1—3]. Polyphenols constitute a very heterogeneous group of compounds, with over 500 different molecules that have different properties and bioavailabilities [4]. This diversity should be considered when studying the health effects of these compounds [5] and hampers the estimation of their content in foods [6]. Polyphenols are divided into five main groups according to structure: phenolic acids, flavonoids, stilbenes, lignans and others (such as secoiridoids) [5,6].

Some studies have used the US Department of Agriculture (USDA) Flavonoid Database [7—10] to estimate flavanoid intake, with the drawback that the limited number of compounds that it contains is far from the wide diversity of polyphenols found in food [11]. In this setting, the aim of this study was to determine the major dietary sources of polyphenols in a Spanish population at high cardiovascular risk (the PREDIMED cohort, PREvención con DIeta MEDiterránea) [12] using the Phenol-Explorer database (www.phenol-explorer.eu), the most complete database currently available, which holds data on 502 polyphenols contained in 452 foods [11]. To our knowledge, this is the first study to report the intake of such a high number of polyphenols in a Spanish population using this tool. The application of this methodology will facilitate further investigation into polyphenol intake and its relation with the incidence of several diseases in the epidemiological observational studies and feeding trials such as the PREDIMED study and may be useful in establishing nutritional recommendations.

Methods

Study population, the PREDIMED cohort

Subjects were participants of the PREDIMED study, which is a large, parallel-group, multicentre, randomised, controlled 5-year feeding trial aimed at assessing the

effects of the traditional Mediterranean Diet (MedDiet) in the primary prevention of cardiovascular diseases (www.predimed.org). The recruitment method and study protocol have been described in detail previously, as well as the characteristics of eligible participants and exclusion criteria [12,13]. The participants provided written informed consent and the study protocol was approved by the institutional review boards of the participating centres.

Estimation of dietary intake

Food intake among the PREDIMED cohort at baseline was estimated using a validated 137-item Food Frequency Questionnaire (FFQ) [14] and physical activity with the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire [15]. Data on other lifestyle factors, including educational level, history of illness and medication use, were collected at baseline through a 47-item questionnaire. Participants also filled in a 14-point score questionnaire on adherence to the traditional MedDiet [16]. Anthropometric and blood pressure measurements were taken. The baseline questionnaires of 7200 participants of the PREDIMED study collected from 2003 to 2009 were used to correlate food consumption with individual and total polyphenol intake.

Estimation of polyphenol intake

Data on the polyphenol content in foods were obtained from the Phenol-Explorer database (www.phenol-explorer.eu), which has been previously described [4]. The correspondence between food items in the FFQ and the Phenol-Explorer database was assessed with the following five steps: 1) all foods with no or only traces of polyphenols were excluded; 2) the yearly FFQ was converted into 24-h dietary recall interviews; 3) recipes were separated according to their ingredients; 4) the polyphenol content of each food item was searched in the Phenol-Explorer database as described by Perez-Jimenez et al., 2011 [11]; and 5) weight loss or gain during cooking was corrected using yield factors [17]. Finally, individual polyphenol intake from each food was calculated by multiplying the content of each polyphenol by the daily consumption of each food. Total polyphenol intake was calculated as the sum of all individual polyphenol intakes from all food sources reported by the FFQ.

The data used to calculate polyphenol intake correspond to normal phase high performance liquid chromatography (HPLC) for all phenolic compounds. In the case of lignans, and phenolic acids in certain foods (cereals, olives and beans), data corresponding to HPLC after hydrolysis were also collected, since these treatments are needed to release phenolic compounds that otherwise could not be analysed.

Dietary sources of polyphenols

A ratio of daily total or individual polyphenols provided by the specific food or food group over the total intake of polyphenols from all foods was used to calculate the contribution of each food or food group to the daily total intake of polyphenols.

Statistical analyses

The mean polyphenol intake was calculated for the 7200 participants who had completed the baseline FFQ, and those who had no missing values in the other questionnaires used. The Stata Statistical Package (version 10.1, Stata Corp., TX, USA) was used for the analyses. Data are presented as means (±SD) for continuous variables and frequencies, and percentages for categorical variables.

Table 1 General characteristics of the studied PREDIMED population (n = 7200).

Characteristics	Mean ± SD	Minimum	Maximum
Age (y)	67.1 ± 6.1	53	82
Weight (Kg)	76.6 ± 11.6	40.0	130.0
BMI (Kg/m^2)	29.9 ± 3.6	17.8	40.0
Systolic BP[a] (mmHg)	148.7 ± 19.2	69.0	234.5
Diastolic BP[a] (mmHg)	82.8 ± 10.3	39.0	145.0
Heart rate (bpm)	71.0 ± 10.8	35.7	128.5
Energy intake (Kcal/d)	2274.9 ± 606.7	587.8	6007.6
Energy consumed due to physical activity in leisure time (METS-min/d)	232.6 ± 240.5	0.0	2975.1
14-point questionnaire of adherence to the traditional MedDiet[b] (score)	8.7 ± 1.9	0	14
Carbohidrates intake (g/d)	239.2 ± 81.0	41.3	749.2
Protein intake (g/d)	92.5 ± 23.1	15.3	369.9
Total fat intake (g/d)	98.7 ± 30.4	16.5	281.5
Fibre intake (g/d)	25.6 ± 9.1	0.9	82.7

[a] BP: blood pressure.
[b] MedDiet: Mediterranean diet.

Table 2 Total polyphenol, flavonoid and phenolic acid intake from the different food groups in the PREDIMED cohort, relative contribution of each food group and main food sources.

Food group	Total polyphenols (mg/d)	Flavonoids (mg/d)	Phenolic acids (mg/d)	Main food sources (% contribution to polyphenol intake in the food group)
Fruit	360 ± 217	255 ± 167	72 ± 61	Oranges (33), apples (28), olives (15), cherries (8)
Non-alcoholic beverages	192 ± 140	23 ± 39	168 ± 133	Coffee (88), orange juice (7), tea (3), other juices (1)
Vegetables	104 ± 40	67 ± 31	37 ± 18	Potatoes (35), spinach (20), onions (12), lettuce (8)
Alcoholic beverages	67 ± 126	52 ± 101	10 ± 18	Red wine (95), beer (3), rosé wine (0.8), white wine (0.7)
Cereals	43 ± 48	19 ± 18	12 ± 14	Refined wheat-flour bread (44), whole-grain wheat-flour bread (31), whole-grain flour biscuits (10), breakfast cereals (5)
Oils	22 ± 11	0.36 ± 0.33	0.12 ± 0.05	Virgin olive oil (62), olive oil (38)
Cocoa products	16 ± 41	15 ± 40	0.3 ± 0.7	Chocolate (73), cocoa powder (27)
Nuts and seeds	10 ± 14	6.4 ± 9.3	3.8 ± 6.0	Walnuts (66), other nuts (34)
Legumes	5.6 ± 4.8	4.8 ± 4.2	0.8 ± 0.7	Beans (97), lentils (2), peas (1)
Total	820 ± 323	443 ± 218	304 ± 156	Coffee (18), oranges (16), apples (12), olives and olive oil (11), red wine (6)

Results

Total polyphenol intake

A brief description of the PREDIMED cohort is detailed in Table 1. According to the Phenol-Explorer database, 93 of the 137 food items from the FFQ contain a total of 290 different polyphenols from 18 polyphenol subclasses. Table 2 shows, in decreasing order, the mean intakes of total polyphenols, total flavonoids and phenolic acids (mg day^{-1} and %) from different types of food, as well as the main contributors within each type of food.

The mean total polyphenol intake was 820 ± 323 mg day^{-1}, 443 ± 218 mg of which were flavonoids, 304 ± 156 mg phenolic acids and 73 mg belonged to other polyphenol groups. Fruits were the main source of polyphenols, providing almost 44% of the total polyphenol intake, more than half of total flavonoids and 23% of total phenolic acids.

The non-alcoholic beverages group provided 55% of total phenolic acids, principally coffee. Vegetables provided more than 12% of total polyphenol intake and were the third source of phenolic acids. Alcoholic beverages, cereals, olive oil, cocoa products, nuts and seeds and legumes each contributed less than 10% of the total intake of polyphenols.

Considering individual foods, coffee was the main source of total dietary polyphenols (18%), followed by two fruits: oranges (16%) and apples (12%). Olives and olive oil were the fourth source, together providing 11% of total polyphenol intake, followed by red wine, which contributed 6%.

Intake of different classes of polyphenols

The consumption of the different classes of polyphenols was also calculated and the main food contributors determined (Table 3). Hydroxycinnamic acids were the most consumed type of polyphenol (33%), mainly provided by coffee. Flavanones were the second most consumed polyphenols, with oranges and their products being almost the single food source. Proanthocyanidins, mainly coming from red wine and apples, were the third most consumed polyphenol group, followed by flavonols, flavones and anthocyanins. Olives provided 90% of phenolic acids other than hydroxybenzoic and hydroxycinnamic acids. The remaining polyphenols were grouped into a wide class of 'other polyphenols', including tyrosols, alkylphenols, hydroxybenzaldehydes, furanocoumarins and hydroxycoumarins, among others, representing 8.7% of total polyphenol intake, and being mainly provided by olives and olive oil (37% and 29%, respectively).

Intake of individual polyphenols

The mean individual polyphenol intake was also calculated. Of the 290 polyphenols, 86 were consumed in amounts >1 mg day^{-1}. A list of the 35 most consumed polyphenols (intake > 4 mg day^{-1}) is given in Appendix 1 Table 1 in decreasing order, along with the polyphenol subclass to which they belong, the mean intake expressed in milligrammes per day, and the main food contributors in percentage.

These 35 major polyphenols included seven hydroxycinnamic acids, five proanthocyanidins, five flavonols, four flavones, three flavanones, two anthocyanins and two

Table 3 Polyphenol intakes according to main polyphenol subclasses in the 7200 participants of the PREDIMED cohort and main food sources.

Polyphenol subclass	Total polyphenols (mg/d)	Main food contributors (% contribution to intake of the polyphenol subclass)
Hydroxycinnamic acids	276 ± 146	Coffee (62), potatoes (9), apples (7), olives (5)
Flavanones	132 ± 125	Oranges (91), orange juice (8), red wine (0.5), tomatoes (0.1)
Proanthocyanidins	117 ± 81	Red wine (35), apples (33), peaches (12), plums (7)
Flavonols	80.4 ± 32.7	Spinach (24), beans (17), onions (14), lettuce (6)
Flavones	41.6 ± 26.1	Oranges (39), whole-grain wheat-flour bread (23), refined-grain wheat-flour bread (19), whole-grain wheat-flour biscuits (5)
Anthocyanins	38.5 ± 37.4	Cherries (30), red wine (29), olives (12), strawberries (10)
Catechins	26.7 ± 19.6	Apples (24), red wine (21), tea (11), peaches (10)
Hydroxybenzoic acids	19.1 ± 16.8	Olives (46), red wine (21), walnuts (10), beer (5)
Dihydroxychalcones	2.95 ± 2.57	Apples (100)
Dihydroflavonols	2.82 ± 5.39	Red wine (98), rosé wine (1), white wine (1)
Stilbenes	1.84 ± 3.39	Red wine (94), white wine (2), grapes (1), strawberries (1)
Lignans	0.85 ± 0.36	Olive oil (47), virgin olive oil (25), whole-grain wheat-flour bread (6), refined-grain wheat-flour bread (5)
Theaflavins	0.33 ± 1.36	Tea (100)
Isoflavonoids	0.003 ± 0.003	Beans (97), beer (3)
Chalcones	0.0006 ± 0.0019	Beer (100)
Other phenolic acids	7.56 ± 11.3	Olives (90), red wine (6), beer (2), virgin olive oil (1)
Other polyphenols	71.2 ± 46.7	Olives (37), virgin olive oil (18), olive oil (11), whole-grain wheat-flour bread (9)

catechins, while seven did not belong to any of the aforementioned groups and were classified as 'other polyphenols'.

As expected from the results described in the previous section, the polyphenols of the hydroxycinnamic acid group headed the list. Notably, 7 of the 35 most consumed polyphenols belong to the 'other polyphenols' group and, moreover, their source was typical MedDiet foods. For example, oleuropein and its aglycone, 3,4-DHPEA-EDA (oleuropein-aglycone di-aldehyde), *p*-HPEA-EDA (ligstroside-aglycone di-aldehyde), 3,4-DHPEA-EA (oleuropein-aglycone di-aldehyde) and hydroxytyrosol, which together represented 39.46 mg day^{-1}, came exclusively from olives and olive oil, except for hydroxytyrosol, since a small amount of which was also provided by red wine [18].

Polyphenols from olives and olive oil: the Mediterranean difference

Olive oil is the main source of fat in the MedDiet [19] and studies have revealed that, as well as being rich in monounsaturated fatty acids, it has a unique phenolic profile with interesting biological properties. The beneficial effects of polyphenols from olives and olive oil on plasma lipid levels and oxidative damage [19,20] have resulted in a positive health claim being accepted by the European Food Safety Authority (EFSA) [21]. We estimated the specific contribution of olives and olive oil to the total polyphenol intake in this cohort. Olives and olive oil provided 21.9 ± 10.9 and 68.5 ± 104.0 mg day^{-1} of polyphenols, respectively, which represented approximately 11% of the total intake, being the fourth polyphenol contributors in the diet and giving the Spanish population a different phenolic profile that could be also characteristic of other Mediterranean countries. Table 4 shows the contribution of olives and olive oil to the intake of different classes of polyphenols, which was more than 98% of 'other phenolic acids' (phenolic acids other than hydroxycinnamic and hydroxybenzoic). This table also presents individual polyphenols ingested only from olives and olive oil according to class. This does not mean that these polyphenols are found only in olives and olive oil but, rather, that other sources are scarcely or not consumed in Spain. For example, 2,4-dihydroxybenzoic acid is also found in American cranberries, isorhoifolin in peppermint, verbascoside in a herb called verbena and *m*-coumaric acid is also present in beers but in very small amounts (data from Phenol-Explorer database).

Discussion

The health effects of polyphenols depend on the amount consumed and their bioavailability [5,22]. However, the essential step towards the understanding of the protective effects of polyphenols against chronic diseases is to estimate their consumption by FFQ or other instruments in order to identify the compounds most likely to provide the greatest protection [5,7,8]. Up to now, very few comprehensive assessments of polyphenol intake in different populations have been performed. Most of the studies on different cohorts published to date have used USDA databases that provide data only on flavonoid intake [3,7]; other studies have included other classes of polyphenols, but are based on internal laboratory data on food polyphenol content [10,23]. Another study on the overall polyphenol intake in a diet was based on spectrophotometric methods, but did not provide information on individual compounds [22]. Thus, estimates vary widely among studies making comparisons difficult. The most exhaustive data available are those obtained in the French SU.VI.MAX cohort [11], allowing comparisons with the current study, since both involved similar methodologies. Although

Table 4 Polyphenol intake from olives and olive oil by the 7200 participants in the PREDIMED cohort.

Polyphenol group	Total intake from… (mg/d)			% Intake derived from olives and olive oil	Individual polyphenols ingested only from olives and olive oil
	Virgin olive oil	Olives	Full diet		
Anthocyanidins	0.00	4.49 ± 6.81	38.5 ± 37.4	11.7	–
Flavones	0.36 ± 0.33	1.51 ± 2.30	41.6 ± 26.1	4.50	Isorhoifolin Luteolin 6-C-glucoside
Flavonols	0.00	2.67 ± 4.06	80.4 ± 32.7	3.32	–
Hydroxybenzoic acids	0.06 ± 0.03	7.62 ± 11.6	19.1 ± 16.8	40.2	2,4-Dihydroxybenzoic acid
Hydroxycinnamic acids	0.04 ± 0.03	19.0 ± 28.8	276 ± 146	7.03	Hydroxycaffeic acid [2] Verbascoside *m*-coumaric acid
Other phenolic acids	0.02 ± 0.01	7.43 ± 11.3	7.56 ± 11.3	98.5	3,4-Dihydroxyphenylacetic acid Dihydro-*p*-coumaric acid[a,c] Homoveratric acid[a,b]
Other polyphenols	20.9 ± 10.5	25.8 ± 39.2	71.2 ± 46.7	65.6	Oleuropein, ligstroside[b], 3,4-DHPEA-EDA[b] and other tyrosols 3,4-Dihydroxyphenylglycol[b,c]
Lignans	0.53 ± 0.30	0.01 ± 0.01	0.85 ± 0.36	63.5	1-Acetoxypinoresinol[c]
Total polyphenols	21.9 ± 10.9	68.5 ± 104.0	820 ± 323	11.0	–

[a] Only described in black olives.
[b] Only described in virgin olive oil.
[c] Only described in green olives.

data on some processed foods (jams, drinks, etc.) are available in the Phenol-Explorer database, general information on changes in polyphenol content in cooked foods is still absent; thus, yield or cooking factors should be included when evaluating the bioavailability of polyphenols in humans [17].

The estimated mean total intake of polyphenols in the PREDIMED cohort in the present study was 820 ± 323 mg day^{-1}, being considerably lower than the 1193 ± 510 mg day^{-1} found by Pérez-Jimenez et al. in the SU.VI.MAX cohort. This could be due to the difference in phenolic acid ingestion, since flavonoid intake was similar in both cohorts (443 in the PREDIMED cohort and 506 mg day^{-1} in the SU.VI.MAX cohort). Phenolic acids were the main polyphenols consumed in a Finnish cohort [10] and in SU.VI.MAX, representing approximately 75% of total phenolic intake, whereas in the Spanish cohort it was only 37%. Coffee is the main source of dietary phenolic acids and even of the total polyphenol intake; therefore, it could be deduced that the consumption of coffee in the Spanish cohort was lower, perhaps due to the mean age of 67 years of the participants.

While coffee is the main food source of hydroxycinnamic acids, tea tends to be the main source of hydroxybenzoic acids, thus also enhancing phenolic acid intake. Consequently, the low intake of these compounds in the PREDIMED cohort compared to SU.VI.MAX could reflect a lack of tea consumption, the main food sources of hydroxybenzoic acids being olives (46%) and red wine (21%).

However, the main characteristic distinguishing the phenolic profile of the Spanish population, and probably of other Mediterranean countries, was the presence of polyphenols provided by olives and olive oil. The intake of these typical MedDiet foods was higher than reported elsewhere and together they provided 90.4 mg of polyphenols daily, constituting 11% of total intake. Some polyphenols, including those derived from tyrosol and hydroxytyrosol, were consumed only as olives and olive oil. This has particular importance as recent studies [24] have demonstrated that minor components of olive oil, particularly hydroxytyrosol and related compounds, together with monounsaturated fatty acids, help to improve plasma lipid levels and repair oxidative damage related to cardiovascular diseases [19,20]. These proven benefits have led EFSA to recently accept a health claim about the role of olive polyphenols in the antioxidant effect on low-density lipoprotein (LDL)-cholesterol [21].

The present work presents certain limitations. First, although the Phenol-Explorer is the most comprehensive database available nowadays, information about some foods widely consumed in Spain is still scarce because they have not been characterised or only poorly characterised (e.g., chickpeas, honey or garlic) and some phenolic groups are also underestimated (e.g., thearubigins and proanthocyanidins) because a suitable quantification method is lacking. It should also be taken into account that the polyphenol content in foods can differ according to ripeness at harvest time, environmental factors, processing and storage and even plant variety [5,25,26]. Another limitation of the study is the absence of information about spices and herbs in the FFQ, which might have resulted in an underestimation of the polyphenol intake as, although consumed in low amounts, they are the richest sources of polyphenols [6]. It should also be borne in mind that the resulting estimation is only valid for the population studied (elderly men and women at high cardiovascular risk). To summarise, this study gives a complete description of the total polyphenol intake and main food contributors of dietary polyphenols in a Spanish population at high cardiovascular risk. To our knowledge, this is the first accurate estimation of polyphenol intake done in Spain. The highly detailed data obtained on dietary

polyphenol intake may serve as a valuable tool to establish the future associations between the amount and type of polyphenols ingested and the risk of chronic diseases, being also useful for setting food and health policies and dietary recommendations for individuals and population groups.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2012.10.008.

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Appendix 1

Table 1: Intake of the 35 most consumed polyphenols by the 7200 participants in the PREDIMED cohort and their main food sources.

Polyphenol	Polyphenol subclass	Intake (mg/day)	Main food contributors (% contribution to intake of the individual polyphenol)
5-Caffeoylquinic acid	Hydroxycinnamic acids	112.83 ± 58.54	Coffee (56), potatoes (23), apples (10), pears (3)
Hesperidin	Flavanones	90.29 ± 85.89	Oranges (100)
3-Caffeoylquinic acid	Hydroxycinnamic acids	49.75 ± 34.18	Coffee (74), plums (12), cherries (7), prunes (3), peaches (2)
4-Caffeoylquinic acid	Hydroxycinnamic acids	42.60 ± 31.79	Coffee (96), tomatoes (2), prunes (1), apples (0,5)
Quercetin 3,4'-*O*-diglucoside	Flavonols	21.12 ± 13.35	Onions (100)
Didymin	Flavanones	20.71 ± 19.70	Oranges (100)
Apigenin 6,8-di-C-glucoside	Flavones	19.33 ± 18.39	Oranges (100)
Narirutin	Flavanones	18.71 ± 17.80	Oranges (100)
Cyanidin 3-*O*-rutinoside	Anthocyanins	15.93 ± 20.66	Plums (60), black olives (25), cherries (15)
(-)-Epicatechin	Catechins	14.34 ± 8.91	Apples (39), red wine (14), peaches (12), cocoa products (9)
Ferulic acid	Hydroxycinnamic acids	14.32 ± 14.35	refined-grain wheat-flour products (77), cocoa products (5), tomatoes (5), olives (4)
Quercetin 4'-*O*-glucoside	Flavonols	13.76 ± 8.70	Onions (100)
Procyanidin dimer B2	Proanthocyanidins	13.76 ± 9.83	Apples (65), red wine (18), plums (3), peaches (3)
Procyanidin dimer B1	Proanthocyanidins	12.32 ± 11.32	Peaches (46), apples (23), red wine (17), plums (6)
Apigenin galactoside-arabinoside	Flavones	11.69 ± 11.25	Whole-grain wheat-flour products (57), refined-grain wheat-flour products (43)
Oleuropein-aglycone	Other polyphenols	10.70 ± 11.69	Olives (71), virgin olive oil (29)
(+)-Catechin	Catechins	9.22 ± 7.76	Red wine (36), peaches (13), apples (11), beans (7)
Quercetin 3-*O*-rutinoside	Flavonols	8.55 ± 6.37	Asparagus (35), olives (34), green beans (10), plums (6)

Phlorin	Other polyphenols	7.76 ± 7.38	Oranges (100)
Apigenin arabinoside-glucoside	Flavones	7.54 ± 6.39	Whole-grain wheat-flour products (52), refined-grain wheat-flour products (48)
5-Feruloylquinic acid	Hydroxycinnamic acids	7.24 ± 5.56	Coffee (99), carrots (0,9)
Hydroxytyrosol	Other polyphenols	7.14 ± 10.06	Olives (92), red wine (4), virgin olive oil (4)
Oleuropein	Other polyphenols	6.93 ± 10.48	Olives (99), virgin olive oil (1)
Malvidin 3-*O*-glucoside	Anthocyanins	6.93 ± 10.46	Red wine (73), red grapes (27)
Procyanidin trimer C1	Proanthocyanidins	6.69 ± 4.86	Apples (43), red wine (21), plums (15), peaches (7)
3,4-DHPEA-EDA[1]	Other polyphenols	6.63 ± 5.07	Virgin olive oil (100)
4-Feruloylquinic acid	Hydroxycinnamic acids	6.17 ± 4.81	Coffee (99), carrots (0,5)
Procyanidin dimer B3	Proanthocyanidins	5.89 ± 9.40	Red wine (78), peaches (7), green beans (2), lentils (2)
Syringic acid	Hydroxybenzoic acids	4.82 ± 4.76	Olives (44), walnuts (43), apples (7), red wine (3)
Verbascoside	Hydroxycinnamic acids	4.61 ± 7.00	Olives (100)
Quercetin	Flavonols	4.56 ± 3.69	Onions (100)
Procyanidin dimer B4	Proanthocyanidins	4.44 ± 7.25	Red wine (82), oranges (10), tea (2), plums (2)
Flavone derivative[2]	Flavones	4.38 ± 4.33	Spinach (100)
3,4-DHPEA-EA[3]	Other polyphenols	4.16 ± 2.71	Virgin olive oil (64), olives (36)
Quercetin 3-*O*-(6"-malonyl-glucoside)	Flavonols	4.13 ± 2.72	Lettuce (100)
p-HPEA-EDA[4]	Other polyphenols	4.10 ± 2.73	Virgin olive oil (100)

[1] Oleuropein-aglycone di-aldehyde
[2] 5,4'-Dihydroxy-3,3'-dimethoxy-6:7-methylenedioxyflavone 4'-*O*-glucuronide
[3] Oleuropein-aglycone mono-aldehyde
[4] Ligstroside-aglycone di-aldehyde

1.2. Publicació 2. Associació inversa entre el consum habitual de polifenols i la incidència d'esdeveniments cardiovasculars en l'estudi PREDIMED

Article 2. Inverse association between habitual polyphenol intake and the incidence of cardiovascular events in the PREDIMED study.

Anna Tresserra-Rimbau, Eric B. Rimm, Alexander Medina-Remón, Miguel A. Martínez-González, Rafael de la Torre, Dolores Corella, Jordi Salas-Salvadó, Enrique Gómez-Gracia, José Lapetra, Fernando Arós, Miquel Fiol, Emilio Ros, Lluis Serra-Majem, Xavier Pintó, Guillermo T. Saez, Josep Basora, José V. Sorlí, José A. Martínez, Ernest Vinyoles, Valentina Ruiz-Gutiérrez, Ramón Estruch, i Rosa M. Lamuela-Raventós. Nutrition, Metabolism and Cardiovascular Diseases. **2014**, 24:639-647.

Resum:

La prevenció de les malalties CV és un objectiu prioritari dels organismes de salut pública del països desenvolupats. Nombrosos estudis han proposat els polifenols com a compostos bioactius capaços de prevenir certes malalties cròniques i alguns factors de risc CV. Fins el moment, però, no s'havia avaluat de forma tan completa i prospectiva la relació entre la ingesta de polifenols i la incidència de malalties CV.

L'objectiu d'aquest estudi era avaluar l'associació entre la ingesta de polifenols totals i dels subgrups de polifenols amb el risc de patir un esdeveniment CV (infart de miocardi, accident vascular cerebral o mort per qualsevol de les causes anteriors). Aquest treball és un estudi observacional i longitudinal emmarcat dins l'estudi PREDIMED.

Durant una mitjana de 4,3 anys de seguiment, es van confirmar 273 casos d'esdeveniments CV entre els 7.200 participants que van completar el QFC d'aliments basal. Es van calcular les ingestes de polifenols total i per grups de cada voluntari i es van ajustar per kilocalories consumides. Per avaluar la ingesta de polifenols a llarg termini es va calcular la mitjana acumulada i aquests valors es van dividir en quintils. Per calcular el valor de l'associació (Hazard ratio, HR) entre la ingesta de polifenols i els esdeveniments CV es van dur a terme regressions de Cox dependents del temps utilitzant variables actualitzades anualment. Totes les anàlisis estadístiques es van realitzar amb el programari SAS, versió 9.3 (SAS Institute, Inc., Cary, NC).

Després d'ajustar per totes les variables necessàries, es va observar una reducció del 46% del risc d'esdeveniment CV en els voluntaris del cinquè quintil d'ingesta de polifenols totals comparats amb el del primer (HR=0.54; IC 95%=0.33-0.91; P-linealitat=0.04). Aquesta associació és més forta quan s'exclouen els que beuen alcohol (HR=0.24; IC 95%=0.09-0.64; P-linealitat=0.002), quan només es tenen en compte els ex-fumadors (HR=0.24; IC 95%=0.10-0.59; P-linealitat=0.006), o en el cas dels participants inclosos en el grup de la dieta baixa en greix (HR=0.47; IC 95%=0.23-0.94; P-linealitat=0.01).

Pel que fa als grups de polifenols, es va observar una associació significativa entre el risc d'esdeveniment CV i la ingesta de lignans (HR=0.51; IC 95%=0.30-0.86; P-linealitat=0.007). Dins dels flavonoids, el grup dels flavanols també es va associar amb el risc CV després d'ajustar per totes les variables (HR=0.40; IC 95%=0.23-0.72; P-linealitat=0.003). Per últim, els àcids hidroxibenzòics, del grup dels àcids fenòlics, es van associar fortament i inversament amb el risc CV (HR=0.47; IC 95%=0.26-0.86; P-linealitat=0.02).

Tot i els esperançadors resultats, els estudis observacionals no permeten demostrar una relació causa-efecte i, per tant, caldria dur a terme estudis clínics d'intervenció per a confirmar aquests resultats i establir recomanacions dietètiques acurades.

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Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study

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Abstract *Background and aims:* Epidemiologic and biological evidence supports an inverse association between polyphenol consumption and the risk of cardiovascular disease (CVD). However, no previous studies have prospectively evaluated the relationship between polyphenol intake and the incidence of CVD in such a comprehensive way. The aim was to evaluate the association between intakes of total polyphenol and polyphenol subgroups, and the risk of major cardiovascular events (myocardial infarction, stroke or death from cardiovascular causes) in the PREDIMED study.
Methods and results: The present work is an observational study within the PREDIMED trial. Over an average of 4.3 years of follow-up, there were 273 confirmed cases of CVD among the 7172 participants (96.3%) who completed a validated 137-item food frequency questionnaire (FFQ) at baseline. Polyphenol consumption was calculated by matching food consumption data from the FFQ with the Phenol-Explorer database on polyphenol content of each reported food. After

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multivariate adjustment, a 46% reduction in risk of CVD risk was observed comparing Q5 vs. Q1 of total polyphenol intake (HR = 0.54; 95% confidence interval [CI] = 0.33–0.91; *P*-trend = 0.04). The polyphenols with the strongest inverse associations were flavanols (HR = 0.40; CI 0.23–0.72; *P*-trend = 0.003), lignans (HR = 0.51; CI 0.30–0.86; *P*-trend = 0.007), and hydroxybenzoic acids (HR = 0.47; CI 0.26–0.86; *P*-trend 0.02).
Conclusion: Greater intake of polyphenols, especially from lignans, flavanols, and hydroxybenzoic acids, was associated with decreased CVD risk. Clinical trials are needed to confirm this effect and establish accurate dietary recommendations. *Clinical trial registry*: International Standard Randomized Controlled Trial Number (ISRCTN of London, England) 35739639.

Introduction

The socioeconomic and health impact of cardiovascular disease (CVD) is well documented [1] and prevention of CVD is a crucial public health objective. Adherence to the Mediterranean Diet (MedDiet) is associated with reduced risk of CVD [2]. However, more intervention trials involving MedDiet and different populations are needed to establish its beneficial effects [3]. The MedDiet is very rich in polyphenols, bioactive compounds mainly found in plant foods, and plant-derived beverages.

To date, numerous studies have examined the association between certain polyphenol subgroups and chronic diseases [4–6], or cardiovascular risk factors [7,8]. However, findings from epidemiologic studies are inconclusive [9]. One possible explanation is that, until recently, epidemiological studies used the USDA (US Department of Agriculture) Flavonoid Database, which captures only a subgroup of polyphenols (the flavonoids) and therefore do not reflect the wide amount and diversity of polyphenols found in food [10].

The aim of our study was to prospectively evaluate the association between total polyphenol intake, polyphenol subgroups and the risk of CVD events. The intake of polyphenols was calculated using the Phenol-Explorer database (www.phenol-explorer.eu), the most complete database currently available [11].

Methods

The PREDIMED trial

The PREDIMED (Prevención con Dieta Mediterránea) study is a randomized, primary prevention trial aimed to assess the effect of the traditional MedDiet on clinical cardiovascular events. Details of the recruitment methods, design and inclusion criteria have been previously described [12]. The 7447 eligible participants were randomized to one of three intervention groups: MedDiet supplemented with extra virgin olive oil (EVOO), MedDiet supplemented with nuts or control diet (low-fat diet). All participants provided written informed consent, and the study protocol was approved by the Institutional Review Boards of the participating centers.

Study population

From the PREDIMED cohort, we excluded 247 participants who did not complete the FFQ at baseline, and 28 with extreme total energy intakes [13]. Thus, data from 7172 participants were available for the analyses.

Assessment population characteristics

Participants filled out the following questionnaires at baseline and yearly thereafter: a general questionnaire, a validated 14-point score questionnaire on adherence to the traditional MedDiet [14], and a validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire [15], to collect information about lifestyle habits, medication use and concurrent diseases.

Assessment of diet and polyphenol intake

A validated 137-item food frequency questionnaire (FFQ) [15] was used to collect information on food habits at baseline and also yearly thereafter. The validity of the FFQ to assess total polyphenol intake was studied using total polyphenol excretion in spot urine samples in a clinical trial ($r = 0.48$, $P < 0.01$) and in a cross-sectional study ($r = 0.26$, $P = 0.04$) [16]. This range is likely an underestimate of the true validity because spot urine polyphenol excretion likely best represents intake from the previous 3–12 h whereas the FFQ captures average intake over the previous year [17]. Daily food and nutrients intake was estimated from the FFQ by multiplying the frequency of consumption by the average portion size.

Data on the polyphenol content in foods were obtained from the Phenol-Explorer database and the correspondence between food items in the FFQ and the database has been described in detail before [10,18]. Individual polyphenol intakes at each cycle were calculated by multiplying the content of each polyphenol in a particular food item (mg/g) by the daily consumption of this food item (g/day) and then summing the product across all food items.

Ascertainment of the outcome

The primary endpoint was the occurrence of the first major cardiovascular event: nonfatal acute myocardial

infarction (AMI), nonfatal stroke or death from cardiovascular causes. All outcomes were reported between October 1, 2003 and December 1, 2010 and were adjudicated following standardized criteria by the end-point adjudication committee of the trial [12].

Statistical analyses

To assess long-term polyphenol intake, we calculated the cumulative average of polyphenol intake at each yearly visit as the average of all previous available dietary questionnaires. We also conducted analyses using weighted means of intake to give more relevance to the latest FFQ, but results were not appreciably different. All foods and nutrients were adjusted for total calories using the residual method. Non-dietary covariates such as smoking, body mass index (BMI), physical activity, and medication use, as well as dietary covariates were updated yearly. Baseline characteristics are presented as means (±SD) for continuous variables and frequencies for categorical variables across quintiles of total polyphenol intake. Differences between quintiles were tested by a one-factor ANOVA test for continuous variables and by the chi square test for categorical variables.

Person-time for each participant was calculated from baseline to the date of diagnosis of a primary event, the date of death, the date of the last visit, or December 1, 2010, whichever came first. Time-dependent Cox proportional hazards regression with updated diet and covariates information was used to estimate hazard ratios (HR) using the lowest quintile of intake as the reference group.

We ran additional stratified analyses to evaluate potential effect modification by the following factors: gender, age, alcohol intake, smoking status, and intervention groups. To test for statistical interactions, we also added interaction terms between total polyphenol intake and each of these factors to the model.

Covariates

In multivariate models, we adjusted for age (<60, 60–64.9, 65–69.9, 70–74.9, ≥75 years), BMI (<25, 25–29.9, or ≥30 kg/m^2), smoking status (never, past and current: cigarettes (<5, 5–19, ≥20 per day) or cigars and pipes (<3, 3–6, >6 per day)), alcohol consumption (0, 0.1–14.9, 15–29.9, ≥30 g/day), physical activity (continuous), energy intake (continuous), saturated, monounsaturated and polyunsaturated fat intake, protein intake and cholesterol intake (continuous), use of aspirin and cardiovascular medication, type-2 diabetes, family history of CVD (yes/no). We did not include in the model other variables that did not change the HR by 10% or more.

To account for potential differences in risk factors, we carried out Cox proportional hazard analyses with stratification for recruitment center, sex, and intervention group.

Statistical analyses were conducted using SAS software, version 9 (SAS Institute, Inc., Cary, NC). All *t* tests were 2-sided and *P* values below 0.05 were considered significant.

Results

Total polyphenol intake and CVD

A greater intake of total polyphenols at baseline was associated with better adherence to the MedDiet (14-point score), more physical activity and higher consumption of alcoholic beverages (mainly wine and beer). A higher polyphenol intake was inversely associated with hypertension, but positively associated with smoking and hypercholesterolemia. There were no differences on polyphenol intakes between the three arms (Table 1). Supplemental Table 1 shows more information about baseline characteristics.

During a mean of 4.3 years of follow-up a total of 273 cases of the primary endpoint were observed among 7172 participants (31,068 person-years of observation). Table 2 presents the HRs for the incidence of cardiovascular events according to quintiles of cumulative intake of total polyphenols and their main classes. After multivariate adjustment, we observed a 46% reduction in the risk of major cardiovascular events comparing participants in the highest vs. the lowest quintile of total polyphenol intake (HR = 0.54; 95% confidence interval [CI] 0.33–0.91; *P*-trend = 0.04).

In stratified analyses (Supplemental Table 2), we found no differences between men (Q5 vs. Q1 HR = 0.61; CI = 0.33–1.12; *P*-trend = 0.05) and women (HR = 0.41; CI = 0.17–0.99; *P*-trend = 0.08; *P*-interaction = 0.93). Likewise, the inverse association did not differ for participants younger than 70 years (HR = 0.41; CI = 0.20–0.82; *P*-trend = 0.02), or older than 70 years or older (HR = 0.71; CI = 0.34–1.47; *P*-trend = 0.38; *P*-interaction = 0.95). After stratification by alcohol intake, we observed substantial difference in the association among non-drinkers (HR = 0.24; CI = 0.09–0.64; *P*-trend = 0.002), and drinkers (HR = 0.82; CI = 0.44–1.53; *P*-trend = 0.57; *P*-interaction = 0.15) although we had limited power with only 38% as non-drinkers. Stratification by smoking showed a strong inverse association in former smokers (HR = 0.24; CI = 0.10–0.59; *P*-trend = 0.006), with no significant results for never smokers and smokers, without a significant interaction. Finally, we conducted analyses by intervention groups (MedDiet + EVOO, MedDiet + nuts, and low fat diet) and the only significant association was between total polyphenol intake and CVD among those in the control arm (HR = 0.47; CI = 0.23–0.94; *P* for trend = 0.01). The small amounts of cases in the MedDiet groups are a possible explanation for this result.

Polyphenol classes and risk of CVD

Polyphenols were divided into five main groups: flavonoids, phenolic acids, stilbenes, lignans and others. After adjusting for confounders, we found a 49% decrease of CVD among subjects in the top quintile of lignan consumption (HR = 0.51; CI = 0.30–0.86; *P*-trend = 0.007) compared with those in the bottom quintile (Table 2).

Table 1 Baseline characteristics of participants in the PREDIMED cohort according to quintiles of total polyphenol intake at baseline (energy-adjusted).[a]

	Q1	Q2	Q3	Q4	Q5	P[b]
No subjects (7172)	1434	1435	1434	1435	1434	
Polyphenol intake (mg/d)	483 ± 108	674 ± 36	794 ± 36	937 ± 50	1235 ± 199	
Sex, women	836 (58.3)	924 (64.4)	712 (60.8)	803 (56.0)	648 (45.2)	<0.0001
Age (y)	67.6 ± 6.2	67.4 ± 6.1	67.4 ± 5.9	66.9 ± 6.0	66.2 ± 6.1	<0.0001
Body mass index (kg/m^2)	30.0 ± 3.7	30.3 ± 3.7	29.7 ± 3.5	29.7 ± 3.7	29.6 ± 3.5	<0.0001
Current smoker	217 (15.1)	210 (14.6)	194 (13.5)	265 (18.5)	317 (22.1)	<0.0001
Physical activity at leisure time (MET-h/d)	3.37 ± 3.56	3.62 ± 3.83	3.77 ± 3.66	4.05 ± 4.25	4.59 ± 4.54	<0.0001
Diabetes	706 (49.2)	680 (47.4)	712 (49.6)	704 (49.1)	668 (46.6)	0.40
Hypertension	1230 (85.8)	1224 (85.3)	1192 (83.1)	1166 (81.3)	1117 (77.9)	<0.0001
Hypercholesterolemia	983 (68.6)	1018 (70.9)	1053 (73.4)	1065 (74.2)	1069 (74.6)	0.001
Family history of CVD	403 (28.1)	290 (20.2)	310 (21.6)	324 (22.6)	446 (31.1)	0.05
Intervention group of MedDiet with EVOO	489 (34.1)	506 (35.3)	477 (33.6)	473 (33.0)	517 (36.1)	0.001
Intervention group of MedDiet with nuts	444 (31.0)	467 (32.5)	454 (31.7)	491 (34.2)	519 (36.2)	
Total energy intake (Kcal/d)	2397 ± 642	2180 ± 589	2161 ± 540	2229 ± 563	2369 ± 577	<0.0001

MedDiet, Mediterranean diet; EVOO, extra virgin olive oil.
[a] Categorical variables: subjects (percentage), continuous variables: mean ± SD.
[b] One-way ANOVA tests (continuous variables) or chi-squared (categorical variables).

Table 2 Association between quintiles of cumulative polyphenol intake (total and main groups) and incident CVD in the PREDIMED study.

	Q1	Q2	Q3	Q4	Q5	*P* for trend
Total polyphenols (mg/d)	562	701	800	917	1170	
No. of CVD cases	66	49	58	49	51	
No. of person years	5312	6668	6905	6629	5554	
Age and sex adjusted	1.00	0.60 (0.38–0.95)[a]	0.62 (0.39–0.97)	0.58 (0.36–0.91)	0.58 (0.36–0.93)	0.04
Model 2[b]	1.00	0.57 (0.36–0.92)	0.60 (0.38–0.95)	0.54 (0.34–0.87)	0.51 (0.30–0.84)	0.02
Model 3[c]	1.00	0.60 (0.38–0.97)	0.67 (0.42–1.07)	0.59 (0.37–0.96)	0.54 (0.33–0.91)	0.04
Total flavonoids (mg/d)	273	362	431	512	670	
No. of cases	55	57	57	50	54	
No. of person years	4927	6491	6895	6956	5799	
Age and sex adjusted	1.00	0.77 (0.49–1.21)	0.66 (0.41–1.04)	0.55 (0.34–0.89)	0.64 (0.40–1.03)	0.06
Model 2	1.00	0.80 (0.51–1.27)	0.66 (0.41–1.06)	0.59 (0.36–0.97)	0.64 (0.39–1.06)	0.07
Model 3	1.00	0.87 (0.55–1.39)	0.74 (0.46–1.19)	0.67 (0.40–1.09)	0.71 (0.43–1.18)	0.16
Phenolic acids (mg/d)	159	229	279	345	453	
No. of cases	63	45	54	56	55	
No. of person years	5663	6560	7002	6721	5122	
Age and sex adjusted	1.00	0.76 (0.48–1.21)	0.66 (0.41–1.05)	0.90 (0.58–1.40)	1.00 (0.64–1.57)	0.69
Model 2	1.00	0.74 (0.46–1.19)	0.64 (0.40–1.03)	0.85 (0.54–1.33)	0.87 (0.54–1.38)	0.83
Model 3	1.00	0.71 (0.44–1.14)	0.64 (0.40–1.04)	0.81 (0.51–1.28)	0.82 (0.51–1.32)	0.69
Stilbenes (mg/d)	0	0.48	1.04	2.04	5.75	
No. of cases	61	47	52	60	53	
No. of person years	5341	6541	6640	6491	6055	
Age and sex	1.00	0.64 (0.40–1.04)	0.81 (0.52–1.27)	0.65 (0.42–1.02)	0.66 (0.42–1.04)	0.22
Model 2	1.00	0.94 (0.46–1.95)	1.17 (0.55–2.47)	0.96 (0.46–2.00)	0.69 (0.31–1.56)	0.19
Model 3	1.00	1.08 (0.53–2.21)	1.36 (0.65–2.84)	1.11 (0.54–2.29)	0.77 (0.35–1.72)	0.20
Lignans (mg/d)	0.44	0.57	0.67	0.77	0.94	
No. of cases	69	57	53	44	50	
No. of person years	4625	6122	6899	6892	6530	
Age and sex	1.00	0.61 (0.40–0.95)	0.55 (0.36–0.86)	0.57 (0.35–0.91)	0.51 (0.31–0.84)	0.004
Model 2	1.00	0.65 (0.41–1.01)	0.55 (0.35–0.87)	0.61 (0.37–0.99)	0.50 (0.29–0.85)	0.007
Model 3	1.00	0.64 (0.41–0.99)	0.54 (0.34–0.85)	0.60 (0.36–0.97)	0.51 (0.30–0.86)	0.007
Other (mg/d)	37	53	66	82	113	
No. of cases	57	66	59	53	38	
No. of person years	4645	6445	7055	7055	5868	
Age and sex	1.00	1.00 (0.65–1.55)	0.88 (0.55–1.39)	0.75 (0.46–1.21)	0.67 (0.40–1.11)	0.06
Model 2	1.00	0.97 (0.62–1.53)	0.86 (0.53–1.37)	0.70 (0.43–1.15)	0.63 (0.38–1.05)	0.02
Model 3	1.00	1.08 (0.69–1.71)	0.94 (0.58–1.53)	0.84 (0.51–1.38)	0.74 (0.43–1.26)	0.14

[a] HR (95% CI).
[b] Additionally adjusted for smoking, BMI, alcohol, physical activity, family history of CVD, aspirin use, antihypertensive drugs, cardiovascular drugs, diabetes status, and total energy intake.
[c] Additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol.

Table 3 The relationship between CVD and cumulative flavonoids subclasses intake (in quintiles) in participants from the PREDIMED study.

Flavonoids	Q1	Q2	Q3	Q4	Q5	*P* for trend
Anthocyanins (mg/d)	11.8	23.6	32.8	45.7	74.6	
No. of cases	69	57	52	43	52	
No. of person years	5375	6347	6589	6963	5795	
Age and sex	1.00	0.95 (0.65–1.40)[a]	0.62 (0.41–0.94)	0.52 (0.34–0.80)	0.60 (0.39–0.90)	0.004
Model 2[b]	1.00	1.15 (0.74–1.79)	0.82 (0.51–1.33)	0.65 (0.39–1.09)	0.62 (0.36–1.06)	0.03
Model 3[c]	1.00	1.18 (0.76–1.84)	0.85 (0.52–1.38)	0.67 (0.40–1.11)	0.67 (0.39–1.13)	0.05
Dihydrochalcones (mg/d)	0.8	1.8	2.6	3.5	5.8	
No. of cases	47	59	55	57	55	
No. of person years	5036	6268	7563	5524	6677	
Age and sex	1.00	1.11 (0.73–1.67)	0.92 (0.60–1.40)	0.96 (0.62–1.48)	0.62 (0.39–0.99)	0.02
Model 2	1.00	1.25 (0.78–1.99)	0.90 (0.55–1.46)	0.92 (0.56–1.52)	0.61 (0.35–1.05)	0.02
Model 3	1.00	1.24 (0.78–1.99)	0.92 (0.56–1.50)	0.95 (0.57–1.57)	0.63 (0.36–1.08)	0.03
Dihydroflavonols (mg/d)	0.1	1.4	2.3	3.8	9.8	
No. of cases	61	47	52	60	53	
No. of person years	5291	6490	6735	6489	6063	
Age and sex	1.00	0.63 (0.41–0.97)	0.71 (0.47–1.06)	0.66 (0.44–0.98)	0.55 (0.36–0.84)	0.03
Model 2	1.00	1.08 (0.52–2.23)	1.13 (0.53–2.43)	1.04 (0.50–2.19)	0.73 (0.32–1.63)	0.18
Model 3	1.00	1.17 (0.58–2.40)	1.27 (0.60–2.69)	1.16 (0.56–2.41)	0.78 (0.35–1.73)	0.19
Flavanols (mg/d)	90	129	158	192	263	
No. of cases	69	51	59	59	35	
No. of person years	4841	6409	7058	6860	5900	
Age and sex	1.00	0.64 (0.43–0.94)	0.65 (0.44–0.95)	0.55 (0.37–0.82)	0.33 (0.21–0.53)	<0.0001
Model 2	1.00	0.65 (0.41–1.02)	0.70 (0.44–1.09)	0.57 (0.36–0.91)	0.36 (0.20–0.63)	0.0004
Model 3	1.00	0.70 (0.44–1.10)	0.77 (0.49–1.21)	0.66 (0.41–1.05)	0.40 (0.23–0.72)	0.003
Flavanones (mg/d)	28	78	113	157	247	
No. of cases	50	51	49	55	68	
No. of person years	4560	6011	7527	6336	6633	
Age and sex	1.00	0.82 (0.54–1.24)	0.53 (0.34–0.83)	0.74 (0.48–1.13)	0.86 (0.57–1.28)	0.76
Model 2	1.00	0.98 (0.61–1.59)	0.62 (0.37–1.04)	1.01 (0.62–1.64)	1.00 (0.63–1.59)	0.74
Model 3	1.00	1.07 (0.67–1.74)	0.67 (0.40–1.13)	1.11 (0.68–1.82)	1.09 (0.68–1.74)	0.54
Flavones (mg/d)	20	29	37	46	67	
No. of cases	44	45	59	69	56	
No. of person years	4803	6485	6595	7134	6050	
Age and sex	1.00	0.76 (0.49–1.19)	1.00 (0.65–1.54)	0.94 (0.62–1.43)	0.92 (0.59–1.42)	0.99
Model 2	1.00	0.88 (0.53–1.46)	1.30 (0.79–2.13)	1.23 (0.76–1.99)	1.02 (0.61–1.69)	0.81
Model 3	1.00	0.94 (0.56–1.56)	1.37 (0.83–2.27)	1.30 (0.79–2.12)	1.07 (0.64–1.80)	0.72
Flavonols (mg/d)	56	74	88	101	124	
No. of cases	69	57	55	40	52	
No. of person years	5608	6961	6668	6179	5652	
Age and sex	1.00	0.85 (0.58–1.25)	0.63 (0.42–0.95)	0.44 (0.28–0.70)	0.56 (0.35–0.88)	0.002
Model 2	1.00	0.79 (0.51–1.22)	0.69 (0.44–1.09)	0.48 (0.28–0.80)	0.58 (0.34–0.98)	0.02
Model 3	1.00	0.84 (0.54–1.31)	0.74 (0.46–1.17)	0.53 (0.31–0.90)	0.69 (0.40–1.19)	0.08

[a] HR (95% CI).
[b] Model 2 – age, sex, smoking, BMI, alcohol, energy, physical activity, family history of CVD, aspirin use, antihypertensive drugs, cardiovascular drugs, and diabetes status.
[c] Model 3 - model 2 plus intake of proteins, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol.

Flavonoids subclasses and CVD risk

Flavanols, which include proanthocyanidins, catechins, and theaflavins, were the most consumed group of flavonoids (90–263 mg/d). Their intake was strongly inversely associated with CVD after adjustment for potential confounders (HR = 0.40; CI = 0.23–0.72; *P*-trend = 0.003) (Table 3). There was an inverse association for flavonols and CVD when applying model 2 (HR = 0.58; CI = 0.34–0.98; *P*-trend = 0.02), but the association was attenuated after adjustment for nutrients (model 3). A similar trend was observed for anthocyanins (HR = 0.67; CI = 0.39–1.13; *P*-trend = 0.05). Dihydrochalcones (HR = 0.63; CI = 0.36–1.08; *P*-trend = 0.03) were also weakly but inversely associated with CVD. We found no or weaker association between other flavonoids subclasses and CVD.

Phenolic acids subclasses and CVD risk

In decreasing order, the contribution of the different subclasses of phenolic acids to the total intake of polyphenols was: hydroxycinnamic acids (138–422 mg/d), hydroxybenzoic acids (6.9–36.1 mg/d), and other phenolic acids (0.1–17.9 mg/d). Hydroxybenzoic acids were strongly and inversely associated with CVD after controlling for potential confounders (HR = 0.47; CI = 0.26–0.86; *P*-trend: 0.02) (Table 4).

Food sources

To translate these findings into food-based dietary guidelines, we summarize the main food sources of polyphenols in the PREDIMED population as well as the main food

Table 4 The relationship between CVD and cumulative phenolic acids subclasses intake (in quintiles) in participants from the PREDIMED study.

Phenolic acids	Q1	Q2	Q3	Q4	Q5	*P* for trend
Hydroxybenzoic acids (mg/d)	6.9	12.9	17.8	24.1	36.1	
No. of cases	69	62	47	55	40	
No. of person years	5398	6603	6734	6853	5480	
Age and sex	1.00	0.80 (0.54–1.17)[a]	0.60 (0.40–0.90)	0.54 (0.36–0.82)	0.46 (0.29–0.71)	0.0003
Model 2[b]	1.00	0.82 (0.52–1.29)	0.65 (0.40–1.06)	0.59 (0.36–0.97)	0.37 (0.20–0.66)	0.0006
Model 3[c]	1.00	0.91 (0.57–1.43)	0.74 (0.46–1.22)	0.73 (0.44–1.21)	0.47 (0.26–0.86)	0.02
Hydroxycinnamic acids (mg/d)	138	207	252	316	422	
No. of cases	61	50	42	59	61	
No. of person years	5632	6486	6869	6914	5167	
Age and sex	1.00	0.80 (0.53–1.20)	0.55 (0.35–0.87)	0.92 (0.62–1.36)	1.08 (0.72–1.63)	0.40
Model 2	1.00	0.81 (0.51–1.29)	0.57 (0.34–0.96)	0.91 (0.58–1.42)	0.99 (0.62–1.58)	0.71
Model 3	1.00	0.79 (0.49–1.25)	0.58 (0.35–0.97)	0.86 (0.55–1.36)	0.93 (0.58–1.49)	0.93
Other phenolic acids (mg/d)	0.1	2.5	4.6	8.6	17.9	
No. of cases	58	66	47	62	40	
No. of person years	5100	5225	6571	7787	5385	
Age and sex	1.00	1.11 (0.75–1.64)	0.69 (0.45–1.08)	0.79 (0.52–1.21)	0.73 (0.46–1.14)	0.10
Model 2	1.00	1.31 (0.83–2.09)	0.75 (0.44–1.28)	0.88 (0.54–1.42)	0.74 (0.45–1.24)	0.11
Model 3	1.00	1.39 (0.87–2.22)	0.82 (0.48–1.39)	0.92 (0.57–1.51)	0.82 (0.49–1.39)	0.19

[a] HR (95% CI).
[b] Model 2 – age, sex, smoking, BMI, alcohol, energy, physical activity, family history of CVD, aspirin use, antihypertensive drugs, cardiovascular drugs, and diabetes status.
[c] Model 3 - model 2 plus intake of proteins, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol.

sources in general (Table 5). Seasonings were separated from other foods due to the high content of polyphenols. The foods with greatest polyphenol concentration did not always correspond to the main sources in this cohort. For instance, our population consumed flavanols mostly from red wine (32%) and apples (31%), but the flavanol richest foods are those containing cocoa.

Some foods were present in several of the polyphenol groups that were significantly associated with lower CVD risk. Thus, olives and olive oil contributed substantially to the intake of lignans, anthocyanins, hydroxybenzoic acids, and 'others', whereas apples contributed to both dihydrochalcones and flavanols intakes.

Discussion

The present work is an observational study within the PREDIMED trial. We observed a significant reduction of cardiovascular events and cardiovascular mortality with higher intake of total polyphenols, lignans, flavanols and hydroxybenzoic acids. Some of our results of individual phenolic compounds agree with previous studies, but others are contradictory or cannot be compared because many compounds such as benzoic acids have not been studied [19–23].

Previous studies have demonstrated that polyphenols and their metabolites can reduce blood pressure, as well as markers of oxidation and inflammation. They may prevent or improve endothelial dysfunction, not only by reducing the expression of NADPH oxidase, but also by increasing antioxidant enzyme activity, anti-inflammatory effects, and bioavailability of nitric oxide and by inhibiting low density lipoproteins [7].

Within the main polyphenol groups our results indicate a potential role of lignans in reducing CV risk that can be explained due to the high consumption of olive oil in our Mediterranean population. Although olive oil is the main source of this polyphenol group in our population, lignans may be consumed in higher amounts through flax and sesame seeds. Lignans can modulate the action of endogenous estrogens and, therefore, exert potential effects on hormone-related diseases such as CVD, some cancers, osteoporosis and menopausal symptoms [24,25].

We also observed a trend toward a reduction in CV risk with increasing intake of "other polyphenols", a heterogeneous group that includes, for example, tyrosols, alkylphenols, hydroxybenzaldehydes, furanocoumarins and hydroxycoumarins. These compounds are present in olives and virgin olive oil, as well as bran breakfast cereals. Extra virgin olive oil can exert beneficial effects on CV risk factors by diminishing blood pressure, regulating plasma lipid levels, reducing systemic inflammation and repairing oxidative damage [26].

Among flavonoids subclasses, we found a linear inverse association between anthocyanins and risk of CVD which was very similar to a recent large prospective study of anthocyanins and CHD risk in middle aged US women [19]. We had fewer cases so the risk reduction did not reach statistical significance. Two other prospective studies among adults reported that intakes of flavanones and anthocyanins were associated with a decreased risk of CHD, CVD and total mortality [20,27]. Anthocyanins are present in red/blue fruits and vegetables, typically berries, but their consumption in our population came mostly from cherries and red wine.

In our study, a higher intake of flavanols was associated with a 60% reduction in risk of cardiovascular events and mortality. The cardioprotective effect of this subclass of flavonoids was previously reported in a meta-analysis of flavan-3-ol randomized, controlled trials and biomarkers of CVD risk [21]. Other studies have focused on the

Table 5 Main food sources of polyphenols.

Polyphenol groups	Main food sources in the cohort (% of total intake within each polyphenol class)[a]	Main food sources, excluding seasonings (mg/100 g)[b]	Main food sources, only seasonings (mg/100 g)[b]
Total polyphenols	Coffee (18), oranges (16), apples (12), olives and olive oil (11), red wine (6)	Cocoa powder (3448), dark chocolate (1664), black elderberry (1359)	Cloves (15,188), dried peppermint (11,960), star anise (5460)
Flavonoids			
Anthocyanins	Cherries (30), red wine (29), olives (12)	Black elderberry (1316), black chokeberry (878), blackcurrant (592)	–
Dihydrochalcones	Apples (100)	Apple puree (8.8), plum (5.9), apple cider (5.8)	Dried oregano (136)
Dihydroflavonols	Red wine (98)	Red wine (5.4), white wine (0.6), rosé wine (0.4)	Dried oregano (128)
Flavanols	Red wine (32), apples (31), peaches (12)	Cocoa powder (512), dark chocolate (212), broad bean pod (154)	–
Flavanones	Oranges (91)	Grapefruit/pummelo juice (67), orange juice (61), grapefruit juice (51)	Dried peppermint (8740), dried oregano (1050), fresh rosemary (55)
Flavones	Oranges (39), whole-grain wheat-flour bread (23), refined-grain wheat-flour bread (19)	Whole-grain wheat flour (83), globe artichoke (58), black olives (27)	Celery seed (2094), dried peppermint (1486), common verbena (790)
Flavonols	Spinach (24), beans (17), onions (14)	Red onion (158), spinach (119), shallot (112)	Capers (655), saffron (510), dried oregano (272)
Isoflavonoids	Beans (97)	Soy flour (467), soy paste (264), roasted soy bean (247)	Soy sauce (1.5)
Phenolic acids			
Hydroxybenzoic acids	Olives (46), red wine (21), walnuts (10)	Pomegranate juice (168), red raspberry (121), American cranberry (53)	Chestnut (1215), cloves (459), star anise (32)
Hydroxycinnamic acids	Coffee (62), potatoes (9)	Maize oil (557), coffee beverage (278), black fox grape (228)	Dried peppermint (1734), common verbena (1365), dried rosemary (1009)
Other phenolic acids	Olives (90)	Green olive (17), black olive (5.1), refined rye-flour (0.3)	–
Stilbenes	Red wine (94)	Red wine (3.4), red wine from muscadine grapes (3.0), lingonberry (3.0)	–
Lignans	Virgin olive oil (72)	Virgin olive oil (2.4), whole-grain rye flour (1.8), bread from whole-grain rye flour (1.2)	Sesame seed oil (1295), black sesame seed oil (1223), flaxseed (867)
Other polyphenols	Olives (37), virgin olive oil (18), olive oil (11)	Bran breakfast cereals (286), black olive (266), green olive (211)	Cloves (14,668), dried turmeric (5433), star anise (5408)

[a] Contribution to polyphenol intake in the food group at baseline (percentages). Foods listed explain at least 70% of the intake or are the top 3 main sources.
[b] Data from Phenol-Explorer database.

mechanisms that explain these effects [21,28]. In this Mediterranean elderly population, red wine, apples and peaches were the main contributors to flavanol intake.

In a Welsh cohort within the Caerphilly study, flavonol intake was weakly but positively associated with CHD mortality, cancer mortality and total mortality [22]. More recent studies have shown a significant decrease of CHD mortality associated with high intakes of flavonols and flavonol-rich foods that are consistent with our findings [23]. Tea is usually the main source of flavonols, but its consumption was very low in our Spanish cohort, where spinach, beans, and onions were the main sources.

Among phenolic acids, hydroxybenzoic acids were significantly associated with decreased cardiovascular events. Although hydroxybenzoics have not been extensively studied they have shown antioxidant and anti-inflammatory properties [29].

Our study has limitations. Although we controlled for many potential confounders in multivariate models, other unknown or unmeasured confounders may exist. However, misclassification would tend to bias estimates and would attenuate associations toward the null. Limitations with respect to the estimation of polyphenol intake were the following: variability or lack of information about polyphenol content in foods depending on ripeness at harvest time, environmental factors, processing and storage, and plant variety [30]; absence of information about some foods in the FFQ, and bioavailability of these molecules. The number of events in the PREDIMED trial was lower than in many epidemiological studies, which limits

the statistical power on associations of polyphenol subclasses with CVD. Finally, generalization to other populations different from middle-aged to elderly people at high cardiovascular risk might be limited.

Our study has also several strengths, including the prospective design, large sample size with a relatively long follow-up, blinded assessment of the end-points, and comprehensive data on risk factors and confounders for CVD risk. The yearly assessment of the polyphenol intake using validated FFQ enabled us to calculate the cumulative average of dietary exposure to have a better representation of long-term consumption, reducing measurement error and taking into account the changes in the diet due to the intervention. In addition, the use of the most comprehensive polyphenol database available (Phenol-Explorer database) allowed us to assess associations with CVD of total polyphenol intake and flavonoids, as well as all polyphenol subgroups, including phenolic acids, lignans, stilbenes and others.

We conclude that there is an inverse association between total polyphenol intake and risk of cardiovascular-related events that is independent of other dietary and non-dietary CVD risk factors. Similar significant associations were established for lignans, flavanols, and hydroxybenzoic acids. Further randomized controlled trials are needed to confirm the promising protective effects of polyphenols on CVD and establish dietary recommendations and desired minimum levels of intake.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2013.12.014.

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Supplemental table 1. Baseline characteristics of participants in the PREDIMED cohort according to quintiles of total polyphenol intake at baseline (energy-adjusted). [a]

	Q1	Q2	Q3	Q4	Q5	*P*[b]
No subjects (7172)	1434	1435	1434	1435	1434	
Mean daily intake:						
Carbohydrates (g/d)	239.7±45.1	236.5±38.9	235.1±37.4	234.2±41.1	236.0±44.8	0.006
Protein (g/d)	91.9±15.1	92.4±13.8	92.4±13.2	91.5±13.6	90.6±14.9	0.004
SFA (g/d)	26.1±6.7	25.4±5.7	25.1±5.3	24.9±5.5	23.5±5.8	<0.0001
MUFA (g/d)	49.0±12.2	48.8±10.6	48.8±10.7	48.7±11.3	46.6±11.2	<0.0001
PUFA (g/d)	15.6±5.8	15.9±5.1	15.8±5.0	15.8±5.2	15.0±5.2	<0.0001
Fiber (g/d)	21.5±6.1	23.9±6.4	25.5±6.7	26.6±7.4	29.4±8.9	<0.0001
Total cholesterol (mg/d)	372±121	367±103	368±107	360±94	354±122	<0.0001
Alcohol (g/d)	4.10±10.87	6.34±10.07	7.60±10.50	9.27±12.81	14.6±18.9	<0.0001
14-point MedDiet score	8.2±1.9	8.5±1.9	8.7±1.9	8.7±1.9	9.2±1.8	<0.0001
Risk factors:						
Waist to height ratio	0.64±0.06	0.63±0.07	0.63±0.06	0.62 ±0.06	0.62±0.06	<0.0001
Systolic blood pressure (mmHg)	150±19	151±19	149±19	148±18	148±18	0.013
Diastolic blood pressure (mmHg)	83±10	84±9.8	82±9.6	82±9.8	83±9.6	0.003
Hearth rate (beats/min)	71.7±11.0	71.2±10.9	70.7±11.1	70.0±10.5	70.5±10.5	0.016
Glucose (mg/dL), n=4311	118±41	116±39	122±42	123±43	123±43	0.0007
Total cholesterol (mg/dL), n=4286	202±36	206±38	207±39	208±38	207±36	0.003
HDL cholesterol (mg/dL), n=4236	50±11	51±11	51±11	52±12	52±11	0.007
Triglycerides (mg/dL), n=4291	130±67	133±74	137±79	130±63	138±80	0.057

[a] Continuous variables: mean±SD.
[b] One-way ANOVA tests.
SFA, Saturated Fatty Acids; MUFA, Monounsatturated Fatty Acids; PUFA, Polyunsatturated Fatty Acids; HDL, High Density Lipoproteins.

Supplemental table 2. The associations between CVD and cumulative polyphenol intake across strata of risk factors for participants from the PREDIMED Study.

			Q1	Q2	Q3	Q4	Q5	*P*-trend	*P*-int.
Sex	**Men**	TP (mg/d)	556	703	800	916	1130		0.93
		No. of cases	35	22	39	29	38		
		Person-years	2019	2390	2698	2935	3275		
		Model 3[a]	1.00	0.55 (0.29-1.05)[b]	0.76 (0.42-1.35)	0.63 (0.35-1.15)	0.61 (0.33-1.12)	0.05	
	Women	TP (mg/d)	565	700	799	910	1103		
		No. of cases	31	27	19	20	13		
		Person-years	3293	4278	4207	3694	2279		
		Model 3	1.00	0.61 (0.32-1.19)	0.42 (0.20-0.89)	0.47 (0.22-1.00)	0.41 (0.17-0.99)	0.08	
Age	**< 70 y**	TP (mg/d)	561	700	800	914	1120		0.95
		No. of cases	25	23	35	28	28		
		Person-years	3437	4419	4711	4804	4112		
		Model 3	1.00	0.57 (0.28-1.16)	0.72 (0.38-1.36)	0.61 (0.32-1.18)	0.41 (0.20-0.82)	0.02	
	> 70 y	TP (mg/d)	562	702	800	908	1112		
		No. of cases	41	26	23	21	23		
		Person-years	1874	2250	2194	1825	1442		
		Model 3	1.00	0.65 (0.34-1.23)	0.51 (0.26-1.01)	0.53 (0.26-1.09)	0.71 (0.34-1.47)	0.38	
Alcohol	**Non drinkers**	TP (mg/d)	563	699	797	909	1090		0.15
		No. of cases	40	23	20	17	15		
		Person-years	2778	3164	3026	2312	1230		
		Model 3	1.00	0.43 (0.21-0.88)	0.29 (0.14-0.62)	0.35 (0.17-0.75)	0.24 (0.09-0.64)	0.002	
	Drinkers	TP (mg/d)	561	702	801	915	1127		
		No. of cases	26	26	38	32	36		
		Person-years	2533	3505	3880	4316	4324		
		Model 3	1.00	0.78 (0.41-1.51)	0.94 (0.51-1.76)	0.73 (0.39-1.39)	0.82 (0.44-1.53)	0.57	
Smoking habit	**Never**	TP (mg/d)	563	700	799	911	1106		0.15
		No. of cases	36	29	23	25	15		
		Person-years	3742	4593	4486	4087	2611		
		Model 3	1.00	0.73 (0.39-1.39)	0.52 (0.26-1.05)	0.59 (0.29-1.17)	0.58 (0.27-1.27)	0.14	
	Former	TP (mg/d)	562	703	799	914	1125		
		No. of cases	21	10	21	15	18		
		Person-years	950	1348	1624	1679	1864		
		Model 3	1.00	0.32 (0.13-0.83)	0.43 (0.20-0.96)	0.37 (0.16-0.96)	0.24 (0.10-0.59)	0.006	
	Current	TP (mg/d)	549	699	803	917	1134		
		No. of cases	9	10	14	9	18		
		Person-years	620	727	796	862	1079		
		Model 3	1.00	1.20 (0.37-3.90)	1.24 (0.42-3.68)	0.83 (0.25-2.82)	1.52 (0.52-4.50)	0.52	
Intervention group	**MedDiet-VOO**	TP (mg/d)	563	702	800	914	1120		0.72
		No. of cases	22	21	21	10	18		
		Person-years	1661	2605	2703	2481	2028		
		Model 3	1.00	1.11 (0.58-2.11)	1.00 (0.50-1.98)	0.82 (0.40-1.70)	0.87 (0.40-1.89)	0.52	
	MedDiet-Nuts	TP (mg/d)	563	700	799	909	1126		
		No. of cases	17	12	13	19	16		
		Person-years	1731	2063	2177	2214	1950		
		Model 3	1.00	0.45 (0.21-0.97)	0.35 (0.17-0.75)	0.68 (0.36-1.32)	0.68 (0.34-1.36)	0.66	
	Low Fat Diet	TP (mg/d)	559	698	800	914	1106		
		No. of cases	27	16	24	20	17		
		Person-years	1919	2001	2025	1934	1576		
		Model 3	1.00	0.78 (0.43-1.40)	0.29 (0.13-0.64)	0.45 (0.23-0.89)	0.47 (0.23-0.94)	0.01	

[a] HR (95% CI).
[b] Adjusted by age, sex, smoking, BMI, alcohol, energy, physical activity, family history of CVD, aspirin use, antihypertensive drugs, cardiovascular drugs, and diabetes status, intake of proteins, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol.
P-int: *P* for interaction, TP: Total Polyphenol intake, MedDiet: Mediterranean Diet, VOO: Virgin Olive Oil

1.3. Publicació 3. Ingesta de polifenols i risc de mortalitat: un re-anàlisi de l'estudi PREDIMED

Article 3. Polyphenol intake and mortality risk: a re-analysis of the PREDIMED trial.

Anna Tresserra-Rimbau, Eric B. Rimm, Alexander Medina-Remón, Miguel A. Martínez-González, M. Carmen López-Sabater, María I. Covas, Dolores Corella, Jordi Salas-Salvadó, Enrique Gómez-Gracia, José Lapetra, Fernando Arós, Miquel Fiol, Emilio Ros, Lluis Serra-Majem, Xavier Pintó, Miguel A. Muñoz, Alfredo Gea, Valentina Ruiz-Gutiérrez, Ramón Estruch, i Rosa M. Lamuela-Raventós. BioMed Central. **2014**, 12(77):1-11.

Resum:

La dieta i un estil de vida saludable són crucials en la prevenció de les malalties cròniques, que en els països desenvolupats causen les majors taxes de mortalitat. Els polifenols són compostos bioactius que poden disminuir el risc de patir certes malalties cròniques gràcies a les seves propietats antioxidants i anti-inflamatòries, així com pels seus efectes beneficiosos sobre la PA, els lípids i la resistència a la insulina.

L'objectiu d'aquest treball ha estat l'avaluació de la relació entre el consum de polifenols totals i per grups i la mortalitat per qualsevol causa en una població d'avançada edat i alt risc cardiovascular (cohort PREDIMED) estimant els polifenols mitjançant els QFC d'aliments i la base de dades de polifenols Phenol-explorer.

La ingesta mitjana acumulada de polifenols es va calcular seguint la metodologia descrita per Tresserra-Rimbau, et al., 2013 i 2014a. Els valors de polifenols totals i per grups es van dividir en quintils de consum i es van associar a la mortalitat total mitjançant regressions de Cox dependents del temps, ajustant per totes les variables necessàries i estratificant per sexe, grup d'intervenció i centre de reclutament. Totes les anàlisis estadístiques es van dur a terme utilitzant el programari SAS, versió 9.3 (SAS Institute, INc., Cary, NC).

Durant una mitjana de 4,8 anys de seguiment, hi va haver 327 defuncions, dels 7172 participants inclosos en l'estudi. D'aquestes, 131 van ser per càncer, 81 per accidents cardiovasculars i 115 per altres causes. Després d'ajustar per totes les variables pertinents, el quintil més alt d'ingesta de polifenols totals es va associar inversament al risc de mortalitat per qualsevol causa tot i que no s'observà linealitat sinó un llindar a partir del qual el benefici es manté (HR=0.63; IC 95%=0.41-0.97; P-linealitat=0.12). Aquesta associació es mantingué significativa després de treure els participants amb només un o dos anys de seguiment. Els anàlisis estratificats mostraren associacions més fortes per a les dones (HR=0.42; IC 95%=0.18-0.98; P-linealitat=0.24), els abstemis (HR=0.39; IC 95%=0.17-0.90; P-linealitat=0.04) i els voluntaris del grup control o grup de la dieta baixa en greix (HR=0.48; IC 95%=0.23-0.98; P-linealitat=0.01). En cap cas, però, les interaccions van ser significatives.

A continuació es van fer anàlisis similars per als diferents grups de polifenols i les diferents classes dins de cada grup. Vam observar una reducció del 52% en el risc de mortalitat entre els participants que consumien més estilbens (HR=0.48; IC 95%=0.25-0.91; P-linealitat=0.04), mentre que la reducció va ser del 40% pels que ingerien més lignans (HR=0.60; IC 95%=0.37-0.95; P-linealitat=0.03). Tot i que no es va observar una associació significativa pels flavonoids, sí que es va trobar per al grup de les isoflavones (HR=0.49; IC 95%=0.28-0.84; P-linealitat=0.009), tot i que la seva ingesta és molt baixa en aquesta població.

La associació inversa entre la ingesta de polifenols totals, lignans, estilbens i isoflavones amb el risc de mortalitat per qualsevol causa és significativa fins i tot després d'ajustar per tots els factors de risc. No obstant, calen més estudis, sobretot d'intervenció per confirmar aquesta relació i establir causalitat.

Tresserra-Rimbau et al. BMC Medicine 2014, 12:77
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RESEARCH ARTICLE Open Access

Polyphenol intake and mortality risk: a re-analysis of the PREDIMED trial

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Abstract

Background: Polyphenols may lower the risk of cardiovascular disease (CVD) and other chronic diseases due to their antioxidant and anti-inflammatory properties, as well as their beneficial effects on blood pressure, lipids and insulin resistance. However, no previous epidemiological studies have evaluated the relationship between the intake of total polyphenols intake and polyphenol subclasses with overall mortality. Our aim was to evaluate whether polyphenol intake is associated with all-cause mortality in subjects at high cardiovascular risk.

Methods: We used data from the PREDIMED study, a 7,447-participant, parallel-group, randomized, multicenter, controlled five-year feeding trial aimed at assessing the effects of the Mediterranean Diet in primary prevention of cardiovascular disease. Polyphenol intake was calculated by matching food consumption data from repeated food frequency questionnaires (FFQ) with the Phenol-Explorer database on the polyphenol content of each reported food. Hazard ratios (HR) and 95% confidence intervals (CI) between polyphenol intake and mortality were estimated using time-dependent Cox proportional hazard models.

Results: Over an average of 4.8 years of follow-up, we observed 327 deaths. After multivariate adjustment, we found a 37% relative reduction in all-cause mortality comparing the highest versus the lowest quintiles of total polyphenol intake (hazard ratio (HR) = 0.63; 95% CI 0.41 to 0.97; *P* for trend = 0.12). Among the polyphenol subclasses, stilbenes and lignans were significantly associated with reduced all-cause mortality (HR =0.48; 95% CI 0.25 to 0.91; *P* for trend = 0.04 and HR = 0.60; 95% CI 0.37 to 0.97; *P* for trend = 0.03, respectively), with no significant associations apparent in the rest (flavonoids or phenolic acids).

Conclusions: Among high-risk subjects, those who reported a high polyphenol intake, especially of stilbenes and lignans, showed a reduced risk of overall mortality compared to those with lower intakes. These results may be useful to determine optimal polyphenol intake or specific food sources of polyphenols that may reduce the risk of all-cause mortality.

Clinical trial registration: ISRCTN35739639.

Keywords: Polyphenol intake, All-cause mortality, PREDIMED, Mediterranean diet, Stilbenes, Lignans

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Tresserra-Rimbau et al. BMC Medicine 2014, 12:77
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Background

Diet and lifestyle are crucial in the prevention of chronic illnesses and therefore substantially lower all-cause mortality in most westernized countries. There is evidence that the Mediterranean diet (MedDiet), a well characterized dietary pattern, is associated with longevity and improved quality of life by reducing the risk of the most frequent chronic diseases such as cardiovascular diseases (CVD), metabolic syndrome, age-related cognitive impairment, type 2 diabetes mellitus (T2DM), cancer and also all-cause mortality [1,2]. The MedDiet is rich in fruits and vegetables, olive oil, nuts, legumes, whole-wheat bread and fish, and wine is consumed in moderate amounts during meals [2]. With respect to nutrients, the MedDiet is very rich in mono- and polyunsaturated fatty acids [3] and also in polyphenols, which are bioactive compounds mainly found in plant foods and plant-derived beverages such as coffee, tea and red wine.

Several studies have examined the association between intake of certain polyphenol subgroups and their sources, and the incidence of chronic degenerative diseases [4], as well as their effects on blood pressure, lipid profile, and endothelial and platelet function [5-7]. If polyphenol intake does protect against the development of chronic diseases such as CVD, cancer or T2DM, we hypothesized that a greater consumption of polyphenols would contribute to lower the risk of all-cause mortality and provide a greater life expectancy.

To date, the association between specific groups of polyphenols and mortality has been described [8], but to our knowledge, neither total polyphenol intake nor that of the different polyphenol subgroups, have been associated with all-cause mortality. We therefore embarked on a study to evaluate the association between the intake of total polyphenols and polyphenol subgroups and the risk of overall mortality, using the Phenol-Explorer database [9] to estimate the polyphenol intake recorded by the food frequency questionnaires (FFQ) administered yearly in the PREDIMED (*Prevención con Dieta Mediterránea*) trial. These results may be useful to determine optimal polyphenol intake or specific food sources of polyphenols that may reduce the risk of all-cause mortality among subjects at high cardiovascular risk.

Methods

The PREDIMED study

The PREDIMED study was a parallel-group, randomized, multicenter, controlled feeding trial aimed at assessing the effects of the MedDiet in the primary prevention of cardiovascular disease. Details of the recruitment method and study design have been described elsewhere [10]. The eligible participants were 7,447 community-dwelling men (55 to 80 years) and women (60 to 80 years) from Spain, who had no cardiovascular disease at enrollment but were at high risk: they had either T2DM or at least three of the following major risk factors: smoking, hypertension, dyslipidemia, overweight or obesity, or a family history of premature coronary heart disease. Starting on 1 October 2003, the eligible participants were randomized in a 1:1:1 ratio to one of three dietary intervention groups: 1) MedDiet supplemented with extra-virgin olive oil (EVOO), 2) MedDiet supplemented with mixed nuts or 3) control diet (low-fat diet). The trial was stopped after a median follow-up of 4.8 years due to the benefit of the MedDiets with respect to major cardiovascular events: myocardial infarction, stroke or death from cardiovascular causes (analysis performed by the Drug and Safety Monitoring Board of the trial), compared to a control low-fat group [2]. All participants provided written informed consent, and the study protocol was approved by the Institutional Review Boards of the participating centers (Hospital Clínic of Barcelona (coordinating centre), Universities of Barcelona, Valencia, Rovira-Virgili, Málaga and Las Palmas, Municipal Institute for Medical Research, Primary Care Division of Barcelona and Sevilla, Institute of Research in Health Sciences (IUNICS) at Palma de Mallorca, Hospital Txangorritxu of Vitoria, and University Hospital of Bellvitge) and registered [11].

Study population and characteristics

The present study was conducted as a re-analysis of an intervention feeding study using polyphenol intake as the exposure. Data came from all participants of the PREDIMED trial, but we excluded 247 individuals with an inadequate FFQ at baseline and 28 with a total energy intake out of the predefined limits (that is, daily energy intake <500 or >3,500 for women and <800 or >4,000 kcal/d for men; n = 28) [12]. Therefore, data from 7,172 participants were available for this analysis.

Participants filled out the following questionnaires at baseline and yearly thereafter: a validated 14-point score questionnaire on adherence to the traditional MedDiet [13], a validated 137-item FFQ [14], and a general questionnaire which included data on lifestyle habits, concurrent diseases and medication used.

Polyphenol intake and dietary assessment

At baseline and yearly thereafter, trained dietitians completed the validated 137-item FFQ [14] in a face-to-face interview with the participant. Energy and nutrient intake were estimated from the FFQ by multiplying the frequency of consumption by the average portion size using Spanish food composition tables.

In a previous study conducted by our group, total polyphenol excreted in spot urine samples was validated as a biomarker of total polyphenol intake from FFQ in a clinical trial ($r = 0.48$, $P < 0.01$) and in a cross-sectional study ($r = 0.26$, $P = 0.04$) [15]. The Phenol-Explorer database

Tresserra-Rimbau et al. BMC Medicine 2014, 12:77
http://www.biomedcentral.com/1741-7015/12/77

[9] was used to obtain information about polyphenol content in foods. This database included 516 polyphenols contained in 456 foods [16] at the time of our analysis, being the most complete database currently available for polyphenol content. Correspondence between food items in the FFQ and the Phenol-Explorer database has been described previously [17]. Individual polyphenol intake was calculated by multiplying the content of each polyphenol in a particular food item (mg/g) by the daily consumption of this food item (g/day) and then summing the product across all food items. Total polyphenol intake was the sum of all individual polyphenol intakes.

Polyphenol and other nutrient intakes were adjusted for total energy intake because it is associated with disease risk and is usually proportional to most nutrient intake [18]. To conduct the analyses, we also used weighted cumulative averages, that is, the polyphenol intake of a given year was the average between the intake of that year and the average of the previous years.

Ascertainment of the outcome

Information on mortality was updated yearly by the endpoint adjudication committee, whose members were unaware of dietary intakes or intervention assignments. The sources of information were the following: yearly questionnaires and examinations from all participants, family physicians, yearly review of medical records and linkage to the National Death Index. All outcomes were reported between 1 October 2003 and 1 December 2010.

Statistical analyses

We calculated the weighted cumulative average of polyphenol intake at each yearly visit to represent long-term polyphenol intake. Polyphenols and other food and nutrient intake were adjusted for total calories using the residual method. Non-dietary covariates such as smoking, body mass index (BMI), physical activity and medication use were updated yearly.

The baseline characteristics of the 7,172 participants were distributed by quintiles of total polyphenol intake. Data were presented as means (±SD) for continuous variables and frequencies, and percentages for categorical variables. We used one-factor ANOVA or Pearson chi-squared tests to compare the quantitative or categorical baseline characteristics of the study participants across quintiles of baseline polyphenol intake. Person-time for each participant was calculated as the time between randomization and the date of death, the date when completing the last interview, 1 December 2010 or date at death, whichever came first. To assess the risk of total mortality by quintiles of polyphenol intake, we ran time-dependent Cox proportional hazard regressions with updated diet and covariates. The referent group was the lowest quintile of polyphenol intake. Results are expressed as hazard ratios (HRs) with 95% confidence intervals (CIs). To show the crude differences in death rates by groups of polyphenol intake, we performed a Nelson Aalen survival function, a non-parametric estimator of the survival function for censored data.

Moreover, we used likelihood ratio tests of interaction in stratified analyses to study the possible interactions among the main risk factors and, as sensitivity analyses, we estimated the fully adjusted HR, excluding participants with less than one or two years of follow-up.

Covariates

To take into account the potential differences in risk factors, all Cox proportional hazard analyses were carried out with stratification for recruitment center, sex and intervention group. In model 2, we adjusted for sex, age (<60, 60 to 64.9, 65 to 69.9, 70 to 74.9, >/=75 years), smoking status (never, past and current: cigarettes (<5, 5 to 19, >20 per day) or cigars and pipes (<3, 3 to 6, >6 per day)), BMI (<25, 25 to 29.9, or >/=30 Kg/m^2), baseline diabetes, alcohol consumption (0, 0.1 to 14.9, 15 to 29.9, >/=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD and/or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycemic agents, insulin and other medication. In model 3, we additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids and cholesterol. We did not include in the model other variables that did not change the HR by 10% or more.

Statistical analyses were conducted using SAS software, version 9.3 (SAS Institute, Inc., Cary, NC, USA). All t tests were two-sided and *P*-values below 0.05 were considered significant.

Results

The baseline characteristics of participants are shown by quintiles of energy-adjusted total polyphenol intake in Table 1. Participants with a greater intake of total polyphenols had a closer adherence to the traditional MedDiet. They also tended to be more physically active, consume more alcoholic beverages (mostly wine and beer) and to have less hypertension. On the contrary, the prevalence of hypercholesterolemia was higher in those who consumed more polyphenols at baseline and they were more likely to be smokers. The groups did not differ in terms of diabetes status, use of medication and distribution into the three arms of the trial.

During a mean of 4.8 years of follow-up among 31,068 person-years, the total number of observed deaths was 327. Of these, 131 were due to cancer, 81 were cardiovascular and 115 were for other causes. The Nelson

Tresserra-Rimbau *et al. BMC Medicine* 2014, 12:77
http://www.biomedcentral.com/1741-7015/12/77

Table 1 Baseline characteristics according to quintiles of total polyphenol intake at baseline (energy-adjusted)

	Q1 (n = 1,434)	Q2 (n = 1,435)	Q3 (n = 1,434)	Q4 (n = 1,435)	Q5 (n = 1,434)	*P*-value*
Polyphenol intake, mean (cutoff values), mg/d	483 (<642)	674 (642 to 749)	794 (750 to 852)	937 (853 to 995)	1,235 (>995)	
Sex, women	836 (58.3)	924 (64.4)	712 (60.8)	803 (56.0)	648 (45.2)	<0.0001
Age, mean (SD), y	67.6 (6.2)	67.4 (6.1)	67.4 (5.9)	66.9 (6.0)	66.2 (6.1)	<0.0001
BMI, mean (SD), Kg/m^2	30.0 (3.7)	30.3 (3.7)	29.7 (3.5)	29.7 (3.7)	29.6 (3.5)	<0.0001
Current smoker	217 (15.1)	210 (14.6)	194 (13.5)	265 (18.5)	317 (22.1)	<0.0001
Former smoker	273 (19.0)	263 (18.3)	317 (22.1)	319 (22.2)	413 (28.8)	
Sports/exercise, mean (SD), MET-h/d	3.37 (3.56)	3.62 (3.83	3.77 (3.66)	4.05 (4.25)	4.59 (4.54)	<0.0001
Diabetes	706 (49.2)	680 (47.4)	712 (49.6)	704 (49.1)	668 (46.6)	0.40
Hypertension	1,230 (85.8)	1,224 (85.3)	1,192 (83.1)	1,166 (81.3)	1,117 (77.9)	<0.0001
Hypercholesterolemia	983 (68.6)	1,018 (70.9)	1,053 (73.4)	1,065 (74.2)	1,069 (74.6)	0.001
Hypolipidemic drug use	660 (46.1)	670 (46.7)	712 (49.7)	716 (50.1)	706 (49.5)	0.09
Antihypertensive drug use	1,071 (74.7)	1,095 (76.4)	1,027 (71.7)	1,030 (72.0)	994 (69.7)	0.0004
Cardiovascular drugs use	118 (8.5)	114 (8.2)	120 (8.6)	110 (7.9)	109 (7.9)	0.94
Insulin use	90 (6.3)	87 (6.1)	115 (8.0)	95 (6.6)	99 (6.9)	0.26
Anti-diabetes drug use, other than insulin	463 (32.3)	454 (31.7)	478 (33.4)	465 (32.5)	439 (30.8)	0.65
Aspirin use	302 (21.1)	326 (22.8)	337 (23.5)	318 (22.2)	324 (22.7)	0.63
Int. Group: MedDiet-EVOO	489 (34.1)	506 (35.3)	477 (33.6)	473 (33.0)	517 (36.1)	0.001
Int. Group: MedDiet-nuts	444 (31.0)	467 (32.5)	454 (31.7)	491 (34.2)	519 (36.2)	
Mean daily intake:						
Total energy intake, mean (SD), Kcal/d	2,397 (642)	2,180 (589)	2,161 (540)	2,229 (563)	2,369 (577)	<0.0001
Carbohydrates, mean (SD), g/d	240 (45)	237 (39)	235 (37)	234 (41)	236 (45)	0.006
Protein, mean (SD), g/d	91.9 (15.1)	92.4 (13.8)	92.4 (13.2)	91.5 (13.6)	90.6 (14.9)	0.004
SFA, mean (SD), g/d	26.1 (6.7)	25.4 (5.7)	25.1 (5.3)	24.9 (5.5)	23.5 (5.8)	<0.0001
MUFA, mean (SD), g/d	49.0 (12.2)	48.8 (10.6)	48.8 (10.7)	48.7 (11.3)	46.6 (11.2)	<0.0001
PUFA, mean (SD), g/d	15.6 (5.8)	15.9 (5.1)	15.8 (5.0)	15.8 (5.2)	15.0 (5.2)	<0.0001
Fiber, mean (SD), g/d	21.5 (6.1)	23.9 (6.4)	25.5 (6.7)	26.6 (7.4)	29.4 (8.9)	<0.0001
Total cholesterol, mean (SD), mg/d	372 (121)	367 (103)	368 (107)	360 (94)	354 (122)	<0.0001
Alcohol, mean (SD), g/d	4.10 (10.9)	6.3 (10.1)	7.6 (10.5)	9.3 (12.8)	14.6 (18.9)	<0.0001
Vegetables, mean (SD), g/d	296 (140)	319 (127)	338 (139)	351 (142)	369 (169)	<0.0001
Fruits, mean (SD), g/d	240 (133)	319 (145)	364 (157)	404 (172)	521 (245)	<0.0001
Legumes, mean (SD), g/d	20.5 (15.3)	20.7 (15.2)	20.3 (10.9)	20.6 (12.4)	20.6 (13.0)	0.93
Dairy products, mean (SD), g/d	398 (226)	391 (216)	389 (208)	380 (219)	353 (217)	<0.0001
Cereals, mean (SD), g/d	247 (98)	233 (81)	227 (78)	219 (79)	209 (80)	<0.0001
Meat or meat products, mean (SD), g/d	135 (60)	132 (54)	132 (50)	130 (50)	129 (55)	0.03
Fish, mean (SD), g/d	94.3 (53.3)	99.9 (46.8)	101 (51.5)	99.6 (45.0)	102 (49.2)	0.0005
Sugar-sweetened soft drinks, mean (SD), g/d	25.0 (84.3)	19.7 (63.3)	17.8 (55.8)	15.4 (56.1)	12.6 (46.3)	<0.0001
Coffee, mean (SD), g/d	25.8 (36.3)	43.6 (40.1)	55.2 (42.9)	70.3 (49.2)	90.1 (63.8)	<0.0001
14-points MedDiet questionnaire score, mean (SD)	8.2 (1.9)	8.5 (1.9)	8.7 (1.9)	8.7 (1.9)	9.2 (1.8)	<0.0001
Risk factors:						
Waist-to-height ratio, mean (SD)	0.64 (0.06)	0.63 (0.07)	0.63 (0.06)	0.62 (0.06)	0.62 (0.06)	<0.0001
Systolic BP, mean (SD), mmHg	150 (19)	151 (19)	149 (19)	148 (18)	148 (18)	0.01
Diastolic BP, mean (SD), mmHg	83 (10)	84 (9.8)	82 (9.6)	82 (9.8)	83 (9.6)	0.003
Hearth rate, mean (SD), beats/min	71.7 (11.0)	71.2 (10.9)	70.7 (11.1)	70.0 (10.5)	70.5 (10.5)	0.02

Table 1 Baseline characteristics according to quintiles of total polyphenol intake at baseline (energy-adjusted) *(Continued)*

Glucose (n = 4,311), mean (SD), mg/dL	118 (41)	116 (39)	122 (42)	123 (43)	123 (43)	0.0007
Cholesterol (n = 4,286), mean (SD), mg/dL	202 (36)	206 (38)	207 (39)	208 (38)	207 (36)	0.003
HDL (n = 4,236), mean (SD), mg/dL	50 (11)	51 (11)	51 (11)	52 (12)	52 (11)	0.007
Triglycerides (n = 4,291), mean (SD), mg/dL	130 (67)	133 (74)	137 (79)	130 (63)	138 (80)	0.06

BMI, Body Mass Index; BP, Blood pressure; MedDiet-EVOO, Mediterranean Diet supplemented with extra virgin olive oil; MedDiet-nuts, Mediterranean Diet supplemented with nuts; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; HDL, High density lipoprotein.
Data are expressed as No. (%) unless otherwise indicated.
*P-values calculated by analysis of variance or χ^2 tests.

Aalen survival function (Figure 1) shows the crude differences in death rates by groups of polyphenol intake: low (<600 mg/d), medium (600 to 750 mg/d) and high (>750 mg/d).

Table 2 shows Cox Proportional HRs and 95% CI for total mortality according to quintiles of cumulative intake of total polyphenols (according to yearly updated assessments). After adjusting for all potential confounders and stratifying by sex, recruitment center and intervention group, the HR for the highest versus the lowest quintile was 0.60 (95% CI, 0.39 to 0.91, P-trend = 0.07). After further adjustment for other dietary confounders, the association was not substantially attenuated (HR 0.63, 95% CI, 0.41 to 0.97, P-trend = 0.12). We did not see a strong inverse linear trend for total polyphenols; instead, the results suggest a modest threshold above the first quintile of intake.

In some cases, follow-ups were too short to assess a mortality endpoint because the ill-health conditions leading to death may influence diet. Therefore, as sensitivity analyses, we estimated the fully adjusted HR for the category of the highest total polyphenol intake vs. the lowest, excluding participants with less than one (31 excluded) or two years of follow-up (75 excluded). In both cases, the association was robust and remained statistically significant: HR 0.57, 95% CI, 0.36 to 0.90, P-trend = 0.07 and HR 0.49, 95% CI, 0.30 to 0.82, P-trend = 0.03, respectively.

We also conducted stratified analyses (Table 3) by the other strong predictors of mortality. In multivariable models, the inverse association between total polyphenol intake and risk of death, comparing the extreme quintiles, was stronger among women (HR 0.42, 95% CI, 0.18 to 0.98, P-trend = 0.24) than men (HR 0.76, 95% CI, 0.46 to 1.26, P-trend = 0.23), although the interaction for sex was not significant (P-interaction = 0.39). We also observed no significant differences by strata of age (<70 vs >/=70 years). However, we noted that those who did not drink alcohol had a stronger inverse association with total polyphenol intake (HR 0.39, 95% CI, 0.17 to 0.90, P-trend = 0.04) than drinkers (HR 0.99, 95% CI, 0.59 to 1.65, P-trend = 0.91), but the interaction was not significant (P-interaction = 0.16). In other stratified analyses, we observed that the inverse association did not change substantially among

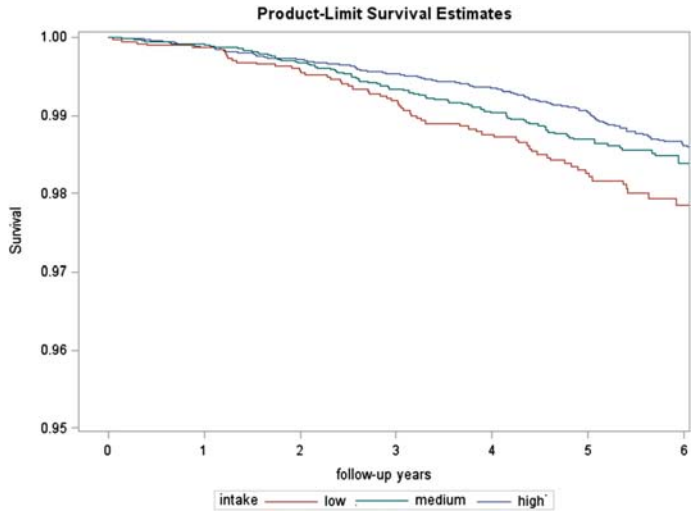

Figure 1 Nelson Aalen estimates of the incidence of death by groups of polyphenol intake.

Tresserra-Rimbau *et al. BMC Medicine* 2014, 12:77
http://www.biomedcentral.com/1741-7015/12/77

Table 2 Cox proportional hazard ratios for total mortality according to quintiles of cumulative total polyphenol intake

	Quintiles of cumulative intake of total polyphenols, mg/d					
	Q1 (535)	Q2 (700)	Q3 (800)	Q4 (917)	Q5 (1170)	*P*-trend
No. of deaths	88	62	52	63	62	
No. of person-years	5,505	6,599	6,767	6,559	5,638	
Age- and sex-adjusted HR (95% CI)*	1.00	0.65 (0.44 to 0.95)	0.55 (0.37 to 0.82)	0.73 (0.50 to 1.06)	0.66 (0.44 to 0.98)	0.12
Multivariable-adjusted HR (95% CI)†	1.00	0.68 (0.46 to 1.01)	0.60 (0.39 to 0.90)	0.75 (0.51 to 1.12)	0.60 (0.39 to 0.91)	0.07
Additionally adjusted HR (95% CI)‡	1.00	0.71 (0.48 to 1.05)	0.62 (0.41 to 0.95)	0.79 (0.53 to 1.17)	0.63 (0.41 to 0.97)	0.12

HR, Hazard ratio; CI, Confidence interval.
*Analyses were stratified by sex, recruitment center and intervention group.
†The multivariable HR has been additionally adjusted for age (<60, 60 to 64.9, 65 to 69.9, 70 to 74.9, >/=75 years), smoking (never, past and current: cigarettes (<5, 5 to 19, >20 per day) or cigars and pipes (<3, 3 to 6, >6 per day)), BMI (<25, 25 to 29.9, or >/=30 Kg/m^2), baseline diabetes, alcohol (0, 0.1 to 14.9, 15 to 29.9, >/=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycaemic agents, insulin, other medication.
‡This model has been additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids and cholesterol (all as continuous variables).

smokers and non-smokers, in those who were physically active or inactive, or in those with or without T2DM or hypertension, and none of these interactions were significant. Finally, we conducted stratified analyses by intervention groups and found a slightly stronger association between total polyphenol intake and death in the control arm of the trial (HR 0.48; CI 0.23 to 0.98; *P*-trend = 0.01) than in the MedDiet + EVOO arm (HR 0.67; CI 0.31 to 1.46; *P*-trend = 0.68) and the MedDiet + nuts arm (HR 0.68; CI 0.34 to 1.35; *P*-trend = 0.81). However, the interaction (*P* = 0.71) was not statistically significant, suggesting no apparent effect modification.

We further investigated the possible effects of the intake of the main polyphenol groups on mortality by any cause (Table 4). Although no significant associations were found for flavonoids or phenolic acids, we observed a 46% reduction in risk of death in participants who consumed more stilbenes (HR 0.48; CI 0.25 to 0.91; *P*-trend = 0.04) and lignans (HR 0.60; CI 0.37 to 0.95; *P*-trend = 0.03). For "other polyphenols", such as tyrosols, alkylphenols, hydroxybenzaldehydes, furanocoumarins and hydroxycoumarins, the association was attenuated after adjustment for other nutrients.

Exploratory analyses (Figure 2) were done for flavonoids (see Additional file 1) and phenolic acid subclasses (see Additional file 2). We found a strong trend towards a reduction in death risk with a higher intake of isoflavones (HR 0.49; CI 0.28 to 0.84; *P*-trend = 0.009). Dihydroflavonols were also inversely associated with the risk of death after multivariable adjustment (HR 0.53; CI 0.28 to 0.99; *P*-trend = 0.05) and the inverse trend was statistically significant after additional adjustment (*P*-trend = 0.04). No other subclasses were associated with mortality by any cause.

Discussion

In this reanalysis of the data of the PREDIMED trial, we observed a 37% reduction of mortality when comparing extreme quintiles of total polyphenol intake. The dose-response trend for the association between total polyphenol intake and all-cause mortality suggested an L-shaped relationship, with an apparent threshold after the first quintile of polyphenol intake, instead of an inverse linear dose-response relationship. Within the polyphenol subclasses, stilbenes and lignans were inversely associated with total mortality.

In stratified analyses we found a stronger association between total polyphenol intake and mortality risk for women and for those who did not drink alcohol. Although the interaction terms were not significant, the observed trend was suggestive, especially for non-drinkers. The relationship between alcohol intake and polyphenols should be the main focus of future studies.

To our knowledge, though previous studies have investigated the association between intake of specific groups of polyphenols and mortality, this is the first study to investigate the association between total polyphenol intake, as well as that of all polyphenol subgroups with all-cause mortality. In addition, we should acknowledge that the effect of polyphenols and polyphenol-rich foods on chronic degenerative diseases and clinical biomarkers has been broadly studied [19-24]. Previous studies have analyzed the association between polyphenols from wine, tea, chocolate, berries, soy and olive oil with several chronic degenerative disease risk or mortality risk [6,25-29]. The reported inverse association, specifically for olive oil and red wine, is consistent with the inverse association we found for stilbenes and lignans [29-31]. The suggestion of an inverse association that we found for several flavonoid compounds is also consistent with previous studies of berries, dark chocolate and soy [6,25,26]. In many of these previously studied populations, intake of any one polyphenol-rich food was not great enough to reduce mortality, but in our study total polyphenol intake was a wider range, coming from several food sources.

Tresserra-Rimbau *et al. BMC Medicine* 2014, 12:77
http://www.biomedcentral.com/1741-7015/12/77

Table 3 HR for total mortality according to quintiles of total polyphenol intake (stratified by risk factors)

Risk factor	No. of deaths	No. of person-years	Multivariable-adjusted HR (95% CI), Quintile 5 vs. 1*	*P*-trend	*P*-interaction
Sex					
Men	203	13,317	0.76 (0.46 to 1.26)	0.23	0.39
Women	124	17,751	0.42 (0.18 to 0.98)	0.24	
Age, y					
<70	142	21,483	0.58 (0.31 to 1.08)	0.21	0.73
≥70	185	9,585	0.70 (0.39 to 1.24)	0.34	
Alcohol intake					
Nondrinkers	133	12,510	0.39 (0.17 to 0.90)	0.04	0.16
Drinkers	194	18,558	0.99 (0.59 to 1.65)	0.91	
Smoking					
Never	144	19,520	0.64 (0.31 to 1.32)	0.47	0.93
Former	111	7,465	0.52 (0.25 to 1.07)	0.29	
Current	72	4,083	0.71 (0.29 to 1.75)	0.21	
Physical activity					
Less than median	203	16,224	0.57 (0.32 to 1.02)	0.17	0.43
More than median	124	14,844	0.77 (0.41 to 1.44)	0.73	
Hypertension					
Yes	184	12,080	0.63 (0.36 to 1.10)	0.24	0.21
No	134	17,721	0.82 (0.44 to 1.55)	0.76	
Diabetes mellitus					
Yes	205	15,345	0.79 (0.47 to 1.33)	0.92	0.52
No	122	15,723	0.60 (0.31 to 1.17)	0.09	
Intervention group					
MedDiet-EVOO	113	11,478	0.67 (0.31 to 1.46)	0.68	0.71
MedDiet-Nuts	108	10,134	0.68 (0.34 to 1.35)	0.81	
Control Diet	106	9,456	0.48 (0.23 to 0.98)	0.01	

HR, Hazard ratio; CI, Confidence interval; MedDiet-EVOO, Mediterranean Diet supplemented with extra virgin olive oil; MedDiet-nuts, Mediterranean Diet supplemented with nuts.
*The multivariable HR has been additionally adjusted for age (<60, 60to 4.9, 65 to 69.9, 70 to 74.9, >/=75 years), smoking (never, past and current: cigarettes (<5, 5 to 19, >20 per day) or cigars and pipes (<3, 3 to 6, >6 per day)), BMI (<25, 25 to 29.9, or >/=30 Kg/m^2), baseline diabetes, alcohol (0, 0.1 to 14.9, 15 to 29.9, >/=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycemic agents, insulin, other medication. Analyses were stratified by sex, recruitment center and intervention group.

Kuriyama *et al.* conducted a prospective cohort study among 40,530 healthy Japanese adults and reported that green tea consumption, a polyphenol-rich beverage, was inversely associated with cardiovascular diseases and all-cause mortality, but not with mortality due to cancer [27]. Other studies have also found an inverse association between polyphenol consumption and CVD and CVD-related mortality [20,25,26,32]. Indeed, it has been demonstrated that some polyphenols and their metabolites exert anti-atherosclerotic effects, improve endothelial function and antioxidant status, increase nitric oxide release, and modulate inflammation and lipid metabolism [5,21,25,33-35].

Polyphenols can also act as chemopreventive agents. For example, resveratrol is a well-known stilbene, mostly found in red wine and grapes, with several health benefits, including inhibition of tumorgenesis [8,36,37]. *In vitro* and *in vivo* studies have shown that epigallocatechin-3-gallate, the major polyphenol of green tea, has anti-carcinogenic effects, such as inhibition of growth proliferation, induction of apoptosis and phase II detoxifying enzymes, and reduction of oxidative damage to DNA [36-38]. Xanthohumol, quercetin, curcumin and genistein are other examples of polyphenols that have shown anti-carcinogenic properties due to their capacity to inhibit tumor growth [8,22,37,38].

Available evidence supports that dietary modifications are able to reduce the risk of T2DM, another highly prevalent chronic disease. Wedick *et al.* found that anthocyanins were inversely associated with the risk of T2DM

Tresserra-Rimbau *et al. BMC Medicine* 2014, 12:77
http://www.biomedcentral.com/1741-7015/12/77

Table 4 Relationship between mortality and intake of the main polyphenol groups (in quintiles)

Main groups	Q1	Q2	Q3	Q4	Q5	*P*-trend
Flavonoids (mg/d)	273	362	431	512	670	
No. of deaths	76	73	42	69	67	
No. of person-years	4,890	6,599	6,755	6,867	5,957	
Age- and sex-adjusted HR (95% CI)*	1.00	0.76 (0.52 to 1.10)*	0.54 (0.36 to 0.81)	0.72 (0.49 to 1.05)	0.70 (0.47 to 1.05)	0.23
Multivariable-adjusted HR (95% CI)†	1.00	0.92 (0.62 to 1.34)	0.69 (0.45 to 1.07)	0.92 (0.62 to 1.36)	0.83 (0.55 to 1.27)	0.70
Additionally adjusted HR (95% CI)‡	1.00	0.96 (0.65 to 1.41)	0.75 (0.48 to 1.16)	0.99 (0.66 to 1.47)	0.89 (0.58 to 1.36)	0.95
Phenolic acids (mg/d)	159	229	279	345	453	
No. of deaths	80	58	62	69	58	
No. of person-years	5,928	6,662	6,716	6,615	5,147	
Age- and sex-adjusted HR (95% CI)*	1.00	0.95 (0.65 to 1.39)	0.78 (0.53 to 1.16)	1.01 (0.70 to 1.47)	0.95 (0.63 to 1.42)	0.64
Multivariable-adjusted HR (95% CI)†	1.00	0.94 (0.64 to 1.39)	0.82 (0.55 to 1.23)	1.07 (0.72 to 1.58)	0.79 (0.51 to 1.22)	0.25
Additionally adjusted HR (95% CI)‡	1.00	0.89 (0.60 to 1.31)	0.77 (0.52 to 1.16)	1.01 (0.68 to 1.50)	0.75 (0.49 to 1.16)	0.20
Stilbenes (mg/d)	0	0.48	1.04	2.04	5.75	
No. of deaths	69	64	47	74	73	
No. of person-years	5,191	6,547	6,840	6,527	5,963	
Age- and sex-adjusted HR (95% CI)*	1.00	0.71 (0.47 to 1.05)	0.66 (0.44 to 0.98)	0.81 (0.56 to 1.18)	0.73 (0.56 to 1.18)	0.44
Multivariable-adjusted HR (95% CI)†	1.00	0.61 (0.33 to 1.11)	0.53 (0.28 to 0.99)	0.68 (0.38 to 1.22)	0.42 (0.22 to 0.81)	0.04
Additionally adjusted HR (95% CI)‡	1.00	0.69 (0.38 to 1.27)	0.62 (0.33 to 1.16)	0.78 (0.43 to 1.40)	0.48 (0.25 to 0.91)	0.04
Lignans (mg/d)	0.44	0.57	0.67	0.77	0.94	
No. of deaths	76	72	57	55	67	
No. of person-years	4,457	6,002	6,737	7,146	6,726	
Age- and sex-adjusted HR (95% CI)*	1.00	0.66 (0.46 to 0.96)	0.58 (0.39 to 0.85)	0.58 (0.39 to 0.87)	0.54 (0.35 to 0.82)	0.002
Multivariable-adjusted HR (95% CI)†	1.00	0.65 (0.44 to 0.99)	0.56 (0.38 to 0.84)	0.56 (0.36 to 0.84)	0.51 (0.32 to 0.79)	0.001
Additionally adjusted HR (95% CI)‡	1.00	0.68 (0.46 to 1.00)	0.60 (0.40 to 0.92)	0.62 (0.39 to 0.98)	0.60 (0.37 to 0.97)	0.03
Others (mg/d)	37	53	66	82	113	
No. of deaths	77	65	72	60	53	
No. of person-years	4,604	6,442	7,320	6,777	5,925	
Age- and sex-adjusted HR (95% CI)*	1.00	0.76 (0.52 to 1.11)	0.78 (0.54 to 1.13)	0.68 (0.46 to 1.01)	0.64 (0.42 to 0.96)	0.04
Multivariable-adjusted HR (95% CI)†	1.00	0.76 (0.51 to 1.13)	0.80 (0.54 to 1.18)	0.67 (0.45 to 1.02)	0.61 (0.40 to 0.93)	0.03
Additionally adjusted HR (95% CI)‡	1.00	0.82 (0.55 to 1.22)	0.86 (0.58 to 1.27)	0.76 (0.50 to 1.16)	0.70 (0.46 to 1.09)	0.13

HR, Hazard Ratio; CI, confidence interval.
*Analyses were stratified by sex, recruitment center and intervention group.
†The multivariable HR has been additionally adjusted for age (<60, 60 to 64.9, 65 to 69.9, 70 to 74.9, >/=75 years), smoking (never, past and current: cigarettes (<5, 5 to 19, >20 per day) or cigars and pipes (<3, 3 to 6, >6 per day)), BMI (<25, 25 to 29.9, or >/=30 Kg/m^2), baseline diabetes, alcohol (0, 0.1 to 14.9, 15 to 29.9, >/=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycaemic agents, insulin, other medication.
‡This model has been additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids and cholesterol (all as continuous variables).

using data from three US prospective cohorts and Muraki *et al.* found similar associations for blueberries, grapes and apples [39,40]. Finally, polyphenols have been proposed as promising phytochemicals for the treatment and prevention of neurogenerative diseases such as Alzheimer's disease, Parkinson's disease and other neurological disorders [29,41].

All of this evidence from chronic disease studies supports the hypothesis that greater polyphenol intake, and the many polyphenol subclasses this represents, serves to extend the life span through multifactorial etiological pathways.

Our study has some limitations. First, we controlled for several confounders in multivariate models, but other unknown or unmeasured confounders may exist. However, if this were the case, we would expect relative risks for all subclasses to be equally over or underestimated and that was not the case. Second, the number of cases of cause-specific deaths was too low to estimate individual relative risks. Others have found the benefits of specific

Tresserra-Rimbau *et al. BMC Medicine* 2014, 12:77
http://www.biomedcentral.com/1741-7015/12/77

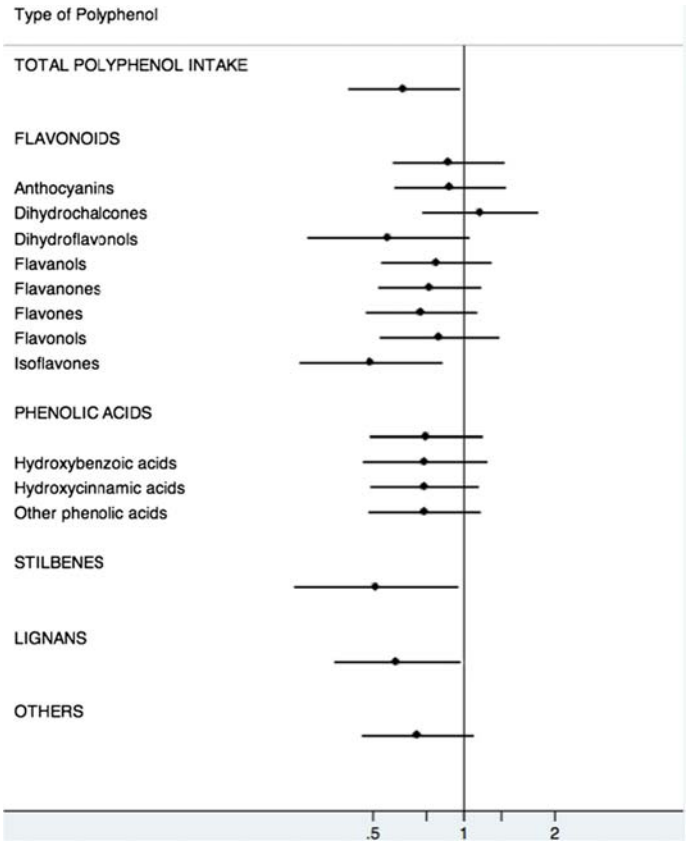

Figure 2 Hazard ratios (95% CI) of total mortality for the highest vs. lowest quintiles of polyphenol intake.

foods are stronger for CVD mortality than cancer or respiratory disease. Future work in this area should include larger studies with estimates of total polyphenol intake. Third, there were limitations with respect to the estimation of polyphenol intake because data were indirectly derived from the FFQs. Although urinary excretion of polyphenols was validated as a biomarker of total polyphenol from the FFQ in two different studies, the values of r were relatively low. The absence of information about some foods in the FFQ could lead to an underestimation of the intake. Moreover, the study did not take into account the bioavailability of these molecules. Finally, these results might be valid only for elderly people at high cardiovascular risk and other studies are needed to generalize the conclusions to other populations.

On the other hand, the main strengths of the study are the prospective design, the large sample size with a relatively long-term follow-up, and comprehensive data on risk factors and confounders. Very importantly, our use of the cumulative average of polyphenol intake across yearly repeated measurements of diet is considered as the best approach to reduce measurement error in nutritional epidemiology [42] and allowed changes in the diet due to the intervention or other secular trends in intake in Spain to be controlled. We also used the most comprehensive polyphenol database currently available (Phenol-explorer database), which allowed risk estimation related not only to intake of total polyphenol but also all the polyphenol subgroups and subclasses. This comprehensive analysis differentiates our paper from previous related studies.

Conclusions

We found an apparent inverse association between total polyphenol intake and the risk of overall mortality, which was independent of other dietary and non-dietary risk factors. This may be helpful in establishing future daily polyphenol intake recommendations. However, more studies are needed to definitively clarify the benefits deriving from long-term consumption of polyphenol-rich foods.

Other PREDIMED Investigators

Other contributors list (Additional file 3).

Additional files

Additional file 1: Flavonoids.doc.
Additional file 2: Phenolic acids.doc.
Additional file 3: Other contributors' list.doc.

Abbreviations

ANOVA: Analysis of Variance; BMI: Body Mass Index; CVD: Cardiovascular diseases; EVOO: Extra Virgin Olive Oil; FFQ: Food Frequency Questionnaire; HR: Hazard ratio; MedDiet: Mediterranean Diet; PREDIMED: Prevención con Dieta Mediterránea; SD: Standard deviation; T2DM: Type 2 diabetes mellitus; 95% CI: 95% Confidence interval.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ATR, RMLR, EBR, RE and MAMG carried out the statistical analyses, interpreted the data and drafted the manuscript. RMLR, RE, MAMG, AMR, MCLS, MIC, DC, JSS, EGG, JL, FA, MF, ER, LSM, XP, MAM, AG and VRG participated in the design of the study and the acquisition of data and contributed to the critical review of the paper. All authors read and approved the final manuscript.

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Additional file 1. The relationship between mortality and flavonoid subclass intake (in quintiles).

Flavonoids	Q1	Q2	Q3	Q4	Q5	*P*-Trend
Anthocyanins (mg/d)	11.8	23.6	32.8	45.7	74.6	
No. of deaths	81	63	53	50	80	
No. of person-years	5886	6488	6503	6409	5782	
Age- and sex-adjusted HR (95% CI) *	1.00	0.76 (0.52-1.10) *	0.68 (0.46-1.01)	0.59 (0.39-0.88)	0.95 (0.66-1.37)	0.95
Multivariable-adjusted HR (95% CI) †	1.00	0.68 (0.46-1.01)	0.71 (0.47-1.08)	0.57 (0.37-0.87)	0.89 (0.58-1.35)	0.79
Additionally adjusted HR (95% CI) ‡	1.00	0.68 (0.46-1.01)	0.73 (0.48-1.12)	0.56 (0.36-0.87)	0.90 (0.59-1.38)	0.84
Dihydrochalcones (mg/d)	0.8	1.8	2.6	3.5	5.8	
No. of deaths	67	59	68	65	68	
No. of person-years	5302	6329	7112	5653	6673	
Age- and sex-adjusted HR (95% CI) *	1.00	0.99 (0.66-1.48)	0.86 (0.57-1.28)	0.99 (0.66-1.49)	0.92 (0.61-1.40)	0.77
Multivariable-adjusted HR (95% CI) †	1.00	1.07 (0.70-1.63)	0.98 (0.66-1.49)	1.04 (0.68-1.60)	1.07 (0.69-1.65)	0.81
Additionally adjusted HR (95% CI) ‡	1.00	1.07 (0.70-1.64)	1.02 (0.67-1.57)	1.13 (0.73-1.74)	1.13 (0.73-1.77)	0.58
Dihydroflavonols (mg/d)	0.1	1.4	2.3	3.8	9.8	
No. of deaths	68	65	48	73	73	
No. of person-years	5130	6577	6880	6528	5954	
Age- and sex-adjusted HR (95% CI) *	1.00	0.91 (0.61-1.34)	0.66 (0.44-1.00)	0.88 (0.60-1.29)	0.79 (0.53-1.18)	0.44
Multivariable-adjusted HR (95% CI) †	1.00	0.90 (0.52-1.54)	0.61 (0.33-1.14)	0.86 (0.49-1.53)	0.53 (0.28-0.99)	0.05
Additionally adjusted HR (95% CI) ‡	1.00	0.97 (0.57-1.66)	0.67 (0.36-1.24)	0.92 (0.52-1.62)	0.56 (0.30-1.04)	0.04
Flavanols (mg/d)	90	129	158	192	263	
No. of deaths	89	50	62	59	67	
No. of person-years	5174	6280	6754	6780	6080	
Age- and sex-adjusted HR (95% CI) *	1.00	0.50 (0.34-0.75)	0.62 (0.43-0.89)	0.60 (0.42-0.87)	0.62 (0.42-0.91)	0.06
Multivariable-adjusted HR (95% CI) †	1.00	0.55 (0.36-0.83)	0.71 (0.48-1.05)	0.67 (0.45-0.99)	0.73 (0.48-1.12)	0.32
Additionally adjusted HR (95% CI) ‡	1.00	0.60 (0.39-0.91)	0.77 (0.52-1.14)	0.75 (0.50-1.12)	0.81 (0.53-1.23)	0.60
Flavanones (mg/d)	28	78	113	157	247	
No. of deaths	84	61	62	54	66	
No. of person-years	4659	5663	7386	6466	6894	
Age- and sex-adjusted HR (95% CI) *	1.00	0.64 (0.43-0.93)	0.54 (0.37-0.78)	0.51 (0.34-0.77)	0.61 (0.42-0.89)	0.02
Multivariable-adjusted HR (95% CI) †	1.00	0.71 (0.48-1.05)	0.68 (0.46-0.99)	0.65 (0.45-0.98)	0.73 (0.50-1.07)	0.15
Additionally adjusted HR (95% CI) ‡	1.00	0.75 (0.51-1.17)	0.70 (0.48-1.04)	0.69 (0.46-1.05)	0.77 (0.52-1.14)	0.25
Flavones (mg/d)	20	29	37	46	67	
No. of deaths	78	69	62	60	58	
No. of person-years	4822	6213	6592	7077	6364	
Age- and sex-adjusted HR (95% CI) *	1.00	0.75 (0.52-1.09)	0.76 (0.52-1.11)	0.71 (0.48-1.04)	0.63 (0.42-0.95)	0.04
Multivariable-adjusted HR (95% CI) †	1.00	0.82 (0.56-1.21)	0.93 (0.63-1.38)	0.85 (0.57-1.27)	0.71 (0.46-1.07)	0.14
Additionally adjusted HR (95% CI) ‡	1.00	0.83 (0.56-1.22)	0.96 (0.64-1.43)	0.87 (0.58-1.31)	0.72 (0.47-1.11)	0.18
Flavonols (mg/d)	56	74	88	101	124	
No. of deaths	84	73	67	50	53	
No. of person-years	6053	6909	6360	6214	5532	
Age- and sex-adjusted HR (95% CI) *	1.00	0.84 (0.58-1.20)	0.80 (0.55-1.17)	0.56 (0.37-0.87)	0.61 (0.40-0.95)	0.01
Multivariable-adjusted HR (95% CI) †	1.00	1.00 (0.69-1.44)	0.89 (0.61-1.31)	0.65 (0.41-1.01)	0.70 (0.45-1.10)	0.06
Additionally adjusted HR (95% CI) ‡	1.00	1.06 (0.73-1.54)	0.96 (0.65-1.42)	0.72 (0.46-1.14)	0.83 (0.53-1.32)	0.26
Isoflavones (mg/d)	0.011	0.018	0.024	0.034	0.050	
No. of deaths	75	74	72	59	47	
No. of person-years	4958	6073	6648	6831	6559	
Age- and sex-adjusted HR (95% CI) *	1.00	0.95 (0.66-1.38)	0.82 (0.57-1.19)	0.49 (0.32-0.75)	0.26 (0.15-0.43)	<0.001
Multivariable-adjusted HR (95% CI) †	1.00	1.01 (0.68-1.51)	0.92 (0.61-1.38)	0.67 (0.43-1.04)	0.35 (0.21-0.60)	<0.001
Additionally adjusted HR (95% CI) ‡	1.00	1.10 (0.73-1.64)	1.03 (0.68-1.55)	0.80 (0.51-1.25)	0.49 (0.28-0.84)	0.009

Abbreviation: HR, Hazard Ratio; CI, confidence interval

* Analyses were stratified by sex, recruitment centre and intervention group.

† The multivariable HR has been additionally adjusted for age (<60, 60-64.9, 65-69.9, 70-74.9, >=75 years), smoking (never, past and current: cigarettes (<5, 5-19, >20 per day) or cigars and pipes (<3, 3-6, >6 per day)), BMI (<25, 25-29.9, or >=30 Kg/m^2), baseline diabetes, alcohol (0, 0.1-14.9, 15-29.9, >=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycaemic agents, insulin, other medication.

‡ This model has been additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol (all as continuous variables).

Additional file 2. The relationship between mortality and phenolic acid subclasses intake (in quintiles).

Phenolic acids	Q1	Q2	Q3	Q4	Q5	*P* -Trend
Hydroxybenzoic acids (mg/d)	6.9	12.9	17.8	24.1	36.1	
No. of deaths	80	74	50	56	67	
No. of person-years	5539	6727	6734	6738	5330	
Age- and sex-adjusted HR (95% CI) *	1.00	0.89 (0.62-1.29) *	0.62 (0.41-0.92)	0.59 (0.39-0.88)	0.70 (0.47-1.04)	0.04
Multivariable-adjusted HR (95% CI) †	1.00	0.83 (0.56-1.22)	0.60 (0.39-0.91)	0.54 (0.35-0.84)	0.58 (0.37-0.93)	0.01
Additionally adjusted HR (95% CI) ‡	1.00	0.90 (0.61-1.34)	0.68 (0.44-1.04)	0.66 (0.42-1.04)	0.74 (0.46-1.20)	0.17
Hydroxycinnamic acids (mg/d)	138	207	252	316	422	
No. of deaths	81	58	57	66	65	
No. of person-years	5941	6621	6543	6776	5186	
Age- and sex-adjusted HR (95% CI) *	1.00	0.71 (0.48-1.04)	0.78 (0.53-1.14)	0.80 (0.55-1.16)	0.97 (0.66-1.43)	0.91
Multivariable-adjusted HR (95% CI) †	1.00	0.67 (0.45-0.99)	0.75 (0.51-1.11)	0.75 (0.51-1.11)	0.78 (0.52-1.18)	0.42
Additionally adjusted HR (95% CI) ‡	1.00	0.63 (0.42-0.93)	0.71 (0.48-1.06)	0.71 (0.48-1.05)	0.74 (0.49-1.12)	0.32
Other phenolic acids (mg/d)	0.1	2.5	4.6	8.6	17.9	
No. of deaths	77	62	58	67	63	
No. of person-years	5199	6485	6608	7555	5221	
Age- and sex-adjusted HR (95% CI) *	1.00	0.76 (0.52-1.12)	0.68 (0.45-1.01)	0.76 (0.52-1.12)	0.83 (0.56-1.24)	0.77
Multivariable-adjusted HR (95% CI) †	1.00	0.64 (0.42-0.97)	0.57 (0.37-0.89)	0.68 (0.45-1.02)	0.68 (0.44-1.03)	0.38
Additionally adjusted HR (95% CI) ‡	1.00	0.67 (0.44-1.03)	0.61 (0.39-0.95)	0.72 (0.48-1.09)	0.74 (0.48-1.13)	0.60

Abbreviation: HR, Hazard Ratio; CI, confidence interval

* Analyses were stratified by sex, recruitment centre and intervention group.

† The multivariate HR has been additionally adjusted for age (<60, 60-64.9, 65-69.9, 70-74.9, >=75 years), smoking (never, past and current: cigarettes (<5, 5-19, >20 per day) or cigars and pipes (<3, 3-6, >6 per day)), BMI (<25, 25-29.9, or >=30 Kg/m^2), baseline diabetes, alcohol (0, 0.1-14.9, 15-29.9, >=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycaemic agents, insulin, other medication.

‡ This model has been additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol (all as continuous variables).

1.4. Publicació 4. Els polifenols excretats per la orina són biomarcadors de la ingesta de polifenols

Article 4. Polyphenols excreted in urine as biomarkers of total polyphenol intake.

Alexander Medina-Remón, Anna Tresserra-Rimbau, Sara Arranz, Ramón Estruch, i Rosa M. Lamuela-Raventós. Bioanalysis. 2012. 4(22):2705-13.

Resum:

Els biomarcadors nutritionals són metabolits que, analitzats en mostres biològiques, s'utilitzen per determinar la ingesta d'un determinat aliment, grup d'aliments o altres constituents no nutricionals. Presenten certes avantatges respecte els QFC d'aliments: són més objectius i precisos i tenen en compte la biodisponibilitat. La quantificació de polifenols en mostres de fluids i teixits és una bona alternativa als QFC per obtenir informació sobre la ingesta de polifenols.

Aquest article fa una revisió dels estudis més recents sobre biomarcadors de consum de polifenols en orina, focalitzant-se en estudis clínics i epidemiològics. També s'hi detalla un mètode ràpid i senzill per determinar polifenols totals en mostres d'orina: una adaptació del clàssic anàlisi colorimètric de Folin-Ciocalteu (F-C). Aquest mètode, tradicionalment utilitzat per la determinació de polifenols en mostres alimentàries es va modificar per tal d'adaptar-se a les mostres d'orina. Va ser necessari filtrar les mostres mitjançant una extracció en fase sòlida per eliminar les interferències habitualment presents en orina: sucres, diòxid de sofre, amines aromàtiques, àcids organics, àcid ascòrbic, Fe(II) i altres compostos orgànics no fenòlics però oxidables. Aquestes substàncies queden retingudes en els cartutxos d'unes plaques de 96 pouets Oasis Max (Waters Corporation, PA, USA). Aquestes plaques, a més, permeten disminuir la quantitat de mostra i els reactius i automatitzar el procés, aconseguint així un estalvi econòmic, de temps i de mostra. El mètode va ser validat utilitzant patrons i orina sintètica.

Aquesta metodologia va ser posteriorment aplicada a mostres d'orina de dos estudis diferents per corroborar que l'anàlisi de polifenols totals en orina era un bon biomarcador de consum de polifenols. El primer, va ser un assaig clínic prospectiu, aleatoritzat i creuat amb 12 voluntaris sans que consumiren dietes altes o baixes en polifenols. El segon estudi es va dur a terme amb una submostra de 60 participants del PREDIMED, els quals se'ls va dividir en tertils de consum de polifenols. En ambdós casos, el consum de polifenols es va relacionar de forma estadísticament significativa amb la quantitat de polifenols excretats en orina, demostrant que aquest sistema era vàlid com a biomarcador.

L'anàlisi de mostres d'orina recollida durant 24 hores dóna resultats més precisos que l'anàlisi de mostres d'orina puntual. No obstant, en estudis amb molts participants o de llarga durada, la utilització d'orina de 24 hores no és possible. En aquest cas, la solució és expressar el resultat en funció de la concentració de creatinina.

RESEARCH ARTICLE

Polyphenols excreted in urine as biomarkers of total polyphenol intake

Background: Nutritional biomarkers have several advantages in acquiring data for epidemiological and clinical studies over traditional dietary assessment tools, such as food frequency questionnaires. While food frequency questionnaires constitute a subjective methodology, biomarkers can provide a less biased and more accurate measure of specific nutritional intake. A precise estimation of polyphenol consumption requires blood or urine sample biomarkers, although their association is usually highly complex. **Results:** This article reviews recent research on urinary polyphenols as potential biomarkers of polyphenol intake, focusing on clinical and epidemiological studies. We also report a potentially useful methodology to assess total polyphenols in urine samples, which allows a rapid, simultaneous determination of total phenols in a large number of samples. **Conclusion:** This methodology can be applied in studies evaluating the utility of urinary polyphenols as markers of polyphenol intake, bioavailability and accumulation in the body.

Polyphenols are the most abundant antioxidants in human diets. They constitute an extremely heterogeneous group of compounds, with over 500 different molecules described in commonly ingested foods and drinks [1]. The estimated mean total intake of dietary polyphenols is approximately 1000 mg/day [2,3], around 100-times higher than that of carotenoid and vitamin E, and ten-times higher than vitamin C.

Polyphenols are generally divided into five main groups according to their structure: phenolic acids, flavonoids, stilbenes, lignans and others (such as secoiridoids) [4,5]. They characteristically all share an aromatic ring bound to at least two hydroxyl groups.

The high variability in consumption, synergism and bioavailability of polyphenols determines their health effects [6]. Numerous clinical and epidemiological studies have shown an inverse association between the risk of myocardial infarction and ingestion of polyphenol-rich food such as fruit and vegetables (F&V) and their derivatives, as well as a close relationship between cancer risk and polyphenol consumption [7–11].

Nutritional biomarkers are metabolites from external components such as foods, assessed in biological samples, and they are used to determine ingestion of a particular food or food group, or nutrient or non-nutrient constituent [12].

Nutritional biomarkers have advantages in acquiring data for epidemiological and clinical studies over traditional dietary assessment tools, such as food frequency questionnaires (FFQs) [13]. While FFQs constitute a subjective methodology, biomarkers can provide a less biased and more accurate measure of specific nutritional intake. The quantification of specific polyphenol biomarkers in accessible fluids or tissues has great potential as an alternative to traditional dietary assessment techniques and provides valuable information about polyphenol intake in humans [14].

The Folin–Ciocalteu (F–C) assay is the most widely used method for the analysis of total polyphenol (TP) content in foods [1,15] and recently in urine samples [16]. The F–C reagents (phosphomolybdic-phosphotungstic acid reagents) reduce polyphenols in alkaline medium. A series of molybdic and tungstic oxides are formed in this redox reaction, giving a blue coloration proportional to the concentration of polyphenols, which is determined by measuring the absorbance at 765 nm [17]. However, polyphenols do not all react with the F–C reagent with the same intensity, which can lead to an underestimation of the amount of polyphenols in the sample.

The F–C method can be hampered by the presence of several water-soluble substances in urine, including sugar, sulfur dioxide, aromatic amines, ascorbic and organic acids, iron(II) and nonphenolic organic substances [15]. This interference was studied by Roura *et al.* in their research on cocoa [16]. After a SPE clean-up procedure with a single cartridge, none of these substances were found in the eluate, thus avoiding the interference reaction with the F–C reagent.

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Key Terms

Polyphenols: This highly diverse class of secondary plant metabolites with potentially beneficial human health effects is found in a range of plant-derived foods, particularly in the skin of fruit and the epidermis of leaves.

Nutritional biomarker: Biological compound that provides a clinical index of nutritional status regarding intake or metabolism of dietary constituents.

96-well plate cartridges: These are used in the SPE clean-up procedure, allowing the simultaneous analysis of a high number of samples.

Oasis® MAX: Mixed-mode anion-exchange and reversed-phase solvent from Waters Corporation (PA, USA).

PREDIMED study: Large, parallel group, multicenter, controlled, randomized 5-year clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease.

The methodology used to determine TP in urine samples has been subsequently further improved for a greater recovery of TPs excreted (TPE) into urine. The present high-throughput method is rapid, simple and allows the simultaneous determination of TP in a large number of samples using 96-well microtiter plates. Its application is potentially useful in studies evaluating the utility of urinary polyphenols as markers of intake, bioavailability and accumulation of these compounds in the body.

In this article, recent research on urinary polyphenols as potential biomarkers of polyphenol intake is described, focusing on clinical and epidemiological studies.

Experimental

■ Urine samples

To determine the bioavailability of polyphenols in intervention studies, concentration is habitually calculated via the area under the curve from multiple blood sampling over a 24-h period, or urinary excretion of polyphenol metabolites, which is generally consistent with plasma kinetic data. It is thought that, 24-h urine samples offer advantages over plasma measurements, mostly because they allow an accurate evaluation of the TP absorbed. Urine samples are mainly appropriate for polyphenols with short plasma half-lives, where plasma measurements may fail to monitor acute intake.

The 24-h urinary determination is quantitative and provides a measure of the total output of polyphenol metabolites over a 24-h period. Being more robust in monitoring daily intake than a single measurement in plasma, it also offers a better index of intake, monitoring the total concentrations of small- and large-intestinal metabolites, without the necessity of taking multiple blood samples.

Although metabolite quantification in 24-h urine samples may be an appropriate methodology in small-scale human intervention studies, it is not realistic in large-scale epidemiological studies, because of the problems involved in organizing the collection of 24-h urine samples from a large-study population. A midway approach could be the quantification of metabolites in spot urine samples but there is little evidence on its suitability. Additionally, urinary data can be deceptive for those polyphenols that use alternative ways of excretion, such as bile from enterohepatic circulation.

■ Interferences from biological fluids

The major drawback, still unresolved, in evaluating dietary polyphenol availability is the difficulty to obtain a biological sample free of any phenolic substance. Consequently, a basal concentration of phenolics is found in urine, even after imposing strict dietary controls (i.e., diets free of those phenolic compounds of interest), and following hours of fasting [18].

Water accounts for approximately 95% of the total volume of urine, the remaining 5% consisting of solutes derived from cellular metabolism and outside sources such as drugs. A wide range of water-soluble compounds, including mineral salts, vitamins, amino acids, enzymes, hormones, antigens, fatty acids, nucleosides, immunoglobulins, pigments, uric acid, urea, hippuric acid and so on, are believed to be normally present in urine, although other substances, such as proteins, glucose, erythrocytes and ketones bodies, can also be found when the body's processes are not operating efficiently [19].

Therefore, without the application of an SPE clean-up procedure, these water-soluble compounds may interfere with the efficiency of the F–C assay in determining TP concentration in urine. Our group recently described a rapid new method, a modified Singleton and Rossi F–C assay [19], to determine TP in complex matrices such as urine samples [20]. SPE with **96-well plate cartridges** (**Oasis® MAX**; Waters Corporation, PA, USA) successfully avoided any interference with the F–C reagent when measuring TPE in urine, as described by Medina-Remón *et al.*, the results being expressed as gallic acid equivalent (mg)/creatinine (g) [20].

In the validation of the method, ten representative polyphenols with varying polarity were prepared in synthetic urine at different concentrations and using different cartridges [20]. The best recoveries were obtained with Oasis MAX cartridges (Table 1), which were consequently selected. These cartridges allowed acidic, basic and neutral compounds to be detected, confirmed and quantified in different fractions in biological fluids. The sensitivity and polarity range of the urinary polyphenols were enhanced and the detection and quantification limits were significantly reduced.

■ SPE clean-up procedure

Briefly, 1 ml of 98% methanol and then 1 ml of sodium acetate 50 mM pH = 7 were loaded to equilibrate the cartridges. A total of 1.2 ml

of the urine samples, previously thawed on an ice bed for 3 h, were centrifuged for 10 min at 4°C, and 1 ml of supernatant was diluted with 1 ml of Milli-Q water, acidified with 34 µl of 35% hydrochloric acid and used to load the cartridges. The cartridges were cleaned with 1 ml of sodium acetate 50 mM pH = 7 containing 5% methanol, and polyphenols were eluted with 1800 µl of methanol containing 2% formic acid. For the F–C reaction, 170 µl of Milli-Q® water (Millipore Corporation, MA, USA), 5 µl of eluted fractions, 12 µl of F–C reagent and 30 µl of 20% sodium carbonate were mixed in Thermo Fisher Scientific microtiter 96-well plates (Nunc™, Roskilde, Denmark). The mixtures were incubated at room temperature in the dark for 1 h and 73 µl of Milli-Q water was added after the reaction period. The absorbance was measured at 765 nm in a UV/visible Thermo Multiskan® Spectrum spectrophotometer (Vantaa, Finland).

■ F–C assay validation

This F–C method was validated with gallic acid and a catechin standard. To evaluate the linearity, a series of calibrators at different concentrations were prepared in synthetic urine. The LOD and LOQ were calculated to evaluate the sensitivity of the method and its accuracy, which was determined by spiking the urine matrix with five different concentrations of standards. Accuracy was estimated from the percentage SDs of added analyte concentrations recuperated from the blank matrix. The CV was calculated by dividing SD by mean concentrations, which were expressed on a percentage basis, to obtain the RSD, calculated to obtain the precision. Additionally, short- and long-term stability as well as stability after freeze–thaw cycles were evaluated in standards and urine samples [20].

■ Creatinine determination

For creatinine determination, the Jaffé alkaline picrate method [21] was adapted to Thermo microtiter 96-well plates. A total of 60 µl of 1% aqueous picric acid solution was mixed with 3 µl of urine and 5 µl of 10% sodium hydroxide. The mixtures were incubated at room temperature in the dark for 15 min. After the reaction time, 232 µl of Milli-Q water was added and the absorbance was measured in the UV/visible spectrophotometer at 500 nm. Creatinine concentrations in urine samples are usually very stable and, in the absence of disease, can be used to estimate some urinary excreted substances in spot urine samples [22–24].

Table 1. Absolute recovery of ten polyphenol standards in Oasis® MAX cartridges after SPE, quantified by HPLC.

Polyphenols	Recovery (%; mean ± SD)
Gallic acid	100.25 ± 3.66
Isoquercetrin	105.13 ± 8.07
Quercetin	95.74 ± 7.12
Catechin	92.52 ± 4.43
Epicatechin	108.05 ± 1.90
4-*O*-methylgallic acid	97.87 ± 1.52
Tyrosol	98.13 ± 2.23
Naringin	101.16 ± 9.45
Caffeic acid	101.07 ± 2.88
Rutin	97.66 ± 0.72

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■ Study designs

Two studies were performed: a clinical trial in order to evaluate the correlation between TP determined in spot urine samples and TP intake and to develop a biomarker of TP intake; and a cross-sectional study in order to corroborate this correlation in a free-living population with 60 volunteers. Validation was based on the results of a clinical trial with different intervention periods.

The clinical trial was a prospective, randomized and crossover study that enrolled 12 healthy adults (four men and eight women; age range 24–54 years old) with no previous relevant illnesses. The volunteers were randomly separated into two groups: the first one began with a high polyphenol diet (HPD) and then changed to a low polyphenol diet (LPD), each lasting 3 days, and separated by a 3-day midway period following a normal diet. The other group began with the LPD, followed by the midway period and finishing with the HPD. The volunteers were instructed by a dietician who taught them how to obtain the required polyphenols from a list of restricted or recommended foods and drinks in each of the intervention periods. The daily food intakes of the volunteers were recorded in a diary. The volunteers refrained from taking vitamin supplements and medication for 1 week before and during the study.

The cross-sectional study was performed with a subsample of 60 participants, 29 men and 31 women (56–80 years old) with a BMI between 20.4 and 36.6 kg/m^2 from the **PREDIMED study**. It was a large, parallel-group, multicenter,

randomized, controlled 5-year clinical trial. The primary aim of the trial was to assess the effects of the traditional Mediterranean diet on a composite end point of cardiovascular death, myocardial infarction and stroke, in comparison with a low-fat control diet [101].

Habitual food intake at baseline was estimated using a validated 137-item FFQ [25] and the validated Spanish version of the Minnesota leisure time physical questionnaire [26]. The exclusion and inclusion criteria described by Estruch *et al.* were used [27]. The volunteers from this substudy were divided into tertiles of daily intake of total F&V: F&V consumption. The first tertile included those with a low ratio (less than 508.03 g/d), the second tertile those with a medium ratio (between 508.04 and 640.23 g/d) and the third tertile those with a high ratio (more than 640.24 g/d). Basal urine samples were collected, coded and stored at -80°C until analysis.

In both studies, data on the TP content in foods and drinks (mg/g fresh matter or mg/ml liquid) were calculated according to Saura-Calixto and Goni. TP intake was calculated as the sum of all individual polyphenol intake from all food sources reported in the FFQ corresponding to eight groups: cereals, vegetables, legumes, nuts, chocolate, fruit, oils and phenolic beverages (coffee, tea, wine, beer and fruit juices) [28].

■ Validation of the method & SPE cartridge selection

The analysis was improved and Oasis MAX 96-well cartridges were selected as the most suitable for the SPE, since they allow acidic, basic and neutral compounds to be detected, confirmed and quantified in biological samples. The LOD and LOQ were significantly reduced, and the polarity range and sensitivity of the urinary polyphenols were improved.

The developed F–C and creatinine method using 96-well plates is particularly suitable for clinical and epidemiological studies in which volunteers consume a wide variety of polyphenols in their habitual diet and large batches of samples are analyzed daily. The 96-well plates allowed the use of much larger sample volumes, provided greater sensitivity and avoided troublesome sample preparation due to the more comfortable format. Compared with previous methods, it was more suitable for automated manipulation and faster, allowing the analysis of 96 urine samples in only 3 h, in comparison with 12.5 h in other procedures [15,16,28].

The Oasis MAX cartridges used in the SPE assay increased the recovery of a high number of polyphenols, with a high selectivity and sensitivity for all polyphenols tested, and decreased the interferences previously observed in TP assays with urine samples.

Results & discussion

The main assumption behind dietary biomarkers is that they are objective measures and are independent of all the biases and errors associated with study subjects and dietary assessment methods.

Biomarkers of nutrient exposure have been used for many years as an alternative to traditional dietary assessment tools, offering a semiquantitative index of the exposition of individual food constituents, measured in a fluid or tissue. They constitute an attractive alternative approach to the study of polyphenols, although the relationship between dietary intake and fluid biomarker concentrations is highly complex. Thus, there are very few existing valid biomarkers of any dietary exposure beyond sodium, energy or sugar intake.

Some aspects must be verified before a particular dietary component or its metabolite becomes a sensitive and accurate biomarker of exposure to a specific polyphenol. For a complete comprehension of polyphenol metabolism in human subjects, the time–response relationship between polyphenol intake and the appearance of the biomarker in biological fluids, the precise dose–response relationship between the polyphenol intake and the appearance of its biomarker in plasma or urine, as well as an understanding of the extent to which certain physiological and environmental factors affect the rate of polyphenol metabolism in these subjects. Spencer *et al.* established the optimal criteria for selecting potential compounds to serve as useful nutritional biomarkers: robust methodology, sensitivity, specificity and bioavailability [14].

The use of biomarkers represents a valuable and independent method for the validation of self-reported intake data. Biomarkers may also be very useful in cohort studies when dietary intake has not been measured, when interesting food items are not included in dietary assessment, or when some important information are lacking.

Most dietary polyphenols (75–99%) are not found in urine, and the quantities detected intact vary from one phenolic compound to another [2]. This may be due to their reduced absorption

through the gut barrier, their hydrolysis and/or the extensive metabolization by intestine or liver enzymes after ingestion, their excretion to the bile or their metabolization by colonic microflora [4].

■ Polyphenols absorption

Absorption, tissue distribution, metabolism and urinary, as well as biliary excretion of polyphenols, are separate physiological processes that all contribute to the time-dependent plasma values and determine bioavailability. Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenols, provided as a pure compound, plant extract, or whole food/beverage. Since the concentrations of native and metabolic forms of polyphenols in the circulation are in the nanomolar to low micromolar range and generally symbolize a very small percentage of the quantity intake, a sensitive and reliable analytical methodology is essential for the measurement of specific metabolites as biomarkers of polyphenol intake. Furthermore, due to a lack of suitable standards of metabolic forms, they are difficult to characterize and/or quantify.

Plasma metabolite concentrations observed in bioavailability and/or pharmacokinetic studies after polyphenol consumption vary greatly according to the nature of the polyphenol and the food source. The interactions between polyphenols and other food components could modify the polyphenol absorption. Therefore, the dose–response relationship between polyphenol intake and the concentration of potential biomarker in fluids may be predisposed by the chemical form of the polyphenol and the food composition [14]. The metabolic response to a given dose of a particular polyphenol-rich food could have significant inter-individual variation, especially in the case of metabolites produced by colonic microflora. Metabolite concentrations in urine and plasma are generally low, hence, the importance of collecting biofluids with the highest concentrations of these putative biomarkers.

■ Polyphenol excretion

The advantage of nutritional biomarkers over FFQs in epidemiological and clinical studies has been shown by the significant correlations observed between urinary excretion of polyphenols and food consumption in intervention studies with specific food items [4], even though few studies have evaluated whether TP compounds can be considered as validated biomarkers of TP intake. In the clinical trial performed by our group, TP intake was positively and significantly correlated ($r = 0.48$, $p < 0.01$) with TPE in spot urine samples due to the observed relationship between polyphenol content in ingested food and recoveries in urine [20].

The concentrations of TPE in spot urine after the consumption of different diets are shown in Figure 1. The median values represented in the boxplot graph show the central tendency of the quantitative data distribution, correctly described by this general index. The figure presents the changes in urinary polyphenol excretion in high, normal and LPDs. The error bars (whiskers) represent the smallest (minimum) and the largest (maximum) sample values, since the distributions do not have outliers. Horizontal lines inside the boxes are the lower and the upper quartiles, hence, the length of the box is the interquartile range of the sample.

The Wilcoxon test analysis of related samples for each intervention period exhibited a significant difference between the urinary TPE after the HPD and LPD ($p = 0.002$). However,

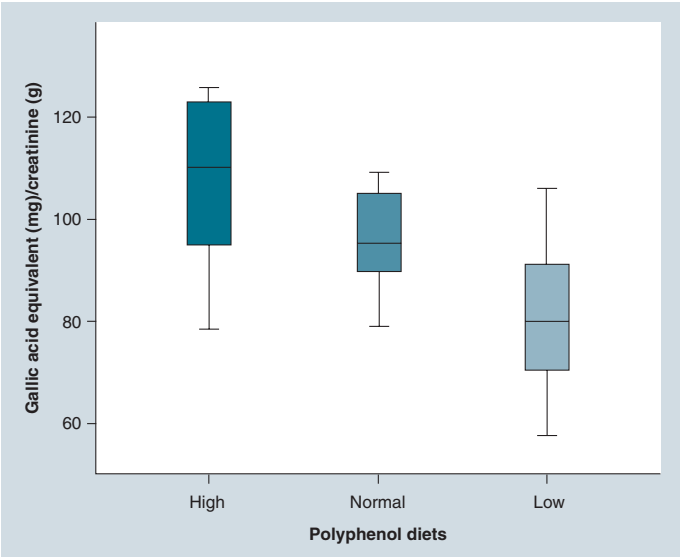

Figure 1. Concentration of total phenolic gallic acid equivalent (mg)/creatinine (g) excreted in morning urines, after the ingestion of the high, normal and low polyphenol diets in the clinical trial.
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when the normal diet was compared with HPDs and LPDs, a tendency to a significant difference was observed (p = 0.06).

In the cross-sectional study, TPE was also a good indicator of TP consumption, as shown by the significant correlation observed between F&V or TP intake and urinary TPE determined by the F–C assay. This was corroborated by the Spearman's rank correlation coefficient (r) analyses, which gave a positive significant correlation between the TPE in spot urine samples and TP intake (r = 0.257, p = 0.04), and the total F&V intake (r = 0.339, p = 0.008) (FIGURE 2). The correlation between TPE in spot urine and TP intake was lower than between TPE in urine and F&V intake, probably due to the translation of F&V and other polyphenol-rich foods from the FFQ into the TP intake. At the time of the study, a complete database with the polyphenol content of all foods, such as the Phenol-Explorer database [1], was not available.

Several authors have reported that phenolic compounds in spot urine samples collected from free-living subjects can be used as biomarkers of specific polyphenol-rich foods: chlorogenic acid for coffee, phloretin for apple, naringenin for grapefruit and hesperetin for orange consumption [29–31]. The combination of several polyphenols (isorhamnetin + hesperetin + naringenin + kaempferol + phloretin) may be a good indicator of total fruit consumption. However, few investigations have correlated TP intake with the concentration of phenolic compounds in spot urine samples to validate their use as biomarkers of polyphenol intake.

Mennen *et al.* studied the correlation between the consumption of polyphenol-rich foods and beverages and the concentration of 13 polyphenols and metabolites in spot urine samples in a free-living population, proposing that some polyphenols measured in spot urine samples can be used as biomarkers of polyphenol-rich food intake [30]. Krogholm *et al.* measured the total flavonoids excreted in urine by LC–MS to determine whether the flavonoid concentration in urine may reflect the intake of F&V [31]. They concluded that the total urinary excretion of flavonoids in 24 h may be used as a biomarker for F&V intake. Roura *et al.* arrived at similar conclusions, finding that the TP concentrations in urine can be correlated to the polyphenol intake from cacao drinks [16]. In their study it was possible to see the relationship between the TP intake and TPE in urine samples, measured by the F–C assay. However, in epidemiological studies, only spot urine samples and rarely 24-h urine samples are collected to investigate the potential beneficial effect of F&V on health.

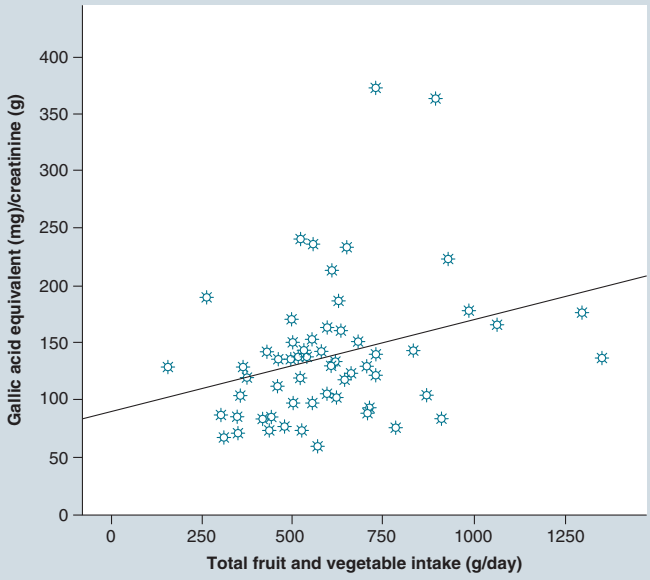

Figure 2. Correlation between total phenolic gallic acid equivalent (mg)/creatinine (g) excreted in morning urine and total fruit and vegetables intake (g/day) in the cross-sectional study.
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■ Epidemiological & clinical studies

Due to the extensive distribution of flavonoids in F&V, various studies have measured their value as biomarkers of F&V intake. In a controlled-dietary intervention study, urinary quercetin, flavanone, and total flavonoids were measured in 24-h urine samples by LC–MS after 6 weeks on a diet with either a low or high content of F&V or berries. Changes in F&V consumption and urinary flavonoid excretion were positively correlated [29], as was the concentration of TP metabolites in 24-h urine samples and F&V consumption, after the implementation of a basic diet supplemented with 300 or 600 g of F&V during 1 day [31].

Similarly, other investigators have analyzed TP in spot urine samples as biomarkers of TP intake, using the F–C assay. Interestingly, urinary polyphenols have been inversely associated with blood pressure and positively associated with a reduction in the risk of coronary heart disease in

another PREDIMED substudy, which included 589 participants, 263 men aged 53–82-years old and 326 women aged 58–82-years old, free of cardiovascular disease at baseline [32].

A significant positive association was obtained between normalized urine TPE expressed as gallic acid equivalent (mg)/creatinine (g) and daily intake of TP (100 mg), F&V (100 g), coffee (100 ml) and wine (100 ml) after adjusting for potential confounding factors ($\beta = 0.116$, $p < 0.001$; $\beta = 0.127$, $p < 0.001$; $\beta = 0.414$, $p < 0.05$; and $\beta = 0.121$, $p = 0.019$; respectively). The **ß coefficients (standardized)** obtained in this model showed that TP and F&V ($\beta = 0.283$ and $\beta = 0.150$, respectively) contributed to urinary TPE more than coffee ($\beta = 0.141$), and all of them contributed to a greater extent than wine ($\beta = 0.120$).

Valls-Pedret *et al.* assessed urinary polyphenols as an objective biomarker of TP intake, and measured the associations between urinary polyphenols and cognitive scores by multiple linear regression models in 447 asymptomatic subjects at high cardiovascular risk enrolled in the PREDIMED study [33]. TP excretion in urine was independently associated with memory performance, with a continuous, dose-related effect; it was also significantly and independently associated with the immediate recall score of the Rey auditory verbal learning test and nearly significantly with the delayed recall Rey auditory verbal learning test score.

Recently, Pedret *et al.* measured the relationship between urinary TPE and oxidative stress biomarkers in 81 healthy adults of different ages from the cross-sectional PAScual MEDicina study [34]. TP intake was quantified from 3-day dietary records using the Phenol-Explorer database, and a significant Pearson correlation was observed between daily TP intake and urinary TPE ($r = 0.281$, $p = 0.012$). In this study the standardized coefficients from a stepwise multivariate model showed that polyphenol intake from fruits is the largest contributor to TP intake; followed by vegetables, coffee and fermented beverages. Additionally, urinary TPE was inversely associated with urinary 8-hydroxydeoxyguanosine ($p < 0.001$) and erythrocyte oxidized glutathione concentrations ($p < 0.05$).

In the InCHIANTI study, Zamora-Ros *et al.* evaluated the relationship between dietary polyphenol intake and TPE, expressed by both 24-h volume and urinary creatinine normalization, in 928 participants [35]. In multiple linear models, the association between dietary TP intake estimated from validated FFQs and both transformed TPE expressions was statistically significant (partial correlation coefficient [pr] = 0.164, $p < 0.001$ for urinary TPE expressed by 24-h volume; pr = 0.113, $p = 0.002$ for urinary creatinine correction TPE; after adjusting for gender, age, BMI, physical activity, energy intake and renal function). Both urinary TPE expression models correlated with polyphenol intake, but it was concluded that the former is the more accurate biomarker.

However, 24-h urine collection is not practical in large-scale epidemiological studies, being tedious for both participants and investigators. In cases where 24-h volume is not available, creatinine-corrected urinary TPE may serve as a suitable biomarker of TP dietary intake in a free-living population [20].

Key Term

ß coefficients (standardized): Regression coefficients that are obtained if the regression model is estimated with the standardized values of the dependent variable.

Conclusion

Urinary polyphenol analysis by a F–C assay, after a SPE clean-up with Oasis MAX 96-well plate cartridges, was pioneered by our group and can be considered as an accurate biomarker of polyphenol-rich food intake [36,20]. This method is simpler, cheaper and more environmentally friendly than others previously described [16].

In addition, our method is especially adapted for the simultaneous analysis of large batches of samples; the 96-well plate system allows the use of much larger sample volumes, provides greater sensitivity, avoids troublesome sample preparation due to its more comfortable format, is more suitable for automated manipulation, and is faster than previous methods.

Future perspective

The development of nutritional biomarkers is a highly complex procedure since the methodology must be robust, sensitive and specific, which depends also on bioavailability of compounds. However, this methodology for assessing nutritional intake has several advantages over dietary data, being more accurate, objective and reliable. Compared with the more subjective information obtained by FFQs, biomarkers provide a more precise measure of the nutritional state since they take into account both the metabolism and bioavailability of the target component.

The quantification of biomarkers in spot urine samples is a good alternative to quantification in 24-h urine samples, which is not a realistic

possibility in large-scale epidemiological studies due to practical issues. However, urinary data can be deceptive for polyphenols that use alternative ways of excretion, such as the bile from enterohepatic circulation. Another limitation is that the colonic microflora catalyze the breakdown of the polyphenol itself to more simple compounds, such as valerolactones or other chemical structures that are not able to further reduce the chemicals present in the F–C reagent, and the behavior of these compounds in SPE has not been studied.

Disclaimer

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The described high-throughput methodology, using 96-well microtiter plates, could potentially be used by the analytical community to detect total polyphenols in urine samples as a biomarker of total polyphenol intake. Accordingly, the measured urinary polyphenol concentration showed a better correlation with clinical data than polyphenol intake obtained by food frequency questionnaires.
- Urinary total polyphenols excreted expressed by 24-h volume is a more accurate biomarker of polyphenol dietary intake than by urinary creatinine normalization, although the latter is more practical in a free-living population study.

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1.5. Publicació 5. Efecte del consum de polifenols sobre la pressió arterial.

Article 5. The effect of polyphenol consumption on blood pressure

Alexander Medina-Remón, Ramón Estruch, Anna Tresserra-Rimbau, Anna Vallverdú-Queralt, i Rosa M. Lamuela-Raventós. Mini-reviews in Medicinal Chemistry. 2013. 13:1137-49.

Resum:

La hipertensió és un greu problema de salut pública, no només per la quantitat de persones afectades, aproximadament 1.000 milions, sinó també perquè és un dels principals factors de risc CV. No obstant, s'ha demostrat que es pot evitar o millorar amb uns bons hàbits alimentaris i un estil de vida saludable. Nombrosos estudis observacionals i d'intervenció han mostrat que hi ha una associació inversa entre les malalties CV i el consum d'aliments rics en polifenols, com ara el cacau, les fruites i verdures en general, el te, el cafè i el vi.

Aquesta revisió bibliogràfica és un recull dels últims resultats obtinguts a partir d'estudis observacionals i d'intervenció en humans sobre l'efecte beneficiós de la ingesta de polifenols sobre la PA. S'hi descriuen, breument, l'estructura i la classificació dels polifenols, així com el seu metabolisme, l'absorció i la biodisponibilitat. En un altre apartat es revisen els biomarcadors de consum de polifenols. A més, també s'hi descriuen breument els mecanismes d'acció que s'han proposat per explicar com els polifenols regulen la PA.

Es conclou que hi ha suficient evidència científica per afirmar que la ingesta de polifenols a través de la dieta ajuda a disminuir la PA i ajuda a prevenir la hipertensió. L'anàlisi de polifenols totals en orina mitjançant el mètode colorimètric de F-C és una manera eficaç de mesurar el consum de polifenols i es pot utilitzar com a biomarcador nutricional. Els polifenols interaccionen amb l'endoteli i augmenten la formació de NO i EDHF, ambdós relacionats amb la disminució de la PA. D'altra banda també actuen com a antioxidants disminuïnt l'estrés oxidatiu que dóna lloc a respostes pro-inflamatòries i pro-trombòtiques a les parets arterials.

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The Effect of Polyphenol Consumption on Blood Pressure

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Abstract: Several observational and intervention studies have found an inverse association between the risk of cardiovascular disease and the consumption of polyphenol-rich foods and beverages such as cocoa, fruit and vegetables, tea, virgin olive oil and wine. We present here an overview of the latest research on the beneficial effect of dietary polyphenols on blood pressure, focusing on the development of urine biomarkers for an accurate estimation of polyphenol intake. Total polyphenols (TP) excreted in spot urine samples have been successfully used as a biomarker of the consumption, bioavailability and accumulation of TP in a cross-sectional clinical trial. In addition, we describe how the vasoprotective effect of dietary polyphenols has been related to their ability to increase endothelial synthesis of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF)-mediated responses.

Keywords: Biomarkers, blood pressure, fruits and vegetables, hypertension, polyphenols, urinary polyphenol.

INTRODUCTION

Hypertension, defined as systolic blood pressure (BP) greater than 140 mm Hg or diastolic BP greater than 90 mm Hg, is a major global public health problem, affecting approximately 1 billion individuals and causing 7.6 million premature deaths, as well as 6% of all cases of disability-adjusted life years worldwide [1]. This disease, which is one of the main cardiovascular risk factors in the elderly, can be managed by following a healthy diet such as the Mediterranean [2] or Dietary-Approaches-to-Stop-Hypertension (DASH) diets [3] and/or by improving other lifestyle factors, such as reducing body weight and increasing physical activity [4]. The Mediterranean and DASH diets are both rich in fruit and vegetables (F&V), which are an abundant source of phytochemicals and are inversely associated with high BP [4-6]. Other ways of controlling hypertension include maintaining a body mass index (BMI) between 18.5 and 24.9 Kg/m^2, reducing sodium intake to less than 2300 mg a day, and performing exercise that raises the heart rate for at least 2½ hours a week. Another measure is to limit alcoholic beverages to 2 drinks a day for men and 1 drink a day for women.

Numerous observational studies have demonstrated an inverse association between polyphenol-rich foods such as cocoa, F&V, tea, olive oil and wine [4, 5, 7-11] and the risk of overall mortality or cardiovascular disease, whereas a high consumption of meat/meat products or refined cereals has been associated with a higher cardiovascular risk [4, 5, 12]. Consumption of fish, another characteristic food of the Mediterranean diet, and low-fat dairy products may also reduce the risk of hypertension [4, 13-17]. The effects of the dietary intake of sodium and potassium, vitamin C or other antioxidant compounds on BP have also been analyzed [18, 19]. However, only one study to date, conducted by our group, has evaluated the role of excreted total dietary polyphenols as a biomarker of total polyphenol intake on BP [20].

Biomarkers of nutrient intake measured in blood and urine are more precise and provide more objective measurements than data obtained from food frequency questionnaires (FFQ); thus, their development is essential for accurate estimations of polyphenol consumption. However, the study of the relationship between dietary intake and nutritional biomarkers is often extremely complex [21].

In the following sections, we present an overview of recent observational studies on the relationship between polyphenol intake and BP, as well as the plausible mechanisms by which polyphenols may exert their cardioprotective role.

POLYPHENOLS: CHEMISTRY AND CLASSIFICATION

Several compounds present in food, known as phytochemicals or phytonutrients, possess the capacity to alter biochemical reactions and thus affect human health. One such group of compounds is the polyphenols, plant secondary metabolites that constitute the most abundant antioxidants in the human diet. Polyphenols may be classified into different groups according to the number of phenol rings they bear and the structural elements that bind these rings to one another. They are generally divided into

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two main groups: flavonoids and non-flavonoids. The flavonoid group comprises compounds with a C6-C3-C6 structure: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones and proanthocyanins. This classification is based on the oxidation of the central ring and the type of substituents in the heterocyclic ring [22]. Flavonoids have a skeleton of diphenyl propanes, two benzene rings (A and B) connected by a three-carbon chain forming a closed pyran ring with the benzene A ring.

As well as presenting a high structural diversity, polyphenols may be associated with various carbohydrates and organic acids and with one another. In plants, they usually occur glycosylated, mainly with glucose or rhamnose, but they can also be linked with galactose, arabinose, xylose, glucuronic acid or other sugars. The number of glycosil moieties usually varies from one to three, although flavonoids have been identified with four and even five moieties [23].

The non-flavonoid group of polyphenol compounds is classified according to the number of carbons they bear and comprises the following subgroups: simple phenols, phenolic acids, hydrolysable tannins, acetophenones and phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans and secoiridoids.

Simple phenols (C6) are formed with an aromatic ring substituted by an alcohol in one or more positions. Phenolic acids (C6-C1), which have the same structure as simple phenols, have a carboxylic group linked to the benzene. Hydrolyzable tannins are mainly glucose esters of gallic acid. Hydroxycinnamic acids are included in the phenylpropanoid group (C6-C3) and are formed with an aromatic ring and a three-carbon chain. They consist of four basic structures: the coumaric acids, caffeic acids, ferulic acids and sinapic acids. In nature, they are usually associated with other compounds such as chlorogenic acid, which is the link between caffeic acid and quinic acid.

Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. They may also be found in nature in combination with sugars such as glycosides. They can be categorized as simple, furanocoumarins, pyranocoumarins and coumarins substituted in the pyrone ring [24]. Chalcones with a C6-C3-C6 structure are flavonoids lacking a heterocyclic C ring. Generally, plants do not accumulate chalcones.

METABOLISM, ABSORPTION, AND BIOAVAILABILITY OF POLYPHENOLS

Polyphenols are the most abundant antioxidants in the human diet and are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate, and beverages such as tea, coffee, or wine [25]. Total dietary polyphenol levels are roughly 1 g/d [26], which is around 10 times higher than the intake of vitamin C and 100 times higher than that of vitamin E and carotenoid. However, various factors make the precise estimation of polyphenol consumption very difficult: polyphenols show a considerable diversity of chemical structures; they are present in a large variety of foods; certain polyphenols such as quercetin are found in all plant products, whereas others, such as flavanones in citrus fruit, isoflavones in soya and phloridzin in apples, are specific to particular foods; their content in a given food can vary widely, due to several factors, and as there are no standardized methods to estimate polyphenols in foods, analytical methods can vary between studies. Since structural changes in polyphenols may result in differences in biological properties, it is important to specify the dietary intake of the various polyphenols separately for an evaluation of their health effects [27].

The health effects of polyphenols depend on both their respective intakes and their variable bioavailability [28]. The most common polyphenols in the human diet are not necessarily the most active *in vivo*, either because they have a lower intrinsic activity than others or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated [23]. In addition, glycosylation of flavonoids and esterification of phenolic acids should be considered, because these modifications will affect their absorption from the gut and bioavailability [29, 30].

Once ingested, polyphenols have several possible fates, including absorption in the small intestine or colon, and/or excretion in the feces or urine. In the small intestine, polyphenols can enter the mucosa through different mechanisms. In some instances, hydrophobic moieties must be cleaved for absorption to take place. In the colon, polyphenols are initially digested into smaller phenolic structures by gut microflora. After this initial digestion is complete, the polyphenols and their metabolites may be absorbed [25, 31]. As a general rule, the metabolites of polyphenols are rapidly eliminated from plasma, which indicates that consumption of plant products on a daily basis is necessary to maintain high concentrations of metabolites in the blood stream [23].

Most dietary polyphenols (75–99%) are not found in urine, and the quantities detected intact vary from one phenolic compound to another [25]. This fact may be due to their reduced absorption through the gut barrier, their hydrolysis and/or metabolization by intestine or liver enzymes, their excretion to the bile or their metabolization by colonic microflora. To acquire high plasma concentrations requires repeated ingestion of polyphenol-rich foods [32].

Absorption, tissue distribution, metabolism, and urinary as well as biliary excretion of polyphenols are separate physiological processes that all contribute to the time-dependent plasma values and determine bioavailability. Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenols, provided as a pure compound, plant extract, or whole food/beverage. The relative urinary excretion ranged from 0.3% to 43% of the ingested dose, depending on the polyphenol. Gallic acid and isoflavones are well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, galloylated tea catechins, and anthocyanins [33].

Anthocyanidins are the only flavonoids that are absorbed from the stomach, and the only ones to occur as glycosides

in plasma. This is reflected in their very low bioavailability, a rapid appearance in plasma after ingestion, and the presence of intact anthocyanidin glycosides in the circulation. The most efficient absorption occurs in the small intestine, because of its large surface area compared with the stomach or colon. Absorption from the small intestine results in peak plasma values within 1–3 h after ingestion. Monomeric flavan-3-ols such as catechin and epicatechin are absorbed from the small intestine, and their relatively low bioavailability may be caused by efflux pumps associated with multidrug resistance, which regulate cellular levels [34]. Galloylated catechins such as epigallocatechin gallate bind to proteins in the gut, which may be another explanation for this low bioavailability [35].

Most flavonoids, with the exception of flavan-3-ols, occur as glycosides in foods; only these, excluding aglycones, are absorbable in the small intestine, and the type of sugar moiety determines whether absorption is possible [36]. Bioavailability is determined by the type of glycoside, for example, quercetin glucoside is absorbed from the small intestine while quercetin rutinoside can be absorbed only from the colon, after the hydrolysis of rutin moiety. After absorption in the small intestine, glucosides are hydrolyzed by lactase phloridzin hydrolase located at the brush border membrane [37].

In the colon, flavonoids that are not absorbed from the small intestine or stomach are subjected to metabolism by microbiota; glycosides are hydrolyzed, which allows absorption, with peak plasma values reached only after 4–6 h [33]. Furthermore, flavonoids are broken down to a range of smaller molecules, the phenolic acids [38], and as a result, the bioavailability of flavonoids absorbed from the colon is generally much lower than those absorbed from the small intestine.

After absorption, polyphenols are readily metabolized in intestinal cells to form glucuronide and sulfate conjugates that appear in the portal blood [39], and methylation of catechol units may also occur [40]. Consequently, generally only conjugated forms of polyphenols are present in blood. Nevertheless, unconjugated forms of anthocyanins, and some phenolic acids [38] may be present in the circulation.

In the liver, additional conjugation and methylation may occur, changing the biological activity of polyphenols, as occurs with the antioxidant activity of quercetin conjugates, being on average about one-half that of the aglycone [41].

Urinary excretion has often been determined in human studies. The total amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations. Our group recently described a rapid new method, a modified Singleton and Rossi Folin-Ciocalteu (F-C) assay [42], to determine total polyphenols in complex matrices such as urine samples, thus providing an accurate biomarker of polyphenol-rich food intake [43].

OBSERVATIONAL STUDIES ON POLYPHENOL INTAKE AND BLOOD PRESSURE

The biological effects of plant polyphenols depend on their bioavailability, kinetics and exposure time [44]. Thus, intestinal absorption and metabolism of polyphenols are a rate-limiting step for their endothelium-dependent protective impact, since the clinical relevance of these compounds depends on their systemic availability. In addition, the complex mixtures of these structurally diverse compounds present in foods and beverages hampers the determination of total phenol content in the diet [45].

Numerous observational studies have reported a protective effect of F&V against cardiovascular disease (CVD) [5, 7], which may be due to the presence of multiple substances. For example, a high F&V intake was correlated with a reduced risk of CVD in a study on 2682 men in Finland [46]. The relationship between F&V intake and CVD risk factors was also examined in urban south Indians by Radhika G. *et al.* [47]. The population of this study comprised 983 individuals aged 20 years or more, selected from the Chennai Urban Rural Epidemiological Study (CURES). Linear regression analysis revealed that after adjusting for potential confounder factors, the highest quartile of F&V intake showed a significant inverse association with systolic BP ($\beta = -2.6$ mm Hg; $P = 0.027$), when compared with the lowest quartile. A high intake of F&V explained 48 % of the protective effect against CVD risk factors.

Similarly, the prevalence of non-previously diagnosed hypertension in the SUN study [5] was inversely correlated with F&V consumption in a Mediterranean population with a very high intake of both fat- and plant-derived foods. In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP, whereas the consumption of refined cereals and meat was directly associated with high systolic BP [48]. In the Chicago Western Electric Study vegetable protein, beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP after an 8-year follow-up in 1714 employed middle-aged men [49]. On the other hand, Hung HC *et al.* [50] evaluated the association of F&V consumption with peripheral arterial disease in a cohort of 44,059 men initially free of cardiovascular disease and diabetes, reporting no evidence that F&V consumption protects against peripheral arterial disease. In the age-adjusted model, men in the highest quintile of F&V had a relative risk of 0.55 (95% CI = 0.38-0.80) for peripheral arterial disease, compared with those in the lowest quintile. However, the associations were greatly weakened after adjustment for smoking and other traditional CVD risk factors.

Compared with other types of foods, the intake of flavonoid-rich juice [51], and flavonoid-rich dark chocolate significantly reduced BP and improved flow-dilated endothelium-dependent vasodilatation in a well-designed double-blind cross-over trial, which contributes to healthy blood flow [10]. Quercetin, one of the most abundant flavonoids present in F&V, reduced BP in several experimental models of hypertension including spontaneously hypertensive rats and rat models of metabolic syndrome; a high dose of quercetin also reduced BP in stage 1 hypertensive patients in a randomized, double-blind, placebo-controlled, crossover study [52].

In a randomized, single-blind, cross-over study [10], 20 men and women (age ~44 years) with never-treated essential hypertension and impaired glucose tolerance, and 15 normotensive subjects consumed daily dark chocolate (100 g) with 500 mg polyphenols (including 66 mg epicatechin and 22 mg catechin) or flavanol-free white chocolate (90 g) for 15 days, each with a 7-day chocolate-free wash-out period in between and after a run-in period which excluded all cocoa foods. Baseline endothelium-dependent - flow-mediated dilation of the brachial artery was significantly lower in hypertensive subjects compared with controls (7.4 ± 1.4% vs. 9.9 ± 0.9%; $P<0.0001$) and significantly increased in hypertensive subjects after consumption of dark chocolate (8.9 ± 1.4%; $P<0.0001$) but not after consumption of white chocolate (7.5 ± 1.3%). Endothelium-dependent -flow-mediated dilation also increased significantly in the control group after consumption of dark chocolate (11.8 ± 1.3%; $P<0.0001$) but not after consumption of white chocolate (10.1 ± 0.9%).

Similarly, in a cross sectional study with Kuna Indians (Panama), the authors observed that daily consumption of flavanol-rich cocoa (from home-grown and Columbian cocoa powder) lowers BP [53], possibly due to the activation of vascular nitric oxide synthase [54]. In the spontaneously hypertensive rat, a polyphenol-rich cocoa powder (up to 300 mg/kg bodyweight) reduced BP to the same extent as 50 mg/kg of captopril, an angiotensin-converting enzyme inhibitor [55]. Cocoa flavanols help maintain endothelium-dependent vasodilation, which contributes to normal blood flow. In order to obtain the claimed effect, 200 mg of cocoa flavanols should be consumed daily. This amount could be provided by 2.5 g of high-flavanol cocoa powder or 10 g of high-flavanol dark chocolate, both of which can be consumed in the context of a balanced diet [56].

In a randomised, controlled, double-blind, cross-over trial [57], 20 patients on secondary prevention for coronary artery disease (64 ± 3 years of age) received a high-flavanol cocoa drink (HF, 375 mg) and a macronutrient- and micronutrient-matched low-flavanol cocoa drink (LF, 9 mg) twice daily (750 mg/day and 18 mg/day, respectively) in a random order over 30 days with one week of wash-out between interventions. By the end of the 30-day periods, flow-mediated dilation values significantly increased to 5.7 ± 0.5% (LF) and 8.4 ± 0.8% (HF, each $P<0.001$ vs. pre-intervention values), and the post-HF values were significantly greater than post-LF values ($P<0.001$ between groups). Also, Berry *et al.* [58] showed that a single dose of high-flavanol (701 mg cocoa flavanols; 139 mg epicatechin) cocoa beverage significantly increased flow-mediated dilation compared to a single dose of a low-flavanol (22 mg cocoa flavanols; 0 mg epicatechin) cocoa beverage (from 3.4 ± 0.5% to 6.1 ± 0.6%) in 21 obese but otherwise healthy volunteers.

In another randomised, double-blind, placebo-controlled, cross-over study [59] the flow-mediated dilation was unchanged 1 h and 2 h after placebo ingestion (0 g cocoa) but significantly increased 1 h and 2 h after consumption of 2, 5, 13 and 26 g of cocoa in a dose-dependent manner. The increase in flow-mediated dilation 1 h and 2 h after consumption of 5, 13 and 26 g of cocoa, but not after consumption of 2 g of cocoa, was statistically significant compared to the placebo, showing an acute dose-dependent effect of cocoa flavanols on endothelium-dependent - flow-mediated dilation in healthy older adults.

In a randomized crossover trial, which included 13 otherwise healthy individuals, the intake of consecutive daily doses of 100 g of polyphenol-rich dark chocolate (500 mg of polyphenols) decreased both systolic and diastolic BP in patients with mild isolated systolic hypertension [60]. In another similar study, Taubert *et al.* determined the effects of low doses of polyphenol-rich dark chocolate on BP in a randomized, controlled, investigator-blinded, parallel-group trial involving 44 adults aged 56 to 73 years (24 women, 20 men) with untreated upper-range prehypertension or stage 1 hypertension without concomitant risk factors. Participants were randomly assigned to receive for 18 weeks either 6.3 g (30 kcal) of dark chocolate per day containing 30 mg of polyphenols or matching polyphenol-free white chocolate. From baseline to 18 weeks, the dark chocolate intake reduced mean (SD) systolic BP by –2.9 (1.6) mm Hg ($P <$ 0.001) and diastolic BP by –1.9 (1.0) mm Hg ($P < 0.001$). In addition, hypertension prevalence declined from 86% to 68% [61].

In another study conducted with 60 volunteers who had fasting blood glucose levels of > or = 6.1 mmol/L or nonfasting blood glucose levels of > or = 7.8 mmol/L, supplementation with green tea-extract powder produces a borderline significant reduction in diastolic BP and no significant changes in systolic BP. The intervention group consumed a packet of green tea-extract powder containing 544 mg polyphenols (456 mg catechins) daily for the first 2 months and then entered the 2-month nonintervention period [62]. Erlund *et al.* investigated the effects of berry consumption on BP in 72 middle-aged unmedicated subjects with CVD risk factors during 8 weeks in a single-blind, randomized, placebo-controlled intervention trial. In this study, berry consumption significantly decreased ($P = 0.050$) systolic BP, the decrease mostly occurring in subjects with high baseline BP (7.3 mm Hg in highest tertile; $P = 0.024$) [63].

Morand *et al.* investigated the effect of orange juice and its major flavonoid, hesperidin, on BP. Twenty-four healthy, overweight men (aged 50-65) were included in a randomized, controlled, crossover study during three 4-week periods. Volunteers daily consumed 500 mL orange juice, 500 mL control drink plus hesperidin, or 500 mL control drink plus placebo. Diastolic BP was significantly lower after the 4-week consumption of orange juice or the control drink plus hesperidin than after consumption of the control drink plus placebo ($P < 0.03$; both) [64]. Significant differences ($P <$ 0.05) were found in diastolic BP between the control drink plus placebo and control drink plus hesperidin, as well as between the control drink plus placebo and orange juice. Regardless of the experimental dietary group, systolic BP was similar after the 4-week supplementation period. Intake of 5.5 ml/kg body weight/day of Concord grape juice daily for 8 weeks by hypertensive Korean patients also reduced both systolic and diastolic BP by an average of 7.2 and 6.2 mm Hg, respectively, at the end of the 8 weeks of intervention [65].

The coronary flow-velocity reserve was increased in 10 healthy volunteers (aged 24–37 years) after drinking a polyphenol-rich beverage (1g/kg ethanol as red wine) but not after drinking the same quantity of alcohol as vodka or white wine, a polyphenol-free beverage or an alcoholic beverage with medium polyphenol content, respectively [66]. After acute intake of 500 mL of red wine and de-alcoholized red wine [67], endothelium-dependent vasodilatation was also improved. A reduction in total and saturated fatty acid intake and an increase of extra-virgin olive oil intake favourably affected BP in hypertensive patients [68]. All these studies support the view that polyphenol-rich diets may reduce BP and improve endothelium function in hypertensive subjects.

Among various studies using pomegranate juice, a consistent 5% reduction in systolic BP was reported when Aviram *et al.* [69] gave 50 mL/d pomegranate juice to 10 hypertensive individuals for 2 weeks, whereas a 21% reduction in systolic BP was observed when the same volume of juice was given to a larger group of participants with asymptomatic severe carotid artery stenosis for a year [51]. By contrast, Sumner *et al.*, [70] reported a reduction in stress-induced ischemia, but no effect on BP after the consumption of 240 mL/d of pomegranate juice, a greater volume than in the Aviram study, for a longer period (90 d) in a much larger group of participants (n = 45) with ischaemic coronary disease [71]. A possible reason for these discrepant results is that the juices used in the studies were derived from different sources and, therefore, differed in their polyphenolic content. Sumner's group used a commercial pomegranate juice, which undergoes technological processing that may affect the polyphenolic composition, while Aviram's group produced an in-house concentrated form of pomegranate juice, which was chemically analysed.

In a double-blind, placebo-controlled, parallel trial, Naruszewicz *et al.* analysed 44 patients (11 women and 33 men, mean age 66 years) who had survived myocardial infarction and received statin therapy for at least 6 months. The subjects were randomized to receive either 3 x 85 mg/day of chokeberry flavonoid extract (*Aronia melanocarpa E*) or a placebo for a period of 6 weeks. Compared to the placebo, the flavonoids significantly reduced systolic and diastolic BP by an average of 11 and 7.2 mm Hg, respectively [72].

A meta-analysis of randomized controlled trial data [73] showed that consumption of chocolate reduced systolic (–5.88 mm Hg; –9.55, –2.21) and diastolic (–3.30 mm Hg; –5.77, –0.83) blood pressure. A soy protein isolate significantly reduced diastolic BP (–1.99 mm Hg; 95% CI: –2.86, –1.12), while the effect on systolic BP was not significant (–1.60 mm Hg; 95% CI: –3.62, 0.42). The consumption of black tea caused an acute increase in systolic BP (5.69 mm Hg; 95% CI: 1.52) and diastolic BP (2.56 mm Hg; 95% CI: 1.03, 4.10), but these increases may be due to the known effects of caffeine on BP observed in another meta-analysis [74]. All this epidemiological evidence supports the argument that a diet rich in F&V or cocoa may prevent BP from increasing and help to reduce high BP levels.

A cross-sectional trial [43], which was performed within a larger clinical trial, the PREDIMED study [2, 75] also correlated total polyphenols (TP) intake with BP levels and/or with the prevalence of hypertension in elderly individuals at high CVD risk. Given that a greater excretion of polyphenols in urine is determined by a high TP consumption, we suggested that the inverse association observed between the objectively measured total polyphenols excreted (TPE) in urine samples with BP may be related to a favourable effect of TP intake on BP levels (see below).

Finally, the relationship between BP and polyphenol-rich food patterns, such as the DASH and Mediterranean diets, has also been examined. The DASH diet is widely promoted in the USA for the prevention and treatment of hypertension [3]. In a free-living UK population [6], systolic and diastolic BP were found to decrease significantly ($P < 0.05$) by 4.6 and 3.9 mm Hg, respectively, in those who followed a DASH-style diet. After 3 weeks on the DASH diet, systolic and diastolic BP in obese hypertensive patients was lower than that on the usual diet (–7.6 ± 1.4/ –5.3 ± 1.4 mm Hg, $P < 0.001/0.02$) or the usual diet supplemented with potassium, magnesium and fibre (–6.2 ± 1.4/–3.7 ± 1.4 mm Hg, $P < 0.005/0.06$), whereas BP did not differ significantly between the usual and supplemented diets [76]. In lean normotensives, BP values did not differ among the three diets. In another study [77] with 27 men and women who followed a DASH or a control diet, the DASH-diet group showed a significant reduction in systolic ($P < 0.001$) and diastolic ($P = 0.005$) BP.

In the SUN study [78], adherence to the Mediterranean diet was associated with reduced mean levels of systolic (moderate adherence, –2.4 mm Hg; high adherence, –3.1 mm Hg) and diastolic BP (moderate adherence, –1.3 mm Hg; high adherence, –1.9 mm Hg) after a 6-year follow-up, but it was not associated with hypertension. Estruch *et al.* [2] compared the short-term effects of 2 Mediterranean diets versus those of a low-fat diet on intermediate markers of cardiovascular risk in an intervention feeding trial (the PREDIMED study). Participants included in the Mediterranean diet groups supplemented with either olive oil or nuts showed a significant decrease in systolic and diastolic BP measurements after 3 months of intervention compared to the low-fat diet group. In a crossover study, Vinson *et al.* [79], observed a significant decrease of 4.3% in diastolic BP and 3.5% in systolic BP when 18 hypertensive subjects received either six to eight small microwaved purple potatoes twice daily or no potatoes during 4 weeks [33].

BIOMARKERS OF TOTAL POLYPHENOL INTAKE

In observational assays nutritional markers have several advantages over FFQ for obtaining dietary data, which has been shown by the significant correlations observed between urinary excretion of polyphenols and food consumption in intervention studies with specific food items [23]. However, few studies have evaluated whether TP in spot urine samples can act as valid biomarkers of TP intake. In a clinical trial performed by our group [43], TP consumption was positively and significantly correlated with TPE in spot urine samples, based on the observed relationship between polyphenol content in ingested food and recoveries in urine.

Several authors [80-82] have reported that phenolic compounds in spot urine samples collected from free-living subjects can be used as biomarkers of specific polyphenol-rich foods: chlorogenic acid for coffee, phloretin for apple, naringenin for grapefruit, resveratrol for wine and hesperetin for orange consumption. The presence of a combination of several polyphenols (isorhamnetin + hesperetin + naringenin + kaempferol + phloretin) may be a good indicator of total fruit consumption.

Recently, Vinson *et al.,* [79] in a single-dose study with eight normal fasting subjects who received six to eight microwaved potatoes with skins or a comparable amount of refined starch in cooked biscuits, determined urine antioxidant capacity due to polyphenol content measured by Folin-Ciocalteu (F-C) reagent after correction for nonphenolic interferences with a solid phase (Polyclar) procedure, and plasma antioxidant capacity, measured by ferric reducing antioxidant power (FRAP). In this study, potato caused an increase in plasma and urine antioxidant capacity, whereas refined potato starch caused a decrease in both; purple potato consumption caused a 92% increase in 24 h urine polyphenols, whereas the refined starch produced a small net decrease (3.5%).

Only a few authors [80-82] have examined the usefulness of urinary concentrations of polyphenols as non-specific biomarkers of F&V consumption. Since flavonoids are widely distributed in F&V, some investigators have studied their value as biomarkers of F&V intake. In a controlled dietary intervention study, total urinary excretion of quercetin, flavanone, and total flavonoids was measured in 24-h urine samples by LC-MS after six weeks on a diet either low or high in F&V and berries. A significant positive correlation between changes in F&V intake and in urinary flavonoid excretion was observed [80].

Mennen *et al.* [81] and Krogholm *et al.* [82] studied the correlation between the consumption of polyphenol-rich foods and beverages and the concentration of polyphenols in urine samples determined by LC-MS/MS. Their results suggest that several polyphenols measured in urine samples can be used as biomarkers of polyphenol-rich food intake. A positive correlation was also found between TP metabolites in 24-h urine samples and F&V intake following 1 d consumption of a basic diet supplemented with 300 or 600 g of F&V [82]. However, in observational studies, only spot urine samples and, rarely, 24-h urine samples have been collected to investigate the potential beneficial effect of F&V on health.

Our group pioneered a rapid new method to determine TP in complex matrices such as urine samples, which may contain many interfering substances. A modified Folin-Ciocalteu (F-C) method was applied to determine TP in urine using Oasis® MAX 96-well plate cartridges for solid phase extraction (SPE) to avoid any interference with the F-C reagent in the urine samples [43]. In this way, TPE can be considered as an accurate biomarker of polyphenol-rich food intake. Roura *et al.* [83] arrived at similar conclusions, finding that TPE in urine, measured by the F-C assay, can be correlated to polyphenol consumption from cacao drinks.

In 928 participants of the InCHIANTI study, Zamora-Ros *et al.* [84] evaluated the relationship between dietary polyphenol intake and TPE, expressed by both 24-h volume and urinary creatinine normalization. Both urinary TPE expression models correlated with polyphenol intake, but it was concluded that the former is the more accurate biomarker. However, 24-h urine collection is not practical in large-scale epidemiological studies, being tedious for both participants and investigators. In cases when 24-h volume is not available, creatinine-corrected urinary TPE may serve as a suitable biomarker of TP dietary intake in a free-living population [43].

Our high-throughput F-C method, further improved to detect TPE in creatinine-normalized urine, is particularly suitable for clinical and observational studies in which volunteers consume a wide variety of polyphenols in their habitual diet. The use of 96-well microtiter plates allows the simultaneous determination of TP in large batches of samples for daily analysis. The method is rapid and simple, and cheaper and more environmentally friendly than others previously described. It has potentially useful application in studies evaluating the utility of urinary polyphenols as markers of intake, bioavailability and accumulation of these compounds in the body.

Interestingly, urinary polyphenols have been inversely associated with BP and positively associated with a reduction in the risk of coronary heart disease in another PREDIMED substudy [20], which included 589 participants, 263 men aged 53 to 82 years and 326 women aged 58 to 82 years, free of CVD at baseline. In this study an inverse association was observed between urinary TPE, an objective measurement of polyphenol intake, and the risk of hypertension in a large Spanish cohort of elderly participants at high cardiovascular risk. Systolic and diastolic BP measurements correlated negatively with urinary TPE after adjustment for potential confounders. The results from this study also provide evidence that total phenol, F&V, coffee and wine intake in the Mediterranean diet is positively correlated with the excretion of TPs in spot urine samples. The standardized coefficients (Beta) showed that the intake of F&V contributed more to urinary TPE than coffee and wine consumption.

In a multivariate logistic regression analysis for cardiovascular risk factors according to quartiles of TPE expressed as mg gallic acid equivalent (GAE)/g creatinine, using the lowest quartile group as the reference category, the participants in the lowest quartile of TPE (<88.99 mg GAE/ g creatinine) had a significantly reduced prevalence of hypertension (OR = 0.64, $P = 0.015$) compared with those in the highest quartile (>160.23 mg GAE/ g creatinine), after adjustment for all possible confounding factors. Participants in the highest quartile had a 36% reduced odds ratio (OR) of hypertension, compared to those in the lowest quartile [20].

PLAUSIBLE MECHANISMS OF ACTION

Blood vessels have the ability to self-regulate tone and adjust blood flow and distribution in response to changes in the local environment due to their capacity to respond to physical and chemical stimuli in the lumen. Numerous blood

vessels respond to an increase in flow, or more specifically shear stress, by dilating, a phenomenon known as flow-mediated dilation. Endothelium-dependent vasodilation contributes to the maintenance of an adequate blood flow to body cells and tissues.

Numerous studies indicate that regular intake of polyphenol-rich beverages and foods are associated with a protective effect on the cardiovascular system. The health benefits of polyphenols have been attributed to their ability to reduce vascular oxidative stress, not only through their direct superoxide anion ($O_2^{\bullet-}$) scavenging properties and interaction with other reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$) and peroxy radicals [85-87] but also through their stimulatory effect on endogenous antioxidant enzymes and their inhibitory effect on xanthine and NAD(P)H oxidases, two major enzymes generating large amounts of ROS [86]. The OH-groups located in the B-ring of the flavonoid molecule are essential determinants for inhibition of $O_2^{\bullet-}$ release. Flavonoids methylated at a single OH-group in the B-ring are only inhibitory when they react with activated neutrophils in the presence of myeloperoxidase [88].

Particular structural groups determine polyphenol radical-scavenging and antioxidant potential, as reviewed by van Acker *et al.*, [89], who showed the existence of multiple mesomeric structures for aroxyl radical species of polyphenols. The *O*-dihydroxy (catechol) structure in the B ring, the obvious radical target site for all flavonoids with a saturated C2-C3 double bond (flavan-3-ols, flavanones, cyanidin chloride) confers great scavenging ability. A pyrogallol (trihydroxy) group in ring B of a catechol produces even higher activity; the C2-C3 double bond of the C ring appears to increase scavenger activity because it confers stability to the phenoxy radicals produced. The C2-C3 double bond in conjunction with a 4-oxo (keto double bond at position 4 of the C ring) increases scavenger activity by delocalizing electrons from the B ring. The 3-OH group on the C ring generates an extremely active scavenger and the 5-OH and 7-OH groups may also add scavenging potential in certain cases.

In addition to the antioxidant effects of polyphenols, some studies, notably by Furchgott and Zawadzki [90], indicate that polyphenols might also protect the cardiovascular system by improving the endothelial function. The endothelium plays a key role in the control of vascular tone by releasing several vasorelaxing factors, which have been recognized as nitric oxide (NO) and the endothelium-derived hyperpolarizing factor (EDHF) [91-95].

Experiments with isolated arteries have revealed that polyphenols cause NO-mediated endothelium-dependent relaxations and increase the endothelial formation of NO. Wine, grape juice, and grape skin extracts induce concentration-dependent relaxation in rat aortic rings with endothelium, but only minor relaxation in rings without endothelium [96]. The grape-derived products increased the endothelial NO synthase activity leading to the formation of NO, and successively relaxed the vascular smooth muscle via the cyclic guanosine monophosphate (cGMP) mediated pathway (Fig. **1**); the polyphenol-induced relaxation associated with an increase in the c-GMP content in intact aortic rings and both the relaxation and the formation of c-GMP are prevented by NO synthase inhibitors. In addition, the endothelium-dependent relaxation appears to be strongly correlated with the concentration of polyphenols in red wines [97]. These endothelium-dependent relaxations induced by polyphenols from grape-derived products have been subsequently observed in various types of animal blood vessels [98-100]. Moreover, polyphenols from several other sources such as cocoa, tea or honey, have also been shown to induce endothelium-dependent NO-mediated relaxations in arteries [101-104].

The phenolic composition of berries is thought to be determinant for their vasorelaxant activity since endothelium-dependent relaxations were observed in response to anthocyanin-enriched extracts of chokeberry and bilberry, although only a minor effect was observed with elderberry [105]. Endothelium-dependent relaxations have also been detected in response to some authentic polyphenolic compounds including resveratrol [106] or soy isoflavones [107].

The calcium signal (Fig. **1**) is an important signal pathway leading to the activation of endothelial NO synthase (eNOS). Red wine polyphenols and delphinidin, at a concentration of 10 mg/L, have been shown to activate eNOS by increasing the intracellular free calcium concentration ($[Ca^{2+}]_i$) in bovine aortic endothelial cells [108]. The phosphatidylinositol 3-kinase/Akt (PI3-kinase/Akt) pathway is another significant signal pathway leading to the activation of eNOS. Red wine polyphenols [109] and the polyphenol-rich fraction of black tea in porcine aorta [110] induced the activation of the PI3-kinase/Akt pathway in endothelial cells, producing the phosphorylation of eNOS at Ser1177 (an activator site) and dephosphorylation of eNOS at Thr495 (an inhibitor site), which increased the formation of NO. This stimulatory effect is calcium-dependent, involving both intracellular and extracellular calcium, and involves the p38 mitogen-activated protein kinase (p38 MAPK) upstream of the PI3-kinase/Akt pathway. Low concentrations of grape and wine polyphenols (resveratrol) are able to activate estrogen receptors resulting in the activation of p38 MAPK and eNOS in endothelial cells [110]. A calcium-dependent activation of eNOS has been shown in response to the tannin 1-*α*-*O*-galloylpunicalagin, which is related with the PI3-kinase/Akt pathway [111]. Consequently, changes in cytosolic $[Ca^{2+}]_i$ in endothelial cells probably contribute to the redox-sensitive activation of eNOS in response to polyphenols via the PI3-kinase/Akt-dependent pathway. Other investigations have identified Src kinase as a redox-sensitive mediator, which plays upstream of the PI3- kinase/ Akt pathway leading to eNOS activation in response to grape-derived polyphenols [112].

Caveolin-1 is a major negative regulator of eNOS activity, and green tea polyphenols down-regulate caveolin-1 gene expression, both time- and dose- dependently, via the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and inhibition of p38 MAPK signaling pathways (Fig. **1**) in bovine aortic endothelial cells [113], increasing eNOS activation.

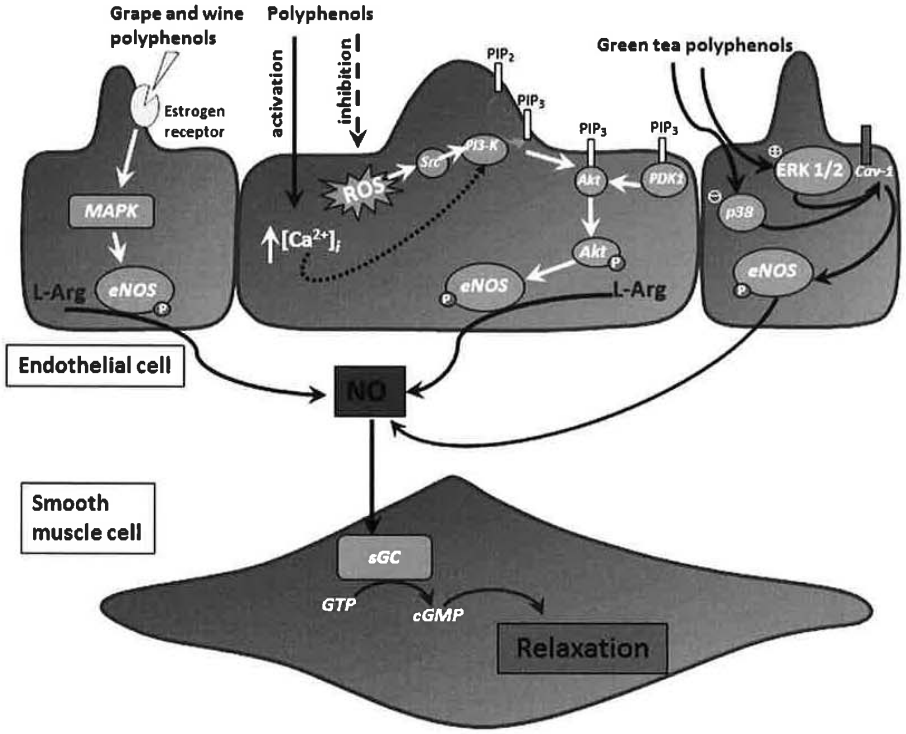

Fig. (1). Intracellular signaling pathways of polyphenols as potent inducers of the endothelial formation of nitric oxide.
$[Ca^{2+}]_i$:Cytosolic calcium concentration; Cav-1: Caveolin-1; cGMP: Cyclic GMP; eNOS: Endothelial NO synthase; ERK1/2: Extracellular signal-regulated kinase ½; GTP: guanosine triphosphate; L-Arg: L-Arginine; MAPK: Mitogen-Activated Protein Kinases; NO: Nitric oxide; P: Phosphorus; PDK1: Phosphoinositide-dependent kinase 1; PI3-K: Phosphatidylinositol 3-kinase; PIP_2: phosphatidylinositol -4,5-diphosphate; PIP_3: phosphatidylinositol-3,4,5-triphosphate; ROS: Reactive oxygen species; sGC: Soluble guanylyl cyclase.

The expression level of eNOS has also been enhanced by polyphenols in endothelial cells, leading to an increased formation of NO; for example, resveratrol, whose stimulatory effect is mainly mediated by an increase in the activity of the eNOS promoter and a stabilization of eNOS mRNA [114].

Furthermore, polyphenols induce EDHF in several types of arteries. The role of polyphenols in endothelium-dependent EDHF-mediated relaxations was first observed in isolated porcine coronary arteries [99]. Red wine polyphenols at concentrations ranging from 1 to 100 mg/L produced concentration-dependent relaxations and hyperpolarizations of vascular smooth muscle cells (Fig. **2**). It was also demonstrated that Concord grape juice, a rich non-alcoholic source of grape-derived polyphenols, is capable of inducing endothelium-dependent EDHF-mediated relaxations of porcine coronary arteries [112]. EDHF-mediated endothelium-dependent relaxations have also been observed in the isolated mesenteric arterial bed in response to alcohol-free lyophilized Brazilian red wine [115]. Resveratrol has been shown to activate IKCa channels in pancreatic islet endothelial cell lines by increasing their open probability [116]. Red wine polyphenols induced EDHF-mediated relaxation of porcine coronary arteries by the redox-sensitive activation of PI3-kinase leading to Akt phosphorylation in endothelial cells [117]. However, the option that the PI3-kinase/Akt pathway modulates myo-endothelial gap junctions and/or potassium channel activity remains to be investigated.

In addition, polyphenols also prevent the development of an endothelial dysfunction by normalizing the excessive vascular formation of superoxide anions, which react with NO to form peroxynitrites. The protective effect of polyphenols on the endothelial function is explained by their ability to prevent the increased vascular expression of NADPH oxidase, a major vascular source of superoxide anions, and the cyclooxygenase-dependent formation of endothelium-derived contracting factors [118]. On the other hand, the beneficial effect might also be due to the downregulation of the angiotensin II type I receptor (AT1) in the arterial wall [119].

CONCLUSIONS

Experimental and observational data support the argument that a polyphenol-rich diet may have a beneficial effect on BP, helping to lower high BP and prevent it from increasing. With the aim of providing more precise and objective measurements of polyphenol consumption than can be obtained from FFQ, measurements of TPE in spot urine

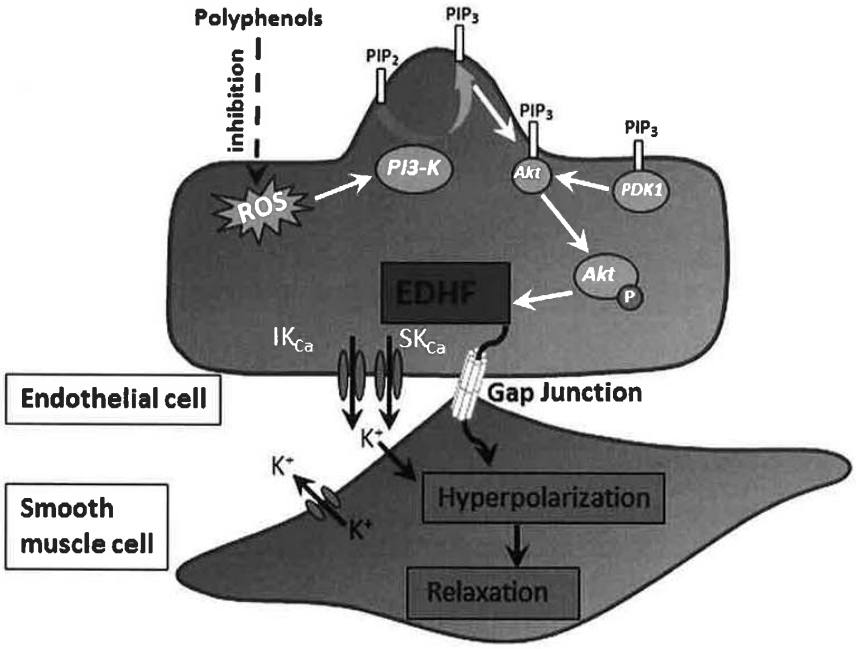

Fig. (2). Intracellular signaling pathways of polyphenols as potent inducers of the endothelial formation of endothelium-derived hyperpolarizing factor (EDHF) via the phosphatidylinositol 3-kinase/Akt pathway.
IK_{Ca}: intermediate-conductance Ca^{2+} -activated K^+; P: phosphorus; PDK1: phosphoinositide-dependent kinase 1; PI3-K: phosphatidylinositol 3-kinase; PIP_2: phosphatidylinositol -4,5-diphosphate; PIP_3: phosphatidylinositol-3,4,5-triphosphate; ROS: reactive oxygen species; Sk_{Ca}: small-conductance Ca^{2+} -activated K^+.

samples have been used as a biomarker of intake, bioavailability and accumulation of TP in a cross-sectional feeding trial. The ability of polyphenols to directly or indirectly increase endothelial formation of NO and EDHF-mediated responses, thus preventing the oxidative stress-induced inactivation of NO, has been reported as a contributory factor in their vasoprotective effect. Overall, these experimental and observational studies highlight the potential of a polyphenol-rich diet to improve or restore vascular protection by enhancing the two major endothelial vasoprotective mechanisms: the formation of NO and EDHF-mediated responses, and also by reducing oxidative stress in the arterial wall, which stimulates pro-inflammatory and pro-thrombotic responses.

Future intervention studies should include a detailed assessment of the bioavailability of polyphenols. Beyond feeding trials carried out with polyphenol-rich foods, more studies with pure polyphenols are also required to establish their role in the prevention of cardiovascular diseases.

CONFLICT OF INTEREST

The authors confirm that the content of this article has no conflicts of interest.

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ABBREVIATIONS

BP	=	blood pressure
$[Ca^{2+}]_i$	=	cytosolic calcium concentration
CVD	=	cardiovascular disease
cGMP	=	cyclic guanosine monophosphate
DASH	=	Dietary-Approaches-to-Stop-Hypertension
EDHF	=	endothelium-derived hyperpolarizing factor
eNOS	=	endothelial nitric oxide synthase
ERK 1/2	=	extracellular signal-regulated kinase 1/2
F-C	=	Folin-Ciocalteu

FRAP	=	ferric reducing antioxidant power
FFQ	=	food frequency questionnaires
F&V	=	fruit and vegetables
GAE	=	gallic acid equivalent
$^{\bullet}OH$	=	hydroxyl radicals
NO	=	nitric oxide
OR	=	odds ratio
p38 MAPK	=	p38 mitogen-activated protein kinase
PI3-kinase/Akt	=	phosphatidylinositol 3-kinase/Akt
ROS	=	reactive oxygen species
SPE	=	solid phase extraction
$O_2^{\bullet-}$	=	superoxide anion
TP	=	total polyphenols
TPE	=	total polyphenol excreted

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1.6. Publicació 6. Efectes dels polifenols en els nivells d'òxid nítric plasmàtic i la pressió arterial en una cohort d'alt risc cardiovascular. L'estudi aleatoritzat PREDIMED després d'un any

Article 6. Effects of total dietary polyphenol on plasma nitric oxide and blood pressure in a high cardiovascular risk cohort. The PREDIMED randomized trial.

Alexander Medina-Remón, Anna Tresserra-Rimbau, Antoni Pons, Josep Antoni Tur, Miquel Martorell, Emilio Ros, Pilar Buil-Cosales, Emilio Sacanella, M. Isabel Covas, Dolores Corella, Jordi Salas-Salvadó, Enrique Gómez-Gracia, Valentina Ruiz-Gutiérrez, José Lapetra, M. García-Valdueza, Fernando Arós, Guillermo T. Saez, Lluis Serra-Majem, Xavier Pintó, Ernest Viñoles, Ramón Estruch i Rosa M. Lamuela-Raventós. Nutrition, Metabolism and Cardiovascular Diseases. **2014**, In press.

Resum:

La hipertensió és un dels principals factors de risc cardiovascular i les xifres de prevalença en els països desenvolupats són alarmants. Mantenir un estil de vida saludable, fent exercici de forma regular i seguint una dieta sana, ajuda a disminuir les probabilitats de tenir hipertensió.

En aquest treball es va avaluar si el consum de polifenols disminuïa la PA a través de l'increment de la producció de NO plasmàtic. Per a fer-ho es van quantificar els polifenols totals de mostres d'orina puntuals de 200 voluntaris de l'estudi PREDIMED, a l'inici de l'estudi i al cap d'un any de seguiment. Els polifenols excretats a través de la orina, analitzats seguint el mètode colorimètric de Folin-Ciocalteu i ajustats per creatinina, són un bon biomarcador del consum de polifenols. Paral·lelament es van prendre dades clíniques i antropomètriques dels voluntaris i es va analitzar el NO en plasma.

Es dugué a terme un anàlisi lineal multivariable amb el programari SPSS v. 19.0 per avaluar la relació entre quartils de canvi en l'excreció urinària de polifenols (variable d'exposició) i els canvis en el NO plasmàtic (variable dependent). Després d'un any d'intervenció s'observà una correlació positiva i significativa entre les dues variables després d'ajustar per edat, sexe i IMC (r=0.173; P=0.026).

Addicionalment es va realitzar una ANCOVA per determinar els efectes de les dues DM (factors fixos), comparades amb la dieta control, en la PAS i la PAD després d'un any d'intervenció (variables dependents), utilitzant les mesures basals com a covariables i altres mesures com a variables addicionals. Havent ajustat per totes les variables de confusió, s'observà un augment significatiu de l'excreció de polifenols en orina i de NO en plasma entre els voluntaris que seguien la DMOO i la DMFS respecte els valors basals. Després de la intervenció amb DMOO i DMFS, també es va observar una reducció significativa de la PAS (-5.79 mmHg i -7.26 mmHg, respectivament) i de la PAD (-3.43 mmHg i -3.26 mmHg) dels voluntaris respecte el grup control.

Aquests resultats augmenten l'evidència que els polifenols protegeixen el sistema cardiovascular gràcies a la millora de la funció endotelial, expressada com un augment de la síntesi endotelial de NO.

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Effects of total dietary polyphenols on plasma nitric oxide and blood pressure in a high cardiovascular risk cohort. The PREDIMED randomized trial

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Abstract *Background and aim:* Hypertension is one of the main cardiovascular risk factors in the elderly. The aims of this work were to evaluate if a one-year intervention with two Mediterranean diets (Med-diet) could decrease blood pressure (BP) due to a high polyphenol consumption, and if the decrease in BP was mediated by plasma nitric oxide (NO) production.
Methods and results: An intervention substudy of 200 participants at high cardiovascular risk was carried out within the PREDIMED trial. They were randomly assigned to a low-fat control diet or to two Med-diets, one supplemented with extra virgin olive oil (Med-EVOO) and the other with nuts (Med-nuts). Anthropometrics and clinical parameters were measured at baseline and after one year of intervention, as well as BP, plasma NO and total polyphenol excretion (TPE) in urine samples. Systolic and diastolic BP decreased significantly after a one-year dietary intervention

Abbreviations: BMI, body mass index; BP, blood pressure; CHD, coronary heart disease; CI, confidence interval; DASH, Dietary-Approaches-to-Stop-Hypertension; EVOO, extra virgin olive oil; FFQ, food frequency questionnaire; GAE, gallic acid equivalent; Med-diet, Mediterranean diet; Med-EVOO, Mediterranean diet-extra virgin olive oil; Med-nuts, Mediterranean diet-nuts; NO, Nitric oxide; PREDIMED, prevention with Mediterranean diet study; SD, standard deviations; TP, total polyphenols; TPE, total polyphenols excreted.

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with Med-EVOO and Med-nuts. These changes were associated with a significant increase in TPE and plasma NO. Additionally, a significant positive correlation was observed between changes in urinary TPE, a biomarker of TP intake, and in plasma NO (Beta = 4.84; 95% CI: 0.57–9.10).
Conclusions: TPE in spot urine sample was positively correlated with plasma NO in Med-diets supplemented with either EVOO or nuts. The statistically significant increases in plasma NO were associated with a reduction in systolic and diastolic BP levels, adding to the growing evidence that polyphenols might protect the cardiovascular system by improving the endothelial function and enhancing endothelial synthesis of NO.

Introduction

Hypertension is one of the main cardiovascular risk factors in the elderly. Hypertension can be managed by following a healthy diet rich in fruits and vegetables, such as the Mediterranean [1] or DASH (Dietary-Approaches-to-Stop-Hypertension) diets, and improving other lifestyle factors [2].

Several epidemiological studies have demonstrated an inverse association between adherence to traditional Mediterranean diet (Med-diet) and death from coronary heart diseases (CHD) [3,4]. This protective effect has been partially attributed to a high content of bioactive compounds such as phytosterols, and phenolic compounds [4], which seems to be inversely associated with BP [5,6]. In a previous study, polyphenol intake, assessed via total polyphenols excreted (TPE) in urine, was negatively associated with BP levels in an elderly Mediterranean population at high cardiovascular risk. In addition, epidemiological studies have concluded that polyphenol-rich food intake may decrease systolic and diastolic BP in humans [7–9]. A plausible mechanism for this effect afforded by polyphenol-rich foods is an induction of vasodilation via activation of the NO system [5,10].

The aims of this study were to evaluate within the PREDIMED trial if a high polyphenol consumption, measured via TPE in spot urine samples, in a one-year intervention with a traditional Med-diet supplemented with either extra virgin olive oil (Med-EVOO) or nuts (Med-nuts), would decrease systolic and diastolic BP compared with a control diet, and if the decrease in BP was mediated by plasma nitric oxide (NO) production.

Methods

Subjects

The PREDIMED study is a large, parallel-group, multicenter, randomized, controlled clinical trial of 4.8-year duration aimed at evaluating the preventative effects of the traditional Med-diet on cardiovascular events (www.predimed.org). The detailed recruitment method and study protocol have been described previously [3,11]. For this substudy, between October-2003 and July-2004 we selected 200 randomly participants recruited from five primary health centers. Eligible participants were community-dwelling men aged 55–80 years and women aged 60–80 years. This study was approved by an institutional review committee and the subjects gave informed consent. This trial has been registered with the International Standard Randomized Controlled Trial Number (ISRCTN of London, England) 35739639. The Ethical Committee for Human Experimentation of the Hospital Clinic at Barcelona University, Spain approved the study.

Assessment and intervention

At baseline all participants completed a validated semi-quantitative food frequency questionnaire (FFQ) with 137 items, the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire; a validated 14-point Med-diet score; and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use [11]. Trained dieticians were responsible for all aspects of the intervention.

Participants in both Med-diet intervention groups were given personalized advice about dietary changes, aimed at achieving a diet close to the traditional Med-diet. Med-EVOO participants received free EVOO (1 L/wk) and Med-nuts group was provided with mixed nuts (30 g/d, as 15 g walnuts, 7.5 g almonds, and 7.5 g hazelnuts). Participants assigned to the control diet received personal advice together with a leaflet with written recommendations to follow a low-fat diet, according to the American Heart Association guidelines [11].

Clinical measurements

Trained nurses measured height and weight with a wall-mounted stadiometer and calibrated scales, respectively. BP was measured in triplicate with the participant in a seated position after resting quietly for 5 min, using a validated semi-automatic oscillometer (Omron HEM-705CP [12]; Hoofddorp, The Netherlands) with a 5-min interval between each reading. Energy and nutrient intake was derived from Spanish food composition tables.

Urine and blood samples were obtained after an overnight fast; they were coded, shipped to a central laboratory, and frozen at −80 °C until analysis. Analysis of TPE and creatinine in urine samples were performed following

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the procedure described by Medina-Remón et al. [13]; TPE was expressed as mg gallic acid equivalent (GAE)/g of creatinine.

For total plasma NO determination, plasma samples were previously washed two times with 200 μL distilled water in order to eliminate nitrite and nitrate contamination of filters, and centrifuged for 30 min at 14,000 g in 10 K filters to remove proteins. The supernatant was recovered and used to measure nitrite and nitrate concentration by detecting liberated NO in a gas-phase chemiluminescence reaction with ozone using an NO-analyzer, as described previously [14]. Nitrate levels were determined following an adaptation of the method described by Braman and Hendrix [15]. Nitrite levels were determined following an adaptation of the method described by Castegnaro et al. [16].

Statistical analysis

Analyses were performed using IBM SPSS software v19.0(Chicago, USA). Baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD). Variables were examined for normality and skewness (Kolmogorov and Levene tests, respectively). ANOVA-one factor was used for analysis of continuous variables and χ^2-test for categorical variables. Changes in all outcomes were assessed with repeated-measures analysis of variance for the 2 factors: diet (Med-EVOO, Med-Nuts and Control diet) and time (baseline and 1-year), and their interaction, with the Bonferroni post-hoc test to compare differences in the effects of each intervention within and between groups. Within- and between-group differences are expressed as mean percent difference [95% confidence interval (CI)]. A multivariate linear regression analysis was performed to assess the relationship between change in TPE, as a biomarker of total polyphenols (TP) intake (exposure variable), and changes in plasma NO(dependent variable), adjusted according to sex, age and body mass index (BMI).

We used the General Linear Model (GLM) approach to ANCOVA to determine the effects of both Med-diet interventions (fixed factors), compared with the control diet, on systolic and diastolic BP after one year (dependent variables), using the baseline measurements as covariates and others as additional covariates. Model 1 was unadjusted; Model 2 was adjusted for baseline BP, change in plasma NO, sex, age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs), supplements taken in the last month, sodium, potassium, total energy, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and, saturated fat acid (SFA) intake. *P*-values$<$0.05 (two-tailed) were considered statistically significant.

Results

Baseline characteristics of the total group according to intervention are shown in Table 1. No differences were observed between intervention groups at baseline. By study design, participants were over 54 years old [mean age (SD): 67.6 (6.0)], 56.5% women, mostly overweight [mean BMI(SD): 29.0 (3.4) kg/m^2], and with a sizeable burden of cardiovascular risk factors (62.5% diabetics, 77.0% hypertensive, 72.0% with dyslipidemia, 16.5% active smokers and 46.3% with a family history of early-onset CHD). Most of them were taking antihypertensive drugs (66.5%), and nearly half were taking oral statins or other hypolipidemic drugs (46.5%), and oral hypoglycemic drugs (41.2%).

After one year, the consumption of foods by the participants of this substudy was similar to the overall PREDIMED population following the Med-diets or control diet. The main dietary changes in the respective Med-diet groups were a substantial increase in the consumption of EVOO or total nuts, an increased intake of fruit, vegetables and legumes in both Med-diet groups, and a reduced intake of meat or meat products by all participants, as well as a reduced intake of cereals in the Med-EVOO and control diet groups (Table 2). EVOO intake was lower in the control diet than in either Med-diet group. Changes in consumption of other types of food were not statistically significant, although both Med-diet interventions significantly increased the Med-diet score. Table 3 shows changes in energy, total polyphenols intake and, daily nutrient intake at baseline and after one year in each

Table 1 Baseline characteristics of the study participants completing 1 year of follow-up.

	Med-EVOO	Med-nuts	Control diet	*P*[a]
No. of subjects	67	64	69	
Age (y), mean (SD)	68.0 (6.0)	67.7 (6.3)	67.1 (5.8)	0.631
Women, *n* (%)	39 (58.2)	33 (51.6)	41 (59.4)	0.621
BMI, (kg/m^2), mean (SD)	28.2 (3.2)	29.3 (3.9)	29.4 (3.1)	0.071
Overweight or obese (BMI ≥25 kg/m^2), *n* (%)	58 (86.6)	58 (90.6)	64 (92.8)	0.476
Hypertension, *n* (%)	49 (73.1)	50 (78.1)	55 (79.7)	0.639
Diabetes, *n* (%)	41 (61.2)	38 (59.4)	46 (66.7)	0.661
Dyslipidemia, *n* (%)	46 (68.7)	46 (71.9)	52 (75.4)	0.684
Current smoker, *n* (%)	9 (13.4)	11 (17.2)	13 (18.8)	0.686
Family history of CHD, *n* (%)	33 (49.2)	29 (45.3)	30 (43.5)	0.170
Medication, *n* (%)				
Antihypertensive	44 (65.7)	43 (67.2)	46 (66.7)	0.983
Statins or other hypolipidemic drugs	35 (52.2)	28 (43.8)	30 (43.5)	0.513
Insulin	6 (9.2)	3 (4.7)	3 (4.3)	0.424
Oral hypoglycemic drugs	28 (42.4)	20 (31.3)	34 (49.3)	0.105
Aspirin or other antiplatelet drugs	7 (10.8)	7 (10.9)	6 (8.7)	0.891
Vitamins or supplements, *n* (%)	3 (4.5)	4 (6.3)	3 (4.3)	0.861

[a] ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); CHD: coronary heart disease; SD: standard deviation. Med: Mediterranean diet; EVOO: extra virgin olive oil.

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Table 2 Changes in daily intake of key foods and Mediterranean diet score after 1 year.[a]

				P[b]		
	Med-EVOO	Med-nuts	Control diet	Time[c]	Group[d]	Interaction[e]
Total nuts (g)						
Baseline	13.7 (14.2)	13.3 (12.3)	13.1 (15.4)	<0.001	<0.001	<0.001
1 year	13.5 (15.0)a	49.6 (15.6)**,b	12.2 (14.2)a			
EVOO (g)						
Baseline	16.3 (20.3)	14.3 (21.8)	11.1 (16.8)	<0.001	<0.001	<0.001
1 year	52.6 (16.3)**,a	15.7 (22.6)b	7.9 (12.8)c			
Fruits (g)						
Baseline	465.6 (202.3)	487.6 (242.7)	483.6 (246.4)	0.076	0.508	0.991
1 year	523.9 (175.7)	496.5 (180.5)	509.3 (228.8)			
Vegetables (g)						
Baseline	427.4 (153.3)	331.7 (153.3)	380.5 (140.5)	0.028	<0.001	<0.001
1 year	491.1 (176.3)**,a	401.2 (140.6)**,b	332.5 (128.7)*,c			
Legumes (g)						
Baseline	20.6 (8.7)	19.3 (8.6)	21.4 (11.3)	0.016	0.025	0.505
1 year	24.2 (13.0)*	24.4 (9.2)**	20.1 (10.3)			
Fish or seafood (g)						
Baseline	112.8 (52.0)	104.6 (41.7)	110.0 (36.6)	0.756	0.096	0.722
1 year	111.9 (42.6)	115.4 (57.0)	103.3 (34.5)			
Meat or meat products (g)						
Baseline	158.9 (56.4)	156.2 (62.7)	167.0 (55.6)	<0.001	0.709	0.616
1 year	139.4 (57.3)*	132.0 (55.1)**	139.0 (50.3)**			
Cereals (g)						
Baseline	263.1 (113.9)	256.8 (106.2)	277.6 (121.8)	0.014	0.034	0.401
1 year	219.7 (93.6)**	267.4 (106.9)	245.5 (112.1)*			
Milk and dairy products (g)						
Baseline	339.7 (178.2)	353.7 (231.7)	409.8 (274.8)	0.426	0.251	0.103
1 year	359.6 (172.7)	312.5 (188.6)a	395.8 (186.9)b			
Pastries, cakes or sweets (g)						
Baseline	12.4 (14.6)	18.4 (27.1)	13.7 (19.2)	0.053	0.017	0.845
1 year	18.4 (23.2)*	14.8 (29.7)	18.4 (16.5)**			
Alcohol (g)						
Baseline	11.0 (16.1)	8.1 (13.0)	8.8 (13.2)	0.678	0.396	0.701
1 year	10.2 (14.8)	9.7 (13.3)	8.8 (12.8)			
Tea (mL)						
Baseline	8.5 (28.6)	3.3 (10.2)	4.3 (12.7)	0.909	0.689	0.038
1 year	9.9 (26.0)a	1.4 (5.3)b	5.3 (19.2)			
Coffee (mL)						
Baseline	31.9 (49.0)	21.0 (39.2)	32.2 (47.9)	0.048	0.123	0.863
1 year	33.1 (45.6)	38.3 (58.6)**	34.6 (44.2)			
Med diet score						
Baseline	8.9 (1.8)	8.3 (1.9)	8.9 (1.8)	<0.001	<0.001	0.015
1 year	10.3 (1.7)**,a	10.3 (1.6)**,a	8.8 (1.5)b			

[a] Data are given as means (SD); $P < 0.05$ indicates statistical significance. Med: Mediterranean diet; EVOO: Extra Virgin Olive Oil. Values with asterisks are statistically different from baseline by Bonferroni post-hoc test ($P < 0.05$): *$P < 0.05$; **$P < 0.01$. Different letters in rows shows significant difference between interventions by Bonferroni post-hoc test ($P < 0.05$).
[b] Data analyzed by repeated-measures 2-factor ANOVA.
[c] Comparison between before and after intervention.
[d] Comparison between the 3 diet groups.
[e] Comparison between measures obtained before and after intervention and between the 3 diet groups.

intervention group. Total fat intake significantly increased in the two Med-diet groups, fundamentally due to an increased consumption MUFA, which was attributed partly to the habitual use of olive oil, and PUFA; total cholesterol intake decreased in all groups. The other nutrient changes were generally not statistically significant, with the exception of sodium intake that decreases in the Med-EVOO group, magnesium and, potassium intakes that decreases in the Med-nut group. No significant changes were observed in energy expenditure in physical activity.

In multivariate linear regression analyses, a significant positive correlation (Beta = 4.87; 95% CI:0.66–9.08) was observed between quartiles of change in urinary TPE (exposure variable) and change in plasma NO(dependent variable). After adjustment for sex, age and BMI, the significance of this positive correlation increased (Beta = 4.84; 95% CI:0.57–9.10). Figure 1 shows that the higher the change in urinary TPE (>25.69), the greater the change in plasma NO concentration (mean = 4.70). As well as, mean changes in systolic and diastolic BP, TPE in spot urine samples, and plasma NO after one year with the different interventions are shown in Fig. 1. Both Med-diets significantly increased TPE and plasma NO, resulting in a significant decrease in systolic and diastolic BP. No

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Table 3 Changes in energy, total polyphenols intake and, daily nutrient intake after 1 year [a]

				P[b]		
	Med-EVOO	Med-nuts	Control diet	Time[c]	Group[d]	Interaction[e]
Total energy, Kcal/d						
Baseline	2417.5 (554.1)	2364.3 (680.2)	2417.4 (615.6)	0.246	0.009	0.645
1 year	2403.7 (495.2)	2580.4 (668.2)**	2352.0 (423.4)			
Total polyphenol intakes (mg GAE/day)						
Baseline	941.6 (304.9)	874.8 (344.6)	906.0 (282.0)	0.001	0.186	0.385
1 year	1016.5 (340.6)*	969.3 (322.6)**	920.9 (292.5)			
Total protein (g)						
Baseline	102.4 (21.0)	99.2 (24.8)	106.1 (26.4)	0.026	0.003	0.962
1 year	98.3 (20.6)	102.5 (24.2)	96.6 (17.4)**			
Total carbohydrate (g)						
Baseline	250.6 (71.0)	260.9 (97.2)	259.3 (76.2)	0.062	0.608	0.384
1 year	233.9 (60.8)	258.1 (93.9)	244.5 (58.1)			
Fibre (g)						
Baseline	28.6 (8.3)	28.5 (10.3)	28.1 (7.3)	0.245	0.002	0.051
1 year	29.2 (7.8)	32.0 (7.7)**,a	26.2 (6.4)b			
Total fat (g)						
Baseline	103.2 (28.8)	96.4 (28.1)	99.4 (30.8)	<0.001	<0.001	0.017
1 year	111.5 (24.2)*,a	118.9 (28.5)**,a	94.4 (21.2)b			
SFA (g)						
Baseline	27.2 (9.0)	26.2 (8.6)	26.2 (10.0)	0.227	0.311	0.373
1 year	27.0 (8.0)	26.3 (8.9)	24.1 (7.3)*			
MUFA (g)						
Baseline	50.0 (15.1)	46.3 (15.0)	48.1 (14.9)	<0.001	<0.001	0.001
1 year	58.9 (12.6)**,a	57.3 (13.2)**,a	44.7 (12.7)b			
PUFA (g)						
Baseline	17.2 (6.4)	16.1 (6.6)	16.1 (6.3)	<0.001	<0.001	<0.001
1 year	16.9 (4.9)a	25.8 (6.6)**,b	16.3 (4.8)a			
Cholesterol (g)						
Baseline	398.7 (122.0)	398.3 (172.8)	388.6 (105.5)	0.036	0.846	0.937
1 year	368.4 (106.0)	377.7 (171.4)	372.8 (89.8)			
Magnesium (mg)						
Baseline	422.1 (101.6)	415.9 (133.6)	434.1 (110.1)	0.260	<0.001	0.125
1 year	423.7 (97.1)a	477.7 (104.2)**,b	396.0 (71.3)**,a			
Potassium (mg)						
Baseline	5099.5 (1028.8)	4888.9 (1387.9)	5079.5 (1025.1)	0.943	0.004	0.399
1 year	5163.6 (1078.0)	5187.2 (1114.2)*,a	4734.1 (888.8)*,b			
Sodium (mg)						
Baseline	2735.2 (937.7)	2547.1 (940.6)	2621.0 (1153.6)	0.121	0.240	0.939
1 year	2487.4 (802.3)*	2584.9 (1006.4)	2513.6 (740.3)			
Energy expenditure in physical activity (kcal/d)						
Baseline	339.2 (160.7)	294.7 (206.0)	276.1 (178.3)	0.066	0.494	0.012
1 year	335.5 (303.9)	243.7 (170.5)	239.7 (199.3)			

[a] Data are given as means (SD); $P < 0.05$ indicates statistical significance. Med: Mediterranean diet; EVOO: Extra Virgin Olive Oil; GAE: gallic acid equivalent; MUFA: monounsaturated fat acids; PUFA, polyunsaturated fat acids and SFA: saturated fat acid. Values with asterisks are statistically different from baseline by Bonferroni post-hoc test ($P < 0.05$): *$P < 0.05$; **$P < 0.01$. Different letters in rows shows significant difference between interventions by Bonferroni post-hoc test ($P < 0.05$).
[b] Data analyzed by repeated-measures 2-factor ANOVA.
[c] Comparison between before and after intervention.
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statistically significant changes were observed in the control group.

Table 4 shows the 1-year changes in systolic and diastolic BP associated with changes in plasma NO. After the covariate analysis of the differences in systolic and diastolic PB after one year with respect to baseline, with systolic and diastolic BP at year one as the dependent variables, the intervention groups (Med-EVOO, Med-nuts and control diet) as the fixed factor, and plasma NO and other measurements as additional covariates, we observed the effects of the different Med-diet interventions with respect to the control diet.

In Table 4, non-standardized coefficient (B) represents the differences in the Med-diet interventions with respect to the control diet. In model 2, adjusted by all possible covariates, participants with the same systolic BP at baseline experienced a statistically significant reduction of −5.79 mmHg and −7.26 mmHg after the Med-EVOO and Med-nuts interventions, respectively, compared with the control diet. In this model, participants with the same

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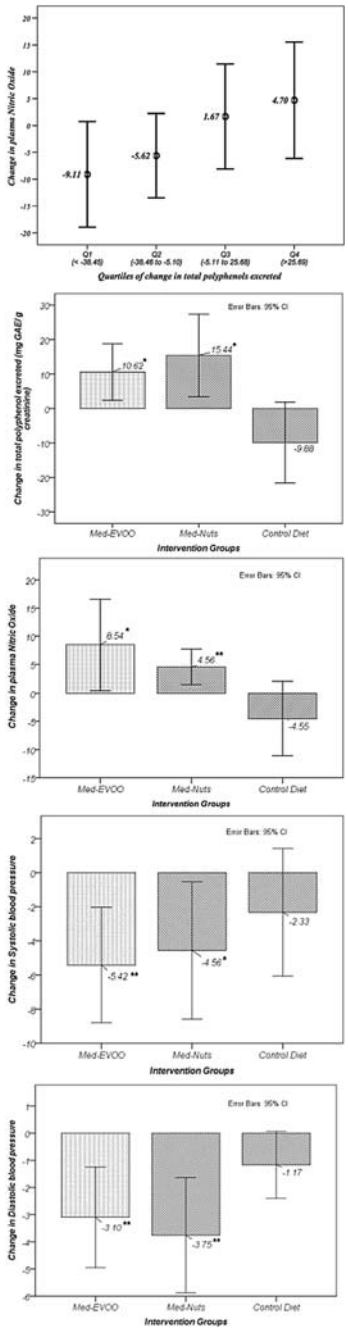

diastolic BP at baseline, after the Med-EVOO and Med-nuts interventions experienced a reduction of −3.43 mmHg and −3.26 mmHg, respectively, compared with the control diet, being statistically significant in all comparisons.

Discussion

In the elderly participants at high cardiovascular risk included in the PREDIMED trial, we observed that the changes in plasma NO were associated with significantly lower systolic and diastolic BP after one-year interventions with Med-diets supplemented with EVOO or nuts, compared with the control diet. We also observed a significant positive correlation between changes in urinary TPE, as a biomarker of TP intake, and changes in plasma NO.

Olive oil is the main natural fat in the Med-diet [4], and EVOO has higher antioxidant phenolic content than other types. Nuts are also typical of the traditional Med-diet and are a rich source of phytochemicals such as phytosterols and phenolic compounds [17], which can account for their multiple cardiovascular benefits.

Non-glycosylated polyphenols contained in nuts skin, such as monomeric flavanols and dimeric procyanidins, would be directly absorbed in the small intestine, where they would be first conjugated and later metabolized in the liver into methyl, glucuronide, and sulfate derivatives by phase II enzymes. Nevertheless, proanthocyanidins are not absorbed and reach the colon, where they are metabolized by the intestinal microbiota into hydroxyphenylvalerolactones and various phenolic acids, including phenylpropionic, phenylacetic, and benzoic acid derivatives, that can be further absorbed and then conjugated in the liver [18,19].

The Med-diet after a 4-year intervention significantly reduced BP compared with the control group [6]. Recently, in another PREDIMED sub-study, Med-Diets reduced 24-h ambulatory systolic and diastolic BP after a 1-year intervention [20]. In the current study we have shown that at least part of the hypotensive effects of Med-Diets may be due to their high polyphenol content via an increase in plasma NO concentration. Consistently with these previous results, we observed that adherence to a traditional Med-diet may be able to reduce cardiovascular risk factors.

Epidemiological evidence suggests that a polyphenol-rich diet may help to prevent BP from increasing and reduce high BP levels in people with normal-to-high BP or hypertension [9]. On the other hand, daily consumption of flavanol-rich cocoa decreased BP [21], possibly due to the activation of vascular NO synthase [10]. Cocoa flavanols

Figure 1 Change in plasma nitric oxide (10^{-6} mol/L (μM)) according to quartiles of change in total polyphenols excreted (mg GAE/g creatinine) and, mean ± SD changes in systolic and diastolic blood pressure (mmHg), total polyphenols excreted in spot urine samples and, plasma nitric oxide (10^{-6} mol/L (μM)), after 1-year with different interventions. Med: Mediterranean diet; EVOO: extra virgin olive oil; GAE: gallic acid equivalent; $^{**}P < 0.01$, $^{*}P < 0.05$ indicates statistical significance between the baseline and after a 1-year intervention period with a confidence interval (CI) of 95%.

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Table 4 Changes in systolic and diastolic blood pressure (mmHg) after one year associated with changes in plasma nitric oxide (uM).

	Model	B	*P*	95% CI
Systolic blood pressure	*Model 1*			
	Med-EVOO vs. control diet	−6.14	0.042	−12.04 to −2.33
	Med-Nuts vs. control diet	−2.69	0.372	−8.62 to −3.24
	Model 2			
	Med-EVOO vs. control diet	−5.79	0.038	−11.24 to −0.31
	Med-Nuts vs. control diet	−7.26	0.012	−12.92 to −1.59
Diastolic blood pressure	*Model 1*			
	Med-EVOO vs. control diet	−5.23	0.001	−8.20 to −2.25
	Med-Nuts vs. control diet	−1.74	0.253	−4.73 to 1.25
	Model 2			
	Med-EVOO vs. control diet	−3.43	0.009	−6.00 to −0.86
	Med-Nuts vs. control diet	−3.26	0.017	−5.92 to −0.60

B: non-standardized coefficient; CI: confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by baseline blood pressure, change in plasma nitric oxide, sex, age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) supplements taken in the last month, sodium, potassium, total energy, monounsaturated fat acids, polyunsaturated fat acids and saturated fat acid intake. Med: Mediterranean diet; EVOO: extra virgin olive oil.

help maintain endothelium-dependent vasodilation, which contributes to normal blood flow. Berry consumption during 8 weeks significantly decreased systolic BP, mostly in subjects with high baseline BP [22], and 85 mg/d of chokeberry flavonoid extract (*Aronia melanocarpa E*) significantly reduced systolic and diastolic BP [23]. At least part of this lowering blood pressure effect of polyphenol-rich foods may be related to an improvement of endothelial function [24].

The endothelium plays a key role in the control of vascular tone by releasing several vasorelaxing factors, which include NO and the endothelium-derived hyperpolarizing factor (EDHF) [25,26]. In experiments with isolated arteries, polyphenols caused NO-mediated endothelium-dependent relaxations and increased the endothelial formation of NO. Wine, grape juice, and grape skin extracts induced concentration-dependent relaxation in rat aortic rings with endothelium, but only minor relaxation in rings without endothelium [27]. The grape-derived products increased the endothelial NO synthase activity leading to the formation of NO, and successively relaxed the vascular smooth muscle via the cyclic GMP-mediated pathway. These endothelium-dependent relaxations induced by polyphenols from grape-derived products have been subsequently observed in animal blood vessels [28]. Moreover, polyphenols from several other sources, such as wine or tea, have also been shown to induce endothelium-dependent NO-mediated relaxations in arteries [5,29]. This change in plasma NO subsequently significantly reduced systolic and diastolic BP. These results add to the increasing body of evidence pointing to an enhancing effect of dietary polyphenols on the endothelial synthesis of NO.

All this epidemiological, clinical and laboratory evidences support the view that polyphenol-rich diets may prevent BP from increasing and help to lower high BP levels in hypertensive subjects. In addition, a previous study performed by our group [30] a biomarker of TP intake, determined in spot urine samples, correlated with BP measurements and prevalence of hypertension. Taking into account that a high polyphenol excretion in urine is determined by a high TP consumption, it was suggested that the inverse association observed between the objectively measured TPE in urine samples and BP may be related to a favorable effect of TP intake on BP levels. In the current study, performed in a Spanish high-risk population, we observed a significant positive correlation between TPE, as a biomarker of TP intake, and changes in plasma NO after a one-year intervention with Med-diets supplemented with polyphenol-rich foods, that is, EVOO or nuts.

The present study has several limitations. First, since our subjects were elderly people at high risk of cardiovascular disease, the results may not be generalized to other populations. However, since participants of the PREDIMED trial most were hypertensive, our results confirm the usefulness of polyphenol-rich diets in the management of hypertension. A second limitation is the size of the study population, which was relatively small in comparison with other studies.

The present study also has several strengths, including the randomized controlled clinical trial, which is considered as the most rigorous method of determining whether a cause-effect relationship exists between an intervention and outcome. In this intervention study, designed to evaluate the effect of a Med-diet treatment, the subjects were followed prospectively to compare the interventions with the control. The main advantage of randomized studies is that the conclusions reached achieve the highest level of scientific evidence. Another strong point of the current work was the use of TPE as a biomarker of TP intake, since this is more precise than self-reported information based on recalled dietary assessment, thus providing a more objective measurement of specific nutrient intake than the subjective information obtained by an FFQ.

In conclusion, a dietary pattern with a high intake of polyphenols, such as Med-diet may help to decrease BP in elderly hypertensive populations and consequently lower their cardiovascular risk throughout an increase in plasma NO. An easy way to improve cardiovascular risk is to include nuts and EVOO in the diets of individuals with normal-to-high BP or hypertension.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2014.09.001.

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1.7. Publicació 7. El consum de vi negre està associat amb un menor risc de síndrome metabòlica a l'estudi PREDIMED

Article 7. Moderate red wine consumption is associated with a low prevalence of metabolic syndrome in the PREDIMED population.

Anna Tresserra-Rimbau, Alexander Medina-Remón, Rosa M. Lamuela-Raventós, Monica Bulló, Jordi Salas-Salvadó, Dolores Corella, Montserrat Fitó, Alfredo Gea, Enrique Gómez-Gracia, José Lapetra, Fernando Arós, Miquel Fiol, Emilio Ros, Lluis Serra-Majem, Xavier Pintó, Miguel A. Muñoz, i Ramón Estruch. British Journal of Nutrition. **2014**, In press.

Resum:

La SM és un trastorn metabòlic que, a causa de l'increment de la obesitat, ha esdevingut un assumpte prioritari de salut pública. Aquesta síndrome és una combinació de factors de risc cardiovascular: obesitat, hipertensió, dislipèmia i hiperglucèmia. Els mals hàbits dietètics en són una causa ben coneguda, però l'efecte del consum d'alcohol encara causa controvèrsia.

L'objectiu d'aquest estudi era mostrar l'associació entre el consum moderat de vi negre, una beguda rica en polifenols, i el risc de SM i els seus components en una població de 5801 participants d'avançada edat inclosos dins l'estudi PREDIMED.

El consum de vi negre es va extreure del qüestionari basal de freqüència de consum d'aliments i es va dividir la població en abstemis, consumidors de 0.1 a 1 UBE/dia, i consumidors de més d'1 UBE/dia. La incidència de SM es va definir seguint el criteri proposat per la *International Diabetes Federation* (IDF) i la *American Heart Association/National Heart, Lung, and Blood Institute* (AHA/NHLBI). Les OR per la SM, i els seus components, i les diferents categories de consum de vi negre es van calcular mitjançant regressions logístiques múltiples, i es va ajustar per totes les variables necessàries. Totes les anàlisis estadístiques es van dur a terme utilitzant el programari IBM SPSS, versió 19.0 (Chicago, USA).

En aquest cas es van estudiar 5801 participants amb dades suficients relacionades amb la SM i els seus components. D'aquests, 3897 reunien els criteris per a ser diagnosticats amb la SM. Un 52% dels voluntaris eren abstemis, un 36% consumien entre 0.1 i 1 UBE/dia, i la resta >1 UBE/dia. Només 111 participants s'accediren de les recomanacions màximes establertes per al consum de begudes alcohòliques.

El consum de més d'1 UBE/dia de vi negre es va associar amb un menor risc de SM comparats amb els abstemis (OR=0.56; IC 95%=0.45-0.68; $P<0.001$). El resultat es mantingué inalterat quan vam extreure els bevedors més extrems (>2 UBE/dia per les dones i >4 UBE/dia pels homes).

Pel que fa als diferents paràmetres que defineixen la SM, els participants que es trobaven al grup de més consum de vi negre presentaven un menor risc de tenir un perímetre de cintura massa elevat (OR=0.59; IC 95%=0.46-0.77; $P<0.001$), un menor risc de tenir el colesterol HDL baix (OR=0.72; IC 95%=0.32-0.53; $P<0.001$) i menys risc de tenir la pressió alta o alts nivells de glucosa plasmàtica en dejú (OR=0.28; IC 95%=0.17-0.45; $P<0.001$ i OR=0.67; IC 95%=0.54-0.82; $P<0.001$, respectivament), comparats amb els abstemis i després d'ajustar per totes les variables. Un ajust addicional per perfil de consum d'altres alcohols no va alterar els resultats. En anàlisis posteriors també es va observar una associació major entre el consum de vi negre i la SM per les dones, pels menors de 70 anys i pels fumadors i els ex-fumadors.

Així doncs, aquest estudi suggereix que un consum de vi negre moderat podria ser protector contra el risc de patir SM, en concret, per disminuir la obesitat abdominal, millorar el colesterol HDL, la PA i els nivells de glucosa plasmàtica en dejú.

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Moderate red wine consumption is associated with a lower prevalence of the metabolic syndrome in the PREDIMED population

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Abstract

Previous studies on the association between alcohol intake and the development of the metabolic syndrome (MetS) have yielded inconsistent results. Besides, few studies have analysed the effects of red wine (RW) consumption on the prevalence of the MetS and its components. As moderate RW drinkers have a better lipid profile and lower incidence rates of diabetes, hypertension and abdominal obesity, all components of the MetS, it was hypothesised that moderate RW consumption could be associated with a lower prevalence of the MetS. In the present cross-sectional study of 5801 elderly participants at a high cardiovascular risk included in the PREDIMED (Prevención con Dieta Mediterránea) study, 3897 fulfilled the criteria of the MetS at baseline. RW intake was recorded using a validated 137-item FFQ. Multiple logistic regression analysis was carried out to estimate the association between RW intake and the prevalence of the MetS. Compared with non-drinkers, moderate RW drinkers (≥1 drink/d) were found to have a reduced risk of prevalent MetS (OR 0·56, 95 % CI 0·45, 0·68; $P<0·001$), a lower risk of having an abnormal waist circumference (OR 0·59, 95 % CI 0·46, 0·77; $P<0·001$), low HDL-cholesterol concentrations (OR 0·42, 95 % CI 0·32, 0·53; $P<0·001$), high blood pressure (OR 0·28, 95 % CI 0·17, 0·45; $P<0·001$) and high fasting plasma glucose concentrations (OR 0·67, 95 % CI 0·54, 0·82; $P<0·001$) after adjusting for several confounders. This association was found to be stronger in female participants, in participants aged <70 years and in participants who were former or current smokers. No significant association was found between RW intake (≥1 drink/d) and TAG concentrations. In conclusion, moderate RW consumption is associated with a lower prevalence of the MetS in an elderly Mediterranean population at a high cardiovascular risk.

Key words: **Red wine: Alcohol: Metabolic syndrome: Glucose: Lipids: Cholesterol: Blood pressure: Obesity: CVD**

Abbreviations: BP, blood pressure; HDL-c, HDL-cholesterol; MedDiet, Mediterranean diet; MetS, metabolic syndrome; PREDIMED, Prevención con Dieta Mediterránea; RW, red wine.

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The metabolic syndrome (MetS), a cluster of metabolic abnormalities that includes abdominal obesity, hypertriacylglycerolaemia, low HDL-cholesterol (HDL-c) concentrations, hypertension and hyperglycaemia, has become a major public health concern[1]. It results from the interaction of multiple factors, including genetic and environmental factors, with the dietary habits playing a crucial role in its development[2].

Previous studies have found both positive and negative effects of alcohol intake on the risk of MetS[3]. However, in a meta-analysis of observational studies, Alkerwi *et al.*[4] showed that a favourable metabolic effect appeared to be restricted to moderate alcohol intake, namely <20 g/d in women and <40 g/d in men. While some authors have found no differences in the incidence rates of the MetS among consumers of various alcoholic drinks, others have reported lower incidence rates among wine drinkers[3,5]. However, using a longitudinal design in the SUN study, Barrio-Lopez *et al.*[5] found that consumers of at least seven alcoholic drinks per week had increased odds of developing the MetS, but they did not find any significant association between wine or liquor consumption and the MetS. Besides containing alcohol, red wine (RW) is rich in polyphenols, which may beneficially influence carbohydrate metabolism[6] and blood pressure (BP)[7,8]. Furthermore, different human intervention studies have shown that other foods rich in polyphenols increase HDL-c concentrations[9–11]; however, the few human studies that have been conducted on the effects of polyphenol-rich foods on abdominal adiposity have yielded conflicting results, with studies showing positive effects[12,13] or no effect[14].

The purpose of the present study was to investigate the association between RW consumption and the prevalence of the MetS and its components in an elderly Mediterranean population at a high cardiovascular risk.

Subjects and methods

Subjects

A cross-sectional study was conducted using baseline data obtained from the cohort of the PREDIMED study. A detailed description of the study has been published previously[15]. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Institutional Review Boards of the participating centres (Clinical Trial Registration: ISRCTN of London, England: 35739639). Written informed consent was obtained from all participants.

Of the 5969 participants from all the PREDIMED centres with complete biochemical analysis data, 140 who did not complete the FFQ at baseline and twenty-eight with extreme total energy intakes were excluded from the present study[16]. Thus, data from 5801 participants were available for the analyses.

Assessment of population characteristics and dietary habits

Dietary habits at baseline were evaluated using a validated 137-item FFQ[17]. Daily food and nutrient intakes were estimated from the FFQ by multiplying the frequency of consumption by the average portion size. A validated 14-point questionnaire was also used[18], but excluding alcohol intake, to assess the adherence to the traditional Mediterranean diet (MedDiet). The participants also filled out a general questionnaire on lifestyle habits, medication use and concurrent diseases and a validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire[19].

Anthropometric measurements and blood analyses

Body weight and height were measured with minimum clothing and no shoes, using calibrated scales and wall-mounted stadiometers, respectively. The BMI was calculated as weight in kg divided by the square of height in m. Waist circumference was measured mid-way between the lowest rib and iliac crest using an anthropometric tape. The waist:height ratio was assessed by dividing the waist circumference by height, as a practical index for assessing central fat distribution. BP was measured in a sitting position, using a semi-automatic sphygmomanometer (Omron HEM-705CP), in triplicate with a 5 min interval between each measurement, and the mean of these values was recorded according to the procedures recommended by the European Hypertension Society[20]. Plasma glucose and lipid profiles were measured using an automatic analyser in a routine laboratory test. For patients with TAG concentrations <400 mg/dl, LDL-cholesterol concentrations were estimated using the Friedewald formula[21].

Categories of red wine intake

The FFQ included questions concerning the intake of wines (RW, rosé wine, white wine and sparkling wine), beer, liquors and spirits. Standard drinks (hereafter referred to as 'drinks'), 10 g of pure alcohol, were used to categorise alcohol intake. The drinks included 100 ml of wine, 250 ml of beer, 65 ml of liquors and 32 ml of spirits. The participants were categorised into three groups based on their RW intake: non-drinkers; 0·1–1 drink/d; >1 drink/d.

Definition of the metabolic syndrome

The MetS was defined in accordance with the updated harmonised International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) criteria[22]. The participants were identified as having MetS The American Journal of Clinical Nutrition AJCN/2014/086991 version 1 when they had at least three of the following components: 125 (1) elevated waist circumference for European individuals (>102 cm in men; >88 in women); (2) elevated TAG concentrations (>150 mg/dl (1·7 mmol/l)) or drug treatment for elevated TAG concentrations; (3) low HDL-c concentrations (≤ 40 mg/dl (1·0 mmol/l) in men; ≤ 50 mg/dl (1·3 mmol/l) in women) or drug treatment for low HDL-c concentrations; (4) elevated BP (systolic ≥ 130 and/or diastolic ≥ 85 mmHg) or antihypertensive drug treatment; (5) elevated fasting glucose concentrations (>100 mg/dl (5·5 mmol/l)) or drug treatment for diabetes.

Statistical analyses

The means of continuous variables among the different RW intake groups were compared using one-way ANOVA with the Bonferroni *post hoc* test. If the variable did not follow a normal distribution (skewness or kurtosis >-2 or <2), the corresponding non-parametrical Mann–Whitney and Kruskal–Wallis tests were performed. The χ^2 test or the Fisher exact test was used for analysing categorical variables.

Pearson's correlation coefficients were calculated to test the linear association between the intake of RW and that of other alcoholic beverages (white and sparkling wines, beer, liquors and spirits), all as continuous variables.

The OR for the MetS and each of its components in the different RW intake categories were calculated using multiple logistic regression analysis, with the outcome as the dependent variable and the intake groups as independent variables. Multivariate models were adjusted for sex, age (continuous), BMI (continuous), smoking status (never, former and current), physical activity during leisure time (yes/no), energy intake (continuous), educational level and adherence to the Mediterranean food pattern excluding wine intake (continuous). Additional analyses stratified by sex, age groups (<70 years and ≥70 years) and smoking status (never/ever) were carried out and the effect modification of these variables with alcohol was evaluated using likelihood ratio tests for the product term introduced in the crude and fully adjusted models. All statistical analyses were conducted using IBM SPSS software version 19.0. All *t* tests were two-sided and *P* values <0·05 were considered significant.

Results

A total of 5801 PREDIMED study participants (2433 men and 3368 women) at a high cardiovascular risk were included in the present study. The baseline characteristics of the study participants according to categories of energy-adjusted RW intake at baseline are summarised in Table 1. More than 50 % of the study population did not consume RW (3037 participants), 36 % consumed <1 drink/d (2086 participants) and 12 % consumed >1 drink/d (678 participants). Of these, only 111 participants consumed ≥5 drinks/d. Participants with the highest RW intake were found to more likely be men, current or former smokers, and more physically active and have a higher educational level. The dietary pattern of the participants distributed into RW intake categories is summarised in Table 2. Those who drank more RW (>1 drink/d) were found to have a higher intake of carbohydrates, protein, SFA, MUFA and PUFA, total cholesterol and total energy. Less frequent consumption of fruits, vegetables and dairy products was observed in the group of moderate/heavy drinkers, although no significant differences were observed in the adherence to the traditional MedDiet among the three groups.

The linear association between the intake of RW and that of other alcoholic beverages was also tested (Table 3). Although the correlation coefficients were significant, only weak linear associations were found among the variables. In general, the intake of RW was found to be positively correlated with the intake of beer, liquors and spirits, but not with that of other types of wines when analysing the entire sample or stratifying the sample by sex.

Red wine and metabolic syndrome

Among the 5801 participants included in the analyses, 3897 (67 %) fulfilled the criteria for the MetS. The metabolic risk parameters according to the RW intake categories are summarised in Table 4. Participants with the highest RW intake had lower BMI and heart rate, but higher BP. No significant differences were observed in total cholesterol or HDL-c concentrations across the groups. However, when these values were translated to MetS components taking into account the medications used to treat triacylglycerolaemia, hypertension or diabetes, fewer MetS cases were observed among the highest RW consumers, as well as a lower prevalence of abnormal waist circumference, high TAG concentrations or lipid-lowering treatment, low HDL-c concentrations, high BP or antihypertensive treatment, and high fasting plasma glucose concentrations or antidiabetic treatment.

Table 1. Baseline characteristics of participants from the PREDIMED (Prevención con Dieta Mediterránea) cohort according to categories of red wine (RW) intake at baseline (energy-adjusted)

(Number of participants; percentages; mean values and standard deviations)

	Non-drinkers		0·1–1 drink/d		>1 drink/d		
Characteristics	*n*	Percentage of the total	*n*	Percentage of the total	*n*	Percentage of the total	*P**
No. of participants (5801)	3037	52·4	2086	36·0	678	11·7	
Sex, women	2299	75·7	973	46·6	96	14·2	<0·001
Current smoker	276	9·1	324	15·5	193	28·5	<0·001
Former smoker	464	15·3	645	30·9	292	43·1	<0·001
Higher education	328	10·8	395	18·9	160	23·6	<0·001
	Mean	SD	Mean	SD	Mean	SD	*P*†
RW intake (units/d)	0	0·0	0·51	0·4	2·9	1·2	<0·001
Age (years)	67·9	6·0	66·3	6·2	65·7	6·1	<0·001
Energy expenditure in physical activity (kJ/d)	829·3	871·1	1042·9	1050·0	1398·6	1147·0	<0·001

* χ^2 tests.
† One-way ANOVA tests.

Table 2. Dietary pattern of 5801 participants from the PREDIMED (Prevención con Dieta Mediterránea) cohort according to categories of red wine (RW) intake at baseline (energy-adjusted)

(Mean values and standard deviations)

	Non-drinkers (*n* 3037)		0·1–1 drink/d (*n* 2086)		>1 drink/d (*n* 678)		
	Mean	SD	Mean	SD	Mean	SD	*P**
Alcoholic beverages (units/d)							
RW	0·00	0·00	0·51	0·38	2·92	1·19	<0·001
White, rosé and sparkling wines	0·10	0·51	0·08	0·36	0·11	0·47	0·36
Beer	0·09	0·37	0·22	0·56	0·37	0·79	<0·001
Liquors/spirits	0·005	0·06	0·01	0·08	0·03	0·10	<0·001
Nutrient intake							
Q10 Total energy intake							<0·001
kJ/d	8875·5	2174	9660	2185·7	10 880	2118·7	
kcal/d	2121·3	519·6	2308·8	522·4	2600·4	506·4	
Carbohydrates (g/d)	228·3	72·4	238·7	72·7	251·3	75·2	<0·001
Protein (g/d)	89·2	21·3	94·3	21·4	95·7	20·3	<0·001
SFA (g/d)	23·7	8·5	26·1	8·3	26·9	8·2	<0·001
MUFA (g/d)	46·0	15·0	50·5	14·9	52·9	14·2	<0·001
PUFA (g/d)	14·8	6·4	16·2	6·6	17·3	6·7	<0·001
Fibre (g/d)	25·0	8·7	25·6	8·7	25·3	7·8	0·066
Total cholesterol (mg/d)	346	119	382	123	385	113	<0·001
Food groups (g/d)							
Fruits	370	204	372	205	352	199	<0·001
Vegetables	332	149	342	152	328	138	0·023
Cereals	137	81	145	83	169	97	<0·001
Meat	125	55	136	55	146	58	<0·001
Fish	96	46	105	58	105	47	<0·001
Legumes	21	14	21	13	20	10	0·162
Dairy products	403	227	374	218	309	197	<0·001
Olive oil	38	18	40	18	42	16	<0·001
Nuts	9	12	11	15	12	14	<0·001
Soft drinks	15	58	20	57	18	61	0·025
13-point MedDiet questionnaire score†	8·34	1·86	8·35	1·90	8·26	1·84	0·56

MedDiet, Mediterranean diet.

* One-way ANOVA tests.

† The 14-point questionnaire of adherence to the traditional MedDiet excluding the question regarding wine intake.

The relative risk was calculated using logistic regression models with non-drinkers as the reference category. Risks calculated using crude models and after adjusting for possible confounders (sex, age, BMI, smoking status, educational level, physical activity, total energy intake and diet) differed significantly (Table 5). Consumption of less than one drink of RW per d was found to be associated with a significantly lower risk of the MetS in both the crude and adjusted Q7 models (OR 0·56, 95% CI 0·45, 0·68; $P<0·001$). The same association was found when the fully adjusted OR for the highest RW intake category was estimated after excluding heavy drinkers (>2 drinks/d for women and >4 drinks/d for men) in sensitivity analyses (data not shown). With regard to individual components of the MetS, participants who were in the highest RW intake category had a 41% lower risk of having an abnormal waist circumference (OR 0·59, 95% CI 0·46, 0·77; $P<0·001$), a 58% lower risk of having low HDL-c concentrations (OR 0·42, 95% CI 0·32, 0·53; $P<0·001$), a 72% lower risk of having high BP (OR 0·28, 95% CI 0·17, 0·45; $P<0·001$) and a 33% lower risk of having high fasting plasma glucose concentrations (OR 0·67, 95% CI 0·54, 0·82; $P<0·001$) compared with non-consumers. A protective effect of RW intake on TAG concentrations that lost significance after adjustment for all potential confounders was also observed.

Analysis performed after stratifying the sample by sex revealed a lower risk of the MetS in women consuming >1 drink/d (OR 0·47, 95% CI 0·30, 0·73; *P* trend <0·001) than in men (OR 0·68, 95% CI 0·53, 0·88; *P* trend 0·004) after multivariate adjustment (Table 6). Analysis performed after stratifying the sample by age groups (<70 years and ≥70 years) revealed that the effects of RW intake to be significant only for the youngest group (OR 0·49, 95% CI 0·38, 0·63; *P* trend <0·001). When analysing the association between the prevalence of the MetS and RW intake among participants stratified by smoking status, a 43% reduction in the risk of the

Q11 **Table 3.** Pearson's correlation coefficients between the intake of different alcoholic beverage groups and that of red wine (drinks/d)

	Entire sample (*n* 5801)		Men (*n* 2433)		Women (*n* 3368)	
	r	*P*	*r*	*P*	*r*	*P*
White, rosé and sparkling wines	0·02	0·23	−0·05	0·018	0·02	0·35
Beer	0·17	<0·001	0·09	<0·001	0·10	<0·001
Liquors	0·12	<0·001	0·08	<0·001	0·10	<0·001
Spirits	0·14	<0·001	0·08	<0·001	0·09	<0·001

MetS in former or current smokers (OR 0·57, 95 % CI 0·43, 0·75; *P* trend < 0·001) and a 40 % reduction among those who never smoked were observed. For all stratum categories, a trend towards a lower risk of the MetS was observed in both the crude and adjusted models, although not all models achieved statistical significance.

The association between the prevalence of the MetS and the intake of different types of alcoholic beverages was also analysed. After adjusting for all confounders, beer intake was found to be associated with an increased risk of the MetS (OR 1·50, 95 % CI 1·08, 2·10; *P* trend 0·005) due to its association with abdominal obesity (OR 1·49, 95 % CI 1·01, 2·19; *P* trend 0·049). However, when the association between beer intake and waist:height ratio (anthropometric index of abdominal fat distribution) was analysed, the association was found to lose the statistical significance (OR 0·67; 95 % CI 0·23, 1·97; *P* trend 0·970). None of the other metabolic criteria varied significantly with beer intake. Analysis of the intake of other alcoholic beverages in relation to the prevalence of the MetS revealed no significant associations for the intake of white, rosé and sparkling wines or for that of liquors and spirits, although the intake of these types of alcoholic beverages was scarce in the study population.

Discussion

In the present cross-sectional study of 5801 elderly participants at a high cardiovascular risk included in the PREDIMED study, 3897 MetS cases were found, representing a prevalence of 67·2 %, which was not unexpected given that only individuals with diabetes or three or more standard cardiovascular risk factors, including overweight or obesity, were eligible for inclusion in the study. In this setting, moderate RW consumption was found to be associated with a decreased prevalence of the MetS, mainly by reducing the risk of having an abnormal waist circumference, high BP, low HDL-c concentrations and high fasting plasma glucose concentrations. This association was stronger in participants aged < 70 years, in participants who were former or current smokers, and also in female participants. In fact, women consuming > 1 drink/d had a lower risk of the MetS than men. Several studies have demonstrated that women are more sensitive to the toxic effects of alcohol than men[23]. Therefore, the recommended upper limit of alcohol consumption for women is half of that recommended for men[24]. On the other hand, several studies have also reported the beneficial effects of moderate alcohol consumption in women even when consuming less amounts of alcohol when compared with men[25]. The results of the present study confirm the greater beneficial effects of moderate RW consumption on the incidence of the MetS in women than in men.

The intake of RW was weakly but positively associated with that of beer, liquors and spirits, but was not associated with the intake of other types of wines. This suggests that RW consumers are more likely to consume beer, liquors and spirits than white wine, rosé wine or sparkling wine. However, although significant, correlation coefficients were too low to draw conclusions. On the other hand, no association was

Table 4. Metabolic risk parameters of 5801 participants from the PREDIMED (Prevención con Dieta Mediterránea) cohort according to categories of red wine intake at baseline (energy-adjusted)

(Mean values and standard deviations; number of participants and percentages)

	Non-drinkers (*n* 3037)		0·1–1 drink/d (*n* 2086)		> 1 drink/d (*n* 678)		
	Mean	SD	Mean	SD	Mean	SD	*P**
BMI (kg/m^2)	30·7	4·1	29·7	3·6	29·2	3·3	< 0·001
Waist:height ratio	0·64	0·07	0·62	0·06	0·61	0·06	< 0·001
Systolic BP (mmHg)	150·4	19·5	148·6	19·2	152·7	19·6	< 0·001
Diastolic BP (mmHg)	82·7	10·3	83·1	10·2	84·7	10·6	< 0·001
Hearth rate (beats/min)	72·3	11·1	69·8	10·1	69·1	10·9	< 0·001
Glucose (mg/dl)	123·1	41·3	118·7	39·0	117·1	33·7	< 0·001
Lipid profile (mg/dl)							
Total cholesterol	206·3	37·6	205·3	37·6	206·8	37·8	0·579
HDL-c	52·8	12·1	52·1	12·4	52·8	12·0	0·099
LDL-c	128·5	33·7	130·8	36·8	131·3	33·5	0·032
TAG	137·3	77·5	127·8	73·6	131·8	73·7	< 0·001
Metabolic syndrome and components	*n*	Percentage of the total	*n*	Percentage of the total	*n*	Percentage of the total	*P*†
Metabolic syndrome	2268	74·7	1267	60·7	362	53·4	< 0·001
Abnormal waist circumference	2560	84·3	1437	68·9	390	57·5	< 0·001
High TAG concentrations or lipid-lowering treatment	1042	34·3	612	29·3	220	32·4	< 0·001
Low HDL-c concentrations	999	32·9	585	28·0	99	14·6	< 0·001
High BP or antihypertensive treatment	2990	98·5	1932	92·6	639	94·2	< 0·001
High fasting plasma glucose concentrations or antidiabetic treatment	2125	70·0	1309	62·7	442	65·2	< 0·001

BP, blood pressure; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol.
* One-way ANOVA tests.
† χ^2 tests.

Table 5. Risk of the metabolic syndrome and individual metabolic syndrome components according to red wine intake categories (0·1–1 drink/d and >1 drink/d groups compared with the non-drinker group)

(Odds ratios* and 95 % confidence intervals)

	0·1–1 drink/d	*P*†	>1 drink/d	*P*†
Metabolic syndrome‡				
Unadjusted OR	0·53	<0·001	0·39	<0·001
95 % CI	0·47, 0·90		0·33, 0·46	
Multivariable OR§	0·64	<0·001	0·56	<0·001
95 % CI	0·56, 0·73		0·45, 0·68	
Abnormal waist circumference (>102 cm in men and >88 cm in women or treatment)				
Unadjusted OR	0·40	<0·001	0·24	<0·001
95 % CI	0·35, 0·45		0·20, 0·29	
Multivariable OR§	0·64	<0·001	0·59	<0·001
95 % CI	0·53, 0·78		0·46, 0·77	
TAG (≥150 mg/dl or TAG-lowering medication)				
Unadjusted OR	0·76	<0·001	0·89	0·18
95 % CI	0·68, 0·86		0·74, 1·06	
Multivariable OR§	0·76	<0·001	0·87	0·18
95 % CI	0·66, 0·86		0·71, 1·06	
HDL-cholesterol (<40 mg/dl in men and <50 mg/dl in women or lipid-lowering treatment)				
Unadjusted OR	0·76	<0·001	0·34	<0·001
95 % CI	0·68, 0·87		0·27, 0·42	
Multivariable OR§	0·85	0·015	0·42	<0·001
95 % CI	0·75, 0·97		0·32, 0·53	
Blood pressure (≥130/85 mmHg or antihypertensive treatment)				
Unadjusted OR	0·20	<0·001	0·26	<0·001
95 % CI	0·14, 0·28		0·17, 0·40	
Multivariable OR§	0·22	<0·001	0·28	<0·001
95 % CI	0·16, 0·31		0·17, 0·45	
Fasting plasma glucose (≥100 mg/dl or antidiabetic treatment)				
Unadjusted OR	0·70	<0·001	0·79	0·01
95 % CI	0·62, 0·79		0·66, 0·95	
Multivariable OR§	0·65	<0·001	0·67	<0·001
95 % CI	0·57, 0·74		0·54, 0·82	

* OR were calculated using logistic regression analysis.
† Two-sided test of significance.
‡ The metabolic syndrome was considered to occur when at least three of the five metabolic criteria were fulfilled.
§ Adjusted for sex, age, BMI, smoking status, educational level, physical activity, total energy intake and diet.

found between the incidence of the MetS and the consumption of alcoholic beverages other than RW.

Wine is considered to be a key component of the traditional MedDiet. Several previous cohort and intervention studies have examined the effects of observed Mediterranean-type diets on the risk of the MetS and its components. The prospective Framingham Heart Study Offspring Cohort study was the first to report a protective effect of the MedDiet on the MetS, as participants in the highest quintile of MedDiet adherence had a 30·1 % incidence rate of the MetS compared with those in the lowest quintile category (38·5 %; $P=0·01$)[26]. Other, albeit not all[27], cross-sectional studies carried out in Mediterranean countries[2,28–31] have reported an inverse association between quartiles of adherence to the MedDiet and the incidence of the MetS. A recent meta-analysis of fifty studies has confirmed that the higher the adherence to the MedDiet, the lower the prevalence and progression of the MetS[32]. Interestingly, intervention studies including MetS patients have confirmed that the MedDiet may favour the regression of the MetS and prevent its progression[33,34].

However, few studies have analysed the role of moderate RW consumption in the prevalence of the MetS. Similar to the present study, a study carried out in the Canary Islands (Spain) showed the intake of wine, as well as that of other components of the traditional MedDiet such as fruits, vegetables and cereals, to be inversely associated with the prevalence of the MetS[23]. Indeed, it has been suggested that not all components of the MedDiet are likely to provide the same level of protection[35].

Several studies with different designs have suggested that the greater health benefits of moderate consumption of RW might be related to its higher polyphenolic content compared with other alcoholic beverages. In fact, polyphenols may provide additional benefits to consumers of other alcoholic beverages by decreasing BP, inhibiting LDL oxidation, improving endothelial function and reducing inflammation and cell adhesion molecule levels[36]. In the present study, an association between regular RW consumption and hypertension was found, contrary to that reported in other studies[37]. In a recent feeding trial, systolic and diastolic BP were found to be significantly decreased after 4 weeks of intervention with dealcoholised RW, but not after RW or gin interventions[8].

The best-established protective factor of alcohol intake is the increase in plasma HDL-c concentrations[38,39]. A meta-analysis of clinical studies assessing the effects of moderate alcohol consumption on HDL-c concentrations has indicated that the intake of 30 g/d of ethanol increases HDL-c concentrations by a mean of 4·0 mg/dl and TAG concentrations by 5·7 mg/dl, irrespective of the alcoholic beverage consumed.

Table 6. Stratified analyses of the risk of the metabolic syndrome according to red wine intake categories* (Odds ratios† and 95 % confidence intervals)

	Non-drinkers	0·1–1 drink/d	>1 drink/d	*P* for trend	*P* interaction
Sex					
Males					
No. of cases/total	482/738	670/1113	313/582		
Unadjusted OR	Ref	0·80	0·61	<0·001	0·41
95 % CI		0·66, 0·97	0·49, 0·78		
Multivariable OR‡	Ref	0·91	0·68	0·004	0·02
95 % CI		0·73, 1·13	0·53, 0·88		
Females					
No. of cases/total	1786/2299	597/973	49/96		
Unadjusted OR	Ref	0·46	0·30	<0·001	
95 % CI		0·39, 0·54	0·20, 0·45		
Multivariable OR‡	Ref	0·52	0·47	<0·001	
95 % CI		0·44, 0·62	0·30, 0·73		
Age (years)					
< 70					
No. of cases/total	1354/1801	851/1414	249/480		
Unadjusted OR	Ref	0·50	0·36	<0·001	0·18
95 % CI		0·43, 0·58	0·29, 0·44		
Multivariable OR‡	Ref	0·61	0·49	<0·001	0·19
95 % CI		0·51, 0·72	0·38, 0·63		
≥ 70					
No. of cases/total	914/1236	416/672	113/198		
Unadjusted OR	Ref	0·57	0·47	<0·001	
95 % CI		0·47, 0·70	0·34, 0·64		
Multivariable OR‡	Ref	0·70	0·72	0·005	
95 % CI		0·56, 0·88	0·50, 1·03		
Smoking status					
Never					
No. of cases/total	1746/2297	675/1117	101/193		
Unadjusted OR	Ref	0·48	0·35	<0·001	0·27
95 % CI		0·41, 0·56	0·26, 0·47		
Multivariable OR‡	Ref	0·60	0·60	<0·001	0·004
95 % CI		0·51, 0·71	0·43, 0·85		
Ever					
No. of cases/total	522/740	592/969	261/485		
Unadjusted OR	Ref	0·66	0·48	<0·001	
95 % CI		0·54, 0·81	0·38, 0·61		
Multivariable OR‡	Ref	0·76	0·57	<0·001	
95 % CI		0·60, 0·95	0·43, 0·75		

Ref, reference.
Q7 * The metabolic syndrome was considered to occur when at least three of the five metabolic criteria were fulfilled.
† OR were calculated using logistic regression analysis.
‡ Adjusted for sex, age, BMI, smoking status, educational level, physical activity, total energy intake and diet.

It has been estimated that an average intake of 30 g of ethanol/d would cause an estimated reduction of 24·7 % in the risk of CHD[40]. In addition, both cross-sectional and intervention studies have shown that moderate RW consumption reduces the plasma concentrations of *in vivo* oxidised LDL[41,42], which has been reported to be associated with the polyphenolic content of RW.

Moderate alcohol consumption has also been reported to be inversely associated with the risk of diabetes in a meta-analysis of observational studies that included data from 477 200 men and women participating in prospective cohort studies. The dose–response trend showed that the alcohol intake of 22–24 g/d had the strongest inverse association, but alcohol intake became deleterious over 60 g/d in men and over 50 g/d in women[43,44].

In addition, randomised clinical trials have also demonstrated that moderate alcohol intake (30 g/d) has beneficial effects on insulin and TAG concentrations and insulin sensitivity in non-diabetic postmenopausal women[45], suggesting that moderate alcohol consumption decreases the risk of CVD and type 2 diabetes by improving insulin sensitivity.

Similar to that observed in other studies[46,47], RW drinkers had significantly reduced BMI and waist circumference Q7 when compared with non-drinkers in the present study. Thus, moderate alcohol consumption, as observed in the Tromso Study[47], as well as moderate RW consumption, as observed in the Danish Diet Cancer and Health Study[46], exerts a beneficial effect by lowering the risk of abdominal obesity in women. Dietary factors including animal fat and refined carbohydrates are postulated to induce oxidative stress that stimulates inflammation in obesity. By contrast, some foods including wine, fruits, vegetables, nuts and others exert antioxidant and anti-inflammatory effects that may prevent the development of the MetS[48,49].

The present study has a few limitations. First, as the study participants were elderly Spanish people at a high risk

of CVD, findings from the study cannot be extrapolated to younger lower-risk populations from other countries. Furthermore, studying high-cardiovascular risk individuals is a limitation rather than an advantage for testing our hypothesis. Another limitation is the cross-sectional nature of the study, which does not allow inferring causal relationships between the MedDiet and the MetS. The present study also has strengths, such as the large sample size and the high number of participants with the MetS, the use of a validated FFQ and the ability to control for potential confounders due to recording of comprehensive data on risk factors, diet and sociodemographic variables. On the other hand, the study population is not representative of the general Spanish population. Although many potential confounders were controlled for in multivariate models, other unknown or unmeasured confounders may exist.

Conclusions

Compared with non-drinkers, moderate RW drinkers from an elderly population at a high cardiovascular risk have a lower risk of developing the MetS and having abnormal waist circumference, low HDL-c concentrations, high BP and hyperglycaemia, four of the five individual metabolic criteria included in its definition.

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The authors' contributions are as follows: A. T.-R., A. M.-R., R. M. L.-R. and R. E. performed the statistical analyses, interpreted the data and wrote the first draft of the manuscript. All authors contributed to the writing and revision of the manuscript and approval of the final version to be published.

Conflicts of interest: R. M. L.-R. reports serving on the board of and receiving lecture fees from Research Foundation on Wine and Nutrition (FIVIN); receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from PepsiCo. J. S.-S. reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council; receiving consulting fees from Danone; and receiving grant support through his institution from Eroski and Nestlé. F. A. reports receiving payment for the development of educational presentations from Menarini and AstraZeneca. E. R. reports serving on the board of and receiving travel support, as well as grant support through his institution, from the California Walnut Commission; serving on the board of the Flora Foundation (Unilever); serving on the board of and receiving lecture fees from Roche; serving on the board of and receiving grant support through his institution from Amgen; receiving consulting fees from Damm and Abbott Laboratories; receiving consulting fees and lecture fees, as well as grant support through his institution, from Merck; receiving lecture fees from Danone, Pace, AstraZeneca, and Rottapharm; receiving lecture fees and payment for the development of educational presentations, as well as grant support through his institution, from Ferrer; receiving payment for the development of educational presentations from Recordati; and receiving grant support through his institution from Sanofi-Aventis, Takeda, Daiichi Sankyo, Nutrexpa, Feiraco, Unilever, and Karo Bio. L. S.-M. reports serving on the boards of the Mediterranean Diet Foundation and the Beer and Health Foundation. X. P. reports serving on the board of and receiving payment for the development of educational presentations, as well as grant support through his institution, from Ferrer; receiving consulting fees from Abbott Laboratories; receiving lecture fees, as well as grant support through his institution, from Merck, Menarini, Unilever, and Roche; receiving lecture fees from Esteve, Lacer, and AstraZeneca; receiving payment for the development of educational presentations from Rubio; and receiving grant support through his institution from Sanofi-Aventis, Amgen, Pfizer, and Boehringer Ingelheim. R. E. reports serving on the board of and receiving lecture fees from the FIVIN; serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. No other potential conflicts of interest relevant to this article are reported.

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DISCUSSIÓ GLOBAL

V. Discussió global

Una dieta rica en fruites, hortalisses i els seus derivats té nombrosos efectes beneficiosos per a la salut. A part de les vitamines, els minerals i altres oligoelements, els productes d'origen vegetal ens aporten polifenols, que són components del metabolisme secundari de les plantes. La hipòtesi d'aquesta tesi plantejava que els polifenols de la dieta poden jugar un paper important en la prevenció de malalties cròniques en una població d'edat avançada amb factors de risc CV (la població de l'estudi PREDIMED). L'objectiu, doncs, era avaluar la certesa de la hipòtesi mitjançant uns estudis més concrets.

En primer lloc, es va establir un mètode per estimar detalladament el consum de polifenols d'aquesta població utilitzant una nova base de dades molt completa, el Phenol-explorer, i identificar els polifenols que més contribuïren a la ingesta. A continuació es van estudiar les associacions entre les ingestes de polifenols i els esdeveniments CV (infart o ictus, amb resultat final de mort o no), així com amb la mortalitat per qualsevol causa. De forma paral·lela es va realitzar un subestudi per relacionar el consum de polifenols, estimat mitjançant l'excreció de polifenols en orina, amb una disminució de la PA i un increment de la producció endotelial de NO en un subgrup de la població del PREDIMED. Donat que el vi és un dels aliments que aporta més polifenols en el PREDIMED, també es va estudiar la influència del consum d'aquesta beguda en la incidència de SM. Addicionalment, es van dur a terme dues revisions bibliogràfiques: la primera, sobre biomarcadors de consum de polifenols, i la segona sobre la influència d'aquests en la PA i els mecanismes d'acció que explicarien aquest efecte.

La ingesta de polifenols i les principals fonts alimentàries es van estimar per a 7200 persones, homes i dones, d'entre 55 i 80 anys, participants de l'estudi PREDIMED, utilitzant la base de dades Phenol-explorer i els QFC basals. Aquesta ingesta a l'inici de l'estudi, calculada com a suma de polifenols individuals, va ser de 820±323 mg/dia. Aquest valor diferia dels 1193±510 mg/dia obtinguts, utilitzant la mateixa metodologia, en una cohort d'adults francesos en el marc de l'estudi SU.VI.MAX[144]. Donat que la ingesta de flavonoids era molt similar en les dues poblacions (546 i 506 mg/dia, respectivament), la diferència era deguda als àcids fenòlics, ja que la mitjana de la població espanyola era 248 mg/dia mentre que en el cas de la francesa era 639 mg/dia. Trobem una valors d'àcids fenòlics similars als dels francesos en un estudi dut a terme amb una cohort d'adults finesa (647 mg/dia)[145]. Una possible explicació seria que el consum de cafè o te, begudes amb un alt contingut d'àcids fenòlics, era més elevat a les poblacions franceses i fineses que en la del PREDIMED, que era una població amb una mitjana d'edat de 67 anys.

La comparació dels resultats obtinguts amb els d'altres autors és difícil per diverses raons. En primer lloc, la majoria d'estudis previs se centraven només en un determinat grup de polifenols, normalment, en els flavonoids[31,146–150]. També cal tenir en compte la base de dades de polifenols utilitzada (USDA, Food Composition Database, Phenol-explorer, etc.), així com la forma d'obtenció de les dades de consum a partir dels diferents qüestionaris. A més, les cohorts estudiades també eren molt diferents entre elles pel que fa a edats, països, característiques de la població, etc. Aquesta heterogeneïtat limita la comparació de les dades.

Generalment, el cafè, el te, el vi, les pomes i altres fruites com els cítrics i les baies apareixen en les primeres posicions de la llista d'aliments que més contribueixen al consum de polifenols. En el cas de la població del PREDIMED, hi havia coincidències en el cafè, les taronges, les pomes i el vi, però en sisena posició hi havia l'oli d'oliva i les olives, que representaven l'11%

del total de polifenols consumits (90.4 mg/dia), fet que no es repetia en cap dels articles publicats anteriorment i que, per tant, distingia la cohort estudiada de les altres.

Pel que fa a les subclasses de polifenols, els àcids hidroxicinàmics van ser els més abundants en la dieta espanyola (276 mg/dia), seguits de les flavanones (132 mg/dia) i les proantocianidines (117 mg/dia). Aquest perfil s'assemblava al de les cohorts francesa i danesa, on els àcids hidroxicinàmics també eren els més consumits, seguits, en aquests casos, de les proantocianidines i de les catequines. La diferència amb aquest últim grup es podia explicar de nou per les diferències en el consum de te.

Pel que fa als polifenols individuals cal destacar que 3 dels 5 més consumits eren isòmers d'àcid cafeoilquínic (que venen del cafè). Cal destacar que 7 dels 35 polifenols més consumits pertanyien al grup dels "altres polifenols" i la seva font eren aliments típicament mediterranis com l'oli d'oliva. Per exemple, l'hidroxitirosol, l'oleuropeïna i els seus derivats provenien únicament de les olives i oli d'oliva, excepte una part de l'hidroxitirosol que es troba també al vi negre. Aquest fet és especialment rellevant ja que estudis recents han demostrat que aquests polifenols de l'oli d'oliva, juntament amb els àcids grassos monoinsaturats, milloren els nivells lipídics en plasma i reparen els danys derivats de l'oxidació[151,152]. Aquests beneficis demostrats han portat a la EFSA (European Food Safety Administration) a acceptar, l'any 2011, una declaració de propietats saludables sobre l'efecte antioxidant dels polifenols de l'oli d'oliva, concretament l'hidroxitirosol i els seus derivats, en el colesterol LDL[153].

Segons les variables sociodemogràfiques, es posà de manifest que els homes consumien més polifenols que les dones. Això coincidia amb quasi tots els estudis[144–148] tot i que en una de les cohorts era al revés[149,150]. Alguns estudis, però, dividien el consum de polifenols entre les calories consumides per corregir el fet que els homes solen menjar més. Fent aquesta correcció en el PREDIMED es mantenia que els homes consumien més polifenols, principalment degut al vi i al cafè.

Curiosament, els fumadors i els que consumien més alcohol també tenien ingestes superiors de polifenols. El fet s'explica perquè en els dos casos consumien més begudes alcohòliques (sobretot vi) i més cafè. En altres estudis[146,148] els fumadors, al tenir dietes menys saludables, eren els menors consumidors de polifenols. En tots els casos[144,148–150] els polifenols es correlacionaren positivament amb el nivell educatiu.

Estudis previs han demostrat que els polifenols i els seus metabòlits poden reduir la PA, així com marcadors d'inflamació i d'oxidació. També milloren la disfunció endotelial a través de diferents mecanismes[154]. Per tots aquests motius es creu que un major consum d'aquests compostos pot estar relacionat amb una millora de les condicions de salut, per exemple, disminuint el risc de patir malalties cròniques i, per tant, allargant l'esperança de vida[34,155]. En la població del PREDIMED es van trobar associacions inverses entre el consum de polifenols totals i malalties CV o mortalitat per qualsevol causa. Comparant els quintils extrems, la reducció va ser del 46% pels esdeveniments CV i del 37% per la mortalitat total, tot i que en aquest últim cas no hi havia una tendència lineal (P-tendència=0,12).

Associacions similars es van trobar per lignans i esdeveniments CV i mortalitat total quan es va valorar el consum dels diferents grups de polifenols. Aquestes associacions es poden explicar per l'elevat consum d'oli d'oliva i olives d'aquesta cohort. Els lignans modulen l'acció dels estròsgens i, per tant, poden afectar en les malalties relacionades amb les hormones, com les CV, el càncer, la osteoporosis o els símptomes de la menopausa[156,157]. Recentment, s'ha observat que aquests compostos poden disminuir el càncer de bufeta[158] i la incidència de diabetis tipus 2 en dones[159]. De fet, en la mateixa direcció, s'ha demostrat que els polifenols de l'oli d'oliva milloren els factors de risc de malalties CV[151,152]. Altres estudis s'han centrat en l'efecte del consum de vi[160] i del seu polifenol més característic, el resveratrol[12], amb resultats que també són consistents amb l'associació inversa que es va trobar pels estilbens i

la mortalitat.

En la cohort PREDIMED, els flavanols es van associar a una disminució del 60% del risc de malaltia i mort CV, però no de mortalitat total. Això es correspon amb els resultats d'un meta-anàlisi sobre flavanols i biomarcadors de risc CV[30]. En una cohort gal·lesa, la ingesta de flavonols es va associar amb la mortalitat CV, la mortalitat per càncer i la total[33]. Recentment, els flavonols també s'han associat amb una menor incidència de càncer de bufeta en la cohort de l'estudi EPIC[158]. Geleijnse i col. també van trobar resultats similars pel consum de te, els flavonoids i l'infart de miocardi[161]. Pel que fa als àcids fenòlics, els hidroxibenzoics també es van associar amb els esdeveniments CV, però no hi ha altres estudis epidemiològics per comparar. No obstant, s'ha vist que l'àcid protocateqüic té propietats antioxidants i antiinflamatòries[162].

L'efecte dels polifenols sobre la PA està avalat per molts dels estudis d'intervenció en humans resumits a la **Taula 1.4** de la introducció. Nombrosos aliments rics en polifenols com el raïm, el cacau, el te i la soja han demostrat la seva capacitat de disminuir la PA en hipertensos mitjançant la millora de la funció endotelial. En efecte, els polifenols poden induir la vasorelaxació de les artèries a través de la producció de NO i de EDHF[163–165]. Així doncs, els polifenols no només tenen un efecte antioxidant, sinó que també participen en vies de senyalització que intervenen en la funció cel·lular[166].

La SM és un problema de salut creixent en els països desenvolupats que, a més, comporta un augment dels casos de malalties CV i diabetis tipus II. Nombrosos estudis han demostrat que alguns patrons dietètics, així com certs grups d'aliments o nutrients poden disminuir la incidència de SM a través de la millora dels seus components[167,168].

En diversos estudis amb humans, els polifenols del raïm i de la xocolata milloraven els símptomes associats a la SM[53,169]. En un altre estudi transversal realitzat en una població iraniana de 2618 adults de totes les edats, un consum elevat de flavonoids es va associar amb un menor risc de tenir SM, així com un perímetre de cintura elevat, hipertrigliceridèmia, hiperglucèmia, el colesterol HDL baix, i hipertensió. Per contra, el consum elevat de lignans es va associar amb un major risc de hipertrigliceridèmia i hiperglucèmia i, el d'estilbens, amb un major risc d'hipertensió[97].

Les begudes alcohòliques sempre han estat motiu de controvèrsia ja que el benefici-risc està estretament lligat al tipus de beguda i, sobretot, a la dosi. L'estreta unió entre el consum d'alcohol i l'estil de vida fan difícil l'estudi dels seus efectes en la salut[170]. El consum moderat de begudes alcohòliques sembla afavorir el metabolisme lipídic i els mecanismes que regulen la glucosa però, d'altra banda, l'alcohol és una font de calories extra ja que se sol afegir a la dieta en comptes de substituir-lo per algun altre aliment[171]. A més, alguns estudis han relacionat el consum d'alcohol amb un major risc d'hipertrigliceridèmia[172] però un menor risc d'hipertensió. Pel que fa al risc d'obesitat, les dades no són concloents ja que depenen, sobretot, de la quantitat i el tipus de beguda consumida[173].

En relació a la SM, els resultats de diferents estudis són divergents: mentre alguns han trobat una relació lineal inversa[174–176] o en forma de U[177] entre el consum de begudes alcohòliques i la SM que és consistent amb els nostres resultats, altres no han trobat cap associació[178] o bé una associació positiva[179–182]. Aquesta divergència es deu, en part, als diferents tipus de poblacions estudiades, al tipus de beguda alcohòlica (alta o baixa graduació) i a la quantitat ingerida.

Un dels punts fort d'aquests treballs és el nivell de detall obtingut en l'estimació del perfil fenòlic consumit, amb grups de polifenols que rarament s'havien estimat abans, i aprofundint fins al consum de polifenols individuals. Això va ser possible gràcies a la utilització d'un QFC ben validat i una base de dades de compostos fenòlics molt completa. La metodologia que es

va fer servir es va aplicar per quantificar el consum de polifenols d'una població relativament gran, de més de 7000 persones, no només a l'inici de l'estudi sinó al llarg dels anys, i es van calcular les ingestes de polifenols acumulades i ajustades per calories. Aquest disseny prospectiu, la mida de la cohort i una extensa informació sobre variables d'ajust també formen part dels punts forts de la tesi, així com la comprovació del esdeveniments CV, que els va realitzar un comitè extern.

D'altra banda, també es va utilitzar un biomarcador de consum de polifenols en orina per contrastar part dels resultats obtinguts. Així doncs, es va demostrar que, efectivament, podien influir en una de les principals variables de risc CV mitjançant l'increment de la producció de NO, fet que comporta una disminució de la PA.

Les conclusions derivades d'aquesta tesi estan condicionades a les limitacions pròpies dels estudis. En primer lloc, hi ha les limitacions inherents a l'estimació de la ingesta de polifenols, que és el primer pas que es va dur a terme. Tot i que la base de dades Phenol-explorer és la més completa d'avui en dia, hi mancava informació sobre alguns aliments que eren àmpliament consumits en la nostra cohort, com per exemple els cigrons, la mel o l'all. A més, alguns àcids fenòlics com les proantocianidines es van subestimar donada l'absència de dades fiables a la literatura[144]. Tampoc es tenien dades sobre la maduresa de les fruites, les condicions de cultiu i emmagatzematge o el mètode de cocció, que se sap que afecten el contingut fenòlic[6,7], així com la freqüència d'ús d'espècies que, tot i que es consumeixen en poques quantitats tenen una concentració de polifenols molt alta. Per últim, s'han de tenir en ment les limitacions derivades dels QFC anuals que, tot i estar validats, mai són una fotografia perfecta de la situació real.

En els dos articles on s'estudià l'associació entre consum de polifenols i malalties cròniques, la primera limitació fa referència a l'estimació de polifenols, com ja s'ha comentat en el paràgraf anterior. En segon lloc trobem les limitacions relacionades amb el disseny de l'estudi: al tractar-se d'un post-anàlisi de les dades d'un estudi d'intervenció on aquesta era diferent de l'exposició estudiada no es va poder establir cap relació causa-efecte. Parlarem, per tant, d'associacions, tendències o resultats que senyalen cap a una direcció determinada. D'altra banda, tot i que els models es van ajustar per moltes variables de confusió, no es pot descartar l'existència d'altres variables desconegudes que poguessin afectar als resultats. L'ús de QFC en comptes de biomarcadors va limitar el coneixement de l'efecte de la biodisponibilitat dels polifenols. Per últim, el nombre de casos no va ser prou elevat per estudiar, per exemple, la influència del consum de polifenols sobre la mortalitat per diferents causes: càncer, malalties respiratòries, malalties neurodegeneratives, etc.

La principal limitació del sisè article referent a la determinació de polifenols en orina és la mida de la mostra, molt menor que la utilitzada en tots els altres casos degut, sobretot, al cost d'analitzar els polifenols totals i els nivells de NO plasmàtic. S'hi han d'afegir també altres limitacions pròpies de l'ús de biomarcadors, com la variabilitat interindividual, l'error propi de l'anàlisi i assumir que els valors de polifenols excretats es corresponen amb els ingerits.

Pel que fa a l'article on es relaciona el consum de vi amb la SM, la primera limitació és l'ús de dades transversals en comptes de longitudinals. A més, el fet d'estudiar una població amb una prevalença tan elevada de SM fa que les dades no siguin extrapolables a la població general.

Aquesta restricció és comuna en tots els estudis que es van realitzar, ja que la cohort utilitzada té unes característiques que, si bé són força habituals entre la gent gran, no són representatives de la societat. El fet d'estudiar una població malalta incrementa els beneficis de les intervencions mentre que és més difícil millorar la salut de les persones sanes. No obstant, els productes d'origen vegetal, rics en polifenols, ja formen part de les recomanacions generals que els professionals de la salut donen als seus pacients per reduir el risc de malalties

crònіques.

El que aquesta tesi aporta és una visió global, però amb detall, dels possibles efectes dels polifenols en la salut, mentre que d'altres estudis se centren en un polifenol concret o en un sol subtipus. De moment, el missatge que podríem donar a la població seria que cal augmentar el consum d'aliments rics en polifenols, especialment d'aquells que continguin flavanols, àcids hidroxibenzoics, lignans, estilbens, i isoflavones, traduït a aliments, això significa incrementar, per exemple, el consum de fruits secs, oli d'oliva verge, cereals sense refinar, soja o altres llegums, vi, i fruits vermells.

Aquests resultats pretenen ser una guia per estudis futurs on, prioritzant aquells grups de polifenols que han donat millors associacions, es poden dissenyar estudis d'intervenció per comprovar si existeix una relació causal. A més, s'haurien d'estudiar amb detall les interaccions amb el sexe, l'alcohol, el tabac i amb altres aliments. També seria interessant estudiar l'efecte del consum de polifenols sobre altres malalties d'elevada taxa de mortalitat com el càncer, les malalties neurodegeneratives o la diabetis.

V. Global discussion

A diet rich in fruits, vegetables and their products has numerous health benefits. Besides vitamins, minerals and other trace elements, plant-derived products are a source of polyphenols, which are plant secondary metabolites. The hypothesis raised in this thesis is that dietary polyphenols can play a crucial role in the prevention of chronic diseases in an elderly population at high cardiovascular risk (the PREDIMED population). Therefore, our objective was to evaluate the truth of the hypothesis by specific studies.

Firstly, we set a method to carefully estimate the polyphenol intake of the cited population using the newly launched and comprehensive database, the Phenol-explorer, and to identify which food most contributed to this intake. We then studied the associations between polyphenol intake and cardiovascular events (myocardial infarction or stroke, ending with death or not), as well as all-cause mortality. Simultaneously, we conducted a substudy on the relationship between polyphenol intake, estimated by the excretion of polyphenols in urine, and reduced BP and an increase of endothelial NO production in a subsample of the PREDIMED cohort. Wine was an important source of polyphenols in the PREDIMED cohort, so their consumption was also correlated with the risk of MS in this population. Additionally, we performed two bibliographic revisions: the first one was about biomarkers of polyphenol intake, and the second one was about the influence of polyphenol intake on BP and the mechanisms of action that can explain this effect.

Intake of polyphenols and their main food sources were estimated for 7200 participants of the PREDIMED study, men and woman aged between 55 and 80 years, using the Phenol-explorer database and baseline FFQ. Polyphenol intake at baseline, calculated as the sum of individual polyphenols, was 820±323 mg/day. This value differed from the 1193±510 mg/day obtained, using the same method, in a cohort of French adults within the SU.VI.MAX study[144]. Since the intake of flavonoids was very similar in the two populations, the difference was due to the intake of phenolic acids: 248 mg/day for the Spanish cohort and 639 mg/day for the French[145]. We found very similar values of phenolic acids in a cohort of Finnish adults (647 mg/day)[145]. One possible explanation could be that the consumption of coffee or tea, beverages with a high polyphenol content, was greater in the French and Finnish populations than in the older PREDIMED population, with an average age of 67 years.

Comparing these results with those of other studies is difficult for several reasons. First of all, most of the studies were focused only on a specific group of polyphenols, usually the flavonoids[31,146–150]. Other factors include the use of different databases (USDA, Food Composition Database, Phenol-explorer, etc.), as well as the type of questionnaire for assessing dietary intake. Moreover, the studied cohorts were very different in terms of age, country, population characteristics, etc. This heterogeneity limits the comparison of the data.

Generally, coffee, tea, wine, apples, and other fruits such as citrus and berries are at the top of lists of food that most contribute to polyphenol intake. This was also the case in the PREDIMED population, with coffee, oranges, apples, and wine in the first positions, but in sixth place was olive oil and olives, which represented 11% of total polyphenol intake (90.4 mg/day). This was a distinguishing characteristic of the studied cohort.

Among the polyphenol subclasses, hydroxycinnamic acids were the most abundant subclass in the Spanish diet (276 mg/day), followed by flavanones (132 mg/day) and proanthocyanidins (117 mg/day). This profile was similar to the French and Danish cohorts, where hydroxy-

cinnamic acids were also the most consumed subclass, followed by proanthocyanidins and catechins. Differences in catechin consumption could also be explained by differences in tea drinking.

With respect to individual polyphenols, we noticed that 3 of the 5 most consumed were caffeoylquinic acid isomers in coffee. Also notable was that 7 of the 35 most consumed polyphenols belonged to the "other polyphenols" group and their sources were typically Mediterranean foods such as olive oil. For instance, the only source of hydroxytyrosol, oleuropein and their derivatives was olive oil and olives, except for hydroxytyrosol, which is also found in red wine. This is noteworthy since recent studies have demonstrated that polyphenols from olive oil, together with monounsaturated fatty acids, improve lipid levels in plasma and repair oxidation-derived damage[151,152]. On the basis on these proven benefits, in 2011 EFSA (European Food Safety Administration) accepted a health claim about the antioxidant effects of olive oil polyphenols, specifically hydroxytyrosol and its derivatives, on LDL cholesterol[153].

Depending on sociodemographic variables, men were found to consume more polyphenols than women, in agreement with all other studies[144–148] except for one cohort[149,150]. Some studies adjusted polyphenol intake by calories. After this correction in our cohort, men still consumed more polyphenols, mainly due to the consumption of wine and coffee.

Curiously, smokers and individuals who consumed more alcohol also had higher intakes of polyphenols, because both groups consumed more alcoholic beverages, mainly wine and coffee. On the contrary, other studies[146,148] have reported that smokers, who generally have poorer diets, consumed fewer polyphenols than non-smokers. Polyphenols were always positively correlated with the educational level[144,148–150].

Previous studies have demonstrated that polyphenols and their products can reduce BP, as well as acting as markers of inflammation and oxidation. They also improve endothelial dysfunction through different mechanisms[154]. For these reasons, polyphenol intake can be associated with an improvement of health conditions, for instance, by decreasing the odds of developing chronic diseases and, therefore, extending the lifespan[34,155]. In the PREDIMED population we found inverse and significant associations between total polyphenol intake and CV diseases or all-cause mortality. Comparing extreme quintiles, reduction was 46% for CV events and 37% for total mortality, although in this case we did not find a lineal trend (P-trend=0.12).

When studying polyphenol subgroups, similar associations were found between lignans and CV events and total mortality, which can be explained by the high consumption of olive oil and olives in this cohort. Lignans modulate estrogen action, so they can affect hormone-related diseases such as CV diseases, cancer, osteoporosis or menopausal symptoms[156,157]. Recently, two differents groups found that lignans could reduce bladder cancer risk[158] and type-2 diabetes in women[159]. Similarly, it has been demonstrated that polyphenols from olive oil improve CV risk factors[151,152]. Other studies have focused on the consumption of wine[160] and its well-known polyphenol, resveratrol[12], with results that are also consistent with the inverse association we found for stilbenes and mortality.

In the PREDIMED cohort, flavanols were also associated with a 60% decrease in risk of CV event or CV mortality, but not with all-cause mortality. These results agree with the conclusion of a meta-analysis of flavanols and CV risk factors[30]. In a Welsh cohort, intake of flavonols was associated with lower CV and cancer mortality and all-cause mortality[33]. Recently, flavonols has also been associated with a lower risk of bladder cancer in the EPIC study cohort[158]. Geleijnse et al. found a similar relationship between the consumption of flavonoids in tea and myocardial infarction[161]. Regarding phenolic acids, hydroxybenzoics were also inversely associated with CV events, and although there is no other study to compare these findings, protocathecuic acid has proven antioxidant and anti-inflammatory

properties[162].

The effects of polyphenols on BP have been demonstrated by several human intervention studies, summarized in the introduction **Table 1.4**. Many polyphenol-rich foods, such as grapes, cocoa, tea or soy, are able to decrease BP in hypertensive subjects by improving endothelial function. Indeed, polyphenols induce arterial vasodilation through the production of NO and EDHF[163–165]. Thus, polyphenols not only have antioxidant properties, but also participate in cellular signaling pathways[166].

MS is a growing public health concern in developed countries that implies an increase of the incidence of CV diseases and type-2 diabetes. Several studies have demonstrated that certain dietary patterns, as well as groups of food and nutrients, can decrease the incidence of MS through an enhanced intake of their components[167,168].

In human studies, polyphenols from grapes and chocolate improved MS-related symptoms[53,169]. In a cross-sectional study within an Iranian cohort of 2618 adults of all ages, a high intake of flavonoids was associated with a decreased risk of MS, abnormal waist circumference, hypertriglyceridemia, hyperglycemia, low HDL-cholesterol, and hypertension. On the other hand, high consumption of lignans was associated with a major risk of hypertriglyceridemia and hyperglycemia and stilbenes, with hypertension[97].

Alcoholic beverages have always been controversial because the risk-benefit ratio is closely related to the type of drink and, above all, to the dose. The effects of alcohol on health are difficult to quantify due to the tight union between alcohol consumption and lifestyle[170]. Moderate alcohol consumption seems to enhance lipid metabolism and the mechanisms that regulate glucose, yet alcohol is an extra source of calories in that it is usually added to the diet without replacing any other food[171]. Other studies have associated alcohol consumption with a greater risk of hypertriglyceridemia but a lower risk of hypertension[172]. With respect to obesity, data are not conclusive since they depend on the dose and type of alcoholic drink[173].

Related to MS, results from different studies diverge: while some have reported an inverse linear[174–176] or U-shaped[177] relationship between alcoholic beverages and MS, which is consistent with our results, others have found no association[178] or a positive relationship[179–182]. This divergence is partially due to the differences in studied populations, the type of beverage (high-grade or low-grade) and the amount consumed.

The main strength of the work presented here is the level of detail obtained in the estimation of the dietary phenolic profile, which included polyphenol groups never studied before, and provided more in-depth data about individual polyphenols. This was achieved through the use of a well-validated FFQ and a comprehensive polyphenol database. We applied the same method to quantify the phenol intake of a relatively large population, more than 7200 people, not only at the beginning of the study, but also over the years. Moreover, we calculated the cumulative polyphenol intake adjusted by calories. The prospective design, the cohort size and comprehensive information about the adjustment variables are other strengths of this thesis, as well as the verification of the CV events, performed by an external committee of experts.

Additionally, a biomarker of polyphenol intake in urine was used to verify some of our results. We were therefore able to demonstrate that polyphenols can indeed influence one of the main CV risk factors by enhancing NO production, which leads to a decrease in BP.

The conclusions derived from this thesis are conditioned by the intrinsic limitations of the studies. These include the limitations related to the estimation of polyphenols, the first step of the work. Although the Phenol-explorer is the most complete database available today, information on some foods extensively consumed in our cohort, such as chickpeas,

honey or garlic, is still scarce. Moreover, some phenolic acids like proanthocyanidins were underestimated due to the lack of reliable data in the literature[144]. Neither did we have information about fruit maturity, cultivation and storage conditions, or cooking methods, all being features that affect the phenolic content[6,7], or the use of condiments and spices, which although consumed in small amounts, have a very high phenolic content. Lastly, the limitations of the yearly FFQ should be taken into account, since these can never provide a perfect picture of reality despite validation.

In the two articles about polyphenol intake and chronic illnesses, the first limitation concerns the polyphenol estimation, as discussed in the previous paragraph. The study design was also found to have limitations: no cause-effect relationship could be established because the exposure variable differed from the intervention. Therefore, we can only talk about associations, trends or results that point in a given direction. Furthermore, even though all models were adjusted for all confounder variables, the influence of other unknown variables cannot be discarded. The use of FFQ instead of biomarkers limits the understanding of polyphenol bioavailability. Lastly, the number of cases was too low to study, for example, the influence of polyphenols on mortality due to specific causes, such as cancer, respiratory diseases, neurodegenerative diseases, etc.

The main limitation of the article about polyphenols in urine and NO, the sixth, is sample size, which was smaller than those from other studies due to the high cost of the analysis of total polyphenols and plasma NO. We should also add the intrinsic limitations of the biomarkers: individual variability, measurement error of the analysis and the assumption that the amount of polyphenols excreted in urine corresponds to the intake.

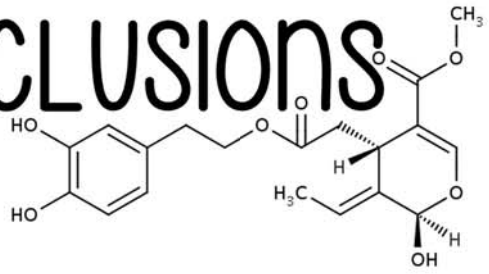
In the study about wine consumption and MS, the first limitation is that the design is cross-sectional rather than longitudinal. Moreover, the results cannot be extrapolated to the general population because the prevalence of MS in our cohort was very high.

This latter restriction is shared by all our studies, due to the characteristic profile of the PREDIMED cohort. Although these characteristics are common among the elderly, they are not representative of the general population. Studying non-healthy populations improves the benefits of the interventions, while it is difficult to improve the risk factors in a healthy population. However, products of plant origin, rich in polyphenols, are currently part of the general recommendations that health professionals give to their patients to reduce the risk of chronic diseases.

This thesis provides not only a global but also a detailed vision of the possible effects of polyphenols on our health, in contrast with other studies, whose focus has been limited to a particular polyphenol or a single subgroup. For now, the message we can give to the population is that we should increase our intake of polyphenol-rich foods, especially those containing flavanols, hydroxybenzoic acids, lignans, stilbenes, and isoflavones. Translating this information into food items this means increasing the consumption of nuts, virgin olive oil, whole-grain cereals, soy and other legumes, wine and berries.

These results aim to serve as a guide for future studies that will give priority to those polyphenols found to have the best associations. Intervention studies should be performed to evaluate causal relationships. Moreover, the interactions between polyphenols and sex, alcohol, smoking habits, and other foods should be studied in detail. It would be very interesting to elucidate the effect of polyphenol intake on other diseases with high mortality outcomes, such as cancer, neurodegenerative diseases and diabetes.

CONCLUSIONS



VI. Conclusions

Conclusions generals

✓ S'ha estimat de forma detallada la ingesta de polifenols i les seves principals fonts alimentàries per part d'una població d'edat avançada i en risc cardiovascular: l'estudi PREDIMED.

✓ La ingesta elevada de polifenols, estimada mitjançant qüestionaris de freqüència de consum i la base de dades Phenol-explorer, s'ha associat de forma inversa amb la incidència d'accidents cardiovasculars i amb la mortalitat per qualsevol causa en un anàlisi longitudinal de la cohort PREDIMED.

✓ El consum de polifenols, estimat mitjançant l'anàlisi colorimètric de Folin-Ciocalteu en mostres d'orina puntuals, s'ha associat amb una disminució de la PA i amb un increment de la producció d'NO en plasma en la població del PREDIMED al cap d'un any.

✓ El consum moderat de vi està associat amb una disminució de la prevalença de SM i amb alguns dels seus principals components en un anàlisi transversal de la cohort PREDIMED.

Conclusions específiques

✓ El consum de polifenols totals abans de la intervenció per part de la població del PREDIMED, calculats com a suma de polifenols individuals a partir de les dades obtingudes dels FFQ i de la base de dades Phenol-explorer, va ser de 820±323 mg/dia. La meitat d'aquests eren flavonoids i el 37%, àcids fenòlics.

✓ El grup d'aliments que més contribueixen a la ingesta de polifenols són les fruites (44%), les begudes no alcohòliques (23%), les verdures (13%) i les begudes alcohòliques (8%). Tenint en compte els aliments individualment, els que més contribuïren foren el cafè (18%), les taronges (16%), les pomes (12%), l'oli d'oliva i les olives (11%) i el vi negre (6%).

✓ Els àcids hidroxicinnàmics van ser la subclasse de polifenols més abundants en la dieta de la cohort estudiada (276 mg/dia), seguits de les flavanones (132 mg/dia), les proantocianidines (117 mg/dia) i els flavonols (80 mg/dia).

✓ Alguns dels polifenols del grup "altres polifenols", com l'oleuropeïna i l'hidroxitirosol, que es troben en olives i oli d'oliva, fan que el perfil fenòlic de la població espanyola sigui molt diferent dels de cohorts d'altres països com França i Finlàndia.

✓ Es va observar una reducció significativa del 46% del risc d'esdeveniment cardiovascular en els voluntaris del cinquè quintil d'ingesta de polifenols totals comparats amb el del primer, així com una reducció del 37% del risc de mortalitat per qualsevol causa.

✓ Tenint el compte els grups de polifenols, els lignans, els flavanols i els àcids hidroxibenzòics es van associar significativament i inversament amb els esdeveniments cardiovasculars, mentre que els estilbens i els lignans es van associar amb la mortalitat total. Sembla que el consum de isoflavones també pot tenir un efecte beneficiós però el seu consum en la població estudiada és massa baix per treure'n conclusions.

✓ Els polifenols excretats a través de la orina, analitzats seguint el mètode colorimètric de Folin-Ciocalteu i ajustats per creatinina, són un bon biomarcador del consum de polifenols. Gràcies a això s'ha relacionat de forma objectiva l'increment de polifenols totals en orina amb la disminució de la PA i amb l'augment de la producció de NO plasmàtic.

✓ El consum moderat de vi negre s'ha associat amb un menor risc de patir síndrome metabòlica i, en concret, s'ha relacionat amb una disminució de la obesitat abdominal, de la pressió arterial i dels nivells de glucosa plasmàtica en dejú, i amb una millora del colesterol HDL.

VI. Conclusions

General conclusions

✓ The polyphenol intake and its main food sources in an elderly population at high cardiovascular risk were comprehensively estimated.

✓ A high polyphenol intake, estimated by use of food frequency questionnaires and the Phenol-explorer database, was inversely associated with the incidence of cardiovascular events and all-cause mortality in a longitudinal analysis within the PREDIMED cohort.

✓ Total polyphenol intake, estimated in spot urine samples using the Folin-Ciocateu colorimetric analysis, was associated with a decrease in blood pressure and an increased NO production in plasma within the PREDIMED population after one year of follow-up.

✓ Moderate wine consumption was associated with a lower prevalence of metabolic syndrome and some of its main components in a cross-sectional analysis of the PREDIMED study.

Specific conclusions

✓ The total polyphenol intake by the PREDIMED population at baseline, calculated as the sum of individual polyphenols using data from the FFQ and the Phenol-explorer database, was 820±323 mg/day. Half of these were flavonoids and 37% were phenolic acids.

✓ The food groups which mostly contributed to this intake were fruits (44%), non-alcoholic beverages (23%), vegetables (13%), and alcoholic beverages (8%). Taking into consideration the food items individually, coffee was the main contributor (18%), followed by oranges (16%), apples (12%), olive oil and olives (11%) and red wine (6%).

✓ Hydroxycinnamic acids were the most abundant polyphenol subclass in the diet of the studied cohort (276 mg/day), followed by flavanones (132 mg/day), proanthocyanidins (117 mg/day) and flavonols (80 mg/day).

✓ Some of the polyphenols from the "other polyphenols" group, such as oleuropein and hydroxytyrosol, found in olives and olive oil, gave the phenolic intake of the studied population a very different profile from that of the cohorts of other countries such as France or Finland.

✓ We observed a 46% reduction in the risk of cardiovascular events when comparing the fifth with the first quintile of total polyphenol intake, and a 37% reduction in the risk of all-cause mortality.

✓ Among the polyphenol groups, lignans, flavanols and hydroxybenzoic acids were significantly and inversely associated with cardiovascular events, whereas stilbenes and lignans were associated with all-cause mortality. Health benefits for isoflavones were also suggested, but their intake in our population was too low to draw conclusions.

✓ Polyphenols in urine analyzed by the Folin-Ciocalteu colorimetric method and adjusted by creatinine are a suitable marker of polyphenol intake. As a result, the increase of total

polyphenols in urine was objectively related with a decrease in blood pressure and an increase of NO production in plasma.

✓ Moderate wine consumption was associated with a decreased risk of metabolic syndrome, particularly a decrease in abdominal obesity, blood pressure and fasting plasma glucose and higher HDL cholesterol.

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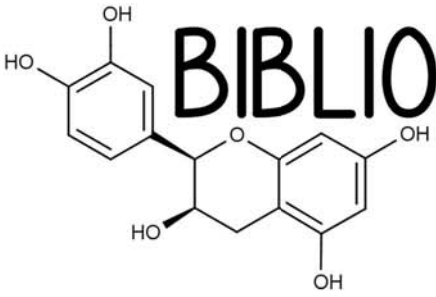

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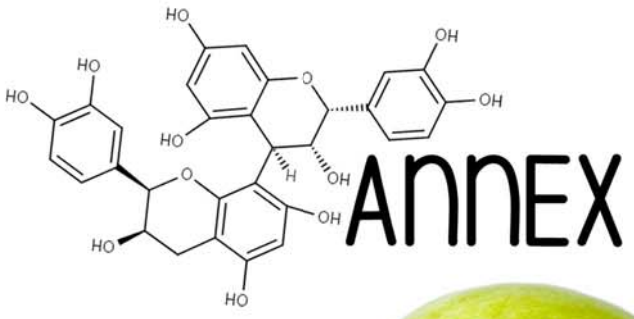

ANNEX

A. Altres publicacions en revistes/Other research articles

En aquest apartat s'hi inclouen altres publicacions en les quals també he col·laborat però no s'han inclòs en el treball de la tesi doctoral.

A.1. Publicació 8. Caracterització del perfil fenòlic del raïm Albariño mitjançant espectometria de masses

Article 8. Characterization of the phenolic profile of Albariño grapes using mass spectrometry

Giuseppe Di Lecce, Sara Arranz, Olga Jáuregui, Anna Tresserra-Rimbau, Paola Quifer-Rada, i Rosa M. Lamuela-Raventós. Food Chemistry. **2014**, 145: 874-82.

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Analytical Methods

Phenolic profiling of the skin, pulp and seeds of Albariño grapes using hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry

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ABSTRACT

This paper describes for the first time a complete characterisation of the phenolic compounds in different anatomical parts of the Albariño grape. The application of high-performance liquid chromatography coupled with two complementary techniques, hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry, allowed the phenolic composition of the Albariño grape to be unambiguously identified and quantified. A more complete phenolic profile was obtained by product ion and precursor ion scans, while a neutral loss scan at 152 u enabled a fast screening of procyanidin dimers, trimers and their galloylated derivatives. The compounds were confirmed by accurate mass measurements in QqToF-MS and QqToF-MS/MS modes at high resolution, and good fits were obtained for all investigated ions, with errors ranging from 0.2 to 4.5 mDa. To the best of our knowledge, two flavanol monomer hexosides were detected in the grape berry for the first time.

1. Introduction

Albariño (*Vitis vinifera* L.) is the most important white grape variety grown in the northwest of Spain (Galicia), notably in the Rías Baixas Denomination of Origin. Although there are five different varieties of grape cultived in this region, Albariño grape provide 95% of the annual harvest obtaining a total of 40 million kg of grapes to produce 286,000 hectoliters of wine annually. Albariño wine is characterised by an appreciated aromatic profile and organoleptic properties (Diéguez, Lois, Gómez, & de la Peña, 2003; Vilanova, Genisheva, Masa, & Oliveira, 2010). Masa, Vilanova, and Pomar (2007) and Rodríguez-Bernaldo de Quirós, Lage-Yusty, and López-Hernández (2009) determined the flavonoid profile of Albariño grape skin and the antioxidant activity of Albariño wines by high performance liquid chromatography (HPLC) (Masa et al., 2007; Rodríguez-Bernaldo de Quirós et al., 2009).

Phenolic compounds are responsible for the colour, astringency and bitterness of wines and it has been demonstrated that the sensory perception of coarseness increases with the degree of galloylation of proantocyanidins (Vidal et al., 2003).

Grape phenols consist of a wide range of structures diversely distributed in every part of the berry (Adam, 2006), but they are present mainly in the skin and seed (Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006; Dietmar, Achim, Reinhold, & Schieber, 2004).

The most abundant phenolic compounds in white grape skin are flavonols, while flavan-3-ol monomers such as (+)-catechin and (−)-epicatechin, as well as dimers, trimers and polymeric forms, also called procyanidins (2–10 units), are present mainly in grape seed. These compounds may contain subunits of gallic acid, epigallocatechin or epicatechin gallate linked by an interflavan bond (Hayasaka, Waters, Cheynier, Herderich, & Vidal, 2003).

In last two decades liquid chromatography mass spectrometry has been widely employed for the characterisation of several food matrices (Justesen, Knuthsen, & Leth, 1998; Zhou, Xu, & Choi, 2009). Electrospray ionisation has proven to be a powerful tool that facilitates the analysis of non-volatile, thermally labile compounds. Different mass analysers, triple-quadrupole instruments (Sánchez-Rabaneda et al., 2004; Cavaliere et al., 2008), ion-trap mass analysers, and high-resolution instruments such as time-of-flight (or the hybrid configuration quadrupole-time-of-flight, Vallverdú-Queralt,

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Jáuregui, Di Lecce, Andrés-Lacueva & Lamuela-Raventós, 2011b) and Fourier trasformation mass spectrometry (FTMS, Vallverdu-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010) have been used for chemical characterisation of food matrices.

Specifically, polyphenol composition in food has been analysed using triple quadrupole instruments, applying MS/MS techniques such as product ion scan (PIS), precursor ion scan (PrI), and neutral loss scan (NL) (Sánchez-Rabaneda et al., 2004; Vallverdu-Queralt et al., 2010). A quadrupole instrument in full scan mode shows a poor signal-to-noise-ratio (if compared with the ratio of a high resolution instrument) but MS/MS techniques such as PrI or NL allow polyphenol families to be screened. In addition, hybrid high-resolution instruments such as Qq-ToF and IT-FTMS can produce high-quality MS/MS spectra, including high-resolution data for the determination of molecular formulae. Both MS and MS/MS experiments can be performed for high accuracy and high resolution analysis.

As far as we know, recent studies of the phenolic profile of the Albariño *cultivar* have only described the flavonoid composition. The aim of this paper is to report the first study on the qualitative and quantitative characterisation of phenolics in the different anatomical parts of the Albariño grape using two complementary QqToF and QqQ instruments to determine structures based on fragmentation patterns.

2. Materials and methods

2.1. Chemicals

The standards were handled without exposure to light. Vanillic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, *trans*-caffeic acid, *trans*-ferulic acid, protocatechuic acid, *m*-, *o*- and *p*-coumaric acids, gallic acid, homovanillic acid, (+)-catechin, (−)-epicatechin, *trans*-resveratrol, *trans*-piceid, kaempferol, myricetin, apigenin, (−)-epigallocatechin, (+)-catechin-gallate, quercetin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside and L-tryptophan were purchased from Sigma–Aldrich (St Louis, MO, USA). Kaempferol-3-*O*-glucoside, procyanidin dimers A2, B1 and B2, trimer C1, (−)-epicatechin-gallate, ethyl gallate, catechin-gallate and tyrosol were purchased from Extrasynthèese (Genay, France). *cis*-Resveratrol and *cis*-piceid were obtained after exposure of the *trans*-isomer standards to UV light (Romero-Pérez, Ibern-Gómes, Lamuela-Raventós, & de la Torre-Boronat, 1999), whereas *trans*-caftaric and *trans*-coutaric acids were isolated from grapes (Vrhovšek, 1998). HPLC-grade acetonitrile and acetic acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain), while ultrapure water was obtained from a Millipore system (Millipore, Bedford, MA, USA).

2.2. Extraction procedure

Albariño grape berries were harvested at the Miguel Torres winery in Vilafranca del Penedès (Barcelona, Spain). The samples were collected at 22 ± 0.6 Brix and immediately frozen (−20 °C) until analysis. Sample extraction was performed in a dark room with a red safety light to prevent oxidation of the analytes during the process. Frozen grapes were manually separated into skin (0.5 g), pulp (5 g) and seeds (0.5 g). The extraction procedure, for the three fractions, was carried out under low temperature, as previously described by our research group (Vallverdú-Queralt et al., 2011a), using 5 mL of ethanol/water 80:20 (v/v) at pH 3.5 (acetic acid). The homogenates, obtained by an Ultra Turrax (IKA, Staufen, Germany), were centrifuged (2500*g*, 20 min at 4 °C), and the supernatants were collected; the extraction procedure was repeated two times. Both fractions were combined and the ethanolic portion was evaporated with a sample concentrator (Techne, Duxford, Cambridge, UK) at room temperature under a stream of nitrogen gas. After filtration of the aqueous extracts with 0.45 μm PTFE syringe filters (Waters Corporation, Massachusetts, USA), the samples were stored at −20 °C and then injected into the HPLC-UV-QqToF and QqQ systems.

2.3. HPLC-UV and mass spectrometry conditions

The chromatography was performed with an HPLC Agilent 1200 RRLC (Santa Clara, CA, USA), using a Nucleosil 120 C18 column (250 mm × 4 mm, 5 μm particle size, Teknokroma, Barcelona, Spain). A constant flow rate of 0.8 mL min^{-1} was used with two solvents: solvent A consisted of water with 0.8% acetic acid (pH 2.65), and solvent B was 20% solvent A mixed with 80% acetonitrile; injection volume was 20 μL. The column was kept at 40 °C and the separation of phenolic compounds was carried out in 45 min under the following conditions: 0 min, 100% A; 5 min, 98% A; 10 min, 96% A; 15 min, 90% A; 30 min, 80% A; 35 min, 70% A; 40 min 0% A and 45 min, 100% A (Betés-Saura, Andrés-Lacueva, & Lamuela-Raventós, 1996). The column was equilibrated for 5 min prior to each analysis. The chromatograms were monitored, with UV detector Agilent SL Plus, at three wavelengths: 280, 320, and 365 nm. Each wavelength was suitable for each group of compounds: 280 nm was used for hydroxybenzoic acids, flavan-3-ols and the oligomeric procyanidins, 320 nm for hydroxycinnamic acids and their tartaric esters, and 365 nm for flavonols.

Individual compounds were quantified using a calibration curve of the corresponding standard compound. When reference compounds were not available, the calibration of structurally related substances was used. All analysis were performed in triplicate.

2.3.1. QqToF analysis

The HPLC system was coupled on-line to a hybrid quadrupole time-of-flight QSTAR Elite (ABSciex, Concord, Ontario, Canada). The MS acquisition was performed using negative ionisation between *m/z* 100 and 1050 with the Turbo Ionspray source. In addition, QqToF was used to obtain product ion information. The MS parameters were: ion spray voltage, −4200; declustering potential (DP), −60; focusing potential (FP), 190; declustering potential two (DP2), 15; ion release delay (IRD), 6 V; ion release width (IRW), 5 ms; nebulizer gas, 50 (arbitrary units), curtain gas, 60 (arbitrary units), and auxiliary gas N_2, 6000 cm^3 min^{-1} heated at 400 °C. The QqToF-MS instrument was calibrated after every three samples injected using two external reference compounds at *m/z* 112.9854 (CF_3COO^-) and *m/z* 1033.9880 ($P_3N_3(OCH_2(CF_2)CF_2H)_6$-$CF_3COO^-$), respectively (1 pmol μL^{-1}, ESI Tuning Mix Agilent solution). The MS/MS acquisition was also performed using information-dependent acquisition (IDA) between *m/z* 100 and 1050. IDA experiments were done at a fixed collision energy of 30 V and modified if no-fragmentation (or excessive) was produced. Acquisition and analysis of data were performed with Analyst QS 2.0 software (ABSciex, Concord, Ontario, Canada).

2.3.2. QqQ analysis

An API 3000 triple quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) equipped with a Turbo Ionspray source in negative-ion mode was used to obtain product ion and neutral loss information. Turbo Ionspray source settings were as follows: ion spray voltage, −3500 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 4 (arbitrary units); focusing potential, −200 V; entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 mL min^{-1}. The DP and collision energy (CE) were optimised for (+)-catechin (DP −50 and CE −25 V), procyanidin B1 (DP −50

and CE −35 V), *trans*-caffeic acid (DP −40 and CE −20 V), and quercetin-3-*O*-glucoside (DP −60 and CE −30 V) in infusion experiments. Individual standard solutions (10 μg mL^{-1}) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 μl min^{-1}, using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Data acquisition was performed scanning from *m/z* 100 to 1050 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In NL experiment, loss of 162 u corresponds to the loss of a glucose or galactose, while loss of 152 u, derived from the product of Retro-Diels–Alder rearrangement, correspond to dimer and trimer procyanidins as well as flavanol galloyl derivatives. Neutral loss experiments at 162 u and 152 u, were performed by scanning within the range of 300–600 u and from 250–900 u, respectively.

3. Results and discussion

3.1. HPLC–ESI-QqToF-MS and HPLC–ESI-QqQ-MS for the determination of phenolic compounds in grape skin, pulp and seed extracts

Phenolic extracts of skin, pulp and seed of Albariño grapes were analysed with two complementary QqToF and QqQ instruments to determine structures based on fragmentation patterns, using QqToF in PIS mode and QqQ in PrI and NL mode. In addition, information-dependent acquisition (IDA) by QqToF was used to generate a peak list of ions present in the spectrum at the time of analysis; this peak list was subjected to a series of user-defined criteria to select precursor ions of interest based on filters such as intensity threshold, charge state, isotope pattern and others. In general, we observed the deprotonated molecule $[M-H]^-$ and its characteristic product ions by MS/MS experiments. The 43 compounds are depicted in Fig. 1 and listed in Table 1 along with their retention time, molecular formulae and mDa of error between the experimental mass and the theoretical mass of each phenol investigated.

Thus, bearing in mind the importance of phenolic compounds as taxonomical markers (Vallverdu-Queralt et al., 2010), a precise characterisation of Albariño grape was obtained.

3.1.1. Hydroxybenzoic acid and its derivatives

Gallic acid (*m/z* 169) was the first compound to elute in skin and seed extract chromatograms. The product ion scan of the deprotonated molecule $[M-H]^-$ showed the typical loss of CO_2, giving an ion at *m/z* 125 $[M-H-44]^-$ as the characteristic fragment. This compound was confirmed by comparison with the calculated mass error (0.9 mDa) and reference compound.

LC-QqToF-MS analysis of seeds showed ions at *m/z* 331 and 315 (peaks 2, 14 and 4, 10, respectively), which were tentatively identified as deprotonated molecules of isomers of gallic acid hexose and protocatechuic acid hexose, respectively. Product ion scan of both ions showed the loss of hexose $[M-H-162]^-$, followed by the loss of CO_2 $[M-H-162-44]^-$. It was not possible to differentiate between the isomers on the basis of fragments and relative intensities in MS/MS spectra in PIS mode (Table 1).

Gallic acid dihexose (*m/z* 493) was also tentatively identified in seed and skin extracts: the PIS of the deprotonated molecule (*m/z* 493) showed two ions at *m/z* 331 and 169 derived from the loss of one and two hexose units, respectively. Furthermore, a very low mass error (0.3 mDa) was obtained with the QqToF instrument. The identification hypothesis was strengthened by the information obtained with the QqQ instrument, through neutral loss scan of 162 u and precursor ion scan of *m/z* 169.

3.1.2. Hydroxycinnamic acid and its derivatives

The skin extract revealed the presence of *p*-coumaric acid (*m/z* 163), which was corroborated by product ion scan experiment showing a predominant ion at *m/z* 119 (loss of CO_2). The presence of coumaric acid hexose (*m/z* 325) was also detected in skin and pulp. The PIS of this ion showed a characteristic fragmentation involving cleavage of the intact sugar $[M-H-162]^-$ (*m/z* 163), and an ion corresponding to the loss of a methyl (*m/z* 148) and CO_2 from aglycone (*m/z* 119). Peak identification was accomplished by comparing MS/MS fragmentation with reported data obtained by LC-ESI-MS in negative mode (Vallverdu-Queralt et al., 2010). NL of 162 u and PrI of *m/z* 163 by QqQ were useful for providing an unequivocal identification of hydroxycinnamic hexose.

3.1.3. Hydroxycinnamic tartaric esters

The skin and pulp extract chromatograms showed two ions at *m/z* 311 (peaks 7 and 8), two ions at *m/z* 295 (peaks 15 and 17), and one ion at *m/z* 325 (peak 21). These deprotonated molecules $[M-H]^-$ were tentatively identified as hydroxycinnamic acid tartaric esters. The ions at *m/z* 311 were identified as *cis* and *trans*-caffeoyl tartaric acid (caftaric acid); their PIS revealed a fragment at *m/z* 179 corresponding to caffeic acid, after the cleavage of the ester bond, and a low intensity signal at *m/z* 135 was ascribed to decarboxylated caffeic acid. MS/MS data were corroborated by comparison with reference compounds isolated from grape pomace (Vrhovšek, 1998). The ions at *m/z* 295 showed a fragmentation pattern similar to caftaric acid and were identified as *cis* and *trans*-coumaroil tartaric acid (coutaric acid) after comparison with the reference compound. The PIS produced only one ion fragment at *m/z* 163, which was ascribed to coumaric acid. In contrast, the deprotonated molecule at *m/z* 325 showed a fragment at *m/z* 193 attributed to ferulic acid, and an ion at *m/z* 149 that indicated a loss of CO_2 from the free ferulic acid. It was not possible identify the isomeric configuration of feruloyl tartaric acid, otherwise known as fertaric acid. The PrI at *m/z* 179, 163 and 193 confirmed the presence of the described peaks. Only *cis* and *trans*-caftaric acid and fertaric acid were found in the pulp extract. Among the aforementioned compounds, *trans*-caftaric acid is considered a major substance for coupled oxidation and enzymatic browning reactions in grape processing (Kroon & Williamson, 1999).

3.1.4. Flavan-3-ols

Reverse phase HPLC procedures provided a good baseline resolution for the flavan-3-ols, which consisted of (+)-catechin, (−)-epicatechin, their condensed product and corresponding galloylated derivatives that exhibited monomeric, dimeric and trimeric degrees of polymerisation. When the degree of polymerisation increased, the procyanidins were eluted as a single peak at the end of the chromatogram (Fig. 1). The resolved procyanidins present in Albariño seed, skin and pulp are mainly dimers (*m/z* 577) and trimers (*m/z* 865) in which the elemental units are linked by C4-C8 interflavan bonds (B-type). Structural variations in procyanidin oligomers may also occur with the formation of a second interflavanoid bond by C–O oxidative coupling to form A-type oligomers. Due to the complexity of this conversion, A-type procyanidins are not as frequently encountered in nature as B-type oligomers. Procyanidin A-type linkage shows a different fragmentation pathway than B-type linkage (Flamini, 2003), and in Albariño grapes no procyanidin A-type linkages were observed.

Peaks 23 and 28 (*m/z* 289) were identified as (+)-catechin and (−)-epicatechin, respectively, after comparison with the authentic standard. Both flavan-3-ols were identified in the three different fractions, with mass errors below 0.7 mDa. Up to four procyanidin dimers (*m/z* 577, peaks 19, 20, 26 and 27) were identified in the seed extract. The PIS at *m/z* 577 showed a Retro-Diels–Alder

(RDA) product with a neutral loss of 152 u $[M-H-152]^-$ followed by loss of a water molecule $[M-H-152-H_2O]^-$. Other fragments at *m/z* 289 and 245, derived from the interflavanic bond cleavage, were also observed (Table 1). Procyanidins B1 and B2 were corroborated by reference compounds, while the elution order of dimers B3 and B4 were assigned referring to the study by Monagas, Suárez, Gómez-Cordovés, and Bartolomé (2005).

LC-QqToF-MS analysis of the seed extract revealed four peaks (6, 24, 25 and 30) at *m/z* 865. Product ion scan showed a base peak at *m/z* 289 and two minor ions at *m/z* 577 and 425, which were also observed for the reference procyanidin C1. Additionally, an ion at *m/z* 695 was registered due to the RDA and successive loss of water $[M-H-152-H_2O]^-$. Peaks 24, 25 and 30 were tentatively identified as procyanidin trimer isomers, but complete identification was not possible without standards.

Thus, on the basis of information obtained by PIS, when a NL experiment of 152 u was conducted by QqQ, the total ion current showed deprotonated molecules belonging to dimer (*m/z* 577) and trimer procyanidins (*m/z* 865, Fig. 3). Various studies of oligomeric and polymeric procyanidins in grape seed extracts have proposed a fragmentation scheme of ions derived from B-type procyanidins (Zhao, Pang, & Dixon, 2010; Sun & Miller, 2003; Gu et al., 2003).

Waterhouse, Ignelzi, and Shirley (2000) demonstrated that in grape seeds, the single unresolved peak, at the end of the chromatogram, corresponds to a mixture of high molecular mass procyanidin polymers.

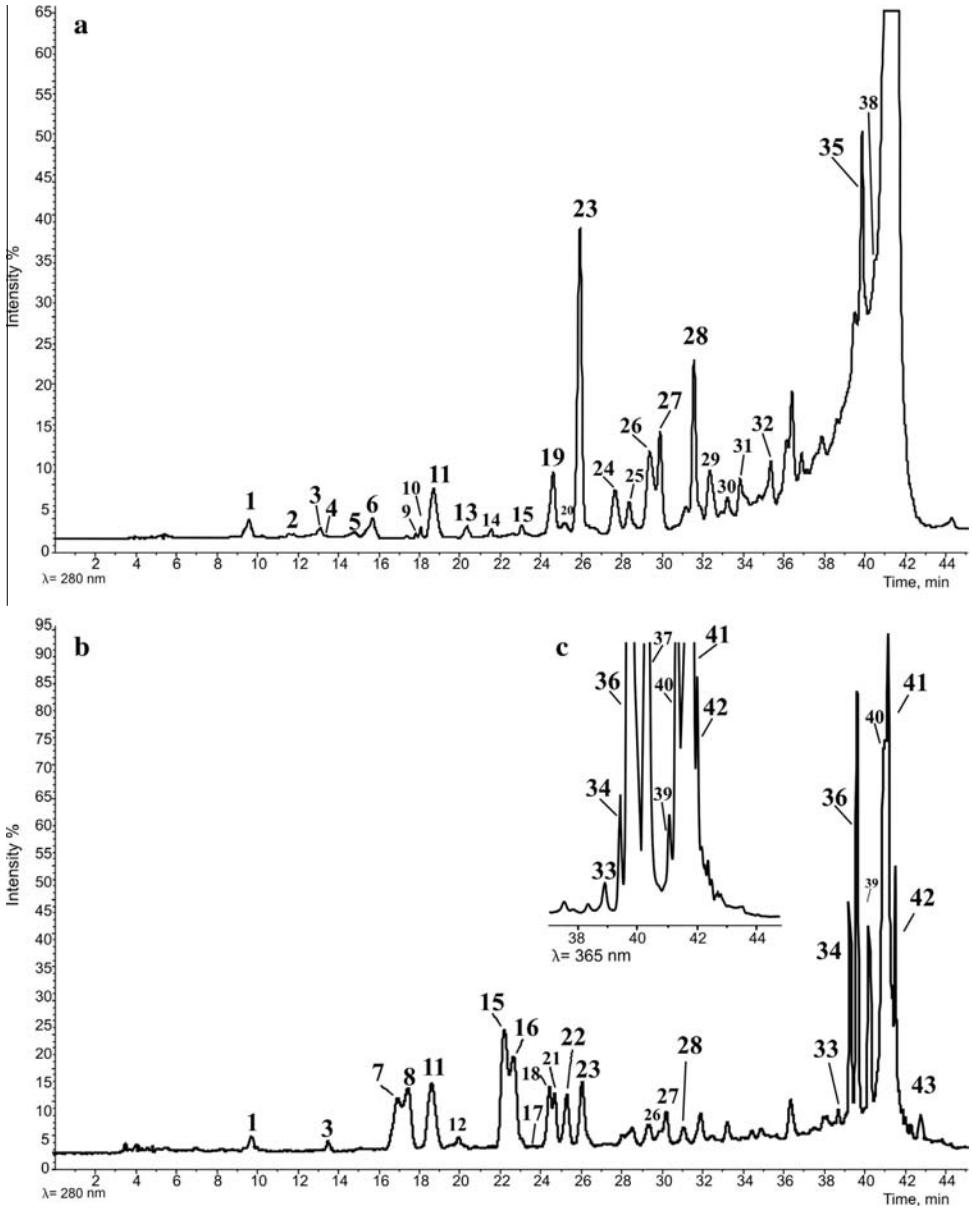

Fig. 1. HPLC-DAD chromatograms at $\lambda = 280$ nm of phenolic compounds identified in seed (a) and skin (b) of Albariño grape. (c) Flavonol profile ($\lambda = 365$ nm). Peak identification is shown in Table 1.

Table 1
List of compounds identified in three fractions of Albariño grape.

Peaks	Retention time	Compounds	Fractions	[M-H]$^-$	Fragments *m/z* (% intensities)	MS/MS experiments			Theoretical mass	Mass difference (mDa)[a]	Formula
						PIS (Qq-ToF)	NL	PrI (QqQ)			
1	9.35	Gallic acid*	se, sk	169.0151	169 (100), 125 (25)	169			169.0142	0.9	$C_7H_6O_5$
2	11.82	Gallic acid hexose I	se	331.0700	331 (5), 169 (70), 125 (100)	331	162	169	331.0670	3.0	$C_{13}H_{16}O_{10}$
3	13.08	Gallic acid dihexose	se, sk	493.1195	493 (9), 331 (100), 169 (100)	493		169	493.1198	−0.3	$C_{19}H_{26}O_{15}$
4	13.21	Protocatechuic acid-*O*-hexoside	se	315.0723	153 (100), 109 (40)	315	162	153	315.0721	0.2	$C_{13}H_{16}O_9$
5	14.76	(epi)gallocatechin-(epi)catechin	se	593.1332	423 (78), 305 (100), 289 (28)	593			593.1300	3.2	$C_{30}H_{26}O_{13}$
6	15.72	Procyanidin trimer C1	se	865.1950	865 (37), 695 (100), 577 (1), 407 (64), 289 (42)	865	152	289	865.1985	−3.5	$C_{45}H_{38}O_{18}$
7	16.90	*cis*-caftaric acid	sk, pu	311.0413	179 (100), 135 (54)	311		179	311.0418	0.5	$C_{13}H_{12}O_9$
8	17.49	*trans*-caftaric acid*	sk, pu	311.0411	179 (100), 135 (40)	311		179	311.0414	0.3	$C_{13}H_{12}O_9$
9	17.84	(epi)gallocatechin-3-gallate	se	457.0783	305 (100), 169 (65)	457			457.0790	0.7	$C_{22}H_{18}O_{11}$
10	18.01	Protocatechuic acid-*O*-hexoside	se	315.0751	153 (100), 109 (40)	315	162	153	315.0781	3.0	$C_{13}H_{16}O_9$
11	18.64	L-tryptophan*	se, sk, pu	203.0847	142 (9), 116 (100)	203			203.0868	2.1	$C_{11}H_{12}N_2O_2$
12	19.82	Epigallocatechin*	sk	305.0699	261 (100), 221 (25), 179 (34)	305			305.0732	3.3	$C_{15}H_{14}O_7$
13	20.23	(epi)catechin-hexose	se	451.1266	289 (100), 245 (40)	451	162	289	451.1287	2.1	$C_{21}H_{24}O_{11}$
14	21.56	Gallic acid hexose II	se	331.0700	331 (12), 169 (100), 125 (84)	331	162	169	331.0670	3.0	$C_{13}H_{16}O_{10}$
15	22.35	*cis*-coutaric acid	sk	295.0469	163 (32), 119 (100)	295		163	295.0479	1.0	$C_{13}H_{12}O_8$
16	22.83	(epi)catechin-hexose	se	451.1266	289 (100), 245 (54)	451	162	289	451.1287	2.1	$C_{21}H_{24}O_{11}$
17	22.91	*trans*-coutaric acid	sk	295.0460	163 (53), 119 (100)	295		163	295.0461	0.1	$C_{13}H_{12}O_8$
18	23.34	Coumaric acid-*O*-hexoside	sk, pu	325.0919	163 (50), 148 (30), 119 (100)	325	162	163	325.0928	0.9	$C_{15}H_{18}O_8$
19	24.51	Procyanidin B3	se, pu	577.1331	407 (75), 289 (81), 245 (67)	577	152	289	577.1311	−2.0	$C_{30}H_{26}O_{12}$
20	24.75	Procyanidin B1*	se	577.1334	407 (75), 289 (70), 245 (45)	577	152	289	577.1317	−1.7	$C_{30}H_{26}O_{12}$
21	24.45	Fertaric acid	sk, pu	325.0585	193 (100), 149 (30)	325		193	325.0605	2.0	$C_{14}H_{14}O_9$
22	24.82	*p*-Coumaric acid*	sk	163.0418	163 (20), 119 (100)	163			163.0436	1.8	$C_9H_8O_3$
23	25.93	(+)-Catechin*	se, sk, pu	289.0710	245(100), 205 (65)	289			289.0703	−0.7	$C_{15}H_{14}O_6$
24	27.72	Procyanidin trimer I	se	865.1954	865 (55), 695 (80), 577 (68), 425 (88), 289 (81)	865	152	289	865.1923	−3.1	$C_{45}H_{38}O_{18}$
25	28.30	Procyanidin trimer II	se	865.1971	865 (28), 695 (19), 577 (33) 575 (21), 289 (100)	865	152	289	865.1957	−1.4	$C_{45}H_{38}O_{18}$
26	29.24	Procyanidin B4	se, sk	577.1332	407 (93), 289 (73), 245 (59)	577	152	289	577.1313	−1.9	$C_{30}H_{26}O_{12}$
27	29.85	Procyanidin B2*	se, sk	577.1331	407 (93), 289 (73), 245 (59)	577	152	289	577.1311	−2.0	$C_{30}H_{26}O_{12}$
28	31.58	(−)-Epicatechin*	se, sk, pu	289.0712	245 (100), 205 (60)	289			289.0707	−0.5	$C_{15}H_{14}O_6$
29	32.42	(epi)catechin-(epi)catechingallate I	se	729.1476	577 (26), 451 (34), 407 (96), 289 (100)	729		289	729.1491	1.5	$C_{37}H_{30}O_{16}$
30	33.26	Procyanidin trimer III	se	865.1959	865 (42), 695 (49), 577 (52), 407 (70), 289 (100)	865	152	289	865.1933	−2.6	$C_{45}H_{38}O_{18}$
31	33.81	(epi)catechin-(epi)catechingallate II	se	729.1472	577 (37), 407 (100), 289 (70)	729	152	289	729.1483	1.1	$C_{37}H_{30}O_{16}$
32	35.42	(epi)catechin-(epi)catechingallate III	se	729.1473	577 (43), 407 (100), 289 (94)	729	152		729.1485	1.2	$C_{37}H_{30}O_{16}$
33	38.76	Quercetin-3-*O*-rutinoside*	sk	609.1466	609 (67), 301 (100)	609	308	301	609.1471	0.5	$C_{27}H_{30}O_{16}$
34	39.30	Quercetin-3-*O*-glucuronide	sk	477.0636	301 (72), 151 (100),	477	176	301	477.0598	−3.8	$C_{21}H_{18}O_{13}$
35	39.44	(−)-Epicatechin-3-*O*-gallate	se	441.0866	289 (100), 271 (47), 169 (85), 125 (6)	441			441.0905	3.9	$C_{22}H_{18}O_{10}$
36	39.65	Quercetin-3-*O*-glucoside*	sk	463.0857	301 (67), 151 (100)	463	162	301	463.0833	−2.4	$C_{21}H_{20}O_{12}$
37	40.27	Dihydroquercetin-3-*O*-rhamnoside	sk	449.1109	303 (100), 151 (75)	449		303	449.1129	2.0	$C_{21}H_{22}O_{11}$
38	40.52	Dimer digallate	se	881.1975	881 (25), 729 (93), 407 (100)	881	152		881.2016	4.1	$C_{45}H_{38}O_{19}$
39	40.95	Quercetin-3-*O*-pentoside	sk	433.0731	301 (100), 151 (77)	433			433.0692	−3.9	$C_{20}H_{18}O_{11}$
40	41.21	Kaempferol-3-*O*-glucuronide	sk	461.0770	285 (100), 257 (18), 229 (25) 135 (8)	461	176		461.0815	4.5	$C_{21}H_{18}O_{12}$
41	41.40	Kaempferol-3-*O*-glucoside*	sk	447.0972	447(30), 285(100)	447	162	285	447.1012	4.0	$C_{21}H_{20}O_{11}$
42	41.56	*trans*-piceid*	sk	389.1246	227 (100), 185 (17), 143 (6)	389	162	227	389.1251	0.5	$C_{20}H_{22}O_8$
43	42.40	*trans*-resveratrol*	sk	227.0750	185 (13), 143 (100)	227			227.0786	3.6	$C_{14}H_{12}O_3$

* Comparison with standard. sk, skin; pu, pulp; se, seeds; PIS, product ion scan, NL, neutral loss; PrI, precursor ion scan; [M-H]$^-$ mass found.
[a] Obtained as theoretical mass − experimental mass.

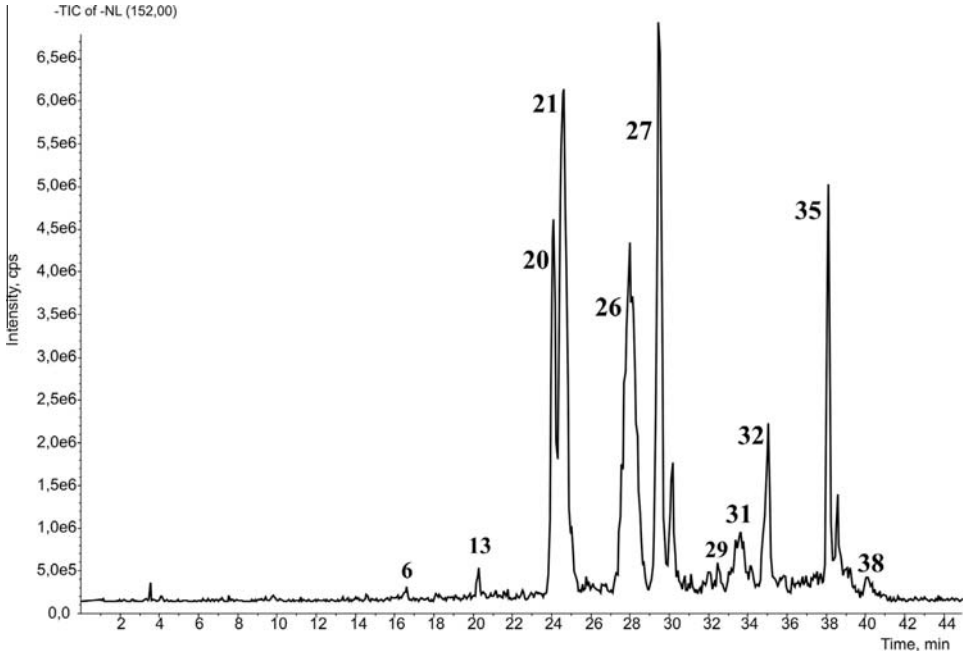

Fig. 2. TIC of seed extract in neutral loss scan mode of 152 u. Peak identification: 6, procyanidin trimer C1; 13, (epi)catechin-hexose; 19, 20, 26 and 27, procyanidin dimers; 29, 31 and 32, (epi)catechin-(epi)catechingallate; 35, (−)-epicatechin-3-*O*-gallate; 38, dimer digallate.

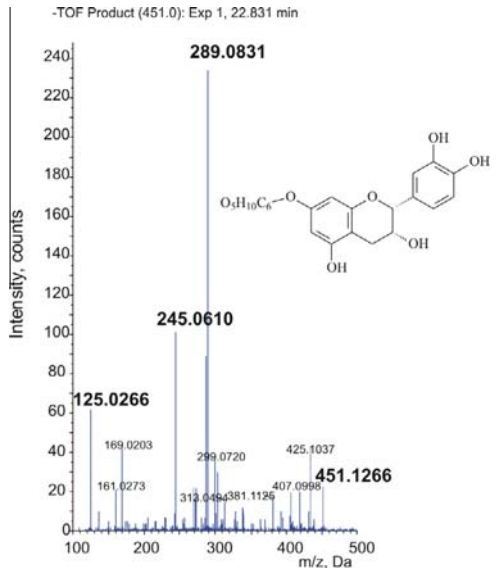

Fig. 3. The MS/MS product ion scan of *m/z* 451 (epi)catechin-hexose.

3.1.4.1. Flavanol hexosides. In Albariño seed, two flavan-3-ol hexoside isomers (peaks 13 and 16) at *m/z* 451 were tentatively identified as (+)-catechin or (−)-epicatechin-hexose. In PIS mode these flavanol hexosides showed a peak at *m/z* 289 due to the hexose moiety and the fragment at *m/z* 245 was attributed to (+)-catechin or (−)-epicatechin fragmentation (Fig. 2); the accurate mass measurements presented the same error for both compounds (2.1 mDa). The NL of 162 u and the PrI at *m/z* 289, in LC-ESI-QqQ-MS, confirmed the tentative identification of flavanol hexosides.

To the best of our knowledge, this is the first time that flavanol monomer hexosides have been detected in grapes, although they have been reported in other plants. Two research groups previously described the presence of flavanol hexose in barley (Wolfgang & Rudolf, 2002) and lentils (Dueñas, Sun, Hernández, Estrella, & Spranger, 2003).

Catechins are associated with health benefits, but they are unstable during storage, processing and during gut transit (Zho et al., 2002). However, recent evidence suggests that catechin-glucoside is more stable (between pH 4 and 8) than (+)-catechin. Raab et al., (2010) have shown that (+)-catechin-3'-*O*-β-D-glucopyranoside presents the greatest stability.

3.1.4.2. Flavan-3-ol galloylated derivatives. An ester derivative identified as (−)-epigallocatechin was found only in the skin. In fact the skin profile gave a peak at *m/z* 305, which represented a flavanolic unit; its confirmation was possible by comparing the chromatographic information with an authentic standard. As far as we know, flavan-3-ol galloylated derivatives have not been previously described in Albariño grape skin. Several monomeric and oligomeric flavanols linked to gallic acid were detected in the seed extract. Peak 9 showed an ion at *m/z* 457 which in PIS mode generated a preponderant fragment at *m/z* 305, probably produced by the loss of a galloyl group to epigallocatechin or gallocatechin. It was not possible to confirm the molecular structure of epigallocatechin-gallate or gallocatechin-gallate due to the lack of reference compounds. Three isomers of (epi)catechin-(epi)catechin-gallate, commonly known as dimer gallate (*m/z* 729), were also tentatively identified in seeds with mass errors below 1.5 mDa. The PIS of *m/z* 729 generated an ion at *m/z* 577 corresponding to the loss of gallic acid, while the more intense fragment at *m/z* 289 was due to the loss of (+)-catechin-gallate or a (−)-epicatechin-gallate unit. For the dimer gallates, the PrI by LC-ESI-QqQ-MS at *m/z* 289 showed peaks at *m/z* 729, thus providing further useful information for checking characteristic phenolic compounds of grape seed.

Another peak present only in the seed fraction at *m/z* 441 was identified as (−)-epicatechin-3-*O*-gallate as its retention time and mass spectra matched the standard. Moreover, the PIS showed two fragment ions resulting from the cleavage of the ester bond at *m/z* 289 for deprotonated (−)-epicatechin and at *m/z* 169 for a deprotonated gallic acid moiety. A prodelphynidin compound was also found in both seed and skin. LC-ESI-QqToF-MS analysis revealed the existence of a deprotonated molecule at *m/z* 593 and the PIS showed fragments at *m/z* 423, 305 and 289, which confirmed the presence of (epi)gallocatechin-(epi)catechin. In addition, another ion was detected in the seed extract at *m/z* 881, which was tentatively identified as (epi)catechin-(epi)gallocatechin-(epi)catechin or (epi)gallocatechin-(epi)catechin-(epi)catechin due to another (epi)catechin linked to the molecular structure. The PIS experiment suggests that the more abundant fragment was at *m/z* 729, which corresponds to the gallate unit moiety (Lazarus, Adamson, Hammerstone, & Schmitz, 1999). As depicted in Fig. 3, the total ion current of NL at 152 u could also be used for a fast screening of flavan-3-ol galloylated derivatives.

As described by Flamini (2003), dimer gallates were first identified in Niagara grapes. Other authors have confirmed that the grape seed phenolic profile is characterised by the presence of flavanol derivatives esterified with gallic acid and their occurrence can be considered typical of grape seeds (Santos-Buelga, Francis-Aricha, & Escribano-Bailón, 2005; Rodríguez Montealegre et al., 2006).

3.1.5. Flavonols

In the skin fraction three flavonol-*O*-hexosides (peaks 36, 37 and 41), two -*O*-glucuronides (peaks 34 and 40), one -*O*-rutinoside (peak 33) and one -*O*-pentoside (39) were plausibly identified (Fig. 1b and c). In this work, we found only -*O*-glycoside derivatives arising from cleavage of the glycosidic bond and loss of the sugar moieties (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2010). LC-ESI-QqToF-MS analysis of the skin phenolic extract revealed mass signals at *m/z* 609, 477 and 463, corresponding to deprotonated quercetin-3-*O*-rutinoside (also called rutin), quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucoside (quercitrin), for peaks 33, 34 and 36, respectively. The deprotonated molecule at *m/z* 609 in PIS mode showed an intense fragment at *m/z* 301 due to the rutinoside moiety, and a fragment at *m/z* 151 typical for quercetin. Similar MS^2 experiments have been done for quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucoside, showing the same fragment at *m/z* 301 for the glucuronide and glucoside moieties. The presence of the three quercetin derivatives was also checked by PrI at *m/z* 301 and by matching the retention time and mass spectra with data of available standard compounds. The presence of another quercetin derivative was detected by LC-ESI-QqToF-MS which showed a deprotonated molecular $[M\text{-}H]^-$ at *m/z* 433 and the PIS showed a fragment at *m/z* 301. In this case, NL and PrI experiments did not yield any information due to the low intensity of the peak. This compound was tentatively assigned to quercetin-3-*O*-pentose and, as far as we know, this is the first time that this phenolic compound has been reported in Albariño grape skin. The chromatogram of the skin extract in QqToF-MS also showed a deprotonated molecule at *m/z* 449 with an error of 2.0 mDa. The PIS of *m/z* 449 gave a product ion at *m/z* 303, which suggested the probable presence of diihydroquercetin-3-*O*-rhamnoside, as described by Masa and Vilanova (2008) for Albariño skin. Analysis of the skin phenolic profile also revealed the presence of two ions at *m/z* 461 and 447 corresponding to kaempferol-3-*O*-glucuronide and kaempferol-3-*O*-glucoside, respectively. The PIS in LC-ESI-QqToF-MS bore out these results, in both cases showing a preponderant fragment at *m/z* 285. Additionally, as described above, NL and PrI experiments characterised kaempferol-3-*O*-glucuronide and kaempferol-3-*O*-glucoside, which were corroborated by comparison with the standard compounds (Table 1).

Flavonoid-*C*-glycosides, which show a different fragmentation pattern from -*O*-glycosides (Sanchez-Rabaneda et al., 2003; Han et al., 2008), were not detected in the Albariño grape.

3.1.6. Stilbenes

Stilbenes were eluted in the final part of the Albariño skin chromatogram. Peaks 42 and 43 showed deprotonated molecules at *m/z* 389 and 227 $[M\text{-}H]^-$, and were identified as *trans*-piceid and *trans*-resveratrol, respectively. Both stilbenes were corroborated by comparison with the reference compounds. The presence of resveratrol and its glucoside in red as well as white grapes has been ascribed to ultraviolet irradiation or stress, especially plant interaction with pathogens (Romero-Pérez, Lamuela-Raventós, Andreés-Lacueva, & de la Torre-Boronat, 2001).

Furthermore, as reported by Lamuela-Raventos, Romero-Pérez, Waterhouse and de la Torre-Boronat, (1995), *trans* isomers are transformed to the *cis* forms when grapes are exposed to UV radiation. Probably due to the extraction procedure, which was performed in a dark room with a red safety light, we did not detect cis isomer forms in Albariño skin. Stilbenes have been extensively studied as critical contributors to the health benefits of grapes and wine (Lamuela-Raventós & Waterhouse, 1999).

3.2. A nitrogen compound with phenolic structure: L-tryptophan

A nitrogen compound identified as L-tryptophan (peak 11) was found in all the anatomical parts of Albariño grapes. In LC-ESI-QqToF-MS, this peak showed an ion at *m/z* 203 and the PIS revealed two ions at *m/z* 116 and 142. The compound identity was confirmed by comparing its mass spectra with those of an authentic standard. L-tryptophan can be present in white must and is ascribed to the metabolic pathway of 2-aminoacetophenone, a causal agent of an 'untypical ageing off-flavour' in wine. In another study, Mattivi, Vrhovšek, and Versini, (1999) found levels of L-tryptophan in Chardonnay musts and wines ranging between 62 and 417 μg L^{-1}. (Mattivi et al., 1999).

3.3. Quantification of phenolic compounds found in skin, pulp and seed

The most abundant class of phenols found in Albariño grape berries were the monomeric and oligomeric form of flavan-3-ols, which were present in hypodermal layers of skin and in the soft parenchyma of the seed. The total content of flavanols was 611 mg $*$ 100 g^{-1} of fresh matter (Table 2), while the compounds at the highest concentration were (+)-catechin and (−)-epicatechin.

Albariño grape skin exhibited a predominance of flavanols and flavonols but a considerable amount of hydroxycinnamates was also found. The major hydroxycinnamic acid present was *cis*-coutaric acid (see Table 2), followed by caftaric isomers. Flavonols were always found in glycoside form, principally as 3-glucosides; small amounts of rutinoside and glucuronide flavonols were also detected. The content of flavonols ranged between 0.39 and 12.4 mg $*$ 100 g^{-1} of fresh matter for quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside, respectively. As reported by Downey, Harvey, and Robinson (2004), the flavonol content cannot be considered as characteristic of a grape *cultivar* because the flavonol concentration is strongly affected by the degree of illumination of the grape cluster (Downey et al., 2004).

The phenolic content in the pulp was very low. Hydroxycinnamic acids were the most representative compounds, with a total content of about 1.63 mg $*$ 100 g^{-1} of fresh matter. Small amounts of catechin, epicatechin and procyanidin B3 were also found.

Table 2
Content of phenolic compounds in the different anatomical parts of Albariño grape (mg * 100 g^{-1} of fresh matter). Mean values (±standard errors); nd, not detected.

Compounds	Skin	Pulp	Seed
Gallic acid	1.19	nd	1.92
Gallic acid hexose I	nd	nd	0.79
Gallic acid dihexose	1.13	nd	1.25
Protocatechuic acid-*O*-hexoside	nd	nd	0.45
Gallic acid hexose II	nd	nd	1.36
Protocatechuic acid-*O*-hexoside	nd	nd	1.54
Hydroxybenzoic acids	2.32 ± 0.1	nd	7.31 ± 0.3
cis-caftaric acid	2.53	0.11	nd
trans-caftaric acid	4.04	0.37	nd
cis-coutaric acid	6.23	nd	nd
trans-coutaric acid	0.27	nd	nd
Coumaric acid-*O*-hexoside	2.27	1.03	nd
Fertaric acid	1.68	0.12	nd
p-coumaric acid	1.96	nd	nd
Hydroxycinnamic acids	18.98 ± 0.8	1.63 ± 0.1	nd
(epi)gallocatechin-(epi)catechin	nd	nd	3.58
Procyanidin trimer C1	nd	nd	12.65
(epi)gallocatechin-3-gallate	nd	nd	1.54
Epigallocatechin	2.09	nd	nd
(epi)catechin-3-hexose	nd	nd	1.49
(epi)catechin-3-hexose	nd	nd	3.52
Procyanidin B3	nd	0.57	44.65
Procyanidin B1	nd	nd	3.09
(+)-catechin	11.45	0.55	106.5
Procyanidin trimer I	nd	nd	31.43
Procyanidin trimer II	nd	nd	18.54
Procyanidin B4	8.04	nd	58.39
Procyanidin B2	8.65	nd	64.53
(−)-epicatechin	2.67	0.23	77.51
(epi)catechin-(epi)catechingallate I	nd	nd	26.76
Procyanidin trimer III	nd	nd	13.54
(epi)catechin-(epi)catechingallate II	nd	nd	23.73
(epi)catechin-(epi)catechingallate III	nd	nd	21.43
(−)-epicatechin-3-*O*-gallate	nd	nd	76.54
Dimer digallate	nd	nd	21.43
Flavanols	32.9 ± 2.7	1.35 ± 0.1	610.8 ± 35.8
Quercetin-3-*O*-rutinoside	0.42	nd	nd
Quercetin-3-*O*-glucuronide	0.98	nd	nd
Quercetin-3-*O*-glucoside	12.43	nd	nd
dihydroquercetin-3-*O*-rhamnoside	5.65	nd	nd
Quercetin-3-*O*-pentoside	0.23	nd	nd
Kaempferol-3-*O*-glucuronide	3.21	nd	nd
Kaempferol-3-*O*-glucoside	8.43	nd	nd
flavonols	31.45 ± 1.6	nd	nd
trans-piceid	6.93	nd	nd
trans-resveratrol	1.43	nd	nd
Stilbenes	8.36 ± 0.4	nd	nd
Total	94.21 ± 5.1	2.98 ± 0.2	618.1 ± 36.1

As described by other authors, the concentration of phenolics and their profile in grapes, depends on the grapevine variety as well as intrinsic factors such as genetics and extrinsic aspects linked to viticulture and the environment. The degree of ripeness and berry size are also influential (Rodríguez Montealegre et al., 2006). In accordance with previous papers, the results presented in this study demonstrate that grape berries generally present a very high polyphenolic content, which contributes to their value as an agricultural crop (Rodríguez Montealegre et al., 2006; Dietmar et al., 2004).

4. Conclusions

Using a combination of spectrometric techniques we were able to identify up to 43 compounds, two of which, (+)-catechin or (−)-epicatechin hexosides, as far as we know, have never been reported before in the grape berry. The QqToF-MS was very useful for its combination of high sensitivity, high resolution and high mass accuracy, also allowing the characterisation of deprotonated molecules from PIS experiments in HRMS mode. Good fits were obtained for all investigated ions, with errors ranging from 0.2 to 4.5 mDa. The QqQ system was effective for obtaining information about the phenolic composition of grapes through NL and PrI experiments that allowed a first screening of families of compounds. In particular, an NL of 152 u was found to be helpful for a rapid screening of procyanidin dimers and trimers and gallate flavanols.

A wide range of phenolic compounds was found diversely distributed in every part of the Albariño grape berries but mainly in the skin and seed. Thus, this investigation resulted in an exhaustive characterisation of the phenolic profile of the different anatomical parts of the Albariño grape, and provides useful information for selecting suitable by-products for the extraction of potential health-promoting compounds.

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B. Capítols de llibre/Book chapters

En aquest apartat s'hi inclouen tres capítols de llibre en els quals he participat.

B.1. Capítol de llibre 1. Els polifenols del cafè i els paràmetres de risc cardiovascular

Book chapter 1. Coffee polyphenols and cardiovascular risk parameters.

Anna Tresserra-Rimbau, Alexander Medina-Remón, Ramon Estruch, i Rosa M. Lamuela-Raventós. "Chapter 42. Coffee polyphenols and high cardiovascular risk parameters". Dins: Coffee in Health and disease prevention. Editors: Victor R. Preedy. Elsevier. **2014**. In press.

CHAPTER

42

Coffee Polyphenols and High Cardiovascular Risk Parameters

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List of Abbreviations

CVD Cardiovascular diseases
CGA Chlorogenic acid
NO Nitric oxide
EDHF Endothelium-derived hyperpolarizing factor
LDL Low-density lipoproteins
VLDL Very low-density lipoproteins
DCHA Dihydrocaffeic acid
CHD Coronary heart disease
OR Odds ratio
EPIC European Prospective Investigation into Cancer and Nutrition
(BP) Blood pressure
FMD Flow-mediated dilation
HHQ Hydroxyhydroquinone

42.1 INTRODUCTION

The high prevalence of cardiovascular diseases (CVDs) worldwide has an enormous health and socioeconomic impact.[1] CVD includes mainly atherosclerotic and hypertensive diseases, particularly cerebrovascular and ischemic heart diseases.[1] Effective strategies to prevent and reduce CVD involve changes in diet and lifestyle. The Mediterranean diet has been proposed as a dietary pattern that can cut the risk of CVD by 30%.[2] Beyond the traditional Mediterranean diet components, polyphenols and polyphenol-rich foods have been associated with a reduced CVD risk profile.[3]

Polyphenols are bioactive compounds found mainly in plant foods and plant-derived beverages such as wine, tea, or coffee. Numerous studies have associated polyphenol consumption with a reduced risk of certain chronic diseases: certain cancers, type 2 diabetes, cognitive dysfunction, and CVD.[4] Polyphenols are known to improve vascular health, for example, by stimulating vasoprotective factors such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) to promote vasodilatation and platelet activation.[4]

Coffee is one of the most commonly consumed beverages in the world, together with tea, and its popularity is increasing. This infusion of ground roasted coffee beans is a complex chemical mixture of carbohydrates, lipids, nitrogenous compounds, vitamins, minerals, alkaloids, and phenolic compounds.[5] According to the Phenol-Explorer database (www.phenol-explorer.eu), filtered coffee is the most polyphenol-rich beverage, with 214 mg of total polyphenols per 100 ml.[6] In many Western diets, coffee is the main source of total polyphenols, particularly phenolic acids and hydroxycinnamic acids.

Other components of coffee play a crucial role in its beneficial or detrimental effects on the cardiovascular system, although results from epidemiological studies are inconclusive and controversial. The optimal health effects of coffee have been observed with a moderate consumption, while the protective effects revert at higher doses (J-shaped relationship).[7]

The purpose of this chapter is to review the available literature on the relationship between polyphenol intake from coffee and CVD risk in humans.

42.2 COFFEE: AN IMPORTANT SOURCE OF POLYPHENOLS

Coffee beverages are a rich source of bioactive constituents, including methylxanthines, amino acids,

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minerals (magnesium, potassium), and polyphenols.[8] While caffeine (1,3,7-trimethylxanthine) is the most recognized bioactive phytochemical, polyphenols have recently been attracting interest worldwide. The phenolic profile of coffee depends on the variety, type of processing, how the green coffee beans are roasted, and brewing method. The distribution of polyphenols in coffee is shown in Figure 42.1. The most abundant polyphenols are hydroxycinnamic acids (phenolic acids), which represent more than 98% of the total polyphenol content (Table 42.1). The remaining 2% is composed of alkylmethoxyphenols, alkylphenols, methoxyphenols, and other polyphenols such as catechol, phenol, and pyrogallol (data from Phenol-Explorer database, www.phenol-explorer.eu).[15] A 100-ml cup of coffee provides approximately 200 mg of hydroxycinnamic acids and about 43–117 mg of chlorogenic acid (CGA), also known as 5-*O*-caffeoilquinic acid. In Western diets, in areas where coffee is consumed more than tea, this beverage is the main source of total polyphenols and phenolic acids.[8,16–18]

42.3 BIOAVAILABILITY OF COFFEE POLYPHENOLS

Chlorogenic acids are a family of esters formed by the binding of quinic acid and *trans*-cinnamic acids.[8] In coffee, these *trans*-cinnamic acids are caffeic and ferulic acid (Figure 42.2). The most abundant individual polyphenol in coffee, and also the most studied, is 5-caffeoylquinic acid, which is usually known as CGA (Figure 42.3). This polyphenol and its degradation products have been considered as key to the association between coffee consumption and chronic disease prevention, but their bioavailability has not been taken into account.[19]

Plasma pharmacokinetic analyses have shown that CGA is absorbed in both the small and large intestines.[20] Two different studies reported that approximately 33% of CGA is absorbed intestinally.[20,21] The remainder reaches the colon, where it is metabolized by the colonic microflora into metabolites, mainly glucuronide and sulfat derivatives of caffeic acid, which are subsequently absorbed and distributed to tissues. At least ten conjugates, dihydroisoferulic acid 3′-*O*-glucuronide, caffeic acid 3′-sulfate, as well as the sulfate and glucuronide derivatives of 3,4-dihydroxyphenylpropionic acid, have been identified in human plasma and/or urine after coffee consumption in a clinical trial conducted by Fumeaux et al.[22] (Table 42.2).

Studies in rats suggested that the absorption of these microbial metabolites is up to 57% of the CGA consumed.[23] The antioxidant activity of these metabolites is still unclear, often being lower than that of the parent compounds.[8] In this respect, it is important to take into account the food matrix as well as high interindividual variability in absorption, which varied from 7% to 72%.[9] Although information is still scarce, some studies indicate that addition of milk or creamers to coffee may have a minimal impact on the bioavailability of coffee polyphenols.[19]

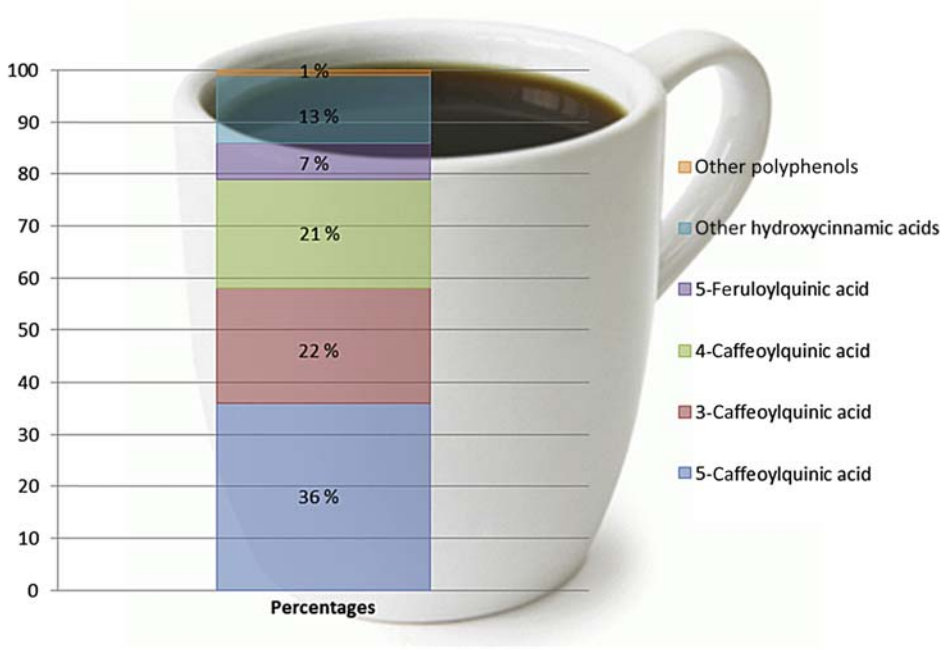

FIGURE 42.1 Polyphenol distribution in coffee.

TABLE 42.1 Phenolic Composition of Coffee Beverages (Means of Different Varieties). Data from Phenol-Explorer Database (www.phenol-explorer.eu)

Polyphenol Class	Polyphenol Subclass	Polyphenol	Mean (Min–Max), mg/100 g	Reference
Phenolic acids	Hydroxycinnamic acids	3,4-Dicaffeoylquinic acid	4.93 (2.66–7.55)	9,10
		3,5-Dicaffeoylquinic acid	3.74 (1.55–6.34)	9,10
		3-Caffeoylquinic acid	47.3 (32.3–57.9)	9–11
		3-Feruloylquinic acid	3.46 (2.74–4.17)	9,10
		4,5-Dicaffeoylquinic acid	3.75 (1.54–8.34)	9,10
		4-Caffeoylquinic acid	43.8 (19.0–60.3)	9–11
		4-Feruloylquinic acid	15.3 (8.57–30.1)	9,10
		5-Caffeoylquinic acid	76.4 (43.1–117)	9–13
		5-Feruloylquinic acid	10.5 (4.64–16.6)	9,10
		Caffeic acid	0.03 (0.03–0.03)	9–13
Other polyphenols	Alkylmethoxyphenols	4-Ethylguaiacol	0.91 (0.64–1.18)	9
		4-Vinylguaiacol	0.61 (0.46–0.75)	10
	Alkylphenols	3-Methylcatechol	0.11 (0.11–0.11)	10,14
		4-Ethylcatechol	0.13 (0.13–0.13)	10,14
		4-Methylcatechol	0.04 (0.04–0.04)	10,14
	Methoxyphenols	Guaiacol	0.22 (0.16–0.27)	10,14
	Other polyphenols	Catechol	0.33 (0.04–0.54)	10,14
		Phenol	0.09 (0.07–0.12)	10
		Pyrogallol	0.46 (0.39–0.54)	10,14

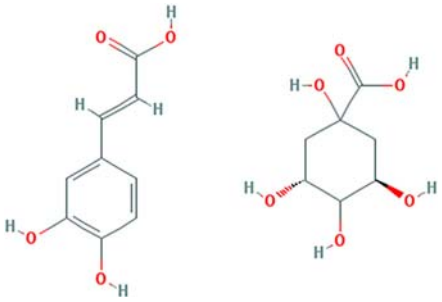

FIGURE 42.2 From left to right, chemical structures of caffeic and quinic acid.

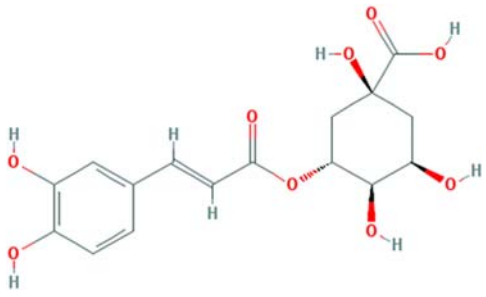

FIGURE 42.3 Chemical structure of chlorogenic acid (CGA).

42.4 IN VITRO AND HUMAN CELL STUDIES

The main source of antioxidants in several Western diets is coffee, due to its high polyphenol content.[24] The in vitro antioxidant properties of CGA, the major polyphenol contained in coffee, are due to its phenolic groups, which can scavenge radicals via proton transfer. Some studies indicate that CGA and caffeic acid can inhibit the oxidation of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) particles, which are the major carriers of cholesterol and triglycerides, respectively.[24–26] CGA provides more effective protection against lipoprotein oxidation than do antioxidant vitamins and gallic acid, although it has lower activity than other polyphenols, such as catechin, quercetin, or caffeic acid.

The powerful antioxidant effect of coffee is also due to the synergism between all the polyphenols it contains.[24] Since coffee and its polyphenols can inhibit the oxidation of atherogenic lipoproteins in vitro, it is logical to think that similar properties may be observed in vivo.

TABLE 42.2 Chlorogenic Acid Metabolites Detected in Human Plasma and Urine after Ingestion of 200 ml of Coffee

	Location	
CGA Metabolite	**Plasma**	**Urine**
Dihydrocaffeic acid 3′-sulfate	X	X
Dihydrocaffeic acid 3′-*O*-glucuronide		X
Caffeic acid 4′-sulfate		X
Dihydroferulic acid 4′-sulfate	X	X
Caffeic acid 3′-sulfate	X	X
Dihydroferulic acid 4′-*O*-glucuronide		X
Ferulic acid 4′-sulfate	X	X
Isoferulic acid 3′-sulfate		X
Dihydroisoferulic acid 3′-*O*-glucuronide		X
Isoferulic acid 3′-*O*-glucuronide		X

Adapted from Fumeaux et al.[22]

CGA is metabolized in human small intestine endothelial cells and liver cells, forming methylation, sulfation, and glucuronidation derivatives.[27,28] Dihydrocaffeic acid, a caffeic acid metabolite quantified in human plasma after ingestion of coffee, also protects human endothelial cells from oxidation by scavenging reactive oxygen species.[29]

On the other hand, NO bioavailability is related to the development of hypertension and other CVD risk factors. In experimental studies using cultured cells, Huang et al.[29] found that dihydrocaffeic acid (DCHA) enhanced NO synthase activity in a dose-dependent manner. NO has also been associated with BP reduction, inhibition of platelet aggregation, and vasoprotective activity.[30]

To sum, preincubation cellular studies with human cells indicate that coffee polyphenols such as CGA, ferulic acid, and/or their metabolites can prevent oxidative damage in vivo and increase levels of NO synthase.

42.5 ANIMAL STUDIES

Since biological processes are essentially similar in the different organisms and many diseases affect both animals and humans, experimental animals are considered as a good model for evaluating the effects of coffee polyphenols on the body. For instance, gut microflora that form microbial metabolites play a key role in the bioavailability of CGA in rats as well as in humans.[23] The effect of CGA in spontaneously hypertensive rats was investigated by Suzuki et al.[31] They observed that dietary CGA reduced BP and oxidative stress and enhanced NO bioavailability through the inhibition of excessive production of reactive oxygen species in the vasculature, which led to the attenuation of endothelial dysfunction. However, the main concern in extrapolating these results to humans is that the lowest dose administered to rats, 30 mg/kg, was much higher than the 5 mg/kg commonly consumed by humans.

Another study analyzed the effect of CGA and caffeic acid (0.02% wt/wt dose) on body fat in high-fat diet–induced obese mice. Both CGA and caffeic acid significantly lowered body weight, visceral fat mass, obesity-related hormone levels (leptin and insulin), triglycerides, and cholesterol concentrations compared to the high-fat control group. The results also suggested that CGA was more effective in reducing body weight and regulating lipid metabolism than caffeic acid.[32]

In type 2 diabetic mice, caffeic acid significantly increased superoxide dismutase, catalase, and glutathione peroxidase and lowered glucose and lipid peroxidation products.[33] A summary of these studies on coffee polyphenols and animals is shown in Table 42.3.

42.6 EPIDEMIOLOGICAL STUDIES

Most of the currently available information about coffee and CVDs has been obtained from human epidemiological studies, whose conclusions raise several issues. First, epidemiological evidence can never prove cause-and-effect but can only be discussed in terms of associations. Problems of misclassification and potential confounders should also be considered when interpreting the results from these studies. Like consumers of alcohol, in many countries coffee drinkers have a significantly less healthy lifestyle than nondrinkers, with a tendency to smoke more, eat less healthy diets, and be more sedentary. When classifying exposure, the collection of consumption data can be skewed by variability in cup size. Another variable factor is caffeine content, which depends on the coffee variety and brewing process. It is also important to distinguish between filtered and nonfiltered (boiled) coffee, since the oils from coffee, which are largely removed by paper filters, are hypercholesterolemic in humans.[8,24] There is also a genetic component that leads to individual variation in the metabolism of compounds in coffee. Perhaps the most important challenge of all is to determine whether the health effects of coffee are related to caffeine, polyphenols, oils, or other compounds or to a synergism between them.

It is not surprising, then, that epidemiological studies conducted in the last three decades have reached conflicting conclusions. The most recent dose–response meta-analysis is a compilation of five independent prospective studies that assessed the association between habitual coffee consumption and the risk of heart failure. The authors observed a statistically significant J-shaped relationship between coffee and heart failure, where the strongest

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TABLE 42.3 Summary of Studies on Coffee Polyphenols in Animal Models

Species	Polyphenol	Dose	Administration	Effect	Reference
Spontaneously hypertensive rats	CGA	0.5% (~300 mg/day)	Oral	↓ Blood pressure ↓ Urinary excretion of hydrogen peroxide ↑ Urinary excretion of NO metabolites ↓ Production of reactive oxygen species in the vasculature	31
20 male C57BL/KsJ-*db/db* mice	Caffeic acid	0.02% (wt/wt)	Oral	↓ Blood glucose ↓ Glycosylated hemoglobin ↓ Plasma glucagon ↑ Plasma insulin ↑ Plasma C-peptide ↑ Plasma leptin ↑ Glucokinase activity ↓ Glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities ↑ Superoxide dismutase, catalase, and glutathione peroxidase activities	32
32 male Diet-induced-obese mice	CGA or caffeic acid	0.02% (wt/wt)	Oral	↓ Body weight ↓ Visceral fat mass ↓ Plasma leptin ↓ Plasma insulin ↓ Triglyceride and cholesterol concentrations	33

inverse association was found for four servings per day. Gender and baseline history of myocardial infarction and diabetes did not change the relationship significantly.[34]

Sofi et al. conducted a meta-analysis of 13 case–control and 10 cohort studies to summarize the relationship between coffee consumption and coronary heart disease (CHD) risk. The summary of odds ratios (ORs) for the case–control studies showed statistically significant associations between a high consumption of coffee (>3 cups/day) and CHD, while no significant association emerged for low daily coffee intake (≤2 cups/day). The analysis of long-term follow-up cohort studies did not show any association between the consumption of coffee and CHD.[35]

Another meta-analysis aimed to summarize the effect of coffee on BP and CVD in hypertensive individuals, using data from controlled trials and cohort studies. In three controlled trials studying the effect of a 2-week intake of coffee, no increase in BP was observed in comparison with a caffeine-free diet or intake of decaffeinated coffee. In seven cohort studies, no association between habitual coffee consumption and a higher risk of CVD was observed.[36]

A recent prospective study not included in the aforementioned meta-analyses has investigated the relationship between coffee consumption and the risk of the most widespread chronic diseases, including type 2 diabetes, cancer, and CVDs such as myocardial infarction and stroke.[37] The authors collected and analyzed data from 42,659 participants (followed during a mean of 9.8 years) in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort using food frequency questionnaires and multivariate Cox regression models. They concluded that coffee consumption, caffeinated or decaffeinated, was not associated with CVD or cancer, but it seemed to be linked to a lower risk of type 2 diabetes.

42.7 CLINICAL TRIALS

As mentioned in the previous section, epidemiological studies can never prove the relationship between cause and effect. However, randomized controlled trials provide the most compelling evidence of a causal relationship between exposure and effect. In clinical trials, the investigators manipulate the administration of a new intervention and measure the effect of that manipulation, whereas epidemiological studies only observe associations between the exposure and the health status or diseases of the participants.

42.7.1 Effects on Antioxidant Activity

Some clinical trials have focused on the antioxidant activity of coffee. Natella et al. compared the effect of coffee and tea on plasma redox homeostasis in humans. The antioxidant capacity of plasma before and after supplementation with 200 ml of coffee (0, 1, and 2 h) was measured by the tartrate-resistant acid phosphatase and crocin tests. The authors concluded that molecules other

than uric acid, probably phenolic compounds, were responsible for the increase in plasma antioxidant capacity after coffee consumption.[38]

The effect of Italian-style coffee consumption on the plasma concentration of plasmatic glutathione and homocysteine was studied by Esposito et al. Plasma glutathione increased by 16% ($p < 0.05$) among participants who consumed five cups of coffee per day for 1 week and returned to the original concentration after the washout period. No significant changes were observed in homocysteine concentration.[39] Another group found similar results for plasma homocysteine concentrations after short- and long-term consumption of filtered coffee.[40]

The most recent study has been performed in Brazil to compare the effects of medium light roast and medium roast (lower CGA and higher caffeine content) paper-filtered coffee on antioxidant capacity and lipid peroxidation in 20 healthy volunteers. Similar effects were observed for both types of coffee consumption: a significant increase in plasma total antioxidant status, catalase activity, and levels of erythrocyte superoxide dismutase and gluthation peroxidase. However, oxygen radical absorbance capacity only increased after medium light roast coffee intake. No significant alteration in lipid peroxidation biomarkers was observed.[41]

42.7.2 Effects on Blood Pressure and Endothelial Function

High blood pressure (BP) and endothelial dysfunction are associated with an increased risk of CVD. Endothelial dysfunction is an early event in the pathogenesis of vascular disease, and NO plays a crucial role in maintaining healthy endothelial function and vascular tone.

In 2005, Noordzij et al. performed a meta-analysis of randomized controlled trials to assess the chronic effects (>7 days) of coffee intake on BP. A significant rise of 2.04 mm Hg (95% confidence interval [CI] 1.10–2.99) in systolic BP and 0.73 mm Hg (95% CI 0.14–1.31) in diastolic BP was found after pooling coffee and caffeine trials, but these increases were no longer significant when caffeine trials were removed from the analysis. This means that caffeine may be chiefly responsible for the BP increase, but it has a lower effect when consumed in coffee.[42]

In order to investigate the effects of coffee on endothelial function, two similar crossover studies were carried out with healthy, nonobese subjects. Brachial artery flow-mediated dilation, the method most commonly used to assess endothelial dysfunction, increased in a dose–response manner after decaffeinated coffee consumption but decreased after intake of caffeinated coffee. Similar results were obtained for BP and heart rate: caffeinated coffee induced unfavorable cardiovascular effects. Again, caffeine seems to blunt the demonstrated health effects of polyphenol components of coffee.[43,44]

Coffee consumption may also affect serum lipids.[45,46] This has been extensively explained in the chapter by Ma et al.

42.7.3 Clinical Trials with CGA

To date, considerable research has been dedicated to the effects of coffee on human health but more clinical trials focusing on the polyphenols in coffee, rather than the complete beverage, are required to establish which coffee components are beneficial or detrimental. In this regard, Watanabe et al.[47] performed a placebo-controlled, randomized clinical trial with 28 subjects with mild essential hypertension. Participants received treatment with either CGA (140 mg/day) from green coffee bean extract or a placebo. Systolic and diastolic BP decreased significantly during the ingestion period only in the CGA group. No difference was found in body mass index and pulse rate between groups.

Green coffee bean extract has been shown to have hypertensive effects in spontaneously hypertensive rats and healthy humans, apparently due to its content of polyphenols, mostly CGA. Volunteers who drank green coffee bean extract for 3–4 months had a higher reactive hyperemia ratio and showed a significant decrease in the plasma total homocysteine level than those who consumed a placebo, and this led to an improvement in vasoreactivity.[42] However, hydroxyhydroquinone (HHQ) or benzene-1,2,4-triol (Figure 42.4), a phenol formed during the roasting of coffee beans, inhibits the antihypertensive effect of chlorogenic acids in brewed coffee. A Japanese research group investigated the effects of HHQ-reduced coffee on hypertension and vasoreactivity in mild hypertensive subjects. In those participants who consumed the HHQ-reduced coffee, that is, coffee with 300 mg of CGA per 184 ml of beverage, endothelium-dependent, flow-mediated vasodilation impairment and systolic BP were significantly improved and urinary isoprostane levels decreased, suggesting a reduced oxidative stress.[48]

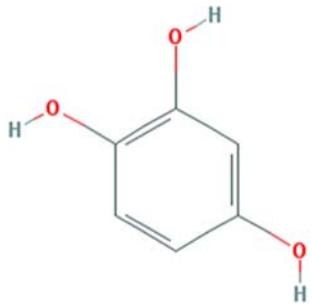

FIGURE 42.4 Chemical structure of hydroxyhydroquinone (HHQ).

More recently, Mubarak et al. designed a randomized, double-blind, placebo-controlled, cross-over trial to investigate the acute effects of CGAs, the most abundant coffee polyphenol, on BP, endothelial function, and NO status. After administration of 400 mg, the equivalent to two cups of coffee, to 23 healthy men and women, they observed a significant reduction of systolic and diastolic BP (−2.41 mm Hg, 95% CI −0.03 to −4.78, p = 0.05; and −1.53 mm Hg, 95% CI −0.05 to −3.01, p = 0.04) compared to the control group. Neither markers of NO status nor endothelial function were significantly influenced.[49] However, more studies are required with a larger cohort and nonhealthy subjects to support these conclusions. Moreover, there is no available evidence about the effects of long-term (chronic) intake of CGA.

42.8 SUMMARY POINTS

- Coffee is one of the most polyphenol-rich beverages consumed worldwide, containing 214 mg of total polyphenols per 100 ml. In Western diets, it is usually the main source of phenolic acids and hydroxycinnamic acids, especially CGA.
- A third of the ingested CGA is absorbed in the small intestine. The remainder, on reaching the colon, is metabolized by the colonic microflora into metabolites such as caffeic acid glucuronide and sulfate.
- CGA has potential cardiovascular benefits via antioxidant mechanisms, related to BP, endothelial function, LDL oxidation, and NO bioavailability.
- Caffeine and lipids from unfiltered coffee may blunt the beneficial effects of polyphenols.
- Considerable research has focused on the effects of coffee as a beverage, but only a few studies, mainly in vitro, cell, and animal, have investigated the specific effects of CGA.
- Further studies are needed to investigate the long-term effects of coffee CGA in humans, especially in subjects with cardiovascular diseases.

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B.2. Capítol de llibre 2. El consum de polifenols i la pressió arterial

Book chapter 2. Polyphenol intake and blood pressure.

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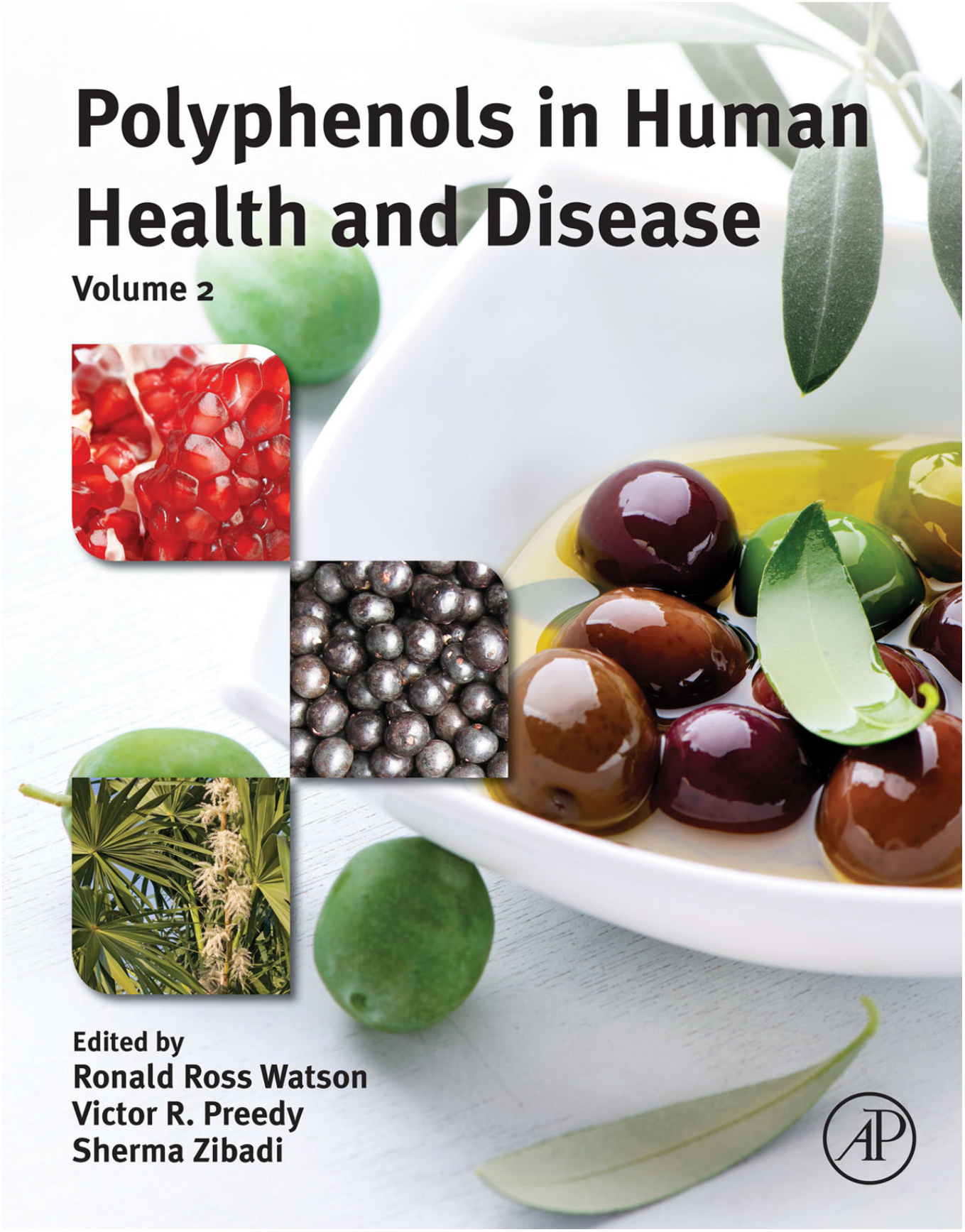
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CHAPTER

75

Polyphenol Consumption and Blood Pressure

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1. INTRODUCTION

Hypertension, defined as diastolic blood pressure (BP) greater than 90 mm Hg or systolic BP greater than 140 mm Hg, is a major public health problem, and a leading cause of premature death and disability in both developed and developing countries, affecting one-quarter of the world's adult population.[1]

High BP, which is one of the main cardiovascular risk factors in the elderly, is the result of interacting genetic and environmental factors. Hypertension can be managed by following a healthy diet such as the traditional Mediterranean diet (TMD)[2] or the Dietary Approaches to Stop Hypertension (DASH) diet,[3] and lifestyle modifications, including smoking cessation, moderate alcohol consumption, limiting alcoholic beverages to two drinks a day for men and one drink a day for women, sodium intake restriction to less than 2300 mg a day, weight reduction, maintaining a body mass index (BMI) between 18.5 and 24.9 Kg/m^2 and regular physical activity for at least 2.5 hours a week to increase the heart rate.[4]

Since the publication of the landmark DASH trial[3] and the ensuing DASH-Sodium trial with added salt restriction,[5] a DASH-type diet, based on vegetables, whole cereals, low-fat dairy products and fish, with restriction of meat and meat products, whole dairies and sweets, has been the epitome of an antihypertensive dietary pattern. More recently, increasing epidemiological and clinical evidence points to the TMD as an alternative dietary pattern for BP control.[6–8] The healthy diets recommended to subjects with or at risk of hypertension should be low in salt[9] and rich in fruits and vegetables (F&V), which are an abundant source of phytochemicals.[10]

Numerous epidemiological studies have shown an inverse association between polyphenol-rich foods such as cocoa, F&V, tea, olive oil, and wine[4,11–16] and the risk of hypertension. Moderate consumption of fish, another characteristic food of the Mediterranean diet, and low-fat dairy products may also reduce the risk of hypertension,[4,17–21] whereas a high intake of refined cereals, meat, and meat products has been associated with a greater cardiovascular risk.[4,12,22] The effects of the dietary intake of sodium and potassium, vitamin C, or other antioxidant compounds on BP have also been analyzed.[23,24] However, only one study to date has evaluated the role of excreted total dietary polyphenols as a biomarker of total polyphenol (TP) intake.[25]

Biomarkers of nutrient intake constitute an established alternative to traditional dietary assessment tools, offering a semi-quantitative index of exposure to individual food constituents, measured in a fluid or tissue. In comparison with food frequency questionnaires (FFQ), biomarkers of nutrient intake measured in blood and urine provide more precise data and more objective measurements, so their development is

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essential for accurate estimations of polyphenol consumption. However, the study of the relationship between dietary intake and fluid biomarker concentrations is highly complex.[26]

In this chapter, we summarize recent observational studies on the relationship between polyphenol intake and BP, as well as the plausible mechanisms by which polyphenols may exert their cardioprotective role.

2. GENERAL CHEMISTRY AND CLASSIFICATION OF POLYPHENOLS

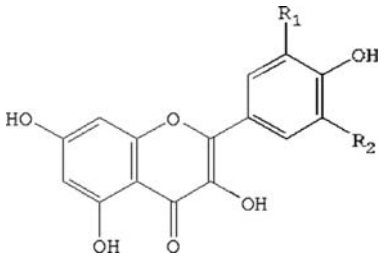
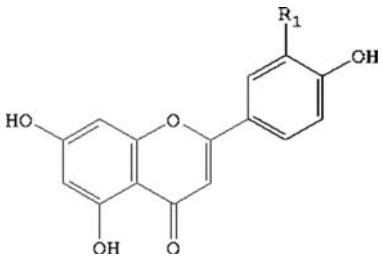
Phytochemicals or phytonutrients are compounds present in food that have the capacity to alter biochemical reactions and consequently affect human health. One such group of compounds is the polyphenols, secondary plant metabolites that constitute the most abundant antioxidants in the human diet. They are classified into different groups, mainly flavonoids and non-flavonoids, according to the number of phenol rings they bear and the structural elements that bind these rings to each other.

The flavonoid group comprises compounds with a C6–C3–C6 structure, and includes flavonols, flavones, flavanones, isoflavones, favan-3-ols or flavanols, anthocyanidins, dihydroflavonols, and proanthocyanins. This classification is based on the oxidation of the central ring and the type of substituents in the heterocyclic ring.[27] Flavonoids have a skeleton of diphenyl propanes, with two benzene rings (A and B) connected by a three-carbon chain forming a closed pyran ring with the benzene A ring. Exhibiting a high structural diversity, flavonoids have the capacity to associate with a variety of carbohydrates and organic acids as well as with one another. In plants, they usually occur in a glycosylated form, generally with glucose or rhamnose, although they can also be linked with galactose, arabinose, xylose, glucuronic acid or other sugars. The number of glycosil moieties usually varies from one to three, but some flavonoids have been identified with four and even five moieties (Table 75.1).[28]

Non-flavonoids are classified according to the number of carbons they bear and comprise the following subgroups: simple phenols, benzoic acids and aldehydes, hydrolyzable tannins, hydroxycinnamic acids, coumarins, stilbenes, chalcones, and lignans. They also include other groups like acetophenones, phenylacetic acids, benzophenones, xanthones, and secoiridoids.

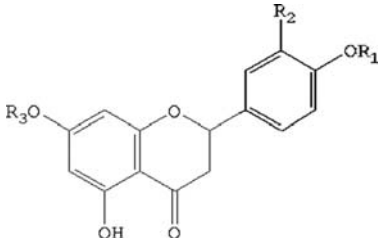
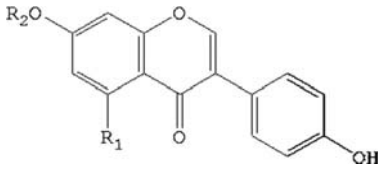
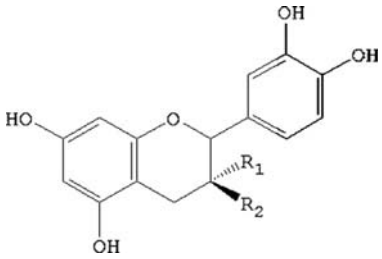
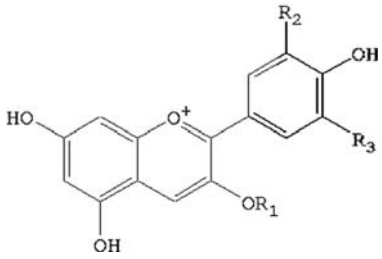
Simple phenols (C6) are formed with an aromatic ring substituted by an alcohol in one or more positions. Hydrolyzable tannins are mainly glucose esters of gallic acid. Hydroxycinnamic acids are included in the phenylpropanoid group (C6–C3) and are formed with an aromatic ring and a three-carbon chain. They consist of four basic structures: the coumaric acids,

TABLE 75.1 Flavonoid Polyphenols Chemistry and Classification

Class	Sub-class	Structure	Name	Source
FLAVONOIDS C6–C3–C6	Flavonols		Quercetin, kaempferol, myricetin, isorhamnetin	External tissues of fruit and vegetables; cappers, onions, berries, asparagus, spices
	Flavones		Apigenin, luteolin, tangeretin	Parsley, thyme, oregano, chicory

(*Continued*)

TABLE 75.1 (*Continued*)

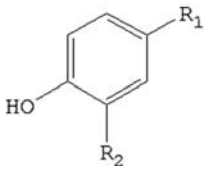
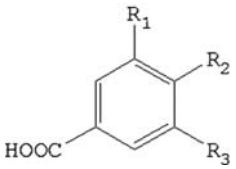
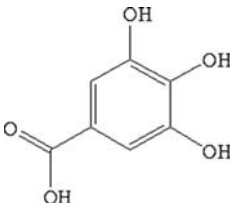
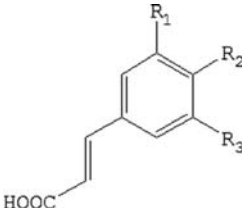
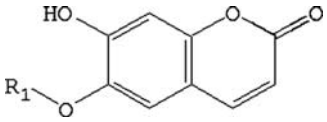
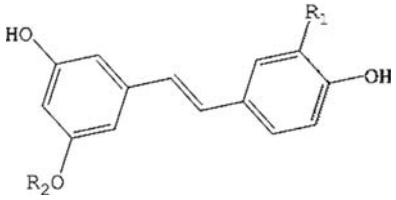
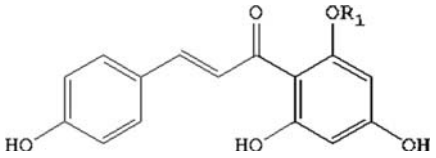
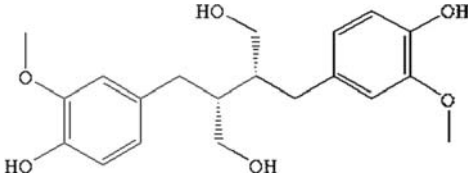
Class	Sub-class	Structure	Name	Source
	Flavanones		Eridictiol, hesperedin, naringenin	Citrus fruits
	Isoflavones		Genistein, genistin, glicetin, daidzein, daidzin	Soy.
	Flavanols or favan-3-ols		Catechin, epicatechin, galocatechin, epigalocatechin, teaflavin, galocatechin	Tea, cocoa, red wine, cherries, walnuts
	Anthocynidins		Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	Berries, purple grapes, cherries

caffeic acids, ferulic acids, and sinapic acids. In nature, they are usually associated with other compounds such as chlorogenic acid, an ester of caffeic acid and quinic acid. Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. They may also be found in nature in combination with sugars such as glycosides. They can be categorized as simple, furanocoumarins, pyranocoumarins and coumarins substituted in the pyrone ring.[29] Chalcones, bearing a C6–C3–C6 structure, are flavonoids lacking a heterocyclic C ring and are generally not accumulated in plants (Table 75.2).

3. ABSORPTION, METABOLISM AND BIOAVAILABILITY OF POLYPHENOLS

Polyphenols are the most abundant antioxidants in the human diet and are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate, and beverages such as tea, coffee, or wine.[30] Roughly, 1000 mg total dietary polyphenols are ingested daily,[31] which is around 10 times higher than the intake of vitamin C and 100 times higher than vitamin E and carotenoids. However, various factors make it difficult to estimate polyphenol consumption: their presence in a wide variety of foods, their diverse chemical structures,

TABLE 75.2 Non-flavonoid Polyphenols Chemistry and Classification

Class	Sub-class	Structure	Examples	Sources
NON-FLAVONOIDS	Simple phenols C6		Hydroxytyrosol, tyrosol, eugenol, guaiacol, vinylguaiacol	Wine, coffee grains, oranges, virgin olive oil
	Phenolic acids and aldehydes C6-C1		*p*-Hydroxybenzoic acid, gallic acid, syringic acid, protocatequic acid, sinapinic acid	Redcurrant, berries, cherries, apple, coffee, black tea, white wine
	Hydrolyzable tannins (C6-C1)n (Gallic acid)n		Ellagitanin, gallotannin	Pomegranate, apple and grape juices, strawberries, berries, seeds
	Hydroxycinnamic acids C6−C3		Caffeic acid, ferulic acid, *p*-coumaric acid, sinapic acid, chlorogenic acid	Fruit and vegetables, mainly in the skin. Coffee, berries, potatoes, tomatoes, grapes, carrots
	Coumarins C6−C3		Furanocoumarin, scopoletin, esculin, merazin	Essential oils, green tea, fruits, carrots, celery
	Stilbenes C6−C2−C6		Piceid, resveratrol, piceatannol	Grapes and wine, peanuts
	Chalcones C6−C3−C6		Chalconaringenin floretin-glucoside	Tomato skin, apples, cider, "orujo"
	Lignans (C6−C3)2		Secoisolariciresinol	Wine, tea, chocolate, soy

and while certain polyphenols, such as quercetin, are found in the majority of plant products, others, such as flavanones, isoflavones, and phloridzin, are specific to a particular food (citrus fruit, soy, and apples, respectively). Polyphenol content in foods can also differ according to the degree of ripeness at harvesting, environmental factors, conditions of processing and storage, and even plant variety. Furthermore, there is no standardized method to estimate polyphenol content in foods, resulting in a variety of analytical approaches among studies. To evaluate polyphenol effects on health, it is necessary to take into account the phenol type as well as the TP content, since structural differences may change biological properties.[32]

The health properties of polyphenols depend on their respective intakes and variable bioavailability.[33] The most common polyphenols in the human diet are not necessarily the most active *in vivo*, either due to a lower intrinsic activity or because they are poorly absorbed, highly metabolized, or rapidly eliminated.[28] Furthermore, some modifications, such as glycosylation of flavonoids and esterification of phenolic acids, should be considered, because they can affect absorption from the gut.[34,35]

After ingestion, polyphenols have several potential fates, including absorption in the small intestine or colon, and/or excretion in the feces or urine. In the small intestine, polyphenols can enter the mucosa through passive diffusion. In some instances, hydrophobic moieties must be cleaved for absorption to take place. In the colon, polyphenols are firstly digested into smaller phenolic structures by gut microflora. After this initial digestion, polyphenols and their metabolites may be absorbed.[30,36] As a general rule, polyphenol metabolites are quickly eliminated from plasma, which indicates that a daily intake of plant products is necessary to maintain high concentrations of these metabolites in the bloodstream.[28]

Most dietary polyphenols (75–99%) are not found in urine, and the quantities detected intact vary from one phenolic compound to another.[30] This may be due to reduced absorption through the gut barrier, hydrolysis and/or metabolization by intestine or liver enzymes, excretion to the bile or metabolization by colonic microflora.[37]

Absorption, metabolism, tissue distribution, and urinary or biliary excretion of polyphenols are separate physiological processes that all contribute to the time-dependent plasma values and determine bioavailability. Numerous researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion after the ingestion of a single dose of polyphenols in adults, supplied as a plant extract, pure compound or whole food/beverage. The relative urinary excretion ranged from 0.3 to 43% of the ingested dose, depending on the polyphenol. Gallic acid and isoflavones are well absorbed, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. Proanthocyanidins, galloylated tea catechins, and anthocyanins are the least well-absorbed polyphenols,[38] being the only flavonoids absorbed from the stomach, and occurring as glycosides in plasma. This is reflected in their very low bioavailability, a rapid appearance in plasma after ingestion, and the presence of intact anthocyanidin glycosides in the circulation.

The most effective absorption takes place in the small intestine, which has a large surface area compared with the stomach or colon, resulting in peak plasma values between 1 and 3 hours after ingestion. Monomeric flavanols such as catechin and epicatechin are absorbed from the small intestine,[39] while galloylated catechins such as epigallocatechin gallate bind to proteins in the gut, which may be another explanation for their low bioavailability.[40]

Most flavonoids, with the exception of flavanols, occur in foods as glycosides, which are the only ones absorbable in the small intestine. The type of sugar moiety determines where absorption can take place,[41] for example, quercetin glucoside is absorbed from the small intestine, whereas quercetin rutinoside (rutin) can only be absorbed from the colon after hydrolysis of the rutin moiety. After absorption in the small intestine, glucosides are hydrolyzed by the lactase phloridzin hydrolase in the brush border membrane.[42]

Flavonoids that are not absorbed from the small intestine or stomach are metabolized by colon microbiota. Glycosides are absorbed in the colon after hydrolysis, with peak plasma values being reached only after 4–6 hours.[38] Flavonoids are also broken down to a variety of smaller molecules, including the phenolic acids,[43] and as a result, the bioavailability of flavonoids absorbed from the colon is usually much lower than those absorbed from the small intestine.

After absorption, polyphenols are readily metabolized in intestinal cells to form glucuronide and sulfate conjugates, which appear in portal blood.[44] Methylation of catechol units may also occur.[45] As a result, only conjugated forms of polyphenols are generally present in blood. Additional conjugation and methylation may occur in the liver, altering polyphenol biological activity, for example, the antioxidant activity of quercetin conjugates, which is on average about half that of aglycone.[46]

The total amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations. A rapid new method, a modified Singleton and Rossi Folin-Ciocalteu (F-C) assay,[47] has been recently described to determine total polyphenols in complex matrices such as urine samples, thus providing an accurate biomarker of polyphenol-rich food intake.[48]

4. EPIDEMIOLOGICAL STUDIES ON POLYPHENOL INTAKE AND BLOOD PRESSURE

Since the biological activity of plant polyphenols depends on their bioavailability, kinetics and exposure time,[49] intestinal absorption and metabolism of polyphenols are rate-limiting steps for their endothelium-dependent protective effects.

Numerous epidemiological studies have provided evidence for the protective effect of F&V against cardiovascular disease (CVD).[11,12] For instance, a relationship between F&V intake and CVD risk factors was examined in urban south Indians by Radhika *et al.*[50] The volunteers of this study, 983 individuals aged 20 years or more, were selected from the Chennai Urban Rural Epidemiological Study (CURES). After adjusting for potential confounders, the linear regression analysis revealed that the highest quartile of F&V intake showed a significant inverse association with systolic BP ($\beta = -2.6$ mm Hg; $p = 0.027$) compared with the lowest quartile. A high intake of F&V explained 48% of the protective effect against CVD risk factors. A high F&V intake was also correlated with a reduced risk of CVD in a study among 2682 men in Finland.[51]

In the Nurses' Health Study,[52] F&V intake was also inversely associated with systolic and diastolic BP, whereas the consumption of refined cereals and meat was directly associated with high systolic BP. Also, the prevalence of non-previously diagnosed hypertension in the SUN study[12] was inversely correlated with F&V consumption in a Mediterranean population with a very high intake of both fat- and plant-derived foods.

In the Chicago Western Electric Study, after an 8-year follow-up of 1714 employed middle-aged men, intake of vegetable protein and beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP.[53] On the other hand, Hung *et al.*[54] evaluated the association of F&V consumption with peripheral arterial disease in a cohort of 44,059 men initially free of CVD and diabetes, reporting no evidence that F&V consumption protects against peripheral arterial disease. In the age-adjusted model, men in the highest quintile of F&V intake had a relative risk of 0.55 (95% CI = 0.38–0.80) for peripheral arterial disease, compared with those in the lowest quintile. However, the associations were greatly weakened after adjustment for smoking and other traditional CVD risk factors.

Quercetin, one of the most abundant flavonoids present in F&V, reduced BP in several experimental models of hypertension, including spontaneously hypertensive rats and rat models of metabolic syndrome. A high dose of quercetin also reduced BP in stage 1 hypertensive patients in a randomized, double-blind, placebo-controlled, crossover study.[55]

The intake of flavonoid-rich juice[56] and flavonoid-rich dark chocolate significantly reduced BP and improved endothelium-dependent flow-dilated vasodilatation, which contributes to healthy blood flow, in a well-designed, double-blind, cross-over trial.[15]

In a randomized, single-blind, cross-over study,[15] 20 men and women (mean age 44 years) with never-treated essential hypertension and impaired glucose tolerance, daily consumed dark chocolate (100 g) containing 500 mg polyphenols (including 66 mg epicatechin and 22 mg catechin) or flavanol-free white chocolate (90 g) for 15 days. Baseline endothelium-dependent flow-mediated dilation of the brachial artery was significantly lower in hypertensive subjects compared with controls ($7.4 \pm 1.4\%$ vs. $9.9 \pm 0.9\%$; $p < 0.0001$) and significantly increased in hypertensive subjects after consumption of dark chocolate ($8.9 \pm 1.4\%$; $p < 0.0001$) but not after consumption of white chocolate ($7.5 \pm 1.3\%$). Endothelium-dependent flow-mediated dilation also increased significantly in the control group after consumption of dark chocolate ($11.8 \pm 1.3\%$; $p < 0.0001$) but not after consumption of white chocolate ($10.1 \pm 0.9\%$).

Similarly, in a cross-sectional study with Kuna Indians (Panama), it was observed that daily consumption of flavanol-rich cocoa (from home-grown and Columbian cocoa powder) lowers BP.[57]

In a randomized, controlled, double-blind, cross-over trial,[58] 20 patients on secondary prevention for coronary artery disease (64 ± 3 years of age) received a high-flavanol (HF) cocoa drink (375 mg), and a macronutrient- and micronutrient-matched low-flavanol (LF) cocoa drink (9 mg) twice daily (750 mg/day and 18 mg/day, respectively) over 30 days, with one week of wash-out between interventions. At the end of the periods, flow-mediated dilation values significantly increased to $5.7 \pm 0.5\%$, $p < 0.001$ for LF and $8.4 \pm 0.8\%$, $p < 0.001$ for HF, compared with pre-intervention values, and the post-HF values were significantly greater than post-LF values ($p < 0.001$ between groups).

In 21 obese but healthy volunteers, Berry *et al.*[59] observed that a single dose of high-flavanol (701 mg cocoa flavanols; 139 mg epicatechin) cocoa beverage significantly increased flow-mediated dilation compared to a single dose of a low-flavanol (22 mg cocoa flavanols; 0 mg epicatechin) cocoa beverage (from $3.4 \pm 0.5\%$ to $6.1 \pm 0.6\%$).

In another randomized, double-blind, placebo-controlled, cross-over study with healthy adults,[60] the flow-mediated dilation was unchanged after placebo

ingestion (0 g cocoa) but significantly increased 1 and 2 hours after consumption of 2, 5, 13 and 26 g of cocoa in a dose-dependent manner.

In a randomized crossover trial, which included 13 healthy individuals, the intake of consecutive daily doses of 100 g of polyphenol-rich dark chocolate (500 mg of polyphenols) decreased both systolic and diastolic BP in patients with mild isolated systolic hypertension.[61] In another similar study, Taubert *et al.*[62] determined the effects of low doses of polyphenol-rich dark chocolate on BP in a randomized, controlled, investigator-blinded, parallel-group trial involving 44 adults aged 56 to 73 years (24 women, 20 men) with untreated upper-range prehypertension or stage 1 hypertension without concomitant risk factors. Participants were randomly assigned to receive for 18 weeks either 6.3 g/day of dark chocolate containing 30 mg of polyphenols or matching polyphenol-free white chocolate. From baseline to 18 weeks, the dark chocolate intake reduced mean (SD) systolic BP by −2.9 (1.6) mm Hg ($p < 0.001$) and diastolic BP by −1.9 (1.0) mm Hg ($p < 0.001$). In addition, hypertension prevalence declined from 86 to 68%.[63]

Cocoa flavanols help maintain endothelium-dependent vasodilation, which contributes to normal blood flow. In order to obtain the claimed effect, 200 mg of cocoa flavanols should be consumed daily, which can be provided by 2.5 g of high-flavanol cocoa powder or 10 g of high-flavanol dark chocolate, both of which can be consumed in the context of a balanced diet.[63]

In another study conducted with 60 volunteers who had fasting blood glucose levels of > or = 6.1 mmol/L or nonfasting blood glucose levels of > or = 7.8 mmol/L, supplementation with green tea-extract powder produced a borderline significant reduction in diastolic BP and no significant changes in systolic BP. The intervention group consumed a packet of green-tea-extract powder containing 544 mg polyphenols (456 mg catechins) daily for the first 2 months and then entered the 2-month nonintervention period.[64] Erlund *et al.*[65] in a single-blind, randomized, placebo-controlled intervention trial with 72 middle-aged unmedicated subjects with CVD risk factors, investigated the effects of berry consumption during 8 weeks on BP. In this study, berry consumption significantly decreased ($p = 0.05$) systolic BP, the decrease mostly occurring in subjects with high baseline BP (7.3 mm Hg in highest tertile; $p = 0.024$).[65]

Morand *et al.*[66] investigated the effect of orange juice and its major flavonoid, hesperidin, on BP in 24 healthy, overweight men (aged 50−65), which were included in a randomized, controlled, crossover study during three 4-week periods. Volunteers consumed 500 mL/day of orange juice, 500 mL control drink plus hesperidin, or 500 mL control drink plus placebo. Diastolic BP was significantly lower after the 4-week consumption of orange juice or the control drink plus hesperidin than after consumption of the control drink plus placebo ($p < 0.03$; both).[66] However, systolic BP was similar after the 4-week supplementation period.

In a similar study with Concord grape juice, both systolic and diastolic BP in hypertensive Korean patients had decreased by an average of 7.2 and 6.2 mm Hg, respectively, at the end of 8 weeks.[67]

In 10 healthy volunteers, aged between 24 and 37 years, the coronary flow-velocity reserve was increased after the intake of a polyphenol-rich beverage (1 g/kg ethanol as red wine) but not after drinking the same quantity of alcohol as vodka (polyphenol-free) or white wine (medium polyphenol content).[68] Endothelium-dependent vasodilatation was also improved after acute intake of 500 mL of red wine and dealcoholized red wine.[69] In hypertensive patients, a reduction in total and saturated fatty acid intake and an increase of extra-virgin olive oil intake favorably affected BP.[70]

Among various studies involving pomegranate juice, Aviram *et al.*[71] observed in 10 hypertensive individuals a consistent 5% reduction in systolic BP after a 50 mL/d pomegranate juice intake for 2 weeks, whereas a 21% reduction in systolic BP was observed when the same volume of juice was given to a larger group of participants with asymptomatic severe carotid artery stenosis for a year.[56] In contrast, Sumner *et al.*[72] described a reduction in stress-induced ischemia but no effect on BP after the intake of 240 mL/d of pomegranate juice, a higher volume than in the Aviram study, by a larger group of participants ($n = 45$), with ischemic coronary disease, for an extensive period (90 days).[73] A possible reason for these discrepant results is that the juices used in the studies were derived from different sources and therefore differed in polyphenolic content. Sumner's group used a commercial pomegranate juice, which undergoes technological processing that may affect the polyphenolic composition, while Aviram's group produced an in-house concentrated form of pomegranate juice, which was chemically analyzed.

In a double-blind, placebo-controlled, parallel trial, Naruszewicz *et al.* analyzed 44 patients (11 women and 33 men, mean age 66 years) who had survived myocardial infarction and received statin therapy for at least 6 months. The subjects were randomized to receive either 3 × 85 mg/day of chokeberry flavonoid extract (*Aronia melanocarpa E*) or a placebo for a period of 6 weeks. Compared with the placebo, the chokeberry flavonoid extract significantly reduced systolic and diastolic BP by an average of 11 and 7.2 mm Hg, respectively.[74]

A meta-analysis of randomized, controlled trial data[75] showed that consumption of chocolate reduced systolic (−5.88 mm Hg; 95% CI: −9.55, −2.21; 5 studies) and diastolic (−3.30 mm Hg; 95% CI: −5.77, −0.83; 4 studies) BP. A soy protein isolate significantly reduced diastolic BP (−1.99 mm Hg; 95% CI: −2.86, −1.12; 9 studies), while the effect on systolic BP was not significant (−1.60 mm Hg; 95% CI: −3.62, 0.42; 9 studies). The consumption of black tea caused an acute increase in systolic BP (5.69 mm Hg; 95% CI: 1.52, 9.86; 4 studies) and diastolic BP (2.56 mm Hg; 95% CI: 1.03, 4.10; 4 studies), but these increases may be due to the known effects of caffeine on BP observed in another meta-analysis.[76]

In a second meta-analysis of 172,567 participants and 37,135 incident cases of hypertension,[77] habitual coffee consumption of >3 cups/day was not associated with an increased risk of hypertension compared with <1 cup/day. However, a slightly elevated risk (9%) appeared to be associated with light-to-moderate consumption of 1−3 cups/day.

Chiva-Blanch *et al*. evaluated the effects of red wine fractions (alcoholic and non-alcoholic) on BP in 67 men at high cardiovascular risk;[78] systolic and diastolic BP decreased significantly (−5.8 mm Hg, $p = 0.0001$ and −2.3 mm Hg, $p = 0.017$, respectively) after the dealcoholized red wine intervention and these changes were correlated with increases in plasma NO.

In a cross-sectional trial[25] involving 589 participants, 263 men aged 53 to 82 years and 326 women aged 58 to 82 years, free of CVD at baseline, performed within a larger clinical trial, the PREDIMED study,[2,79] urinary polyphenols have been inversely associated with BP and positively associated with a reduction in the risk of coronary heart disease (Figure 75.1). After adjustment for different potential confounding factors, in multivariate linear regression analyses with systolic and diastolic BP as dependent variables, and a quartile of total phenol excretion (TPE) in spot urine samples (mg GAE/g creatinine) as the exposure variable, systolic and diastolic BP exhibited a monotonic inverse association with TPE in spot urine samples. The non-standardized coefficients, $\beta = -1.73$ ($p = 0.024$) and $\beta = -1.26$ ($p = 0.003$), represent the expected change in systolic and diastolic BP, respectively, corresponding to an increase in TPE to the upper quartile. A higher polyphenol excretion in urine was associated with lower systolic and diastolic BP.

In a multivariate logistic regression analysis for cardiovascular risk factors according to quartiles of TPE expressed as mg gallic acid equivalent (GAE)/g creatinine, using the lowest quartile group as the reference category, the participants in the highest quartile (>160.23 mg GAE/g creatinine) had a significantly reduced prevalence of hypertension (OR = 0.64, $p = 0.015$) compared with those in the lowest quartile

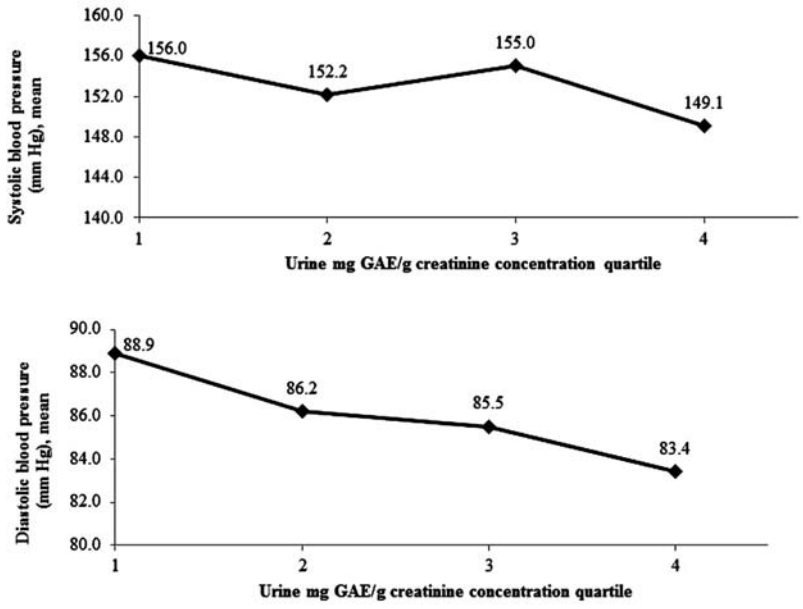


FIGURE 75.1 Changes in systolic and diastolic blood pressure according to quartiles of total polyphenol excretion expressed as mg gallic acid equivalent (GAE)/g creatinine.

of TPE (<88.99 mg GAE/g creatinine) after adjustment for all possible confounding factors. Participants in the highest quartile had a 36% reduced odds ratio of hypertension compared to those in the lowest quartile.

Given that a greater excretion of polyphenols in urine usually means a high TP consumption, we suggested that the inverse association observed between the objectively measured TPE in urine samples with BP may be related to a favorable effect of TP intake on BP levels.

Lastly, the relationship between BP and polyphenol-rich food patterns, such as the DASH and Mediterranean diets, has also been examined. The DASH diet is widely promoted in the USA for the prevention and treatment of hypertension.[3] In a free-living UK population,[80] systolic and diastolic BP were found to decrease significantly ($p < 0.05$) by 4.6 and 3.9 mm Hg, respectively, in those who followed a DASH-style diet. Obese hypertensive patients showed lower systolic and diastolic BP after 3 weeks on the DASH diet than those following the usual diet ($-7.6 \pm 1.4/-5.3 \pm 1.4$ mm Hg, $p < 0.001/0.02$) or the usual diet supplemented with potassium, magnesium and fiber ($-6.2 \pm 1.4/-3.7 \pm 1.4$ mm Hg, $p < 0.005/0.06$), whereas BP did not differ significantly between the usual and supplemented diets. In lean normotensives, BP values did not differ among the three diets.[81] In another study with 27 men and women who followed a DASH or a control diet, the DASH-diet group showed a significant reduction in systolic ($p < 0.001$) and diastolic ($p = 0.005$) BP.[82]

After a 6-year follow-up in the SUN study, adherence to the Mediterranean diet was associated with reduced mean levels of systolic (moderate adherence, −2.4 mm Hg; high adherence, −3.1 mm Hg) and diastolic BP (moderate adherence, −1.3 mm Hg; high adherence, −1.9 mm Hg), but it was not associated with hypertension.[83] In an intervention feeding trial (PREDIMED study) Estruch *et al.*[2] compared the short-term effects of two Mediterranean diets with those of a low-fat diet on intermediate markers of cardiovascular risk. Participants following the Mediterranean diet, supplemented with either olive oil or nuts, showed a significant decrease in systolic and diastolic BP after 3 months of intervention compared to the low-fat diet group. In a crossover study, Vinson *et al.*[84] observed a significant decrease of 4.3% in diastolic BP and 3.5% in systolic BP when 18 hypertensive subjects received six to eight small microwaved purple potatoes twice daily for 4 weeks compared with a control group on a potato-free diet.

All this weight of epidemiological evidence supports the hypothesis that polyphenol-rich diets may prevent BP from increasing and help to reduce high BP levels.

5. BIOMARKERS OF TOTAL POLYPHENOL INTAKE

In epidemiological studies, nutritional markers have several advantages over FFQ for obtaining dietary data, as shown by the significant correlations observed between urinary excretion of polyphenols and food consumption in intervention studies with specific food items.[28] Nevertheless, few studies have assessed whether TP in spot urine samples can act as valid biomarkers of TP intake. In a clinical trial,[48] TP consumption was positively and significantly correlated with TPE in spot urine samples, based on the observed relationship between polyphenol content in ingested food according to FFQ and recoveries in urine samples. Recently, Vinson *et al.*[84] determined the antioxidant capacity of urine due to polyphenol content measured by the F-C reagent after correction for nonphenolic interferences with a solid phase (Polyclar) procedure.

Some authors[85–87] have reported that phenolic compounds in spot urine samples collected from free-living subjects can be used as biomarkers of specific polyphenol-rich foods: chlorogenic acid for coffee, phloretin for apple, naringenin for grapefruit, resveratrol for wine and hesperetin for orange consumption. The presence of a combination of several polyphenols (isorhamnetin + hesperetin + naringenin + kaempferol + phloretin) may be a good indicator of total fruit consumption.

Recently, Vinson *et al.*[84] determined the antioxidant capacity of urine due to polyphenol content measured by the F-C reagent after correction for nonphenolic interferences with a solid phase (Polyclar) procedure. Plasma antioxidant capacity was also measured by ferric reducing-antioxidant power in a single-dose study with eight normal fasting subjects who received six to eight microwaved potatoes with skins or a comparable amount of refined starch in cooked biscuits. In this study, potatoes caused an increase in plasma and urine antioxidant capacity, whereas refined potato starch caused a decrease in both; purple potato consumption caused a 92% increase in 24 hours urine polyphenols, whereas the refined starch produced a small net decrease (3.5%).

Since flavonoids are widely distributed in F&V, some investigators[85–87] have examined the usefulness of urinary concentrations of polyphenols as non-specific biomarkers of F&V consumption. In a controlled dietary intervention study, a significant positive correlation between changes in F&V intake and urinary flavonoid excretion was observed after six weeks on a diet either low or high in F&V and berries. Total urinary excretion of quercetin, flavanone, and total flavonoids in 24-hour urine samples was measured by LC-MS.[85]

Mennen *et al.*[86] and Krogholm *et al.*[87] studied the correlation between polyphenol concentration in urine samples determined by LC-MS/MS and the intake of polyphenol-rich foods and beverages. Their results suggest that several polyphenols measured in urine samples can be used as biomarkers of polyphenol-rich food intake. A positive correlation was also observed between TP metabolites in 24-hour urine samples and F&V intake following one-day consumption of a basic diet supplemented with 300 or 600 g of F&V.[87] However, in observational studies, only spot urine samples, and rarely 24-hour urine samples, have been collected to investigate the potential beneficial effect of F&V on health.

A rapid new method to determine TP in complex matrices such as urine samples, which may contain many interfering substances, was pioneered by our group. A modified F-C method was applied to determine TP in urine using Oasis® MAX 96-well plate cartridges for solid phase extraction (SPE) to avoid any interference with the F-C reagent in the urine samples,[48] and TPE was found to be an accurate biomarker of polyphenol-rich food intake. Roura *et al.*[88] arrived at similar conclusions, correlating TPE in urine, measured by the F-C assay, with polyphenol consumption from cacao drinks.

Zamora-Ros *et al.*[89] evaluated the relationship between dietary TP intake and TPE, expressed by both 24-hour volume and urinary creatinine normalization, in 928 participants from the InCHIANTI study. Both urinary TPE expression models correlated with TP intake, but 24-hour volume was found to be the more accurate biomarker. Nevertheless, 24-hour urine collection is not practical in large-scale epidemiological studies, being tedious for both participants and investigators, and in cases when 24-hour volume is not available, creatinine-corrected urinary TPE may be used as a suitable biomarker of dietary TP intake in a free-living population.[48] The aforementioned high-throughput F-C method, further improved to detect TPE in creatinine-normalized urine, is particularly suitable for clinical and observational studies in which volunteers consume a wide variety of polyphenols in their habitual diet. The use of 96-well microtiter plates allows the simultaneous determination of TP in large batches of samples for daily analysis. This method is rapid, simple, cheaper and more environmentally friendly than others previously described. It has potentially useful application in studies evaluating the utility of urinary polyphenols as markers of intake, bioavailability and accumulation of these compounds in the body.

A PREDIMED substudy[25] also provides evidence that total phenol, F&V, coffee, and wine intake in the Mediterranean diet are positively correlated with the excretion of TP in spot urine samples. The standardized coefficients (β) showed that F&V intake contributed more to urinary TPE than coffee and wine consumption.

6. PLAUSIBLE MECHANISMS OF ACTION

Blood vessels have the ability to self-regulate tone and adjust blood flow and distribution in response to changes in the local environment due to their capacity to respond to physical and chemical stimuli in the lumen. Numerous blood vessels respond to an increase in flow or, more specifically, shear stress, by dilating, a phenomenon known as flow-mediated dilation. Endothelium-dependent vasodilation contributes to the maintenance of an adequate blood flow to body cells and tissues.

Various studies indicate that regular intake of polyphenol-rich beverages and foods is associated with a protective effect on the cardiovascular system. The health benefits of polyphenols have been attributed to their ability to reduce vascular oxidative stress, not only through their direct superoxide anion ($O_2^{\bullet -}$) scavenging properties and interaction with other reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$) and peroxy radicals[90–92] but also through their stimulatory effect on endogenous antioxidant enzymes and their inhibitory effect on xanthine and NAD(P)H oxidases, two major enzymes that generate large amounts of ROS.[91] The OH-groups located in the B-ring of the flavonoid molecule are essential determinants for inhibition of $O_2^{\bullet -}$ release. Flavonoids methylated at a single OH-group in the B-ring are only inhibitory when they react with activated neutrophils in the presence of myeloperoxidase.[93]

Particular structural groups determine polyphenol radical-scavenging and antioxidant potential, as reviewed by van Acker *et al.*,[94] who showed the existence of multiple mesomeric structures for aroxyl radical species of polyphenols. The *O*-dihydroxy (catechol) structure in the B ring, the obvious radical target site for all flavonoids with a saturated C2−C3 double bond (flavan-3-ols, flavanones, cyanidin chloride) confers great scavenging ability. A pyrogallol (trihydroxy) group in ring B of a catechol produces even higher activity; the C2−C3 double bond of the C ring appears to enhance scavenging behavior because it stabilizes the phenoxy radicals produced. The C2−C3 double bond in conjunction with a 4-oxo (keto double bond at position 4 of the C ring) increases scavenger activity by delocalizing electrons from the B ring. The 3-OH group on the C ring generates an extremely active scavenger and the 5-OH and 7-OH groups may also add scavenging potential in certain cases.

Polyphenols might also protect the cardiovascular system by improving the endothelial function.[95] The endothelium plays a key role in the control of vascular tone by releasing several vasorelaxing factors, which have been recognized as nitric oxide (NO) and the endothelium-derived hyperpolarizing factor (EDHF).[96–100]

Polyphenols cause NO-mediated endothelium-dependent relaxations and increase the endothelial formation of NO, as has been seen in experiments with isolated arteries. Wine, grape juice, and grape skin extracts induce concentration-dependent relaxation in rat aortic rings with endothelium, but only minor relaxation in rings without endothelium.[101] The grape-derived products increased the endothelial NO synthase activity leading to the formation of NO, and successively relaxing the vascular smooth muscle via the guanosine cyclic monophosphate (c-GMP)-mediated pathway (Figure 75.2); the polyphenol-induced relaxation associated with an increase in the c-GMP content in intact aortic rings and both the relaxation and the formation of c-GMP are prevented by NO synthase inhibitors. Additionally, the endothelium-dependent relaxation appears to be strongly correlated with polyphenol concentration in red wines.[102] These endothelium-dependent relaxations induced by polyphenols from grape-derived products have been subsequently observed in various types of animal blood vessels.[103–105] Moreover, polyphenols from several other sources such as cocoa, tea, wine or honey, have also been shown to induce endothelium-dependent NO-mediated relaxations in arteries.[78,106–109]

Since endothelium-dependent relaxations have been observed in response to anthocyanin-enriched extracts of chokeberry and bilberry, the phenolic composition of berries is regarded as determinant for their vasorelaxant activity, although only a minor effect was observed with elderberry.[110] Endothelium-dependent relaxations have also been detected in response to some authentic polyphenolic compounds including resveratrol[111] or soy isoflavones.[112]

The calcium signal (Figure 75.2) is an important signal pathway leading to the activation of endothelial NO synthase (eNOS). Red wine polyphenols and delphinidin, at a concentration of 10 mg/L, have been shown to activate eNOS by increasing the intracellular free calcium concentration ($[Ca^{2+}]_i$) in bovine aortic endothelial cells.[113] The phosphatidylinositol 3-kinase/Akt (PI3-kinase/Akt) pathway also significantly activates eNOS. Red wine polyphenols[114] and a polyphenol-rich fraction of black tea in porcine aorta[115] activated the PI3-kinase/Akt pathway in endothelial cells, producing the phosphorylation of eNOS at Ser1177 (an activator site) and dephosphorylation of eNOS at Thr495 (an inhibitor site), which increased the formation of NO. This calcium-dependent stimulatory effect involves both intracellular and extracellular calcium, as well as the p38 mitogen-

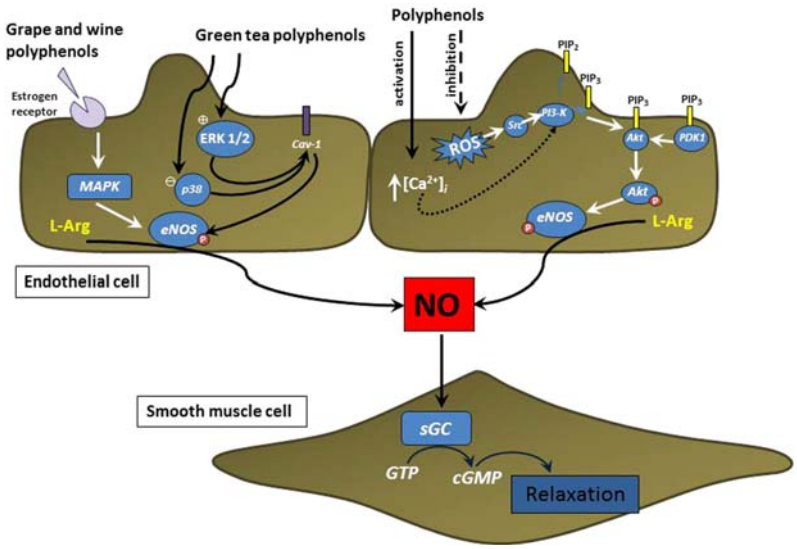


FIGURE 75.2 **Intracellular signaling pathways of polyphenols as potent inducers of the endothelial formation of nitric oxide.** $[Ca^{2+}]_I$, cytosolic calcium concentration; Cav-1, caveolin-1; cGMP, cyclic guanosine monophosphate; eNOS, endothelial NO synthase; ERK1/2, extracellular signal-regulated kinase ½; GTP, guanosine triphosphate; L-Arg, L-Arginine; MAPK, mitogen-activated protein kinases; NO, nitric oxide; P, phosphorus; PDK1, phosphoinositide-dependent kinase 1; PI3-K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-4,5-diphosphate; PIP_3, phosphatidylinositol-3,4,5-triphosphate; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase.

activated protein kinase (p38 MAPK) upstream of the PI3-kinase/Akt pathway. Low concentrations of resveratrol from grape and wine are able to activate estrogen receptors resulting in the activation of p38 MAPK and eNOS in endothelial cells.[115] A calcium-dependent activation of eNOS has been shown in response to the tannin 1-α-*O*-galloylpunicalagin, which is related with the PI3-kinase/Akt pathway.[116] Consequently, changes in cytosolic $[Ca^{2+}]_i$ in endothelial cells probably contribute to the redox-sensitive activation of eNOS in response to polyphenols via the PI3-kinase/Akt-dependent pathway. Other investigations have identified Src kinase as a redox-sensitive mediator, which plays upstream of the PI3-kinase/Akt pathway leading to eNOS activation in response to grape-derived polyphenols.[117]

In bovine aortic endothelial cells, green tea polyphenols down-regulate caveolin-1 gene expression, a major negative regulator of eNOS activity, both time- and dose-dependently, via the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and inhibition of p38 MAPK signaling pathways (Figure 75.2), increasing eNOS activation.[118]

In endothelial cells, the expression level of eNOS has also been enhanced by polyphenols, leading to an increased formation of NO; for example, the stimulatory effect of resveratrol is mainly mediated by an increase in the activity of the eNOS promoter and a stabilization of eNOS mRNA.[119]

Moreover, polyphenols induce EDHF in several types of arteries. The role of polyphenols in endothelium-dependent EDHF-mediated relaxations was first observed in isolated porcine coronary arteries.[104] Red wine polyphenols at concentrations ranging from 1 to 100 mg/L produced concentration-dependent relaxations and hyperpolarizations of vascular smooth muscle cells (Figure 75.3). It was also demonstrated that Concord grape juice, a rich non-alcoholic source of grape-derived polyphenols, is capable of inducing endothelium-dependent EDHF-mediated relaxations of porcine coronary arteries.[117] EDHF-mediated endothelium-dependent relaxations have also been observed in the isolated mesenteric arterial bed in response to alcohol-free lyophilized Brazilian red wine.[120] Resveratrol has been shown to activate IKCa channels in pancreatic islet endothelial cell lines by increasing their opening probability.[121] Red wine polyphenols induced EDHF-mediated relaxation of porcine coronary arteries by the redox-sensitive activation of PI3-kinase leading to Akt phosphorylation in endothelial cells.[122] However, the option that the PI3-kinase/Akt pathway

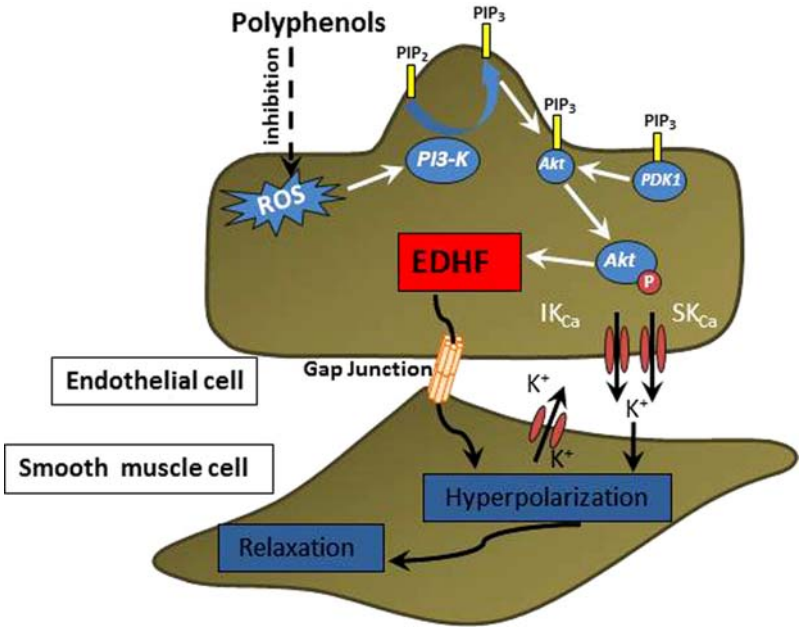


FIGURE 75.3 **Intracellular signaling pathways of polyphenols as potent inducers of the endothelial formation of endothelium-derived hyperpolarizing factor (EDHF) via the phosphatidylinositol 3-kinase/Akt pathway.** IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+; P, phosphorus; PDK1, phosphoinositide-dependent kinase 1; PI3-K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-4,5-diphosphate; PIP_3, phosphatidylinositol-3,4,5-triphosphate; ROS, reactive oxygen species; Sk_{Ca}, small-conductance Ca^{2+}-activated K^+.

modulates myo-endothelial gap junctions and/or potassium channel activity remains to be investigated.

Polyphenols also prevent the development of an endothelial dysfunction by normalizing the excessive vascular formation of superoxide anions, which react with NO to form peroxynitrites. The protective effect of these compounds on the endothelial function is explained by their ability to prevent the increased vascular expression of NADPH oxidase, a major vascular source of superoxide anions, and the cyclooxygenase-dependent formation of endothelium-derived contracting factors.[123] Moreover, the beneficial effect might also be due to the down-regulation of the angiotensin II type I receptor (AT1) in the arterial wall.[124]

7. CONCLUSIONS

Experimental and observational data support the argument that a polyphenol-rich diet may have a beneficial effect on BP, helping to lower high BP and preventing it from increasing. Overall, these studies highlight the potential of dietary polyphenols to improve or restore vascular protection by enhancing the two major endothelial vasoprotective mechanisms: the production of NO and EDHF-mediated responses, and also by reducing oxidative stress in the arterial wall, which stimulates pro-inflammatory and pro-thrombotic responses. Future intervention studies should include a detailed assessment of the bioavailability of polyphenols, beyond feeding trials carried out with polyphenol-rich foods. More studies with individual polyphenols are also required to establish their role in the prevention of CVD.

Acknowledgments

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B.3. Capítol de llibre 3. Els polifenols de les fruites i les verdures disminueixen la pressió arterial

Book chapter 3. Fruit and vegetable polyphenols decrease blood pressure.

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Chapter 26

Fruit and Vegetable Polyphenol Consumption Decreases Blood Pressure

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Hypertension is one of the main cardiovascular risk factors, modifiable by diet. On the other hand, an inverse association between risk for cardiovascular disease and consumption of polyphenol-rich foods has been found in several epidemiologic studies. The aim of the current study was to evaluate the usefulness of total polyphenols excreted in urine as a new biomarker of total polyphenols intake and to correlate it with blood pressure (BP) and the prevalence of hypertension in a large cohort of high-risk subjects included in the PREDIMED trial. Participants in the highest quartile of urinary total polyphenols excreted had a 36% reduced prevalence of hypertension compared to those in the lowest quartile. In addition, systolic and diastolic BP were inversely associated with urinary total polyphenols excreted after adjustment for potential confounders ($P < 0.05$) in an elderly Mediterranean population at high cardiovascular risk. We concluded that high consumption of polyphenol-rich foods reduces the prevalence of hypertension.

Essential hypertension, defined as systolic blood pressure (BP) greater than 140 mmHg or diastolic BP greater than 90 mmHg, is the major cause of cardiovascular morbidity, as well as the major modifiable cause of death in both economically emerging and developed countries (*1*). In population studies, a reduction in the entire distribution of BP by 5 mmHg has been hypothesised to produce a 40% decrease in the incidence of stroke and a 20–25% reduction in CHD (*2–4*).

Lifestyle changes may help to prevent hypertension. These changes include the maintenance of a body mass index (BMI) between 18.5 and 24.9 Kg/m^2, reduction of sodium intake to less than 2,300 mg a day, which is about 1 teaspoon of salt, and to perform exercise that raises heart rate at least 2½ hours a week. Other measures are to follow-up a healthy diet and limit alcoholic beverages to 2 drinks a day for men and 1 drink a day for women. In fact, the Mediterranean (*5*) and the DASH (Dietary-Approaches-to-Stop-Hypertension) diets (*6*) have also demonstrated to reduce high BP. Similarly, other clinical trials have observed that following a diet rich in fruits, vegetables and cocoa also decreases BP (*6–10*). However, up to now, clinical trials focused on the relationship between phenolic compounds from fruits and vegetables (F&V) and BP have reported conflicting results, mainly because the food studied had very different phenolic profile and the effect of microbiota was not well established.

There is evidence suggesting that the high consumption of F&V lowers BP and may protect against CVD and stroke (*11–15*). F&V contains high amounts of polyphenols, micronutrients that have exhibited a broad spectrum of biological activities (*16*) for human health including anti-hypertensive properties (*17*). Table I summarizes the results of different intervention clinical trials with polyphenol-rich foods on BP (*18–33*). It is difficult to draw conclusions since some results are contradictory and many variables may affect the results observed, such as subject characteristics (healthy, hypertensive or with other cardiovascular risk factors), the amount and class of polyphenols given, food matrix, and duration of the study. Thus, for instance, to lower blood pressure is more difficult in normotensive than in hypertensive subjects. Another example of variability is the different results obtained in various studies using pomegranate juice. A consistent 5% reduction in systolic BP was reported when Aviram *et al.* (*18*) gave 50 mL/d pomegranate juice to 10 hypertensive individuals for 2 weeks, whereas 21% reduction in systolic BP was observed when the same volume of juice was given to a larger group of participants with asymptomatic severe carotid artery stenosis for a year (*19*). By contrasts, Sumner and colleagues (*21*) reported a reduction in stress-induced ischaemia, but no effect on BP after 240 mL/d of pomegranate juice, even though a larger volume than Aviram's was consumed for a longer length of time (90 d) in a much larger group of participants (n = 45) with ischaemic coronary disease (*20*). The explanation for these discrepant results is unclear and counterintuitive. A possible reason is that juices used in the studies were derived from different sources and thus had different polyphenolic content. Sumner's group used a commercial pomegranate juice, which suffers more technologic processing that may affect the polyphenolic composition, while Aviram's group produced an in-house concentrated form of pomegranate juice, which was chemically analysed.

The differences in the health status of the participants in the studies and the length of dietary intervention may also explain part of these discrepancies. Thus, available data is weak since it include few and small studies. Therefore, long-term clinical or epidemiological trials are needed to definitively clarify the benefits deriving from long-term consumption of polyphenol-rich foods or a polyphenol-rich diet pattern.

In epidemiological trials, biomarkers of the intake of some nutrients are more precise and provide better objective measures than data obtained from food frequency questionnaires (FFQ). The development of biomarkers, measured in blood or urine, is essential for making accurate estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers has been often highly complex (*34*).

The major and still unresolved drawback in evaluating polyphenol bioavailability is the fact that after strict dietary monitoring (i.e., diets free of those phenolic compounds of interest), and following hours of fasting, it remains impossible to eliminate all phenolic compounds in biological fluids (*35*). Therefore, a basal concentration of phenolics will be found in the urine, even avoiding polyphenol-containing food intake for some days.

Water accounts for about 95% of the total volume of urine and the remaining 5% consisting of solutes derived from cellular metabolism and outside sources. A wide range of water-soluble compounds, including mineral salts, vitamins, amino acids, enzymes, hormones, antigens, fatty acids, nucleosides, immunoglobulins, pigments, uric acid, urea, hippuric acid, etc, are believed to be present in urine normally, although other substances like proteins, glucose, erythrocytes, and ketones bodies can also be found when the body's processes are not operating efficiently (*36*). Thus, the Folin-Ciocalteu (F-C) assay could prove a poor method for determining the total phenolic concentration in urine, due to the above-mentioned interfering elements. However, we reported that the application of a Solid Phase Extraction (SPE) procedure to urine samples can remove such reductant water-soluble compounds. Following this with the Singleton and Rossi F-C assay (*37*) with certain modifications, provides an effective technique to measure total phenolics compounds excreted in urine (*38*). SPE with 96-well plate cartridges (Oasis® MAX) was performed in the urine samples to avoid any interference with F-C reagent. For all the spot urine samples, total polyphenols excreted (TPE) was analyzed as described by Medina-Remón A. *et al* (*38*) and expressed as mg gallic acid equivalent (GAE)/g of creatinine. To ensure that there are not interfering reductant substances, an evaluation of the main reductant substances from urine and the major drugs consumed in Europe was performed (see Table II). Before SPE cleaning up procedure, some interfering substances at the level that may be present in urine react with the F-C reagent (Fe(II), Vitamin C, adrenaline, noradrenaline and dopamine; however after the SPE clean-up any of them react with F-C. From the drugs studied, only paracetamol can give interference, after the cleaning process, on TPE results and therefore paracetamol intake should be registered in all questionnaires and evaluations.

Table I. Effects of food polyphenols on blood pressure in human intervention studies

References	*Type of study*	*No. Ind.* †	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols* ‡	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP*¥
Aviram & Dornfeld (2001) (18)	Chronic, single arm, no control	10 (7m,3f)	Hypertensives	62-77	Pomegranate juice	Tannins,anthocyanins	50 mL (1.5 mmol)	14 days	SBP serum ACE	↓5% ↓36%
Reshef et al. (2005) (19)	Chronic, controlled parallel	12 (8m, 4f)	Stage I Hypertensives	42-62	Sweetie fruit (hybrid between grapefruit and pummelo)	Flavonoids	0.5 L (889 mg/L)	5 weeks	SBP DBP	?
Aviram et al. (2004) (20)	Chronic, controlled parallel	19 (14m,5f)	Patients with asymptomatic severe carotid artery stenosis	65-75	Pomegranate juice	Tannins,anthocyanins	50 mL (2484 mg/L)	1-3 years	SBP (after 1 y) SBP (after 3 y) DBP	↓ 12% No further reduction↔
Sumner et al. (2005) (21)	Chronic, controlled parallel	45 (40m,5f)	Patients with ischaemic coronary disease and myocardial Ischaemia	58-80	Pomegranate juice	Tannins,anthocyanins	240 mL	90 days	BP	↔

References	*Type of study*	*No. Ind.* †	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols* ‡	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP*§
Ruel et al. (2005) (22)	Chronic, single arm, no control	21 (all men)	Healthy	30-46	Cranberry juice	Flavonoids, phenolic acids	7 mL/Kg body wt.	14 days	SBP	↓ 2% (not statistically significant)
Taubert et al. (2003) (23)	Acute, controlled crossover	13 (6m,7f)	Healthy but with stage 1 mild isolated systolic hypertension	55-64	Dark chocolate	Flavonoids	100 g (500 mg)	14 days	SBP DBP	↓ 5.1 mmHg ↓ 1.8 mmHg
Gorinstein et al.(2006) (24)	Chronic, controlled parallel	57	hyperlipidaemics after coronary bypass surgery	39-72	Blond or red grapefruit	Flavonoids, anthocyanins	One fruit (20 mg/100 g fresh wt.)	30 days	SBP DBP	↔ ↔
Naruszewicz et al.(2007) (25)	Chronic, controlled parallel	44 (33m,11f)	myocardial infarction survivors on statins for 6 months	57-75	Chokeberry flavonoid extract	Anthocyanins, procyanidins	3 × 85 mg	42 days	DBP SBP serum ACE	↓ 7.2 mmHg ↓11 mmHg ↓33.3%
Taubert et al. (2007) (26)	Chronic, controlled parallel	44 (20m,24f)	Hypertensives	56-73	Dark chocolate	Flavonoids	6.3 g (30 mg)	18 weeks	SBP DBP	↓2.9 mmHg ↓1.9 mmHg

Continued on next page.

Table I. (Continued). Effects of food polyphenols on blood pressure in human intervention studies

References	*Type of study*	*No. Ind.* †	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols* ‡	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP*¥
Fukino et al. (2008) (27)	Chronic, single arm, no control	60 (49m,11f)	Healthy	32-73	Green tea-extract powder	Catechins	1 packet (544 mg)	2 months	DBP	↓4 mmHg
Wilson et al.(2008) (28)	Acute, controlled parallel	187 (38m,149f)	Healthy	19-20	Cranberry extract or other beverage	Anthocyanins, phenolic acids	480 mL	Postprandial	BP Heart rate (from 0 to 180min)	↔ ↔
Borochov-Neori et al.(2008) (29)	Chronic, single arm, no control	10	Healthy	All ages (adults)	Marula juice	Hydrolysable tannins, catechins	200 mL (56 mg/dL)	21 days	BP	↔
Erlund et al.(2008) (30)	Chronic, controlled crossover	71 (25m,46f)	Subjects with CVD risk factors	51-64	Bilberries, lingonberries, blackcurrant, strawberry puree and raspberry juice	Anthocyanins	150 g (837 mg)	56 days	SBP	↓ by 7·3mmHg in subjects with high baseline BP in the treatment group

References	*Type of study*	*No. Ind.* †	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols* ‡	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP*¥
Monagas et al. (2009) *(31)*	Chronic, controlled crossover	42 (19m,23f)	Healthy	58-81	Cocoa powder (with skim milk)	Flavonoids	40 g (495.2 mg)	28 days	SBP DBP Heart rate	↔ ↔ ↔
Morand et al. (2010) *(32)*	Chronic, controlled crossover	44 (all men)	Healthy overweight	50-65	Orange juice or hesperidin enriched drink.	Flavonoids (Hesperidin)	500 mL (342 mg)	28 days	SBP DBP Pulse pressure	↔ ↓3.2-5.5 mmHg ↔
van Mierlo et al. (2010) *(33)*	Chronic, controlled crossover	35 (all men)	Healthy	18-45	Wine grape or grapes seed extracts (capsules)	Anthocyanins, phenolic acids	6 capsules (800 mg)	14 days	SBP DBP Heart rate	↔ ↔ ↔

SBP, systolic blood pressure; serum ACE, plasma angiotensin 1-converting enzyme; DBP, diastolic blood pressure; BP, blood pressure.* Table is arranged by year in ascending order. † m: male, f: female ‡ Only the top two polyphenols with the highest concentrations are listed. ¥ ↑refers to increase; ↓ refers to decrease; ↔ refers to no change; unless otherwise stated, (%) refers to changes from baseline when test substance given.

Table II. Possible interferences in urine by Folin-Ciocalteu Assay

Reductant compounds (maximum levels in urine)	*F-C Assay*	*F-C post SPE*
Sugars: Glucose (2 mg/L) and Fructose (1 mg/L)	-	-
Fe (II) (1 mg/L)	+	-
Organic acids: Oxalic, Citric and Tartaric acids (100 mg/L)	-	-
Aminoacids: Phe, Tyr, Glut, Arg (1 mg/L)	Weak	-
Vitamin C (100 mg/L)	+	-
Folic acid (100 mg/L)	-	-
Hippuric acid (10 mg/L)	-	-
Epinephrine or adrenaline (0.02 mg/L)	+	-
Norepinephrine or noradrenaline (0.08 mg/L)	+	-
Dopamine (0.4 mg/L)	+	-
Drugs (use)	*F-C Assay*	*F-C post SPE*
Paracetamol (analgesic, antipyretic)	+	+
AAS (analgesic, antipyretic)	-	-
Celecoxib (analgesic, antidysmenorrhoea, antirheumatic, anti-inflammatory)	-	-
Diclophenaco (NSAIDs)	-	-
Ibuprofen (NSAIDs)	-	-
Digoxine (antiarrhytmic, cardiotonic)	-	-
Manidipine (antihypertensive)	-	-
Hidrochlorotiazine (antihypertensive)	-	-
Enalapril Maleate (antihypertensive)	-	-
Losartan (antihypertensive)	-	-
Amlodipino (antihypertensive)	-	-
Atenolol (antihypertensive)	-	-
Doxazocina (antihypertensive)	-	-
Rupatadine (antihistaminic)	-	-
Simvastatine (hypolipidemic)	-	-
Bezafibrate (hypolipidemic)	-	-
Omeprazol (inhibits gastric acidity)	+	-
Alopurinol (reduces uric acid production)	-	-
Glimepiride (oral antidiabetic)	-	-

Continued on next page.

Table II. (Continued). Possible interferences in urine by Folin-Ciocalteu Assay

Drugs (use)	*F-C Assay*	*F-C post SPE*
Metformina (oral antidiabetic)	-	-
Gliclazida (oral antidiabetic)	+	-
Insulina (injectable antidiabetic)	+	+
Paroxetina (anxiolytic)	-	-
Aprazolam (anxiolytic)	-	-
Melatonina (anti jet-lag)	+	-

(+) Substances that react with the F-C. (-) There is no reaction, so this substance does not interfere.

The aim of the current study was to evaluate the usefulness of a new biomarker (TPE) and to correlate it with BP and the prevalence of hypertension (*17*).

Subjects and Design

The PREDIMED (*PREvención con DIeta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639). The detailed recruitment method and study protocol have been described previously (*5*). From October-2003 to July-2004, we selected 612 potential participants in primary health centers. Eligible participants were community-dwelling men aged 55 to 80 years and women aged 60 to 80 years, who were free of cardiovascular disease at baseline and fulfilled at least one of the following two criteria: (1) type-2 diabetes mellitus and/or (2) three or more coronary heart disease (CHD) risk factors (*39*). The participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of the two participating centres.

Measurements

At baseline, all participants completed a validated semiquantitative FFQ with 136-items (*40*), the validated Spanish version (*41*) of the Minnesota Leisure Time Physical Activity Questionnaire, and a 47-item questionnaire about education, lifestyle, history of illnesses and medication used. Trained nurses measured BP thrice with a validated semi-automatic oscillometer (Omron HEM-705CP (*42*); Hoofddorp, The Netherlands). Energy and nutrient intake was derived from Spanish food composition tables (*43*). TP consumption from plant food and beverages (mg/g fresh matter) was quantified according to Saura-Calixto F *et al.* (*44*) and Brat P *et al.* (*45*), from the data of the FFQ. For TPE determination urine samples were thawed on a ice bed for 3 h; they were centrifuged for 10 min at 4

ºC and 1 mL of supernatants, catechin and gallic acid standards for calibrated line (1, 2, 4, 6, and 8 mg L^{-1}) were diluted with 1 mL of water Milli-Q and acidified with 34 µL of hydrochloric acid at 35%; they were used to load the Oasis® MAX 96-well plate SPE cartridges separately. The extraction procedure described above for Oasis®MAX cartridges was applied and 15 µL of the eluted fractions were mixed with 170 µL of Milli-Q water in the thermo microtiter 96-well plate (nunc™, Roskilde, Denmark), adding 12 µL of F-C reagent and 30 µL of sodium carbonate (200 g/L). The multichannel pipette minimized differences in the times (3 s) of the F-C reaction between the eight lines of the 96-well plate, ensuring a similar reaction time for all samples analyzed on the same plate. The 96-well plate permitted fewer reagents to be used in a more environmentally friendly test. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 73 µL of Milli-Q water were added with the multichannel pipette. Absorbance was measured at 765 nm in UV/VIS Thermo Multiskan Spectrum spectrophotometers (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in only 10 s.

For creatinine in urine samples, 3 µL of urine were mixed with 60 µL of aqueous picric acid solution (1%) and 5 µL of sodium hydroxide (10%). After shaking, the mixture was left 15 min in the dark at room temperature; 232 µL of Milli-Q water was added and the absorbance was measured at 500 nm in the UV/VIS spectrophotometers. Total polyphenols were expressed as mg gallic acid equivalent (GAE)/ g creatinine and mg catechin/ g creatinine.

Results and Discussion

Table III shows the average food consumption of study participants divided according to quartiles of urinary TPE expressed as mg GAE/g creatinine. Significant increasing trends across quartiles of TPE were observed for the intake of fruits, vegetables, total F&V, dairy products, fish and TP intake; whereas decreasing trends across TPE quartiles were observed for total alcohol intake, cereals, common olive oil, pastries, cakes or sweets, as well as total energy intake.

The linear regression analyses of TPE in spot urine samples and TP intake (100 mg) and total F&V intake (100g) are presented in Table IV with various models after adjusted for potential confounding factors. We observed a significant positive association between urine TPE and daily intake of F&V in the unadjusted model (β=0.131; P<0.001). After adjusting for potential confounding factors the association remained statistically significant in the multivariate regression analysis. The standardized coefficients (Beta) are the regression coefficients obtained with the regression model using the standardized values (measured in standard deviation units), and are therefore independent of measurement units. The standardized coefficients from this model showed that total phenol intake (Beta=0.283) contributed more to urinary TPE than F&V intake (Beta=0.150)

On multivariate linear regression analyses, systolic and diastolic BP exhibited a monotonic inverse association with TPE in spot urine samples (quartiles) after adjustment for potential confounders (Table V). The non-standardized coefficients, β=-1.731, P=0.024 and β=-1.264, P=0.003 represent the expected

change of systolic and diastolic BP, respectively, corresponding to an increase from a TPE to the upper quartile.

Logistic regression analysis showed an inverse association between urinary TPE (by quartile) and the prevalence of hypertension (Table VI). Compared to the participants in the lowest quartile of TPE (<88.99 mg GAE/g creatinine), participants in the highest quartile (>160.23 mg GAE/ g creatinine) had a significantly reduced prevalence of hypertension (OR=0.71, CI 0.53 to 0.95; P=0.022) in the unadjusted model. In all three models, a significant difference was observed between the top and the bottom quartiles for the prevalence of hypertension, after adjustment for potential confounders. When the analysis was adjusted for all possible confounding factors (model 4), participants in the highest quartile had a 36% reduced odds of hypertension (OR=0.64, CI 0.45 to 0.92; P=0.015), compared to those in the lowest quartile.

Finally, BP correlated better with urinary TPE than with TP intake assessed by FFQ. In fact, a highly significant association between polyphenol intake assessed via TPE in urine and systolic (P for trend = 0.024) and diastolic BP (P for trend = 0.003) was observed, whereas polyphenol intake assessed via FFQ tended to be associated with BP values, but here the association did not reach the statistical significance.

The results from the present study provide evidences that total phenol and F&V intake in the Mediterranean diet are positively correlated with the excretion of TPs in spot urine samples. Thus, F&V consumption is the main contributor to urinary TPE.

Polyphenols are the main dietary represent a wide variety of structures from different subclasses. The biological effects of these compounds depend on their bioavailability, their kinetics and exposure time (*46*). The main sources of these compounds are fruit, vegetables and beverages such as wine, coffee and tea which contain complex mixtures of often poorly characterized polyphenols (*47*), possibly explaining the difficulties in determination of total phenol content in foods (*45*).

The most common polyphenols in the human diet are not necessarily the most active *in vivo*, either because they have a lower intrinsic activity than others or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated (*47*).

The presence of multiple antioxidants in fruit- and vegetable-rich diets may explain the lowering effect on BP observed in hypertensive patients. A protect effect of F&V against cardiovascular disease (CVD) has been observed in numerous epidemiological studies (*7*, *8*). Thus, high intake of F&V correlated with a reduced risk of CVD in a study on 2682 men in Finland (*48*). Radhika G. *et al.* (*49*) also examined the relationship between F&V intake and CVD risk factors in urban south Indians. The study population was comprised of 983 individuals aged 20 years or more, selected from the Chennai Urban Rural Epidemiological Study (CURES). Linear regression analysis revealed that after adjusting for potential confounder factors, the highest quartile of F&V intake showed a significant inverse association with systolic BP ($\beta = -2.6$ mmHg; P = 0.027), when compared with the lowest quartile. A high intake of F&V explained 48% of the protective effect against CVD risk factors.

Table III. Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE /g creatinine. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<89.0)	*Q2 (89.1-119.5)*	*Q3 (119.6-160.2)*	*Q4 (>160.3)*	*P for trend*[1]
Urine total polyphenol (mg GAE/ g creatinine)[2]	72.8 (14.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	< 0.001
No. of subjects	147	147	147	147	0.742
Olive oil (g)	48.7 (14.3)	47.3 (13.7)	45.1 (14.6)	45.0 (15.7)	0.014
Total nuts (g)	8.6 (10.7)	9.2 (11.3)	9.6 (12.2)	9.1 (10.9)	0.680
Vegetables (g)	253.1 (80.2)	261.1 (81.1)	262.6 (84.0)	272.7 (92.1)	0.053
Legumes (g)	17.9 (7.0)	17.9 (7.5)	17.7 (7.0)	16.5 (7.6)	0.107
Fruits (g)	304.6 (143.6)	319.3 (141.3)	315.1 (132.9)	360.8 (151.2)	0.002
Total fruits and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Fish or seafood (g)	86.5 (34.0)	82.2 (35.8)	88.7 (36.9)	94.1 (39.0)	0.030
Meat or meat products (g)	132.0 (49.6)	129.7 (43.7)	134.3 (48.5)	134.2 (47.5)	0.531
Pastries, cakes or sweets (g)	34.8 (31.6)	27.9 (24.8)	25.3 (25.9)	23.7 (28.7)	< 0.001
Cereals (g)	243.7 (106.7)	235.8 (91.6)	228.7 (98.3)	211.3 (83.3)	0.003
Milk and dairy products (mL)	352.1 (214.8)	378.8 (216.5)	396.5 (222.1)	403.1 (215.1)	0.033
Wine (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (48.3)	74.5 (56.0)	0.097
Tea (mL)	3.92 (16.4)	3.25 (11.4)	4.16(12.6)	5.47 (20.4)	0.332
Chocolate (g)	3.1 (6.2)	2.4 (5.2)	2.3 (5.5)	1.9 (4.8)	0.063
Natural orange juice (mL)	23.3 (54.3)	25.19 (57.8)	13.4 (41.2)	19.1 (50.8)	0.199

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Table III. (Continued). Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE /g creatinine.

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<89.0)	*Q2 (89.1-119.5)*	*Q3 (119.6-160.2)*	*Q4 (>160.3)*	*P for trend*[1]
Total polyphenol intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Alcohol (g)	15.7 (17.9)	10.2 (16.0)	12.2 (18.5)	9.9 (17.8)	0.018
Fibre (g)	22.1 (6.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.2)	0.606
Cholesterol (g)	353.6 (119.7)	328.6 (93.7)	340.5 (113.4)	342.1 (89.4)	0.561
Sodium (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potassium (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Total energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	< 0.001

[1] One-factor ANOVA was used for continuous variables and χ^2-test for categorical variables; [2] Mean (standard deviation). GAE: gallic acid equivalent.

In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP, whereas the intake of cereals and meat was directly associated with systolic BP (*50*). In the Chicago Western Electric Study vegetable protein, beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP, after the 8-year follow-up in 1714 employed middle-aged men (*51*). On the other hand, Hung HC *et al.* (*52*) evaluated the association of F&V consumption with peripheral arterial disease in a cohort of 44,059 men initially free of cardiovascular disease and diabetes, reporting no evidence that F&V consumption protects against peripheral arterial disease. In the age-adjusted model, men in the highest quintile of F&V had a relative risk of 0.55 (95% CI = 0.38-0.80) for peripheral arterial disease, compared with those in the lowest quintile. However, the associations were greatly weakened after adjustment for smoking and other traditional cardiovascular risk factors. .

Table IV. Multivariate linear regression analysis with total polyphenol excreted normalized, in spot urine samples (mg GAE/g creatinine) as the dependent variable, and total polyphenol (100mg/d) and total fruit and vegetable (100g/d) as exposure variable, adjusted for potential confounders. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Model	*β*	*SE*	*Beta*	*P*	*95% CI*
Total polyphenol intake (100mg/d)	Model 1	0.073	0.017	0.179	<0.001	0.041 to 0.106
	Model 2	0.110	0.016	0.268	<0.001	0.078 to 0.141
	Model 3	0.117	0.017	0.286	<0.001	0.085 to 0.150
	Model 4	0.116	0.020	0.283	<0.001	0.077 to 0.154
Fruit and Vegetables (100g)	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Model 2	0.112	0.033	0.132	0.001	0.047 to 0.177
	Model 3	0.107	0.034	0.126	0.002	0.040 to 0.174
	Model 4	0.127	0.036	0.150	<0.001	0.056 to 0.198

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2 was adjusted by sex, age and weight; Model 3 as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus total fish/seafood, olive oil, beer, chocolate, natural orange juice, tea, cereals, milk and dairy products, meat or meat products, nuts, pastries/cakes/sweets, fruits and vegetables, wine and coffee intake; GAE: Gallic acid equivalent.

Table V. Multivariate linear regression analyses with systolic blood pressure and diastolic blood pressure as the dependent variables, and quartile of total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as exposure variable, adjusted for potential confounders. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Model	*β*	*SE*	*Beta*	*P*	*95% CI*
Systolic blood pressure	Model 1	-1.743	0.712	-0.104	0.015	-3.141 to -0.345
	Model 2	-1.895	0.741	-0.113	0.011	-3.350 to -0.440
	Model 3	-1.895	0.743	-0.113	0.011	-3.354 to -0.436
	Model 4	-1.731	0.765	-0.103	0.024	-3.233 to -0.228
Diastolic blood pressure	Model 1	-1.705	0.397	-0.180	<0.001	-2.485 to -0.925
	Model 2	-1.438	0.408	-0.152	<0.001	-2.238 to -0.637
	Model 3	-1.405	0.409	-0.148	0.001	-2.208 to -0.602
	Model 4	-1.264	0.422	-0.133	0.003	-2.092 to -0.435

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, and educational level; Model 4 adjusted as in Model 3 plus drug consumed in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

Table VI. Multivariate adjusted odds ratios (95% confidence intervals) for cardiovascular risk factors (hypertension) according to quartiles of total polyphenol excretion expressed as mg GAE/ g creatinine using the lowest quartile group as reference category. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<88.99)	*Q2 (89-119.46)*	*Q3 (119.47-160.22)*	*Q4 (>160.23)*	*P for trend*
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Model 1	1.00	1.82 (0.33-10.13)	0.67 (0.29-1.55)	0.71 (0.53-0.95)	0.021
Model 2	1.00	1.40 (0.21-9.24)	0.61 (0.25-1.47)	0.64 (0.46-0.89)	0.006
Model 3	1.00	1.29 (0.19-8.49)	0.55 (0.22-1.37)	0.65 (0.47-0.91)	0.007
Model 4	1.00	1.39 (0.19-10.34)	0.55 (0.20-1.48)	0.64 (0.45-0.92)	0.047

Model 1, unadjusted; Model 2 was adjusted for sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

All these epidemiological evidence support the hypothesis that a diet rich in F&V may prevent BP from increasing and may help to decrease elevated BP levels. However, only one study (*17*) has attempted to correlate the biomarker of TP intake, determined in spot urine samples, with BP measurements or with the prevalence of hypertension. Taking into account that greater excretion of polyphenols in urine is determined by high TP consumption, we suggested that the inverse association observed between the objectively measured TPE in urine samples with BP may be related to a favorable effect of TP intake on preventing raised BP levels.

The BP lowering effects of fruit- and vegetable-rich diets in hypertensive patients have mainly been attributed to the presence of multiple polyphenols in these foods. In respect to the last point, some studies have suggested that polyphenols contained in foods may exert antihypertensive effects and contribute to the prevention of hypertension, due to its vasodilatation properties related to the increasing release of endothelial-derived nitric oxide (*53*, *54*).

Conclusions

Total polyphenol consumption (TPE) can be used as a polyphenol intake biomarker. It has been found a negatively association between high TPE in urine samples and BP levels in an elderly Mediterranean population (*17*). Thus, in order to lower cardiovascular risk, which is associated to high blood pressure, a high intake of polyphenol-rich diet mainly from fruits and vegetables is recommended. On comparing the participants in the highest with those in the lowest quartile of TPE a 36% reduction was observed in the odds ratio of hypertension. However, the observation that systolic and diastolic BP decreases when TPE increases should be confirmed in further intervention studies.

Acknowledgments

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C. Comunicacions en congressos/Conference communications

C.1. Comunicació 1. Pòster

Títol: Lignans, flavanols, and hydroxybenzoic acids intake decreased cardiovascular risk in the PREDIMED trial.

Autors: Rosa M. Lamuela-Raventós, Anna Tresserra-Rimbau, Eric B. Rimm, Alexander Medina-Remón, Miguel A. Martínez-González, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: Oxygen Club of California World Congress. Davis, USA. 2014.

LIGNANS, FLAVANOLS, AND HYDROXYBENZOIC ACIDS INTAKE DECREASED CARDIOVASCULAR RISK IN THE PREDIMED TRIAL

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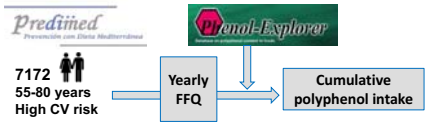
Background and objectives

Cardiovascular diseases (CVD) are the leading cause of mortality and disability in developed countries. Epidemiologic and mechanistic evidence showed an inverse association between flavonoids and CVD. However, data for other polyphenol groups is still scarce.
The aim of this study was to evaluate the association between intakes of all polyphenol subgroups, and the risk of major cardiovascular events (myocardial infarction, stroke or death from cardiovascular causes in the PREDIMED.

Methods



We used Time-dependent Cox proportional hazards regression with updated diet and covariates information was used to estimate the Hazard Risk (HR) to relate polyphenol consumption and risk of cardiovascular event using the lowest quintile as the referent group. All intakes were calories adjusted. Statistical analyses were conducted by using SAS software, version 9.3. All *P* values were 2-sided and differences below the probability level (*P*<0.05) were considered significant. Clinical Trial Registration: International Standard Randomized Controlled Trial Number (ISRCTN of London, England) 35739639.

Results

Over an average of 4.3 years of follow-up, 273 confirmed cases of CVD were reported among the 7172 participants (96.3%) who completed the FFQ. After multivariate adjustment and comparing Q5 vs. Q1 different polyphenol subgroups intake, we observed a significant association for flavanols (HR=0.40; CI 0.23-0.72; P-trend=0.003), lignans (HR=0.51; CI 0.30-0.86; P-trend=0.007), and hydroxybenzoic acids (HR=0.47; CI 0.26-0.86; P-trend 0.02).

Table 1. Baseline characteristics of participants in the PREDIMED cohort according to quintiles of total polyphenol intake at baseline (energy-adjusted).[a]

	Q1	Q2	Q3	Q4	Q5	P[b]
No subjects (7172)	1434	1435	1434	1435	1434	
Polyphenol intake (mg/d)	483±108	674±36	794±36	937±50	1235±199	
Sex, women	836 (58.3)	924 (64.4)	712 (60.8)	803 (56.0)	648 (45.2)	<0.0001
Age (y)	67.6±6.2	67.4±6.1	67.4±5.9	66.9±6.0	66.2±6.1	<0.0001
Body mass index (kg/m^2)	30.0±3.7	30.3±3.7	29.7±3.5	29.7±3.7	29.6±3.5	<0.0001
Current smoker	217 (15.1)	210 (14.6)	194 (13.5)	265 (18.5)	317 (22.1)	<0.0001
Physical activity at leisure time (MET-h/d)	3.37±3.56	3.62±3.83	3.77±3.66	4.05±4.25	4.59±4.54	<0.0001
Diabetes	706 (49.2)	680 (47.4)	712 (49.6)	704 (49.1)	668 (46.6)	0.40
Hypertension	1230 (85.8)	1224 (85.3)	1192 (83.1)	1166 (81.3)	1117 (77.9)	<0.0001
Hypercholesterolemia	983 (68.6)	1018 (70.9)	1053 (73.4)	1065 (74.2)	1069 (74.6)	0.001
Family history of CVD	403 (28.1)	290 (20.2)	310 (21.6)	324 (22.6)	446 (31.1)	0.05
Intervention group of MedDiet with EVOO	489 (34.1)	506 (35.3)	477 (33.6)	473 (33.0)	517 (36.1)	0.001
Intervention group of MedDiet with nuts	444 (31.0)	467 (32.5)	454 (31.7)	491 (34.2)	519 (36.2)	
Total energy intake (Kcal/d)	2397±642	2180±589	2161±540	2229±563	2369±577	<0.0001

[a] Categorical variables: subjects (percentage); continuous variables: mean±SD
[b] One-way ANOVA tests (continuous variables) or chi-squared (categorical variables)
MedDiet, Mediterranean diet; EVOO, extra virgin olive oil.

Table 2. The relationship between CVD and cumulative polyphenols subclasses intake (in quintiles) in participants from the PREDIMED Study.

	Q1	Q2	Q3	Q4	Q5	*P* for trend
Lignans (mg/d)	0.44	0.57	0.67	0.77	0.94	
No. of cases	69	57	53	44	50	
No. of person years	4625	6122	6899	6892	6530	
Age and sex	1.00	0.61 (0.40-0.95)	0.55 (0.36-0.86)	0.57 (0.35-0.91)	0.51 (0.31-0.84)	0.004
Model 2	1.00	0.65 (0.41-1.01)	0.55 (0.35-0.87)	0.61 (0.37-0.99)	0.50 (0.29-0.85)	0.007
Model 3	1.00	0.64 (0.41-0.99)	0.54 (0.34-0.85)	0.60 (0.36-0.97)	0.51 (0.30-0.86)	0.007
Flavanols (mg/d)	90	129	158	192	263	
No. of cases	69	51	59	59	35	
No. of person years	4841	6409	7058	6860	5900	
Age and sex	1.00	0.64 (0.43-0.94)	0.65 (0.44-0.95)	0.55 (0.37-0.82)	0.33 (0.21-0.53)	<0.0001
Model 2	1.00	0.65 (0.41-1.02)	0.70 (0.44-1.09)	0.57 (0.36-0.91)	0.36 (0.20-0.63)	0.0004
Model 3	1.00	0.70 (0.44-1.10)	0.77 (0.49-1.21)	0.66 (0.41-1.05)	0.40 (0.23-0.72)	0.003
Hydroxybenzoic acids (mg/d)	6.9	12.9	17.8	24.1	36.1	
No. of cases	69	62	47	55	40	
No. of person years	5398	6603	6734	6853	5480	
Age and sex	1.00	0.80 (0.54-1.17)[a]	0.60 (0.40-0.90)	0.54 (0.36-0.82)	0.46 (0.29-0.71)	0.0003
Model 2[b]	1.00	0.82 (0.52-1.29)	0.65 (0.40-1.06)	0.59 (0.36-0.97)	0.37 (0.20-0.66)	0.0006
Model 3[c]	1.00	0.91 (0.57-1.43)	0.74 (0.46-1.22)	0.73 (0.44-1.21)	0.47 (0.26-0.86)	0.02

[a] HR (95% CI)
[b] Model 2 – age, sex, smoking, BMI, alcohol, energy, physical activity, family history of CVD, aspirin use, antihypertensive drugs, cardiovascular drugs, and diabetes status.
[c] Model 3 - model 2 plus intake of proteins, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol.

Conclusions

Greater intakes of flavanols, lignans, and hydroxybenzoic acids were associated with a lower risk of CVD in a Spanish cohort of elderly people at high cardiovascular risk.

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C.2. Comunicació 2. Pòster

Títol: A mediterranean diet pattern supplemented with nuts or extra virgin olive oil increases total polyphenol excretion and significantly reduces inflammatory parameters: the predimed randomized trial after one year.

Autors: Alexander Medina-Remón, Rosa Casas, Anna Tresserra-Rimbau, Anna Vallverdú-Queralt, Palmira Valderas-Martínez, Sara Arranz, I. Roth, Miguel A. Martínez-González, Lluis Serra-Majem, Dolores Corella, Jordi Salas-Salvadó, Rosa M. Lamuela-Raventós, i Ramón Estruch.

Congrés: 10th Barcelona International Conference on the Mediterranean Diet. Barcelona, Espanya. 2014.

A MEDITERRANEAN DIET PATTERN SUPPLEMENTED WITH NUTS OR EXTRA VIRGIN OLIVE OIL INCREASES TOTAL POLYPHENOL EXCRETION AND SIGNIFICANTLY REDUCES INFLAMMATORY PARAMETERS: THE PREDIMED RANDOMIZED TRIAL AFTER ONE YEAR.

Alexander Medina-Remón[1,2], Rosa Casas[1,2], Anna Tressserra-Rimbau[2,3], Anna Vallverdú-Queralt[2,3], Palmira Valderas-Martínez[1,2], Sara Arranz[1,2], Irene Roth[1,2], Miguel A. Martínez-González[2,4], Lluis Serra-Majem[2,5], Dolores Corella[2,6], Jordi Salas-Salvadó[2,7], Rosa M. Lamuela-Raventos[2,3], and Ramón Estruch[1,2,*], on behalf of the PREDIMED Study Investigators.

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INTRODUCTION

Hypertension is one of the main cardiovascular risk factors among the elderly. In the earliest stage of hypertension, vascular inflammation is activated by proinflammatory stimuli, resulting in the secretion of inflammatory cytokines that promote the generation of endothelial adhesion molecules. These molecules are subsequently released to the blood circulation, where they mediate the adhesion of circulating monocytes and lymphocytes to the vascular endothelium [1, 2]. Adherences to the traditional Mediterranean diet (DMed) in numerous epidemiological studies have demonstrated an inverse association with a reduction in hypertension; this protective effect has been attributed in part, to the richness of this diet in antioxidants. Olive oil is the main natural fat in the DMed [3], the extra virgin olive oil (EVOO) has the highest antioxidant phenolic content [4]; nuts are also typical from DMed, they are a rich sources of nutrients and antioxidant phytochemicals [5]. Previous studies have demonstrated that polyphenol intake, assessed in urine are correlate with polyphenol consumption [6].

Aims: The aim of this study was to evaluate whether a one-year intervention with two traditional DMed supplemented with extra virgin olive oil (EVOO) or nuts increased total polyphenol excretion (TPE), and their association with circulating inflammatory biomarkers related to atherogenesis in participants at high risk of cardiovascular disease.

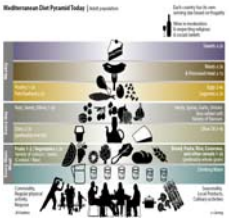
Subjects and Methods

1139 free of CVD at baseline admitted trial participants from the PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial (*www.predimed.org*; ISRCTN35739639).

- Men: 55-80 years old
- Women: 60-80 years old
- High CV risk without CVD
- type 2 diabetics
- 3+ risk factors

1. Smoking
2. Hypertension
3. ↑ LDL
4. ↓ HDL
5. Overweight/obesity
6. Family history CVD

random



Mediterranean diet

One-year

n=394 DMed + EVOO

n=366 DMed + Nuts

n=379 Control Low-fat

Urinary total polyphenols: Analyzed by the Folin-Ciocalteu assay, after a solid phase extraction (SPE) with 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) [9].

Inflammatory biomarkers: Plasma Interleukin-6 (IL-6), Monocyte Chemotactic Protein-1 (MCP-1), Soluble Inter-Cellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1) and Tumor Necrosis Factor Alpha (TNF-α).

Results

Table 1: Baseline of the study participants completing 1 year of follow-up.

	DMed+EVOO	DMed+Nuts	Control Diet	*P* for trend[a]
No. of subjects	394	366	379	
Age, (y) mean (SD)	67.2 (6.1)	67.2 (6.0)	68.3 (5.9)	0.025
Women, n (%)	219 (55.6)	181 (49.5)	228 (60.2)	0.013
BMI, (kg/m2) mean (SD)	29.2 (3.2)	29.3 (3.4)	29.7 (3.5)	0.128
Overweight or obese (BMI ≥25 Kg/m2), n (%)	356 (90.4)	332 (90.7)	340 (89.7)	0.896
Systolic BP (mm Hg), mean (SD)	150.4 (17.5)	152.2 (19.2)	152.5 (17.8)	0.230
Diastolic BP (mmHg), mean (SD)	83.9 (9.7)	85.3 (10.7)	84.0 (9.9)	0.145
Hypertension, n (%)	302 (76.6)	285 (77.9)	314 (82.8)	0.082
Diabetes, n (%)	168 (42.6)	161(44.0)	176 (46.4)	0.561
Dyslipidemia, n (%)	256 (65.0)	242 (66.1)	242 (63.9)	0. 678
Current smoker, n (%)	64 (16.2)	58 (15.8)	64 (16.9)	0.927
Family history of CHD, n (%)	76 (19.3)	64 (17.5)	64 (17.0)	0.875
Energy expenditure in physical activity (kcal/d), mean (SD)	311.9 (241.3)	289.1 (212.3)	240.7 (187.3)	<0.001

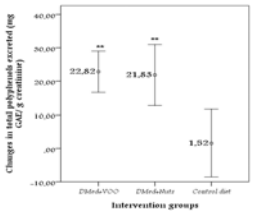
[a]*ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. BMI: body mass CHD: coronary heart disease; BP: blood pressure; DMed: Mediterranean diet, VOO: virgin olive oil.*

Table 3: Baseline level and 1-year changes in adiposity, blood pressure and total polyphenol excreted.[a]

	DMed+EVOO	*P* Value[b]	DMed+Nuts	*P* Value[b]	Control Diet	*P* Value[b]	*P* Value[c]
N	394		366		379		
Urine total polyphenol (mg GAE/ g creatinine)							
Baseline	110.8 (47.1)	< 0.001	127.5 (66.5)	< 0.001	138.2 (81.3)	0.903	<0.027[e,f]
1 year	133.5 (74.7)		149.3 (85.6)		139.7 (87.8)		
Weight (kg)							
Baseline	74.8 (10.7)	0.392	75.3 (11.3)	0.654	75.2 (11.2)	0.195	0.330[d]
1 year	74.7 (10.9)		74.9 (11.3)		78.8 (49.9)		
BMI							
Baseline	29.2 (3.2)	0.384	29.3 (3.4)	0.601	29.7 (3.5)	0.208	0.043[d]
1 year	29.2 (3.3)		29.1 (3.5)		31.3 (22.2)		
Waist, cm							
Baseline	97.2 (9.7)	0.022	97.5 (10.3)	< 0.001	97.9 (10.4)	0.001	0.435[d]
1 year	96.5 (9.6)		96.3 (10.2)		97.2 (10.9)		
Systolic BP (mm Hg), mean (SD)							
Baseline	150.6 (17.4)	< 0.001	152.2 (19.3)	0.001	152.4 (17.8)	0.494	0.001[d]
1 year	147.4 (16.9)		149.3 (18.6)		151.9 (18.2)		
Diastolic BP (mmHg), mean (SD)							
Baseline	84.1 (9.6)	< 0.001	85.5 (10.7)	0.001	84.3 (9.8)	0.538	0.056[f]
1 year	82.6 (9.7)		84.1 (10.1)		84.0 (9.5)		

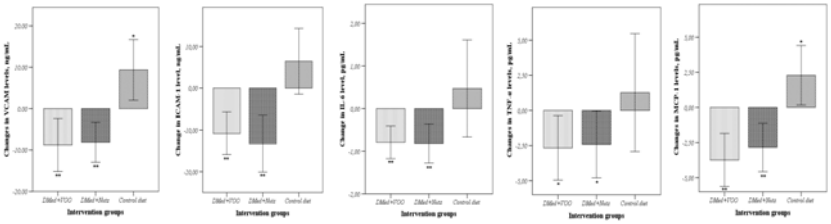
[a] Data are given as means (S.D.); [b] Differences from baseline by no-parametric Wilcoxon test; P<0.05 indicates statistical significance. DMed: Mediterranean diet; VOO: virgin olive oil; GAE: gallic acid equivalent; [c] P values for differences among diets, [d] Between DMed+VOO and Control diet, [e] Between DMed+Nuts and Control diet, [f] Between DMed+VOO and DMed+Nuts.

Figure 1: Mean ± SD changes in total polyphenols excreted in spot urine samples after one-year with different intervention.



***P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%.*

Figure 2: Changes from baseline after 1-year with different intervention in plasma concentration of the inflammatory biomarker.



***P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%. VCAM-1: Vascular Cell Adhesion Molecule-1; ICAM-1: soluble Inter-Cellular Adhesion Molecule-1; IL-6: Plasma Interleukin-6; TNF-α: Tumor Necrosis Factor Alpha; MCP-1: Monocyte Chemotactic Protein-1.*

CONCLUSIONS

Urinary total polyphenols excreted are statistically significant increased in Mediterranean diets supplemented with extra virgin olive oil and nuts (figure 1).

The weight and BMI have not differences from baseline in any intervention groups or between groups after one year.

Both traditional Mediterranean diets supplemented with either EVOO or nuts had an anti-inflammatory effect, inducing significant reductions in the plasma concentrations of VCAM-1, ICAM, IL-6, TNF-α and MCP-1 compared with participants in the Control diet group (figure 2). Both Mediterranean diets exhibited beneficial effects on cardiovascular risk factors.

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SUPPORTED BY

We thank the participants in the PREDIMED study for their valuable cooperation. This study was supported in part by CICYT (AGL2010-22319-C03) and CIBEROBN from the Spanish Ministry of Science and Innovation (MICINN), and Quality Group from Generalitat de Catalunya 2009 SGR 724. The CIBEROBN and RD06/0045 are initiatives of the Instituto de Salud Carlos III (ISCIII), Spain. A. M.-R. thanks the "Juan de la Cierva" postdoctoral program (JCI-2012-13463) from MEC. A.T-R would like to thank the ISCIII for granting her a predoctoral fellowship (FI10/00265).

X Congreso Dieta Mediterránea, Barcelona, Abril 2-3, 2014

C.3. Comunicació 3. Pòster

Títol: Effects of total polyphenol excretion on plasma nitric oxide and blood pressure after one year with mediterranean diet, supplemented with nuts or extra virgin olive oil. The predimed randomized trial

Autors: Alexander Medina-Remón, Anna Tresserra-Rimbau, A. Pons, J.A. Tur, Miguel A. Martínez-González, Lluis Serra-Majem, Dolores Corella, Jordi Salas-Salvadó, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: 10th Barcelona International Conference on the Mediterranean Diet. Barcelona, Espanya. 2014.

EFFECTS OF TOTAL POLYPHENOL EXCRETION ON PLASMA NITRIC OXIDE AND BLOOD PRESSURE AFTER ONE YEAR WITH MEDITERRANEAN DIET, SUPPLEMENTED WITH NUTS OR EXTRA VIRGIN OLIVE OIL. THE PREDIMED RANDOMIZED TRIAL.

Alexander Medina-Remón[1,2], Anna Tresserra-Rimbau[2,3], Antoni Pons[2,4], Josep Antoni Tur[2,4], Miguel A. Martínez-González[2,5], Lluis Serra-Majem[2,6], Dolores Corella[2,7], Jordi Salas-Salvadó[2,8], Ramon Estruch[1,2], and Rosa M. Lamuela-Raventos[2,3,*], on behalf of the PREDIMED Study Investigators.

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INTRODUCTION

Hypertension is one of the main cardiovascular risk factors among the elderly. Hypertension can be managed by following a healthy diet rich in fruits and vegetables, such as the Mediterranean diet (Med-diet) [1, 2, 3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. Many epidemiological studies have demonstrated an inverse association between adherence to the traditional Med-diet and death from coronary heart diseases. This protective effect has been partially attributed to a high content of bioactive compounds. Olive oil is the main natural fat in the Med-diet [5], the extra virgin olive oil (EVOO) has the highest antioxidant phenolic content [6]; nuts are also typical from Med-diet, they are a rich sources of nutrients and antioxidant phytochemicals [7]. In a previous study conducted by our group, polyphenol intake, assessed via total polyphenols excreted in urine, was negatively associated with blood pressure levels in an elderly Mediterranean population at high cardiovascular risk [8].

Subjects and Methods

AIMS: The aims of this work were to evaluate, whether a one-year intervention with a traditional Med-diet increased total polyphenols excreted in spot urine samples compared with a control diet, and evaluate if the decrease in blood pressure due to polyphenol consumption was mediated by plasma nitric oxide production.

200 free of CVD at baseline admitted trial participants from the PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial (www.predimed.org; ISRCTN35739639).

- Men: 55-80 years old
- Women: 60-80 years old
- High CV risk without CVD
  - type 2 diabetics
  - 3+ risk factors

random

1. Smoking
2. Hypertension
3. ↑ LDL
4. ↓ HDL
5. Overweight/obesity
6. Family history CVD

One-year

n=67 →  **Med-diet + EVOO**

n=64 →  **Med-diet + Nuts**

n=69 →  **Control Low-fat diet**

Urinary total polyphenols → **Analyzed by the Folin-Ciocalteu assay, after a solid phase extraction with 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) [9].**

Clinical measures → **Plasma Nitric Oxide**

Systolic and diastolic blood pressure (mmHg)

Results

Table 1: Baseline of the study participants completing 1 year of follow-up.

	Med + VOO	Med + Nuts	Control Diet	P*
No. of subjects	67	64	69	
Age, (y) mean (SD)	68.0 (6.0)	67.7 (6.3)	67.1 (5.8)	0.631
Women, n (%)	39 (58.2)	33 (51.6)	41 (59.4)	0.621
BMI, (kg/m²) mean (SD)	72.5 (9.3)	74.4 (10.1)	76.3 (9.6)	0.074
Overweight or obese (BMI ≥25 Kg/m²), n (%)	58 (86.6)	58 (90.6)	64 (92.8)	0.476
Hypertension, n (%)	49 (73.1)	50 (78.1)	55 (79.7)	0.639
Diabetes, n (%)	41 (61.2)	38 (59.4)	46 (66.7)	0.661
Dyslipidemia, n (%)	46 (68.7)	46 (71.9)	52 (75.4)	0.684
Current smoker, n (%)	9 (13.4)	11 (17.2)	13 (18.8)	0.686
Family history of CHD, n (%)	33 (49.2)	29 (45.3)	30 (43.5)	0.170
Energy expenditure in physical activity (kcal/d), mean (SD)	343.8 (296.4)	246.2 (206.0)	199.6 (178.3)	0.002

*ANOVA-one factor was used for continuous variables and χ2-test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); CHD: coronary heart disease; SD: standard deviation. Med : Mediterranean diet; EVOO: extra virgin olive oil.

Table 2: Change in systolic and diastolic blood pressure at one-year associated to change in plasma nitric oxide.

	Model	B	P	95 % CI
Systolic blood pressure	*Model 1*			
	Med-EVOO vs. Control diet	-6.14	0.042	-12.04 to -2.33
	Med-Nuts vs. Control diet	-2.69	0.372	-8.62 to -3.24
	Model 2			
	Med-EVOO vs. Control diet	-5.98	0.033	-11.48 to -0.48
	Med-Nuts vs. Control diet	-6.30	0.029	-11.96 to -0.65
Diastolic blood pressure	*Model 1*			
	Med-EVOO vs. Control diet	-5.23	0.001	-8.20 to -2.25
	Med-Nuts vs. Control diet	-1.737	0.253	-4.73 to 1.25
	Model 2			
	Med-EVOO vs. Control diet	-3.58	0.007	-6.17 to -0.99
	Med-Nuts vs. Control diet	-2.91	0.033	-5.57 to -0.24

B: Non-standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by baseline blood pressure, change in plasma nitric oxide, sex, age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) supplements taken in the last month, sodium, potassium, and total energy intake. Med: Mediterranean diet; EVOO: extra virgin olive oil.

Figure 1: Correlation between the quartiles of change in total polyphenols excreted, as biomarker of total polyphenols intake, and change in plasma nitric oxide.

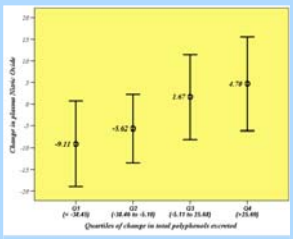
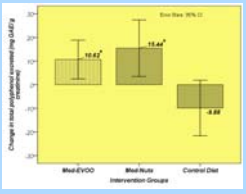
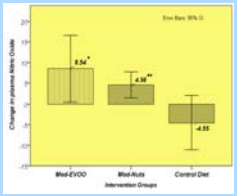
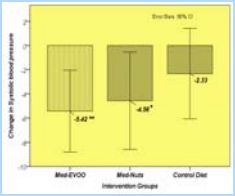


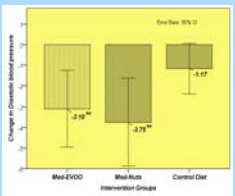
Figure 2: Mean ± SD changes in total polyphenols excreted in spot urine samples, plasma nitric oxide, systolic and diastolic blood pressure after 1-year with different intervention.







*Med: Mediterranean diet; EVOO: Extra virgin olive oil; GAE: gallic acid equivalent;**P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval (CI) of 95%.*



CONCLUSIONS

Total polyphenols excreted in spot urine as biomarker of total polyphenols intake, are positively correlated with plasma Nitric Oxide in Med-diet supplemented with EVOO or nuts.

The statistically significant increases in plasma NO were associated with a reduction in systolic and diastolic Blood Pressure levels, after one year interventions with Mediterranean diets supplemented with EVOO or nuts, compared with the control

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SUPPORTED BY

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X Congreso Dieta Mediterránea, Barcelona, April 2-3, 2014

C.4. Comunicació 4. Pòster (2n premi)

Títol: High polyphenol intake reduces cardiovascular and mortality risk: a longitudinal study using the PREDIMED cohort.

Autors: Anna Tresserra-Rimbau, Eric B. Rimm, Alexander Medina-Remón, Miguel A. Martínez-González, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: 10th Barcelona International Conference on the Mediterranean Diet. Barcelona, Espanya. 2014.

HIGH POLYPHENOL INTAKE REDUCES CARDIOVASCULAR AND MORTALITY RISK: A LONGITUDINAL STUDY USING THE PREDIMED COHORT.

Tresserra-Rimbau A[1,2,3]; Rimm E.B.[4]; Medina-Remón A[1,2,3]; Martínez-González MA[3,5]; Estruch R[2,3,6]; Lamuela-Raventos RM[1,2,3]; on behalf of the PREDIMED Study Investigators.

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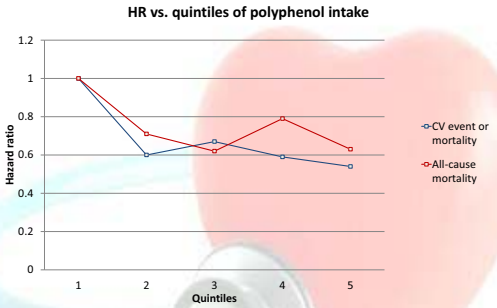
Background and objectives

Cardiovascular diseases (CVD) are the leading cause of mortality and disability in developed countries. Epidemiologic and mechanistic evidence supports an inverse association between the consumption of certain groups of polyphenols and the risk of chronic diseases. We assessed the hypothesis that polyphenol intake is associated with a lower risk of total mortality and cardiovascular death or event in the PREDIMED cohort.

Methods

We used Time-dependent Cox proportional hazards regression to estimate the Hazard Risk (HR) to relate polyphenol consumption and risk of cardiovascular event or death using the lowest quintile as the referent group. All intakes were calories adjusted. Statistical analyses were conducted by using SAS software, version 9. All *P* values were 2-sided and differences below the probability level ($P<0.05$) were considered significant. Clinical Trial Registration: International Standard Randomized Controlled Trial Number (ISRCTN of London, England) 35739639.



Results

Over an average of 4.3 years of follow-up, 273 confirmed cases of CVD and 327 deaths for any cause were reported among the 7172 participants (96.3%) who completed the FFQ. After multivariate adjustment and comparing Q5 vs. Q1 of total polyphenol intake, we observed a significant 46% reduction in risk of CVD and 37% reduction for all-cause death.

Table 1. Baseline characteristics of participants in the PREDIMED cohort according to quintiles of total polyphenol intake at baseline (energy-adjusted).

	Q1	Q2	Q3	Q4	Q5	P*
No subjects (7172)	1434	1435	1434	1435	1434	
Polyphenol intake (mg/d)	483±108	674±36	794±36	937±50	1235±199	
Sex, women	836 (58.3)	924 (64.4)	712 (60.8)	803 (56.0)	648 (45.2)	<0.0001
Age (y)	67.6±6.2	67.4±6.1	67.4±5.9	66.9±6.0	66.2±6.1	<0.0001
Body mass index (kg/m^2)	30.0±3.7	30.3±3.7	29.7±3.5	29.7±3.7	29.6±3.5	<0.0001
Current smoker	217 (15.1)	210 (14.6)	194 (13.5)	265 (18.5)	317 (22.1)	<0.0001
Physical activity at leisure time (MET-h/d)	3.37±3.56	3.62±3.83	3.77±3.66	4.05±4.25	4.59±4.54	<0.0001
Diabetes	706 (49.2)	680 (47.4)	712 (49.6)	704 (49.1)	668 (46.6)	0.40
Hypertension	1230 (85.8)	1224 (85.3)	1192 (83.1)	1166 (81.3)	1117 (77.9)	<0.0001
Hypercholesterolemia	983 (68.6)	1018 (70.9)	1053 (73.4)	1065 (74.2)	1069 (74.6)	0.001
Family history of CVD	403 (28.1)	290 (20.2)	310 (21.6)	324 (22.6)	446 (31.1)	0.05
Total energy intake (Kcal/d)	2397±642	2180±589	2161±540	2229±563	2369±577	<0.0001

Categorical variables: subjects (percentage), continuous variables: mean±SD
* One-way ANOVA tests (continuous variables) or chi-squared (categorical variables)

Table 2. Cox Proportional Hazard Ratios, HR(95%CI), according to quintiles of cumulative total polyphenols intake*.

	Q1	Q2	Q3	Q4	Q5	P-trend
Total polyphenols (mg/d)	562	701	800	917	1170	
Cardiovascular event or mortality						
No. of CVD cases	66	49	58	49	51	
No. of person years	5312	6668	6905	6629	5554	
Age and sex adjusted	1.00	0.60 (0.38-0.95)*	0.62 (0.39-0.97)	0.58 (0.36-0.91)	0.58 (0.36-0.93)	0.04
Model 2†	1.00	0.57 (0.36-0.92)	0.60 (0.38-0.95)	0.54 (0.34-0.87)	0.51 (0.30-0.84)	0.02
Model 3‡	1.00	0.60 (0.38-0.97)	0.67 (0.42-1.07)	0.59 (0.37-0.96)	0.54 (0.33-0.91)	0.04
All-cause mortality						
No. of cases	88	62	52	63	62	
No. of person years	5505	6599	6767	6559	5688	
Age and sex adjusted	1.00	0.65 (0.44-0.95)	0.55 (0.37-0.82)	0.73 (0.50-1.06)	0.66 (0.44-0.98)	0.12
Model 2	1.00	0.68 (0.46-1.01)	0.60 (0.39-0.90)	0.75 (0.51-1.12)	0.60 (0.39-0.91)	0.07
Model 3	1.00	0.71 (0.48-1.05)	0.62 (0.41-0.95)	0.79 (0.53-1.17)	0.63 (0.41-0.97)	0.12

* Analyses were stratified by sex, recruitment centre and intervention group.
† Adjusted for age (<60, 60-64.9, 65-69.9, 70-74.9, >=75 years), smoking (never, past and current: cigarettes (<5, 5-19, >20 per day) or cigars and pipes (<3, 3-6, >6 per day)), BMI (<25, 25-29.9, or >=30 kg/m^2), baseline diabetes, alcohol (0, 0.1-14.9, 15-29.9, >=30 g/day), total energy intake (continuous variable), physical activity (continuous variable), family history of CVD or cancer, aspirin use, antihypertensive drug use, use of cardiovascular medication, use of oral hypoglycaemic agents, insulin, other medication.
‡ Model 2 additionally adjusted for intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol (all as continuous variables).

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X International Conference Mediterranean Diet
Barcelona, Spain April 2-3, 2014

Conclusions

Greater intake of polyphenols was associated with a lower risk of CVD and death for any cause. Clinical trials are needed to confirm this effect and establish accurate dietary recommendations.

Acknowledgements

We would like to thank all the volunteers involved in the PREDIMED study for their valuable cooperation. This study was supported in part by CICYT (AGL2010-22319-C03) from the Spanish Ministry of Science and Innovation (MICINN), and the Instituto de Salud Carlos III, ISCIII (CIBERobn-CB06/03, RD 06/0045, PI1002658, and PI1001407). The CIBERobn is an initiative of the ISCIII, Spain. ATR received support from ISCIII (FI10/00265).

C.5. Comunicació 5. Pòster

Títol: Cardiovascular risk factors and alcohol consumption within an elderly Spanish population at high cardiovascular risk.

Autors: Anna Tresserra-Rimbau, Alexander Medina-Remón, Miguel A. Martínez-González, Jordi Salas-Salvadó, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: I World Forum for Nutrition Research Conference. Reus, Espanya. 2013.

CARDIOVASCULAR RISK FACTORS AND ALCOHOL CONSUMPTION WITHIN AN ELDERLY SPANISH POPULATION AT HIGH CARDIOVASCULAR RISK.

Tresserra-Rimbau A[1,2,3]; Medina-Remón A[1,2,3]; Martínez-González MA[3,5]; Estruch R[2,3,6]; Lamuela-Raventos RM[1,2,3]; on behalf of the PREDIMED Study Investigators.

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Background and objectives

Cardiovascular diseases (CVD) are the leading cause of mortality and disability in developed countries. A healthy diet can improve cardiovascular risk factors. Specifically, polyphenols, bioactive compounds mostly found in fruits, vegetables and derivatives, have been associated with lower risk of CVD. Wine and beer are polyphenol-rich beverages that are also very common in the Mediterranean diet. The aim of this study was to compare the main cardiovascular risk factors between wine and beer consumers and non-consumers from the PREDIMED study.

Methods

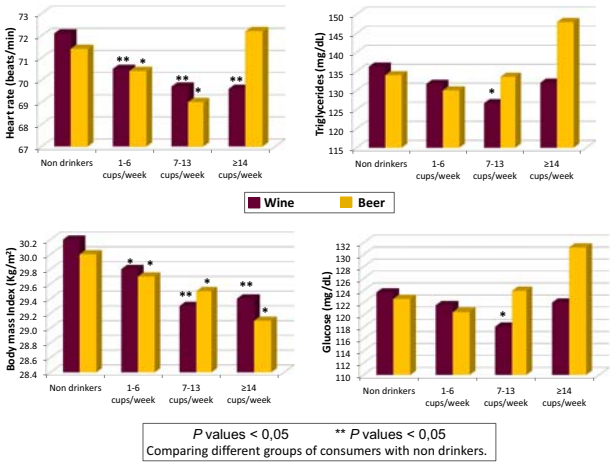
The PREDIMED study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. The 7,447 eligible participants were community-dwelling people aged 55 to 80 years, who were free of cardiovascular disease at baseline.
Wine and beer consumers and non-consumers (data from the first year) were divided into groups to compare cardiovascular risk factors such as triglycerides and glucose levels, heart rate, BMI or blood pressure, among others.
Statistical analyses (ANOVAs and Bonferroni tests) were conducted using SAS software, version 9.3 (SAS Institute, Inc., Cary, North Carolina).

Conclusions

Polyphenol-rich beverages can improve some cardiovascular risk factors. These data will be useful to further investigate about polyphenol intake and the incidence of several pathologies in the PREDIMED cohort.

Results

We observed a decrease in triglycerides levels (-9,6 mg/dL, *P*=0.05) and glucose levels (-5.6 mg/dL, *P*=0.046) comparing moderated wine drinkers with non-drinkers. Heart rate and BMI were significantly lower among wine and beer consumers comparing to non-drinkers (*P*<0.05). There were no changes on SBP and DBP among non-drinkers and moderate wine drinkers, although both pressures were increased among those who drunk >14 cups/week (SBP: +3.3 mmHg, P=0.001; DBP: +2.6 mmHg, *P*<0.001). No significant differences were observed in total cholesterol, HDL, and LDL concentrations.



Acknowledgements

We would like to thank all the volunteers involved in the PREDIMED study for their valuable cooperation. This study was supported in part by CICYT (AGL2010-22319-C03) from the Spanish Ministry of Science and Innovation (MICINN), and the Instituto de Salud Carlos III, ISCIII (CIBERobn-CB06/03, PI1002658, and PI1001407). CIBERobn is an initiative of ISCIII, Spain. AT-R received support from ISCIII (FI10/00265).

C.6. Comunicació 6. Pòster i presentació oral

Títol: A Mediterranean Diet supplemented with nuts or virgin olive oil increases total polyphenol excretion and plasma nitric oxide, and significantly decreases blood pressure.

Autors: Alexander Medina-Remón, Anna Tresserra-Rimbau, Anna Vallverdú-Queralt, Rosa Casas, Palmira Valderas-Martínez, Emilio Ros, Miguel A. Martínez-González, M. Isabel Covas, Lluis Serra-Majem, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: I World Forum for Nutrition Research Conference. Reus, Espanya. 2013.

A Mediterranean diet supplemented with nuts or virgin olive oil increases total polyphenol excretion and plasma nitric oxide, and significantly decreases blood pressure.

Medina-Remón A[1,2]; Tresserra-Rimbau A[1,2]; Pons A[2,3]; Sureda A[2,3]; Capó X[2,3]; Tur JA[2,3]; Martínez-González MA[2,4]; Covas MI[2,5]; Gómez-Gracia E[2,7]; Arós F[2,8]; Estruch R[2,6]; Lamuela-Raventos RM[1,2]; on behalf of the PREDIMED Study Investigators.

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INTRODUCTION


The first step in its management of hypertension is to follow a healthy diet such as the traditional Mediterranean diet (Med-diet) [1, 2, 3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. Adherences to the traditional Med-diet in numerous epidemiological studies have demonstrated an inverse association with a reduction in hypertension; this protective effect has been attributed in part, to the richness of this diet in antioxidants. Olive oil is the main natural fat in the Med-diet [6], the extra virgin olive oil (EVOO) has the highest antioxidant phenolic content [7]; nuts are also typical from Med-diet, they are a rich sources of nutrients and antioxidant phytochemicals [8]. Previous studies have demonstrated that polyphenol intake, assessed in urine are correlate with polyphenol consumption [9].

AIMS: The aims of this study were to evaluate whether a one-year intervention with two traditional Med-diet supplemented with EVOO or nuts increased the total polyphenols excretion (TPE) in spot urine samples; this biochemical analysis was correlated with change in plasma nitric oxide (NO), and this plasma NO change was associated with average systolic and diastolic blood pressure (BP).

Subjects and Methods

200 free of CVD at baseline admitted trial participants from the PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial (www.predimed.org; ISRCTN35739639).

- Men: 55-80 years old
- Women: 60-80 years old
- High CV risk without CVD
  - type 2 diabetics
  - 3+ risk factors

random

1. Smoking
2. Hypertension
3. ↑LDL
4. ↓HDL
5. Overweight/obesity
6. Family history CVD

Mediterranean diet

One-year

- n=67 → Med diet + EVOO
- n=64 → Med diet + Nuts
- n=69 → Control Low-fat diet



Urinary total polyphenols → Analyzed by the Folin-Ciocalteu assay, after a solid phase extraction (SPE) with 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) [9].

Clinical measures → Plasma Nitric Oxide; Systolic and diastolic blood pressure (mmHg)

Results

Table 1: Baseline of the study participants completing 1 year of follow-up.

	Med + VOO	Med + Nuts	Control Diet	P*
No. of subjects	67	64	69	
Age, (y) mean (SD)	68.0 (6.0)	67.7 (6.3)	67.1 (5.8)	0.631
Women, n (%)	39 (58.2)	33 (51.6)	41 (59.4)	0.621
BMI, (kg/m²) mean (SD)	72.5 (9.3)	74.4 (10.1)	76.3 (9.6)	0.074
Overweight or obese (BMI ≥25 Kg/m²), n (%)	58 (86.6)	58 (90.6)	64 (92.8)	0.476
Hypertension, n (%)	49 (73.1)	50 (78.1)	55 (79.7)	0.639
Diabetes, n (%)	41 (61.2)	38 (59.4)	46 (66.7)	0.661
Dyslipidemia, n (%)	46 (68.7)	46 (71.9)	52 (75.4)	0.684
Current smoker, n (%)	9 (13.4)	11 (17.2)	13 (18.8)	0.686
Family history of CHD, n (%)	33 (49.2)	29 (45.3)	30 (43.5)	0.170
Energy expenditure in physical activity (kcal/d), mean (SD)	343.8 (296.4)	246.2 (206.0)	199.6 (178.3)	0.002

*ANOVA-one factor was used for continuous variables and χ2-test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); CHD: coronary heart disease; SD: standard deviation. Med : Mediterranean diet; EVOO: extra virgin olive oil.

Table 2: Change in systolic and diastolic blood pressure at one-year associated to change in plasma nitric oxide.

	Model	B	P	95 % CI
Systolic blood pressure	*Model 1*			
	Med-EVOO vs. Control diet	-6.14	0.042	-12.04 to -2.33
	Med-Nuts vs. Control diet	-2.69	0.372	-8.62 to -3.24
	Model 2			
	Med-EVOO vs. Control diet	-5.98	0.033	-11.48 to -0.48
	Med-Nuts vs. Control diet	-6.30	0.029	-11.96 to -0.65
Diastolic blood pressure	*Model 1*			
	Med-EVOO vs. Control diet	-5.23	0.001	-8.20 to -2.25
	Med-Nuts vs. Control diet	-1.737	0.253	-4.73 to 1.25
	Model 2			
	Med-EVOO vs. Control diet	-3.58	0.007	-6.17 to -0.99
	Med-Nuts vs. Control diet	-2.91	0.033	-5.57 to -0.24

B: Non-standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by baseline blood pressure, change in plasma nitric oxide, sex, age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) supplements taken in the last month, sodium, potassium, and total energy intake. Med: Mediterranean diet; EVOO: extra virgin olive oil.

Figure 1: Correlation between the quartiles of change in total polyphenols excreted, as biomarker of total polyphenols intake, and change in plasma nitric oxide.

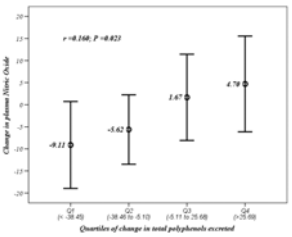
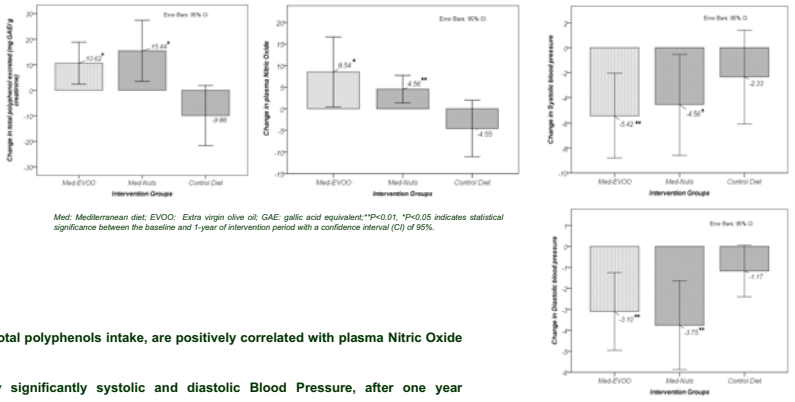


Figure 2: Mean ± SD changes in total polyphenols excreted in spot urine samples, plasma nitric oxide, systolic and diastolic blood pressure after 1-year with different intervention.



Med: Mediterranean diet; EVOO: Extra virgin olive oil; GAE: gallic acid equivalent;**P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval (CI) of 95%.

CONCLUSIONS

Total polyphenols excreted in spot urine as biomarker of total polyphenols intake, are positively correlated with plasma Nitric Oxide in Med-diet supplemented with either EVOO or nuts.

Change in plasma Nitric Oxide, decreased statistically significantly systolic and diastolic Blood Pressure, after one year interventions with Mediterranean diets supplemented with EVOO or nuts, compared with the control diet.

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SUPPORTED BY

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C.7. Comunicació 7. Pòster

Títol: Following a Mediterranean diet pattern supplemented with nuts or virgin olive oil increases total polyphenol excretion and reduces significantly blood pressure and inflammatory parameters. The PREDIMED randomized trial after one year

Autors: Alexander Medina-Remón, Anna Tresserra-Rimbau, Anna Vallverdú-Queralt, Rosa Casas, Palmira Valderas-Martínez, Emilio Ros, Miguel A. Martínez-González, M. Isabel Covas, Lluis Serra-Majem, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya. 2012.

Following a Mediterranean diet pattern supplemented with nuts or virgin olive oil increases total polyphenol excretion and reduces significantly blood pressure and inflammatory parameters. The PREDIMED randomized trial after one year.

Alexander Medina-Remón[1, 2, 3], Anna Tressserra-Rimbau[1, 2, 3], Anna Vallverdú-Queralt[1, 2, 3], Rosa Casas[2, 4], Palmira Valderas-Martinez[1, 2, 4], Emilio Ros[2, 5], Miguel A. Martinez-González[3, 6], María-Isabel Covas[2, 7], Lluis Serra-Majem[3, 8], Ramón Estruch[2, 3, 4], and Rosa M. Lamuela-Raventos[1, 2, 3, *], on behalf of the PREDIMED Study Investigators.

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INTRODUCTION


The first step in its management of hypertension is to follow a healthy diet such as the traditional Mediterranean diet (DMed) [1, 2, 3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. Adherences to the traditional DMed in numerous epidemiological studies have demonstrated an inverse association with a reduction in hypertension; this protective effect has been attributed in part, to the richness of this diet in antioxidants. Olive oil is the main natural fat in the DMed [6], the virgin olive oil (VOO) has the highest antioxidant phenolic content [7]; nuts are also typical from DMed, they are a rich sources of nutrients and antioxidant phytochemicals [8]. Previous studies have demonstrated that polyphenol intake, assessed in urine are correlate with polyphenol consumption [9].

AIMS: The aims of this study was to evaluate whether a one-year intervention with two traditional DMed supplemented with VOO or nuts increased total polyphenols excretion (TPE); and their association with average systolic and diastolic blood pressure (BP) and circulating inflammatory biomarkers.

Subjects and Methods

1139 free of CVD at baseline admitted trial participants from the PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial (www.predimed.org; ISRCTN35739639).

- ✓ Men: 55-80 years old
- ✓ Women: 60-80 years old
- ✓ High CV risk without CVD
  type 2 diabetics
  3+ risk factors

1. Smoking
2. Hypertension
3. ↑LDL
4. ↓HDL
5. Overweight/obesity
6. Family history CVD

random

Mediterranean diet

One-year

- n=394 DMed+ Virgin Olive Oil
- n=366 DMed+ Nuts
- n=379 Control Low-fat



Urinary total polyphenols → Analyzed by the Folin-Ciocalteu assay, after a solid phase extraction (SPE) with 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) [9].

Inflammatory biomarkers → Plasma Interleukin-6 (IL-6), Monocyte Chemotactic Protein-1 (MCP-1), Soluble Inter-Cellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1) and Tumor Necrosis Factor Alpha (TNF-α).

Clinical measures → Systolic and diastolic blood pressure

Results

Table 1: Baseline of the study participants completing 1 year of follow-up.

	DMed+VOO	DMed+Nuts	Control Diet	*P* for trend[a]
No. of subjects	394	366	379	
Age, (y) mean (SD)	67.2 (6.1)	67.2 (6.0)	68.3 (5.9)	0.025
Women, n (%)	219 (55.6)	181 (49.5)	228 (60.2)	0.013
BMI, (kg/m2) mean (SD)	29.2 (3.2)	29.3 (3.4)	29.7 (3.5)	0.128
Overweight or obese (BMI ≥25 Kg/m2), n (%)	356 (90.4)	332 (90.7)	340 (89.7)	0.896
Systolic BP (mm Hg), mean (SD)	150.4 (17.5)	152.2 (19.2)	152.5 (17.8)	0.230
Diastolic BP (mmHg), mean (SD)	83.9 (9.7)	85.3 (10.7)	84.0 (9.9)	0.145
Hypertension, n (%)	302 (76.6)	285 (77.9)	314 (82.8)	0.082
Diabetes, n (%)	168 (42.6)	161(44.0)	176 (46.4)	0.561
Dyslipidemia, n (%)	256 (65.0)	242 (66.1)	242 (63.9)	0. 678
Current smoker, n (%)	64 (16.2)	58 (15.8)	64 (16.9)	0.927
Family history of CHD, n (%)	76 (19.3)	64 (17.5)	64 (17.0)	0.875
Energy expenditure in physical activity (kcal/d), mean (SD)	311.9 (241.3)	289.1 (212.3)	240.7 (187.3)	<0.001

[a]*ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. BMI: body mass CHD: coronary heart disease; BP: blood pressure; DMed: Mediterranean diet; VOO: virgin olive oil.*

Table 2: Covariate analysis with systolic blood pressure and diastolic blood pressure at one-year as the dependent variables; intervention groups as the fixed factor and baseline measures as covariates.

	Model	B	*P*	95 % CI
Systolic blood pressure	*Model adjusted*			
	DMed-VOO vs. Control diet	-3,429	0,002	-5,598 to -1,261
	DMed-Nuts vs. Control diet	-2,268	0,042	-4,450 to -0,086
Diastolic blood pressure	*Model adjusted*			
	DMed-VOO vs. Control diet	-1,382	0,014	-2,479 to -0,285
	DMed-Nuts vs. Control diet	-0,696	0,217	-1,803 to 0,410

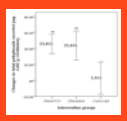
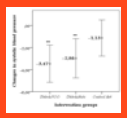
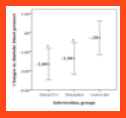
B: Non-standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model adjusted by sex, age, weight, smoking status, physical activity, educational level at baseline, medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate.

Table 3: Baseline level and 1-year changes in adiposity, blood pressure and total polyphenol excreted.[a]

		DMed+VOO	*P* Value[b]	DMed+Nuts	*P* Value[b]	Control Diet	*P* Value[b]	*P* Value[c]
N		394		366		379		
Urine total polyphenol (mg GAE/ g creatinine)								
	Baseline	110.8 (47.1)	< 0.001	127.5 (66.5)	< 0.001	138.2 (81.3)	0.903	<0.027[e,f]
	1 year	133.5 (74.7)		149.3 (85.6)		139.7 (87.8)		
Weight (kg)								
	Baseline	74.8 (10.7)	0.392	75.3 (11.3)	0.654	75.2 (11.2)	0.195	0.330[d]
	1 year	74.7 (10.9)		74.9 (11.3)		78.8 (49.9)		
BMI								
	Baseline	29.2 (3.2)	0.384	29.3 (3.4)	0.601	29.7 (3.5)	0.208	0.043[d]
	1 year	29.2 (3.3)		29.1 (3.5)		31.3 (22.2)		
Waist, cm								
	Baseline	97.2 (9.7)	0.022	97.5 (10.3)	< 0.001	97.9 (10.4)	0.001	0.435[d]
	1 year	96.5 (9.6)		96.3 (10.2)		97.2 (10.9)		
Systolic BP (mm Hg), mean (SD)								
	Baseline	150.6 (17.4)	< 0.001	152.2 (19.3)	0.001	152.4 (17.8)	0.494	0.001[d]
	1 year	147.4 (16.9)		149.3 (18.6)		151.9 (18.2)		
Diastolic BP (mmHg), mean (SD)								
	Baseline	84.1 (9.6)	< 0.001	85.5 (10.7)	0.001	84.3 (9.8)	0.538	0.056[f]
	1 year	82.6 (9.7)		84.1 (10.1)		84.0 (9.5)		

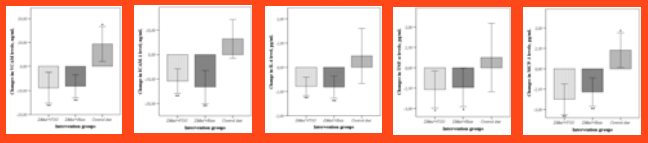
[a]*Data are given as means (S.D.);* [b]*Differences from baseline by no-parametric Wilcoxon test; P<0.05 indicates statistical significance. DMed: Mediterranean diet; VOO: virgin olive oil; GAE: gallic acid equivalent;* [c] *P. values for differences among diets,* [d] *Between DMed+VOO and Control diet,* [e] *Between DMed+Nuts and Control diet,* [f] *Between DMed+VOO and DMed+Nuts.*

Figure 1: Mean ± SD changes in total polyphenols excreted in spot urine samples, systolic and diastolic blood pressure after one-year with different intervention.

***P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%.*

Figure 2: Changes from baseline after 1-year with different intervention in plasma concentration of the inflammatory biomarker.



***P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%. VCAM-1: Vascular Cell Adhesion Molecule-1; ICAM-1: soluble Inter-Cellular Adhesion Molecule-1; IL-6: Plasma Interleukin-6; TNF-α: Tumor Necrosis Factor Alpha; MCP-1: Monocyte Chemotactic Protein-1.*

CONCLUSIONS

Urinary total polyphenols excreted are statistically significant increased in Mediterranean diets supplemented with virgin olive oil and nuts (figure 1).

The weight and BMI have not differences from baseline in any intervention groups or between groups after one year.

Participants in both Mediterranean diets had decreased systolic and diastolic blood pressure (figure 1).

A one-year intervention with a traditional DMed supplemented with either VOO or nuts increases total polyphenols excreted in urine samples from elderly participants, and decreases systolic and diastolic BP. Both DMed exhibited beneficial effects on cardiovascular risk factors.

Both traditional DMed supplemented with either VOO or nuts had an anti-inflammatory effect, inducing significant reductions in the plasma concentrations of VCAM-1, ICAM, IL-6, TNF-α and MCP-1 compared with participants in the Control diet group (figure 2).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SUPPORTED BY

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IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea 27-28 Marzo del 2012

C.8. Comunicació 8. Pòster

Títol: Olives and olive oil make a difference in the polyphenol intake in an elderly Spanish population.

Autors: Anna Tresserra-Rimbau, Alexander Medina-Remón, Jara Pérez-Jiménez, Miguel A. Martínez-González, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea. Barcelona, Espanya. 2012.

Olives and olive oil make a difference in the polyphenol intake in an elderly Spanish population

Anna Tresserra-Rimbau[1,2,3]; Alexander Medina-Remón[1,2,3]; Jara Pérez-Jiménez[4]; Miguel Ángel Martínez-González[3,5]; Ramon Estruch[2,3,6]; and Rosa Mª Lamuela-Raventos[1,2,3]; on behalf of the PREDIMED Study Investigators.

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Introduction & Aim

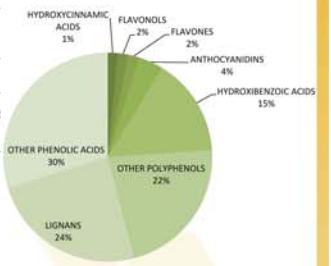
Olive oil is the main fat in the Mediterranean Diet. It contains monounsaturated fatty acids and it is a source of polyphenols, some of them unique. Some studies reveal that the consumption of extra virgin olive oil has important benefits on health, and EFSA has recently accepted a health claim about the role of olive polyphenols in the protection of LDL particles from oxidative damage.

The aim of this study is to estimate the weight of olives and olive oil in the total intake of polyphenols in a Spanish population at high cardiovascular risk (PREDIMED study) by using individual food consumption records and the recently launched Phenol-explorer database.

Subjects & method

Results

POLYPHENOL GROUP	TOTAL INTAKE FROM... OLIVE OIL (mg/day)	OLIVES (mg/day)	OTHER FOODS (mg/day)	% INTAKE DERIVED FROM OLIVE AND OLIVE OIL	INDIVIDUAL POLYPHENOLS INGESTED ONLY FROM OLIVES AND OLIVE OIL
ANTHOCYANIDINS	0,00	4,49	33,8	11,7	-
FLAVONES	0,39	1,51	35,2	5,12	Isorhoifolin Luteolin 6-C-glucoside
FLAVONOLS	0,01	2,67	49,9	5,11	-
HYDROXYBENZOIC ACIDS	0,18	7,62	8,83	46,9	2,4-Dihydroxybenzoic acid
HYDROXYCINNAMIC ACIDS	0,10	10,82	221	4,70	Hydroxycaffeic acid Verbascoside m-coumaric acid
OTHER PHENOLIC ACIDS	0,02	1,21	0,29	91,4	3,4-Dihydroxyphenylacetic acid Dihydro-p-coumaric acid Homoveratric acid
OTHER POLYPHENOLS	21,0	25,8	0,12	67,6	Oleuropein, ligstroside, 3,4-DHPE-EDA and other tyrosols 3,4-Dihydroxyphenylglycol
LIGNANS	0,85	0,01	22,4	74,7	1-Acetoxypinoresinol
TOTAL POLYPHENOLS	22,6	54,1	738	9,41	



POLYPHENOL INTAKE DERIVED FROM OLIVE AND OLIVE OIL (PREDIMED STUDY)

Conclusions

Olives and olive oil daily provide 76,7 mg of polyphenols, which means more than 9% of total intake, being the fifth polyphenol contributor in the diet after coffee (18%), apples (16%), oranges (12%) and beans (10%). That makes the phenolic profile of the Spanish population unique. These data will be useful to investigate about polyphenol intake and the incidence of several pathologies in the PREDIMED cohort.

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C.9. Comunicació 9. Pòster

Títol: Wine phenolic markers identification by ultra-fast chromatography coupled to high resolution mass spectrometry.

Autors: Giuseppe Di Lecce, Sara Arranz, Anna Tresserra-Rimbau, Paola Quifer, Alexander Medina-Remón, Anna Velázquez, Miguel Tubio, i Rosa M. Lamuela-Raventós.

Congrés: 5th International Conference on Polyphenols and Health (ICPH 2011). Sitges, Espanya. 2011.

Wine phenolic markers identification by ultra fast chromatography coupled to high resolution mass spectrometry

Giuseppe Di Lecce[1*], Sara Arranz[2], Anna Tresserra-Rimbau[1, 3], Paola Quifer-Rada[1, 3], Alexander Medina-Remón[1,3] Anna Velàzquez[4], Miguel Tubio[5] and Rosa Mª Lamuela-Raventós[1,3]

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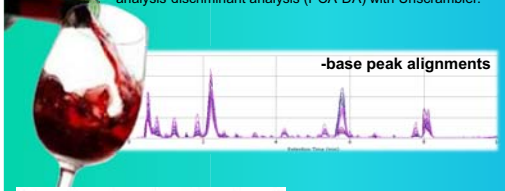
Introduction

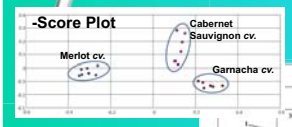
Recent and historical evidence suggests that regular and moderate wine consumption has a positive impact on human health due to its high amount of functional components. Red wine in particular contains a complex mixture of phenolic compounds that also contribute to its organoleptic quality. Current advances in both HPLC and mass spectrometry techniques have a significant impact on resolving complex mixtures such as food and agricultural products. The highest number of polyphenols and possible markers of red wines were described.

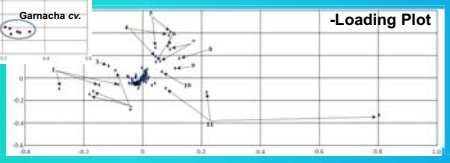
Methods and materials

Twenty one red wines were randomly injected into an UHPLC system (C18 column, 2.1x100mm with 1.7 µm particles, Acquity Waters) using two different binary gradients. UHPLC was coupled to a ESI-LTQ-Orbitrap-MS (Thermo Scientific) operating in positive mode. Resolving power was set at 30000 for full scan analysis, followed by data dependent MS/MS scan on the most intense ion, at 15000 resolving power. SIEVE 1.3 software was used for comparative and trend analyses. The base peaks were aligned and the *m/z* values of ions, showing peaks above a set abundance threshold were extracted together with the retention times and heights. The results were further subjected to principal component analysis-discriminant analysis (PCA-DA) with Unscrambler.



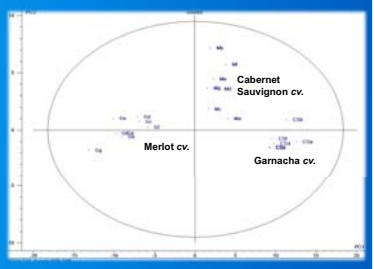
Results

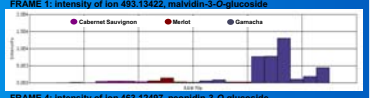


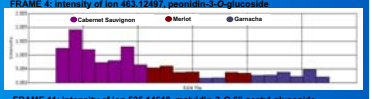


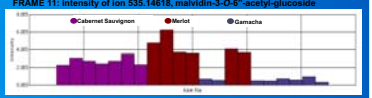
n. cluster	RT	detected mass		assignation	theoretical mass	mass difference (mDa)[a]	formula
				possible markers of Garnacha wine			
1	2.20	493.13422	$[M+H]^+$	malvidin-3-O-glucose	493.13458	0.36	$C_{23}H_{25}O_{12}$
		494.13821	$^{13}C\ [M+H]^+$	isotope	494.14186	3.65	
		331.08188	$[M+H-C_6H_{10}O_5]^+$	malvidin	331.08176	- 0.12	$C_{17}H_{15}O_7$
2	0.50	381.08044	$[M+H]^+$	unidentified			
		337.17245		unidentified			
		258.11093		unidentified			
3	8.00	693.17340	$[M+H]^+$	malvidin-3-O-6"-*p*-coumaroyl-glucoside	693.17138	- 2.02	$C_{32}H_{31}O_{14}$
				possible markers of Merlot wine			
4	1.82	463.12497	$[M+H]^+$	peonidin-3-O-glucoside	463.12404	- 0.97	$C_{22}H_{23}O_{11}$
		301.07317	$[M+H-C_6H_{10}O_5]^+$	peonidin	301.07065	- 2.52	$C_{16}H_{13}O_6$
5	7.79	609.16248	$[M+H]^+$	peonidin-3-O-6"-p-coumaroil-glucoside	609.16082	- 1.66	$C_{32}H_{29}O_{13}$
		301.07251	$[M+H-C_{13}H_{12}O_6]^+$	peonidin	301.07065	- 1.86	$C_{16}H_{13}O_6$
6	1.22	479.11987	$[M+H]^+$	petunidin-3-O-glucoside	479.11895	- 0.82	$C_{22}H_{23}O_{12}$
		317.06638	$[M+H-C_6H_{10}O_5]^+$	petunidin	317.06611	- 0.27	$C_{22}H_{23}O_{12}$
7	0.75	465.10367	$[M+H]^+$	delfinidin-3-O-glucoside	465.10330	- 0.37	$C_{21}H_{21}O_{12}$
		303.05051		delfinidin	303.04992	- 0.59	$C_{15}H_{11}O_7$
8	5.30	505.13608	$[M+H]^+$	peonidin-3-O-6"-acetyl-glucoside	505.13460	- 1.48	$C_{24}H_{25}O_{12}$
9	4.21	521.13055	$[M+H]^+$	petunidin-3-O-6"-acetyl-glucoside	521.12952	- 1.03	$C_{24}H_{25}O_{13}$
10	2.60	507.11330	$[M+H]^+$	delfinidin-3-O-6"-acetyl-glucoside	507.11384	0.54	$C_{23}H_{23}O_{13}$
				possible markers of Cabernet Sauvignon wine			
11	5.79	535.14618	$[M+H]^+$	malvidin-3-O-6"-acetyl-glucoside	535.14514	- 1.04	$C_{25}H_{27}O_{13}$
		536.14880	$^{13}C\ [M+H]^+$	isotope			
		331.08191	$[M+H-C_8H_8O_4]^+$	malvidin	331.08176	- 0.15	$C_{17}H_{15}O_7$

To compare difference in the metabolite profiling of the three monovarietal red wines, unsupervised PCA was performed. The samples were collected in two different region of Spain (north and south). The PCA score plot could be readily divided into three different groups, indicating that the content and distribution of components were highly varied in red wines. The corresponding PCA loading plot was used to identify the different metabolic composition, accountable for the separation among groups. The variety-discriminating markers responsible for the observed clustering patterns were confirmed by accurate mass measurements, MS^n experiments and reference compounds. Good fits were obtained for all investigated ions, with errors ranging from 0.12 to 3.65 mDa. Furthermore PCA-DA was executed to confirm the results obtained with SIEVE software. The intensity of specific frames, characteristics of the three sample of red wines were reported. Malvidin-3-*O*-glucoside is more abundant in Garnacha, Peonidin-3-*O*-glucoside is highest in Cabernet Sauvignon and Malvidin-3-*O*-6"acetyl-glucoside is greater in Merlot wines.









Conclusions

LTQ-Orbitrap-MS^n provided an unambiguous identification and structural characterization of the compounds based on accurate mass measurement and informative levels of fragmentation. The SIEVE software allowed the process of a large number of samples presenting statistical differences between ion population and grape *cultivars*. The processing data outcoming from this study shows that the proposed method could be used to discriminate some red wine varieties.

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C.10. Comunicació 10. Pòster

Títol: Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk.

Autors: Anna Tresserra-Rimbau, Alexander Medina-Remón, Jara Pérez-Jiménez, Miguel A. Martínez-González, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: 5th International Conference on Polyphenols and Health (ICPH 2011). Sitges, Espanya. 2011.

Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk

Anna Tresserra-Rimbau[1,2,3]; Alexander Medina-Remón[1,2,3]; Jara Pérez-Jiménez[4]; Miguel Ángel Martínez-González[3,5]; Ramon Estruch[2,3,6]; and Rosa Mª Lamuela-Raventos[1,2,3]; on behalf of the PREDIMED Study Investigators.

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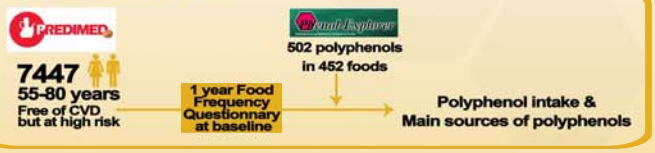
Introduction

Epidemiological data have shown an inverse association between the risk of cardiovascular disease and the consumption of polyphenol-rich foods[1-3]. To date, existing available data about the intake of polyphenols are limited, due mainly to the fact that data concerning the polyphenol content of foods are insubstantial in literature and the existing databases include only a limited number of polyphenols. However, the recently launched Phenol-Explorer database contains information about polyphenol content in foods[4]. The aims of this study are to estimate the quantitative intake of polyphenols in a Spanish population at high cardiovascular risk (Predimed cohort[5]) by using individual food consumption records[6], and to determine major dietary sources of polyphenols.

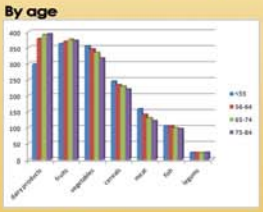
Subjects & method

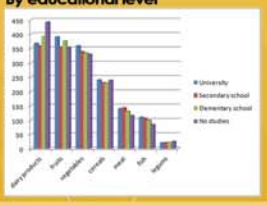


Results

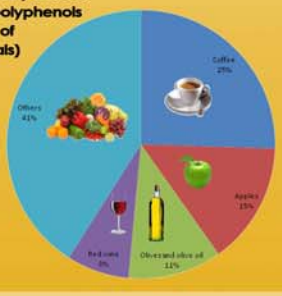
Main dietary sources of polyphenols (by groups)

Food distribution by population characteristics

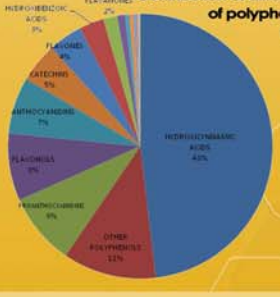




Main dietary sources of total polyphenols (by sum of individuals)



Contribution of different groups of polyphenols to the total intake



Conclusions

Hidroxicynnamic acids are the most largely consumed polyphenols, due mainly to coffee, in the Predimed cohort. Proanthocyanidins, flavonols and anthocyanidins are the three next. Apples, olives, olive oil and red wine also have a great contribution to the total polyphenol intake.
Food distribution by population characteristics shows an association between age and the consumption of dairy products and fruits, and the opposite for vegetables, cereals, meat and fish. Women have a higher consumption of dairy products, fruits and vegetables than men. Data also indicates that population with higher level of studies consumes more fruits, vegetables, meat and fish.
These data will allow further investigations into polyphenol intake and the incidence of several pathologies in the PREDIMED cohort and it can be useful to establish nutritional recommendations.

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Acknowledgments

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C.11. Comunicació 11. Pòster

Títol: Food polyphenols decrease blood pressure

Autors: Rosa M. Lamuela-Raventós, Alexander Medina-Remón, Anna Tresserra-Rimbau, i Ramón Estruch.

Congrés: Diet and optimum health conference. Corvallis, USA. 2011.

Food Polyphenols Decrease Blood Pressure.

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1. INTRODUCTION

The first step in its management of hypertension is to follow a healthy diet such as the traditional Mediterranean diet (TMD) [1, 2, 3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. Adherences to the TMD in numerous epidemiological studies have demonstrated an inverse association with a reduction in hypertension; this protective effect has been attributed in part, to the richness of this diet in antioxidants. Olive oil is the main natural fat in the Mediterranean diet [6], the virgin olive oil (VOO) has the highest phenolic content [7]; nuts are also typical from TMD, they are a rich sources of nutrients and antioxidant phytochemicals [8]. Previous studies have demonstrated that polyphenol intake, assessed in urine are correlate with polyphenol consumption [9]. However, nutritional recommendations on overall food polyphenol consumption should be base in large scale randomized intervention studies in which clinically relevant end-points, such as blood pressure are evaluated.

AIMS: We undertook this substudy in the frame of the PREDIMED study, in order to evaluate the effect of polyphenol intake measured by means of total polyphenols excreted (TPE) in urine, a reliable biomarker of polyphenol intake, on blood pressure; after one-year intervention with two traditional Mediterranean diets supplemented with virgin olive oil (VOO) or nuts.

2. SUBJECTS and METHODS

1139 admitted trial participants from the PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial (*www.predimed.org*; ISRCTN35739639).

- Men: 55-80 years old
- Women: 60-80 years old
- High CV risk without CVD
  - type 2 diabetics
  - 3+ risk factors

All free of CVD at baseline

random

1. Smoking
2. Hypertension
3. ↑ LDL
4. ↓ HDL
5. Overweight/obesity
6. Family history CVD

Mediterranean diet

One-year

- n=394 Med Diet + Virgin Olive Oil
- n=366 Med Diet + Nuts
- n=379 Control Low-fat

Urine samples

solid phase extraction (SPE): 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) to avoid any interference with Folin-Ciocalteu reagent.

Urinary total polyphenols were analyzed by the Folin-Ciocalteu assay, after a solid phase extraction [9].

3. RESULTS

Table 1: Baseline of the study participants completing 1 year of follow-up.

	DMed+VOO	DMed+Nuts	Control Diet	*P* for trend[a]
No. of subjects	394	366	379	
Age, (y) mean (SD)	67.2 (6.1)	67.2 (6.0)	68.3 (5.9)	0.025
Women, n (%)	219 (55.6)	181 (49.5)	228 (60.2)	0.013
BMI, (kg/m2) mean (SD)	29.2 (3.2)	29.3 (3.4)	29.7 (3.5)	0.128
Overweight or obese (BMI ≥25 Kg/m2), n (%)	356 (90.4)	332 (90.7)	340 (89.7)	0.896
Systolic BP (mm Hg), mean (SD)	150.4 (17.5)	152.2 (19.2)	152.5 (17.8)	0.230
Diastolic BP (mmHg), mean (SD)	83.9 (9.7)	85.3 (10.7)	84.0 (9.9)	0.145
Hypertension, n (%)	302 (76.6)	285 (77.9)	314 (82.8)	0.082
Diabetes, n (%)	168 (42.6)	161(44.0)	176 (46.4)	0.561
Dyslipidemia, n (%)	256 (65.0)	242 (66.1)	242 (63.9)	0. 678
Current smoker, n (%)	64 (16.2)	58 (15.8)	64 (16.9)	0.927
Family history of CHD, n (%)	76 (19.3)	64 (17.5)	64 (17.0)	0.875
Energy expenditure in physical activity (kcal/d), mean (SD)	311.9 (241.3)	289.1 (212.3)	240.7 (187.3)	<0.001

[a]ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. BMI: body mass CHD: coronary heart disease; BP: blood pressure; DMed: Mediterranean diet, VOO: virgin olive oil.

Table 2: Covariate analysis with systolic blood pressure and diastolic blood pressure at one-year as the dependent variables; intervention groups as the fixed factor and baseline measures as covariates.

	Model	B	*P*	95 % CI
Systolic blood pressure	*Model adjusted*			
	DMed-VOO vs. Control diet	-3,429	0,002	-5,598 to -1,261
	DMed-Nuts vs. Control diet	-2,268	0,042	-4,450 to -0,086
Diastolic blood pressure	*Model adjusted*			
	DMed-VOO vs. Control diet	-1,382	0,014	-2,479 to -0,285
	DMed-Nuts vs. Control diet	-0,696	0,217	-1,803 to 0,410

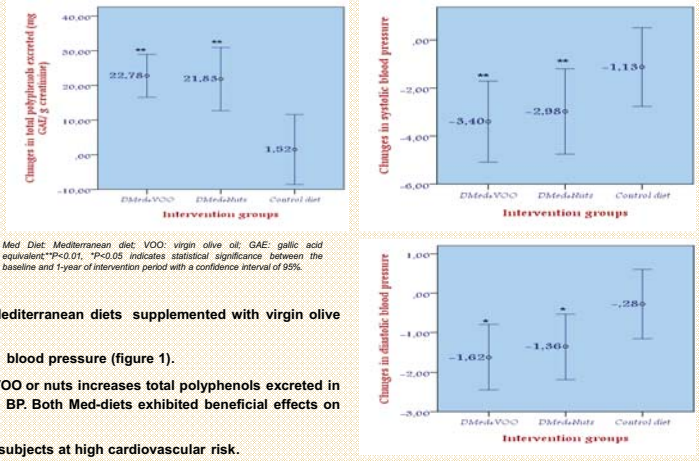
B: Non-standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model adjusted by sex, age, weight, smoking status, physical activity, educational level at baseline, medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate; DMed: Mediterranean diet; VOO: virgin olive oil.

Table 3: Baseline level and 1-year changes in adiposity, blood pressure and total polyphenol excreted.[a]

	DMed+VOO	*P* Value[b]	DMed+Nuts	*P* Value[b]	Control Diet	*P* Value[b]	*P* Value[c]
N	394		366		379		
Urine total polyphenol (mg GAE/ g creatinine)							
Baseline	110.8 (47.1)	< 0.001	127.5 (66.5)	< 0.001	138.2 (81.3)	0.903	<0.027[e,f]
1 year	133.5 (74.7)		149.3 (85.6)		139.7 (87.8)		
Weight (kg)							
Baseline	74.8 (10.7)	0.392	75.3 (11.3)	0.654	75.2 (11.2)	0.195	0.330[d]
1 year	74.7 (10.9)		74.9 (11.3)		78.8 (49.9)		
BMI							
Baseline	29.2 (3.2)	0.384	29.3 (3.4)	0.601	29.7 (3.5)	0.208	0.043[d]
1 year	29.2 (3.3)		29.1 (3.5)		31.3 (22.2)		
Waist, cm							
Baseline	97.2 (9.7)	0.022	97.5 (10.3)	< 0.001	97.9 (10.4)	0.001	0.435[d]
1 year	96.5 (9.6)		96.3 (10.2)		97.2 (10.9)		
Systolic BP (mm Hg), mean (SD)							
Baseline	150.4 (17.5)	0.003	152.2 (19.2)	0.008	152.5 (17.8)	0.071	0.001[d]
1 year	147.5 (16.9)		149.2 (18.6)		151.9 (18.2)		
Diastolic BP (mmHg), mean (SD)							
Baseline	83.9 (9.7)	0.002	85.3 (10.7)	0.021	84.0 (9.9)	0.370	0.056[f]
1 year	82.6 (9.7)		84.1 (10.1)		84.0 (9.5)		

[a] Data are given as means (S.D.); [b] Differences from baseline by no-parametric Wilcoxon test, P<0.05 indicates statistical significance. DMed: Mediterranean diet; VOO: virgin olive oil; GAE: gallic acid equivalent; [c] P values for differences among diets, [d] Between DMed+VOO and Control diet, [e] Between DMed+Nuts and Control diet, [f] Between DMed+VOO and DMed+Nuts.

Figure 1: Mean ± SD changes in total polyphenols excreted in spot urine samples, systolic and diastolic blood pressure after one-year with different intervention.



Med Diet: Mediterranean diet; VOO: virgin olive oil; GAE: gallic acid equivalent;**P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%.

4. CONCLUSIONS

Urinary total polyphenols excreted are statistically significant increased in Mediterranean diets supplemented with virgin olive oil and nuts (figure 1).

Participants in both Mediterranean diets had decreased systolic and diastolic blood pressure (figure 1).

A one-year intervention with a traditional Med-diet supplemented with either VOO or nuts increases total polyphenols excreted in urine samples from elderly participants, and decreases systolic and diastolic BP. Both Med-diets exhibited beneficial effects on cardiovascular risk factors.

Increase in polyphenol-rich foods may be helpful in the management of BP in subjects at high cardiovascular risk.

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6. SUPPORTED BY

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Diet and Optimum Health Conference September 1316, 2011 Corvallis, Oregon

C.12. Comunicació 12. Pòster

Títol: Taninos condensados y polifenoles hidrolizables en uvas tintas de variedad Cabernet Sauvignon: evolución según clima y grado de madurez.

Autors: Sara Arranz, Ana Incer, Ilaria Tedechi, Giuseppe Di Lecce,Anna Tresserra-Rimbau, Paola Quifer, Alexander Medina-Remón, Núria Tobella, Mireia Torres, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Taninos condensados y polifenoles hidrolizables en uvas tintas de variedad Cabernet Sauvignon: Evolución según clima y grado de madurez.

Sara Arranz * [1, 4], Ana Incer [2], Ilaria Tedeschi [2], Giuseppe Di Lecce [2, 3], Anna Tresserra [2, 4], Paola Quifer [2, 4], Alexander Medina-Remón [2, 4], Nuria Tobella [5], Mireia Torres [5], Ramón Estruch [1, 4], Rosa Mª Lamuela [2, 4].

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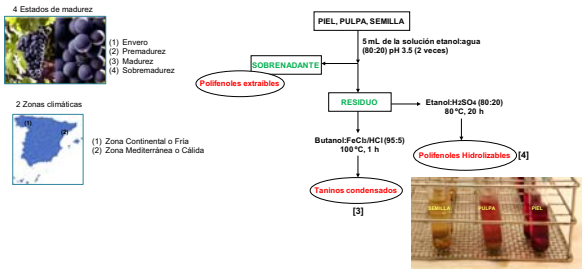
INTRODUCCIÓN

Una extracción de polifenoles habitual se basa en la utilización de disolventes acuoso-orgánicos dando lugar a la obtención de polifenoles extraíbles o de menor peso molecular que se encuentran más accesibles en el fruto. Sin embargo, existen compuestos de mayor peso molecular que no son extraídos con soluciones acuoso-orgánicas generalmente formando polímeros de flavanoles o taninos condensados (TC) y polifenoles hidrolizables (PH) que se encuentran asociados a la matriz vegetal y que poseen menor accesibilidad y biodisponibilidad [1]. La manera de extraer estos compuestos del fruto requiere condiciones más drásticas como son la hidrólisis o la depolimerización a altas temperaturas y en medios ácidos o básicos [2].

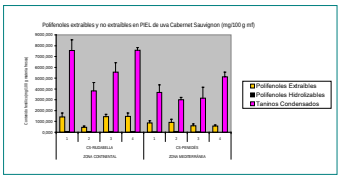
El objetivo de este trabajo consistió en la determinación de polifenoles extraíbles y no extraíbles en las diferentes partes de la uva Cabernet Sauvignon (piel, pulpa y semilla) cultivada en diferentes zonas climáticas (zona continental o fría y zona mediterránea o cálida) y recogida en diferentes puntos de madurez.

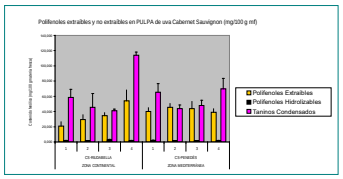
MATERIAL Y MÉTODOS

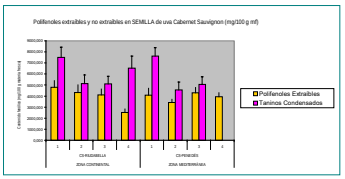


RESULTADOS

Los taninos condensados se encuentran presentes en las tres partes de la uva siendo mucho mayor su contenido en pieles y semillas. Los polifenoles hidrolizables se encuentran en piel y pulpa siendo mayoritarios en piel aunque su contenido es menor que el de taninos condensados. Además el contenido de polifenoles no extraíbles es de hasta diez veces superior al de extraíbles.







En cuanto a las diferencias según zona climática, en la zona continental o más fría se favorece la presencia de taninos condensados y polifenoles hidrolizables en la piel y pulpa mientras que la semilla de la zona cálida posee mayor contenido de taninos condensados. Este tipo de compuestos aumentan en general con el grado de madurez siendo el punto 4 (sobremadurez) el que mayor contenido presenta en comparación con los grados 1 (envero), 2 (prematurez) y 3 (madurez) en piel y pulpa pero disminuye el contenido de los mismos en el caso de la semilla, siendo las semillas en estado envero las que mayor cantidad presentan.

CONCLUSIONES

Es importante tener en cuenta además de los polifenoles extraíbles, la cantidad tan importante de polifenoles no extraíbles que la uva presenta. El clima frío parece favorecer la acumulación de estos compuestos a lo largo de la maduración sobre todo en piel.

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C.13. Comunicació 13. Pòster

Títol: Caracterización del perfil fenólico de la uva blanca de variedad Chardonnay mediante UHPLC-DAD acoplado a detector MS (LTQ Orbitrap)

Autors: Sara Arranz, Giuseppe Di Lecce, Anna Tresserra-Rimbau, Paola Quifer, Alexander Medina-Remón, Núria Tobella, Mireia Torres, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Caracterización del perfil fenólico de la uva blanca de variedad Chardonnay mediante UHPLC-DAD acoplado a detector MS (LTQ Orbitrap).

Sara Arranz * [1, 4], Giuseppe Di Lecce [2, 3], Anna Tresserra [2, 4], Paola Quifer [2, 4], Alexander Medina-Remón [2, 4], Nuria Tobella [5], Mireia Torres [5], Ramón Estruch [1, 4], Rosa Mª Lamuela [2, 4].

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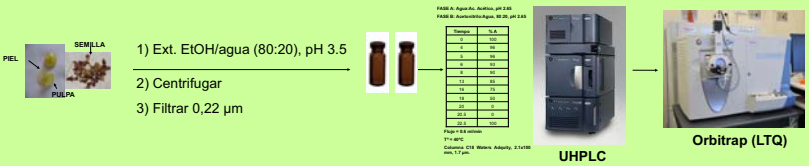
INTRODUCCIÓN

La variedad de uva blanca Chardonnay es una de las más apreciadas en el mundo. Originaria de la Borgoña (Francia), su cultivo ha sido extendido a numerosas zonas del mundo entre las que se encuentra España. La uva Chardonnay es utilizada en la elaboración de vinos blancos secos, cava y champagne. Se caracteriza por producir mostos suaves y aromáticos y por su alto nivel de azúcares [1, 2].

OBJETIVO

El objetivo de este trabajo es la identificación de los compuestos polifenólicos presentes en la piel, pulpa y semilla de la uva Chardonnay de la zona Penedés de España mediante cromatografía de UHPLC-DAD acoplada a un detector de masas con trampa de iones (LTQ-Orbitrap) que proporciona alta resolución y masa exacta de los compuestos analizados como se ha descrito en trabajos anteriores[3, 4, 5].

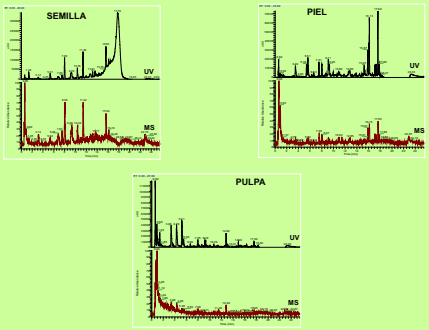
MATERIAL Y MÉTODOS



RESULTADOS

Tabla 1. Compuestos fenólicos identificados en piel, pulpa y semilla de la uva Chardonnay.

	Compuesto identificado	Parte	Masa Ión precursor [M-H]-	Fragmentación	Fórmula
Uva Chardonnay	Ácido gálico	pi, se	169,0151	169, 125	C7H6O5
	Hexosa de ácido gálico	se	331,0732	331, 169, 125	C13H16O10
	(+)-Catequina	pu, pi, se	289,0710	245, 205	C15H14O6
	Epicatequina	pu, pi, se	289,0712	245, 205	C15H14O6
	(-)-Epicatequin-3-O-galato	se	441,0866	289, 271, 169, 125	C22H18O10
	Procianidina B1	se	577,1331	407, 289, 245	C30H26O12
	Procianidina B2	pi, se	577,1331	407, 289, 245	C30H26O12
	Procianidina B3	pu, pi, se	577,1334	407, 289, 245	C30H26O12
	Procianidina B4	pi, se	577,1325	407, 289, 245	C30H26O12
	Dimero galoilado	se, pi	881,1945	881, 729, 407, 289	C45H38O19
	Procianidina Trímera	se	865,1854	865, 695, 577, 407, 289	C45H38O18
	Ácido Cis-Caftárico	pu, pi	311,0413	179, 135	C13H12O9
	Ácido Cis-Cutárico	pi	295,0469	163, 119	C13H12O8
	Ácido Fertárico	pu, pi	325,0585	193, 149	C14H14O9
	Ácido Siringico	pi	197, 0455	197, 153	C9H10O5
	Ácido ferúlico	pi	193,0506	193, 149	C10H10O4
	Quercetin-3-O-rutinósido	pi	609,1495	609, 301	C27H30O16
	Quercetin-3-O-glucurónido	pi	477,0636	301,151	C21H18O13
	Quercetin-3-O-glucósido	pi	463,0857	301,151	C21H20O12
	Canferol-3-O-glucósido	pi	447,0972	447,285	C21H20O11
	Trans-Piceído	pi	389,1256	227, 185, 143	C20H22O8



Los compuestos mayoritarios identificados en piel son flavonoles, flavanoles, ácidos hidroxicinámicos y estilbenos. En la pulpa se identifican algunos flavanoles y ácidos hidroxicinámicos. En la semilla se identifican algunos ácidos benzoicos y principalmente flavanoles del tipo monómero, dímero y trímeros.

CONCLUSIONES

La mayor parte de compuestos fenólicos está presente en la semilla en forma de flavanoles y en la piel en forma de flavonoles y flavanoles mientras que la pulpa posee un bajo contenido fenólico formado principalmente por ácidos hidroxicinámicos. Algunos compuestos como la catequina y la epicatequina se encuentran presentes en las tres partes de la uva.

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C.14. Comunicació 14. Pòster

Títol: Contenido en polifenoles totales y proteínas en uvas de la variedad Albariño en diferentes zonas climáticas durante el proceso de maduración.

Autors: Palmira Valderas, Sara Arranz, Giuseppe Di Lecce, Alexander Medina-Remón, Anna Tresserra-Rimbau, Paola Quifer, Anna Velázquez, Miguel Tubio, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Contenido en polifenoles totales y proteínas en uvas de la variedad Albariño en diferentes zonas climáticas durante el proceso de maduración.

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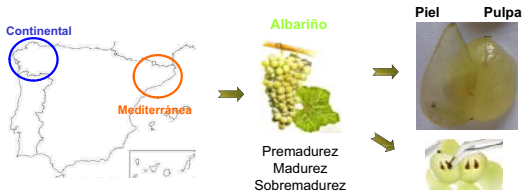
INTRODUCCIÓN

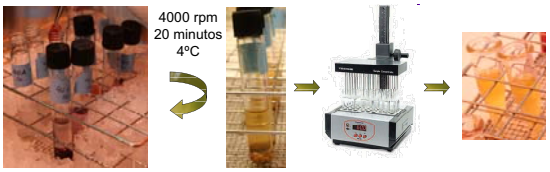
La variedad de uva blanca Albariño es propia de zonas frías y húmedas, y en España se cultiva ampliamente en Galicia dentro de las Denominaciones de Origen Rías Baixas y Ribeiro, aunque también se puede encontrar en Cantabria, Castilla y León y Cataluña. En los últimos años se ha observado que el aumento de la temperatura por el cambio climático influye en la maduración del fruto lo que ha afectado a las características sensoriales del producto final, por este motivo, se están buscando soluciones que permitan mantener el perfil del vino.

OBJETIVO

El objetivo de este trabajo es determinar el contenido de polifenoles totales (PT) y proteínas presentes en uva Albariño de dos zonas climáticas diferentes (continental y Mediterránea) a lo largo de la madurez.

MATERIAL Y METODOS





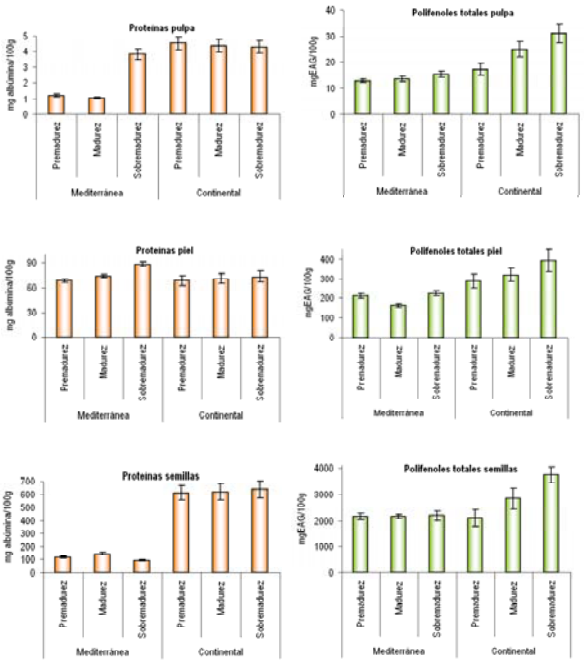
etanol/agua (80:20)
pH 3.5 (ácido acético)

El extracto se evapora y se resuspende en agua

Polifenoles totales (Folin-Ciocalteu)

Proteínas (Bradford)

RESULTADOS



CONCLUSIÓN

Los polifenoles y las proteínas de la baya se encuentran mayoritariamente en la semilla y en la piel. Las bayas recogidas en la zona cálida o mediterránea (Miguel Torres, Finca Fransola) presentan un mayor contenido de polifenoles totales que las de la zona continental (M.Códax). pero se observa el efecto contrario en las proteinas.

De este trabajo podemos concluir que la zona climática influye en el contenido de polifenoles totales y proteínas de la uva de la variedad Albariño.

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Palmira Valderas agradece a la Ayuda al Personal Investigador en Formación de la Universidad de Barcelona.

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Jerez de la Frontera (Cádiz)
1-4 Junio de 2011

C.15. Comunicació 15. Pòster

Títol: Influencia del clima y de la sobremaduración de las uvas de variedad Merlot en el perfil fenólico del vino.

Autors: Paola Quifer, Anna Tresserra-Rimbau, Sara Arranz-Martínez, Alexander Medina-Remón, Giuseppe Di Lecce, Núria Tobella, Mireia Torres, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

INFLUENCIA DEL CLIMA Y DE LA SOBREMADURACIÓN DE LAS UVAS VARIEDAD MERLOT EN EL PERFIL FENÓLICO DEL VINO

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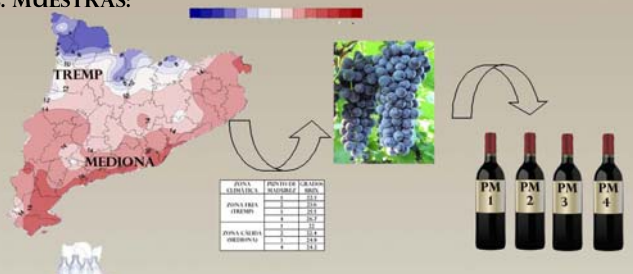
5 Bodegas Miguel Torres. Miquel Torres i Carbó 6, 08720 Vilafranca del Penedès

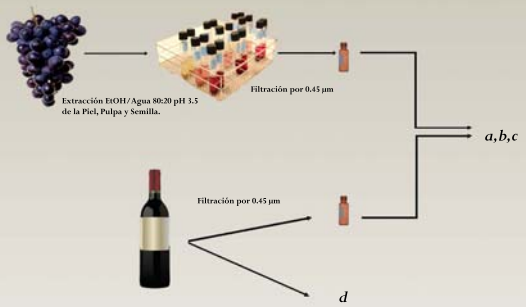
1. INTRODUCCIÓN:

Diversos estudios han demostrado que actualmente la madurez fenólica de la uva no coincide con su madurez fisiológica debido a un aumento de las temperaturas *(1,2)*. Este factor es de especial importancia en la industria vinícola ya que los vinos producidos en estas condiciones podrían adquirir un perfil fenólico inmaduro dando a lugar vinos de menos calidad. El objetivo de este trabajo es estudiar la evolución fenólica desde su punto de madurez a sobremadurez y como esta influye en el perfil fenólico final del vino en uvas merlot cultivadas en dos zonas climáticas diferentes, una zona cálida (prelitoral de Cataluña) y otra fría (Pre-pirineo occidental).

2. MUESTRAS:



3. MATERIAL Y METODOS:



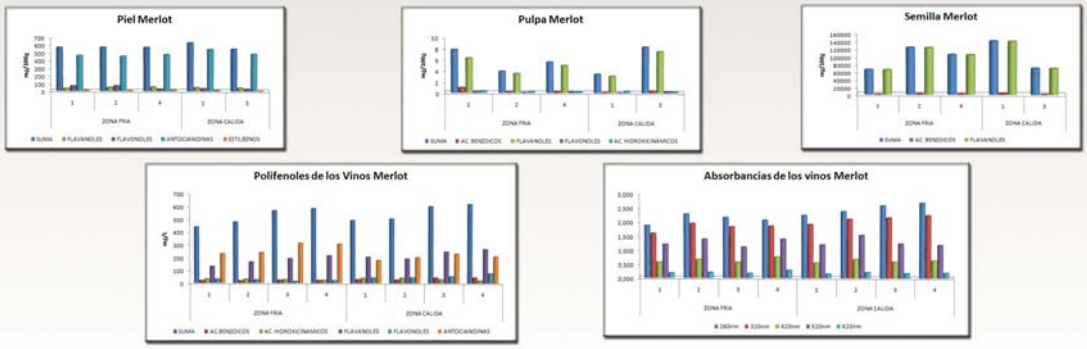
***a)* Determinación de perfil fenólico por HLPC-DAD** *(3)*
Fase A: Agua/Ac.Acético pH 2.65
Fase B: ACN/Fase A 80:20 .
Columna C18 Nucleosil 120 (250x4 mm), Flujo 0.8 mL/min, Vinyeción=50 µL. Temp. 40ºC. λquant: 280,320,365 nm.

***b)* Determinación de Antonianidinas por HPLC-DAD** *(4)*.
Fase A: Agua 5%Ac. Fórmico
Fase B: 100% ACN
Columna C18 Phenomenex Luna (150x2 mm), Flujo 0.8 mL/min, Temp. 40ºC. λquant:520nm.

***c)* Confirmación de los compuestos por HPLC-QqQToF**

***d)* Absorbancia de los vinos:**
cubetas de quarzo de 1 mm a 5 longitudes de onda: 280,320,420,520,620 nm.

4.RESULTADOS:



5. CONCLUSIONES:

Las absorbancias a 280 nm y 320 nm en los vinos de la zona cálida son más elevadas pero no se observaron diferencias significativas en los parámetros de intensidad de color, tonalidad y absorbancia a 520 nm entre las dos zonas. En cuanto al perfil fenólico de los vinos, se observo que los vinos Merlot pertenecientes a la zona cálida presentan un mayor contenido en la suma total de los polifenoles y concretamente en los ácidos benzoicos y las flavanonas. Por el contrario, los de la zona fría presentan niveles mayores en antocianidinas. Por lo que respecta a la maduración, se observa una tendencia al aumento de los flavanoles y las antocianidinas presentando una mayor diferencia entre el grado 4 con el 1 y 2. Por el contrario, los ácidos hidroxicinámicos disminuyen con la sobremaduración presentando para el grado 4 los valores más pequeños.
La piel presenta niveles más elevados de epicatequina, quercetina galactosido y canferol glucósido en la zona fría. La pulpa presenta diferencias en el contenido de ácido cafeico, catequina, epicatequina y ácido p-cumárico siendo mayores en la zona cálida. Por lo que respecta a las semillas, presentan niveles mayores de ácido cafeico, p-cutárico, catequina, epicatequina y canferol glucósido en la zona cálida y de epigalocatequin galato, hexosa de ácido hidroxibenzoico y ácido feruoilglucosido en la zona fría.

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7.AGRADECIEMIENTOS:

A Bodegas Torres y Proyecto CENIT-DEMETER FBG 305131

C.16. Comunicació 16. Pòster

Títol: Caracterización de compuestos fenólicos en piel, pulpa y semilla de uva Albariño por espectrometría de masas con q-TOF y triple cuadrupolo.

Autors: Giuseppe Di Lecce, Sara Arranz-Martínez, Anna Tresserra-Rimbau, Paola Quifer, Alexander Medina-Remón, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Caracterización de compuestos fenólicos en piel, pulpa y semilla de uva Albariño por espectrometría de masas con q-ToF y triple cuadrupolo

Giuseppe Di Lecce[*] [1,3], Sara Arranz[2,3], Anna Treserra[3,5], Paola Quifer[3,5], Alexander Medina-Remón[3,5], María Rosa Pérez [4], Rosa Mª Lamuela[3,5]

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INTRODUCCIÓN

La variedad Albariño (*Vitis vinifera* L.) es la variedad de uva blanca más importante cultivada en el noroeste de España (Galicia) perteneciente a la Denominación de Origen "*Rías Baixas*", siendo la mayor zona geográfica de este cultivo. El conocimiento de la composición fenólica de la uva tiene una importancia fundamental en la elaboración de los vinos debido al papel que juegan directa o indirectamente sobre la calidad. Los compuestos fenólicos se encuentran distribuidos en las diferentes partes anatómicas de la uva y la trasformación tecnológica adoptada condiciona la extracción y el relativo perfil fenólicos de los vinos.

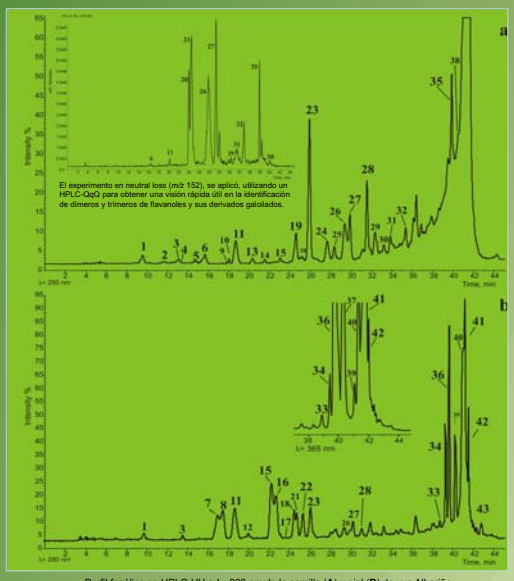
OBJETIVO

El objetivo de este trabajo fue la identificación de los compuestos polifenólicos presentes en la piel, pulpa y semilla de la uva Albariño mediante cromatografía UHPLC-UV acoplada a un detector de masas triple cuadrupolo (QqQ) y a un híbrido cuadrupolo a tiempo de vuelo (q-ToF-MS) que proporciona alta resolución y masa exacta.

MATERIAL Y MÉTODOS

RESULTADOS

Los experimentos de espectrometría de masas, tales como el PIS, el PrI y el NL, fueron útiles para obtener un perfil fenólico inequivocable. Además los polifenoles fueron confirmados por medición de la masa exacta por q-ToF de los iones obtenidos por MS y MS/MS, resultando esencial para la asignación de la composición elemental y por lo tanto para la caracterización de moléculas pequeñas.



Perfil fenólico en HPLC-UV a λ= 280 nm de la semilla (**A**) y piel (**B**) de uva Albariño.

pico	rt	compuestos	fracciones	[M-H]-	fragmento m/z (%)	MS/MS experiments PIS	NL	PrIS	error mDa	formula
1	9.35	gallic acid*	pi, se	169.0151	169 (100), 125 (25)	169			0.9	$C_7H_6O_5$
2	11.82	gallic acid hexose	se	331.0700	331 (5), 169 (70), 125 (100)	331	162	169	3.0	$C_{13}H_{16}O_{10}$
3	13.08	gallic acid dihexose	pi, se	493.1195	493 (9), 331 (100), 169 (100)	493		169	- 0.3	$C_{19}H_{26}O_{15}$
4	13.21	protocatechuic acid-*O*-hexoside	se	315.0723	153 (100), 109 (40)	315	162	153	0.2	$C_{13}H_{16}O_9$
5	14.76	(epi)gallocatechin-(epi)catechin	se	593.1332	423 (78), 305 (100), 289 (28)	593			3.2	$C_{30}H_{26}O_{12}$
6	15.72	procyanidin trimera C1	se	865.1950	865 (37), 695 (100), 289 (42)	865	152	289	- 3.5	$C_{45}H_{38}O_{18}$
7	16.90	*cis*-caftaric acid	pi, pu	311.0413	179 (100), 135 (54)	311		179	0.5	$C_{13}H_{12}O_9$
8	17.49	*trans*-caftaric acid*	pi, pu	311.0411	179 (100), 135 (40)	311		179	0.3	$C_{13}H_{12}O_9$
9	17.84	(epi)gallocatechin-3-gallate	se	457.0783	305 (100), 169 (65)	457			0.7	$C_{22}H_{18}O_{11}$
10	18.01	protocatechuic acid-*O*-hexoside	se	315.0751	153 (100), 109 (40)	315	162	153	3.0	$C_{13}H_{16}O_9$
11	18.64	L-tryptophan*	pi, se, pi	203.0847	142 (9), 116 (100)	203			2.1	$C_{11}H_{12}N_2O_2$
12	19.82	(-)epigallocatechin*	pi	305.0699	261 (100), 221 (25), 179 (34)	305			3.3	$C_{15}H_{14}O_7$
13	20.23	(epi)catechin-3-hexose	pi	451.1266	289 (100), 245 (40)	451	162	289	2.1	$C_{21}H_{24}O_{11}$
14	21.56	gallic acid hexose	pi	331.0700	331 (12), 169 (100), 125 (84)	331	162	169	3.0	$C_{13}H_{16}O_{10}$
15	22.35	*cis*-coutaric acid	pi	295.0469	163 (32), 119 (100)	295		163	1.0	$C_{13}H_{12}O_8$
16	22.83	(epi)catechin-3-hexose	se	451.1266	289 (100), 245 (54)	451	162	289	2.1	$C_{21}H_{24}O_{11}$
17	22.91	*trans*-coutaric acid	pi	295.0460	163 (53), 119 (100)	295		163	0.1	$C_{13}H_{12}O_8$
18	23.34	coumaric acid-*O*-hexoside	pi, pu	325.0919	163 (50), 148 (30), 119 (100)	325	162	163	0.9	$C_{15}H_{18}O_8$
19	24.45	fertaric acid	pi, pu	325.0585	193 (100), 149 (30)	325		193	2.0	$C_{14}H_{14}O_9$
20	24.51	procyanidin B3	pi, se, pu	577.1331	407 (75), 289 (81), 245 (67)	577	152	289	- 2.0	$C_{30}H_{26}O_{12}$
21	24.75	procyanidin B1*	se	577.1334	407 (75), 289 (70), 245 (45)	577	152	289	- 1.7	$C_{30}H_{26}O_{12}$
22	24.82	*p*-coumaric acid*	pi	163.0418	163 (20), 119 (100)	163			1.8	$C_9H_8O_3$
23	25.93	(+)-catechin*	pi, se, pu	289.0710	245(100), 205 (65)	289			- 0.7	$C_{15}H_{14}O_6$
24	27.72	procyanidin trimer	se	865.1954	577 (68), 425 (88), 289 (81)	865	152	289	- 3.1	$C_{45}H_{38}O_{18}$
25	28.30	procyanidin trimer	se	865.1971	577 (33) 575 (21), 289 (100)	865	152	289	- 1.4	$C_{45}H_{38}O_{18}$
26	29.24	procyanidin B4	se, pi	577.1332	407 (93), 289 (73), 245 (59)	577	152	289	- 1.9	$C_{30}H_{26}O_{12}$
27	29.85	procyanidin B2*	pi, se	577.1331	407 (93), 289 (73), 245 (59)	577	152	289	- 2.0	$C_{30}H_{26}O_{12}$
28	31.58	(-)-epicatechin*	pi, se, pu	289.0712	245 (100), 205 (60)	289			- 0.5	$C_{15}H_{14}O_6$
29	32.42	(epi)catechin-(epi)catechingallate I	se	729.1476	577 (26), 407 (96), 289 (100)	729		289	1.5	$C_{37}H_{30}O_{14}$
30	33.26	procyanidin trimera	se	865.1959	865 (42), 577 (52), 289 (100)	865	152	289	-2.6	$C_{45}H_{38}O_{18}$
31	33.81	(epi)catechin-(epi)catechingallate II	se	729.1472	577 (37), 407 (100), 289 (70)	729	152	289	1.1	$C_{37}H_{30}O_{14}$
32	35.42	(epi)catechin-(epi)catechingallate III	se	729.1473	577 (43), 407 (100), 289 (94)	729	152		1.2	$C_{37}H_{30}O_{14}$
33	38.76	quercetin-3-*O*-rutinoside*	pi	609.1466	609 (67), 301 (100)	609	308	301	0.5	$C_{27}H_{30}O_{16}$
34	39.30	quercetin-3-*O*-glucuronide	pi	477.0636	301 (72), 151 (100),	477	176	301	- 3.8	$C_{21}H_{18}O_{13}$
35	39.44	(-)-epicatechin-3-*O*-gallate	se	441.0866	289 (100), 271 (47), 169 (85),	441			3.9	$C_{22}H_{18}O_{10}$
36	39.65	quercetin-3-*O*-glucoside*	pi	463.0857	301 (67), 151 (100)	463	162	301	- 2.4	$C_{21}H_{20}O_{12}$
37	40.27	diidroquercetin-3-*O*-rhamnoside	pi	449.1109	303 (100), 151 (75)	449		303	2.0	$C_{21}H_{22}O_{11}$
38	40.52	dimer digallate	se	881.1975	881 (25), 729 (93), 407 (100)	881	152		4.1	$C_{44}H_{34}O_{18}$
39	40.95	quercetin-3-*O*-arabinoside	pi	433.0731	301 (100), 151 (77)	433			- 3.9	$C_{20}H_{18}O_{11}$
40	41.21	kaempferol-3-*O*-glucuronide	pi	461.0770	285 (100), 257 (18), 229 (25)	461	176		4.5	$C_{21}H_{18}O_{12}$
41	41.40	kaempferol-3-*O*-glucoside*	pi	447.0972	447(30), 285(100)	447	162	285	4.0	$C_{21}H_{20}O_{11}$
42	41.56	*trans*-piceid*	pi	389.1246	227 (100), 185 (17), 143 (6)	389	162	227	0.5	$C_{20}H_{22}O_8$
43	42.40	*trans*-resveratrol*	pi	227.0750	185 (13), 143 (100)	227			3.6	$C_{14}H_{12}O_3$

Compuestos fenólicos identificado: rt, tiempo de retención, fracciones de los extractos de piel (pi), pulpa (pu) y semilla (se); [M-H]- ión molécular deprotonado; fragmentos y porcentaje de los mayoritarios; PIS, product ion scan, PrIS, precursor ion scan, NL, experimento de neutral loss, error milDalton y fórmula molecular; *confirmados con patrones.

CONCLUSIONES

Por primera vez se describe una completa caracterización fenólica de las diferentes partes anatómicas de la uva Albariño. Hasta 20 monómeros y oligómeros de flavan-3-oles y sus derivados galoilados, 13 derivados de ácido hidroxibenzoicos e hidroxicinámicos y 8 flavonoles, así como trans-resveratrol y su glucósido, fueron identificados. La piel resultó ser una rica fuente de ácidos hidroxicinámicos, flavonoles y sus glucósidos mientras que los flavanoles se presentaron principalmente en la semilla. La (+)-catequina y (-)-epicatequina fueron identificados en las tres partes de la uva. Se obtuvieron buenos valores de masa exacta para todos los iones investigados, con errores que van desde 0,2 hasta 4,5 mDA. El valor observado de la desviación de la masa no es mayor que el recomendado para la confirmación de la fórmula molecular mediante la medición de masa exacta.

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C.17. Comunicació 17. Pòster

Títol: Efecto de la disponibilidad de agua y la radiación solar en la uva Albariño.

Autors: Anna Tresserra-Rimbau, Paola Quifer, Sara Arranz-Martínez, Giuseppe Di-Lecce, Alexander Medina-Remón, Anna Velázquez, Miguel Tubio, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Efecto de la disponibilidad de agua y la radiación solar en la uva Albariño

Anna Tresserra [1,2], Paola Quifer [1,2], Sara Arranz [1,3], Giuseppe di Lecce [4], Alexander Medina [1,2], Anna Velàzquez [5], Miguel Tubio [6], Rosa Mª Lamuela * [1,2]

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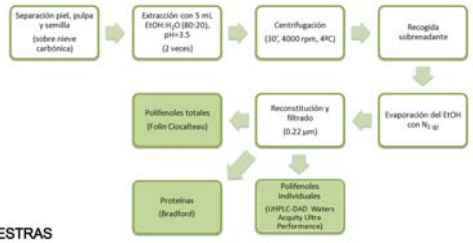
Introducción

La disponibilidad de agua y la temperatura influyen en el crecimiento de la vid y en la calidad de la uva, tanto a nivel químico, como microbiológico y sensorial. Los efectos del cambio climático son el adelantamiento de los periodos de cosecha, un incremento del azúcar de la baya, que se traduce en un mayor grado alcohólico en los vinos, una menor acidez, más compuestos fenólicos y la modificación de algunos compuestos aromáticos. Las altas temperaturas dan lugar a la acumulación de metabolitos secundarios como los polifenoles, que aunque confieren el color al vino y tienen beneficios para la salud, algunos de ellos también son responsables del sabor **astringente y amargo de la uva y el vino.**

En este trabajo se ha estudiado el efecto de la radiación solar y la disponibilidad de agua sobre los compuestos fenólicos y las proteínas de la piel, pulpa y semilla de la uva Albariño.

Material y Métodos



MUESTRAS

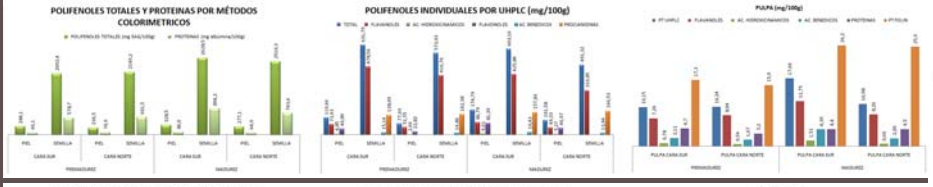
·Uvas albariño recolectadas simultáneamente de dos viñedos gallegos (D.O. Rías Baixas) homogéneos.
·Clasificadas según grado de madurez (por densidad)
·Separadas según la orientación en la vid (cara Norte y Sur)

Resultados

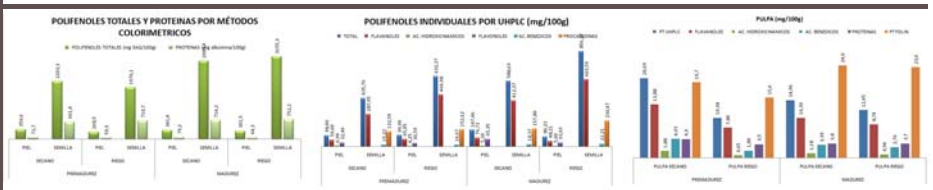
La semilla y la piel son las partes más ricas en prótidos y polifenoles. En todas las partes de la uva los flavanoles son el grupo de polifenoles mayoritario (catequina, epicatequina, procianidinas, dímeros y trímeros). En la piel, destaca también la presencia de flavonoles (quercetina, quercetin-glucosido y caenferol-glucósido) y, en la pulpa, de ácidos benzoicos (ácido gálico, protocateico y hexosas de ácido gálico) y ácidos hidroxicinámicos.

Se ha observado además, que las pieles y las pulpas de las uvas de los viñedos con mayor potencial hídrico y las orientadas hacia el norte presentan, en general, una disminución de compuestos fenólicos y proteínas. Las semillas presentan la misma tendencia respecto a la radiación solar pero inversa en el caso del potencial hídrico.

Radiación solar



Potencial hídrico



Conclusiones

Los polifenoles mayoritarios en todas las partes de la uva son los flavanoles. Tanto los compuestos fenólicos como las proteínas aumentan con la exposición a la radiación solar. En la piel yt la pulpa se observa una disminución de estos compuestos con el riego.

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C.18. Comunicació 18. Pòster

Títol: Consumo de polifenoles del vino en el marco de una dieta mediterránea y su correlación con la excreción de polifenoles totales: el estudio PREDIMED.

Autors: Alexander Medina-Remón, Anna Tresserra-Rimbau, Anna Vallverdú-Queralt, Sara Arranz-Martínez, Palmira Valderas-Martínez, Ramón Estruch, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Consumo de polifenoles del vino en el marco de una dieta Mediterránea y su correlación con la excreción de polifenoles totales: el estudio PREDIMED.

Alexander Medina-Remón [*,1,2], Anna Tresserra-Rimbau [1,2], Anna Vallverdú-Queralt [1,2], Sara Arranz-Martínez [1,3], Palmira Valderas-Martínez [2,3], Ramón Estruch [2,3], Rosa Mª Lamuela-Raventos [1,2]

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1. Introducción

La hipertensión es el problema más importante de salud pública y la principal causa de muerte y discapacidad en los países desarrollados. Una cuarta parte de la población adulta del mundo padece hipertensión [1].
Los polifenoles son cuantitativamente la principal fuente dietética de antioxidantes [2]. Diversos estudios epidemiológicos han demostrado una asociación inversa entre el riesgo de enfermedad cardiovascular y el consumo de alimentos ricos en polifenoles como el vino, frutas verduras y hortalizas (F&V), té, aceite de oliva virgen y el riesgo de hipertensión [4, 5, 6].

El objetivo de este trabajo es evaluar la asociación entre los alimentos ricos en polifenoles en una dieta mediterránea habitual, con los polifenoles totales excretados (PTE), en una población *free living* con elevado riesgo cardiovascular. Explicando en qué medida influye el consumo de vino sobre los PTE y esta a su vez sobre la incidencia de hipertensión.

2. Material y Métodos


Sub-estudio transversal con datos de 584 participantes del estudio PREDIMED (*www.predimed.org*)

263 hombres (55 - 80 años) | **326 mujeres (60 - 80 años)**

Cuestionario semi-cuantitativo de frecuencia de consumo de alimentos (FFQ) con136-ítems.

La versión validada en español del Cuestionario de Tiempo de Actividad Física y Ocio de Minnesota.

Cuestionario de 47 ítems sobre la educación, estilo de vida, historia de enfermedades y el uso de medicamentos.

Medición de la presión arterial y recogida de muestras de orina de la mañana.

La ingesta de energía y nutrientes se obtuvo de las tablas españolas de composición de alimentos.

El consumo total de polifenoles de los alimentos vegetales y bebidas (mg / g de materia fresca) se cuantificó a partir del FFQ.

Se analizaron los PTE en orina basal por el método de Folin-Ciocalteu (FC) [7].

SPE: extracción en fase sólida con placas Oasis® MAX (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) de 96 cartuchos, para evitar interferencias con el reactivo de F-C.

TPE se expresaron como mg de equivalentes de ácido gálico (GAE) / g de creatinina.

Análisis estadístico

Los análisis se realizaron utilizando el software SPSS versión 14.0. Las características basales de los participantes se expresaron como medias o porcentajes y desviaciones estándar (SD).

3. Resultados

Tabla 1: Consumo diario de alimentos seleccionados según cuartiles de polifenoles totales excretados, expresado en mg GAE / g de creatinina.

	Cuartiles de concentración urinaria mg GAE/ g creatinina				
	Q1 (<89.0)	Q2 (89.1-119.5)	Q3 (119.6-160.2)	Q4 (>160.3)	P for trend[1]
Polifenoles totales excretados (mg GAE/ g creatinina)[2]	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	< 0.001
Sujetos	147	148	147	147	0.742
Frutas, verduras y hortalizas totales (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Vino (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Polifenoles totales ingeridos (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Alcohol (g)	15.7 (17.9)	10.2 (16.0)	12.2 (18.5)	9.9 (17.8)	0.018
Fibra (g)	22.1 (6.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.2)	0.606
Sodio (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potasio (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Energía total, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	< 0.001

[1]One-factor ANOVA para variables continuas y χ^2-test para variables categóricas; [2]Media (SD). GAE: equivalentes de ácido gálico.

Tabla 2: Regresión lineal múltiple, relación entre los PTE como variable dependiente y los PT ingeridos, junto con los diferentes grupos de alimentos como variable de exposición.

	Modelos	β	SE	Beta	P	95 % CI
PT ingeridos (100mg)	Modelo 1	0.073	0.017	0.179	<0.001	0.041 to 0.106
	Modelo 2	0.116	0.020	0.283	<0.001	0.077 to 0.154
Frutas, verduras y hortalizas (100g)	Modelo 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Modelo 2	0.127	0.036	0.150	<0.001	0.056 to 0.198
Vino (100 mL)	Modelo 1	-0.090	0.041	-0.090	0.029	-0.171 to -0.009
	Modelo 2	0.121	0.051	0.120	0.019	0.020 to 0.222

β:Coeficiente no estandarizados (coeficiente de regresión línea); SE: error estándar; Beta: coeficiente estandarizado; CI : Intervalo de confianza; Modelo 1: sin ajustar; Modelo 2: ajustado por sexo, edad , peso, consumo de tabaco, actividad física, nivel de educación , medicamentos más consumidos en el último mes, ingesta de sodio y potasio y tasa de filtración glomerular (TFG); GAE: equivalente de ácido gálico.

Figura 1: Cambio en la presión arterial sistólica y diastólica según cuartiles de la excreción de polifenoles totales, expresados como mg GAE / g de creatinina

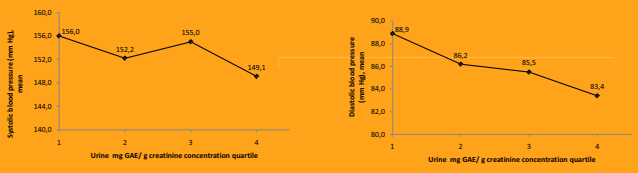

Tabla 3: Análisis de regresión logística, OR (95% intervalo de confianza) para el factor de riesgo cardiovascular (hipertensión), según cuartiles de la excreción de polifenoles totales, expresados como mg GAE / g de creatinina con el cuartil más bajo como categoría de referencia.

	Cuartil de excreción de polifenoles totales (mg GAE/ g creatinina)				
	Q1 (<88.99)	Q2 (89-119.46)	Q3 (119.47-160.22)	Q4 (>160.23)	P for trend
Hipertensión, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Modelo 1	1.00	1.82 (0.33-10.13)	0.67 (0.29-1.55)	0.71 (0.53-0.95)	0.021
Modelo 2	1.00	1.39 (0.19-10.34)	0.55 (0.20-1.48)	0.64 (0.45-0.92)	0.047

OR: odds ratios; Modelo 1: sin ajustar; Modelo 2: ajustado por sexo, edad , peso, consumo de tabaco, actividad física, nivel de educación , medicamentos más consumidos en el último mes , ingesta de sodio y potasio y tasa de filtración glomerular (TFG); GAE: equivalente de ácido gálico.

4. Conclusiones

Los PTE en la orina se correlacionaron positivamente con la ingesta total de polifenoles de la dieta, con el consumo de F&V así como con el consumo de vino.

A su vez los PTE en orina, se asoció negativamente con los niveles de PA y la prevalencia de la hipertensión en una población mediterránea de edad avanzada con alto riesgo cardiovascular.

Una intervención dietética dirigidas a incrementar la ingesta de alimentos ricos en polifenoles, podría ser eficiente en la prevención y el tratamiento de la hipertensión.

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Jerez de la Frontera (Cádiz) 1-4 Junio de 2011

C.19. Comunicació 19. Pòster

Títol: Correlación entre compuestos polifenólicos identificados en vinos tintos y sus diferentes atributos de cata.

Autors: Alexander Medina-Remón, Sara Arranz-Martínez, Anna Tresserra-Rimbau, Paola Quifer, Giuseppe Di Lecce, Núria Tobella, Mireia Torres, i Rosa M. Lamuela-Raventós.

Congrés: XI Congreso Nacional de Investigación Enológica (Gienol 2011). Jerez de la Frontera, Espanya. 2011.

Correlación entre compuestos polifenólicos identificados en vinos tintos y sus diferentes atributos de cata.

Alexander Medina-Remón [*,1,2], Sara Arranz [1,3], Anna Tresserra [1,2], Paola Quifer [1,2], Giusseppe di Lecce [4], Nuria Tobella [5], Mireia Torres [5], Rosa Mª Lamuela [1,2].

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1. Introducción

Las propiedades sensoriales "en boca" de los vinos tintos, abarcan múltiples e interactivas sensaciones de acidez, dulzura, amargor, percepción de aroma retronasal (sabor), viscosidad, calor, y astringencia. Estas propiedades sensoriales se describen a menudo por catadores experimentados utilizando términos generales y subjetivos.
La importancia de los catadores es lograr una comprensión común de los términos que describen el vino en boca [1]; el término sensación bucal que no ha sido adecuadamente definido, sustancialmente reduce el valor comunicativo de estas descripciones.
El extensivo uso de una rueda de aroma del vino [2] sugiere que existe una necesidad similar de un panel sensorial que describa "en boca" la astringencia y otras sensaciones de vino tinto.

El objetivo de este trabajo es correlacionar las diferentes cualidades de cata de vinos tintos, determinada por expertos catadores, y los compuestos fenólicos que podemos encontrar en vinos de diferentes variedades.

2. Material y Métodos

Polifenoles totales se determinaron usando el método de Folin-Ciocalteu [3].

Absorbancia a 280, 320, 420, 520 y 620 nm en cubetas de cuarzo de 1 mm de paso óptico

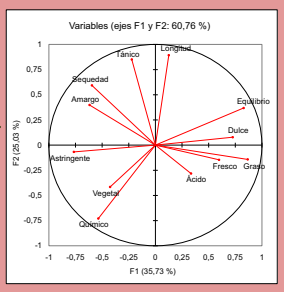
Polifenoles por HPLC

columna C18 Nucleosil 120 (250x4 mm)
micropartícula de 5µm
flujo de 0.8 ml/min, 45 min

Fases (A) agua:ácido acético, pH 2.65 y (B) 80% acetonitrilo: 20 % A.

la separación cromatográfica se desarrolló siguiendo el método de Bétes-Saura et al. [4]

7 CATADORES



3. Resultados

Correlaciones

ASTRINGENCIA

Con la absorbancia a 280 nm, con la copigmentación, con ácido gálico, tirosol, procianidina B4, epicatequina, ácido feruoilglucósido y con el canferol.
Con la tonalidad del vino, el grado de madurez y el gusto vegetal y amargo.

Con el ácido cafeico, ácido cutárico, ácido protocatequico o la quercetina y la absorbancia a 520 nm.
Con el gusto suave, dulce y tánico, y con los parámetros de intensidad, equilibrio, ácido málico y tartárico.

VEGETAL

Con los ácidos cafeico, caftárico y cutárico, tirosol, procianidina y la suma de ácidos benzoicos.
Con la astringencia, el gusto amargo y el ácido tartárico.

Con las absorbancias a 420, 520 y 620 nm, con epicatequina, epigalocatequin galato, cianidinas, peonidinas y petunidinas y con la copigmentación.
Con la intensidad, el grado alcohólico y el ácido málico.

AMARGOR

Con la absorbancia a 280 nm, ácido gálico, tirosol, epicatequina y suma de ácidos benzoicos.
Con la tonalidad, la astringencia y el gusto vegetal.

Con la quercetina y con el gusto dulce, tánico y fresco, con equilibrio y ácido málico.

ACIDEZ

Con gusto vegetal y ácido tartárico

Con absorbancias a 320, 420, 520 y 620 nm, copigmentación y ácido protocatequico, ácido feruoilglucósido, epicatequina, procianidina B4, epigalocatequin galato, cianidin glucósido, petunidin glucósido, peonidin glucósido, delfinidin glucósido, malvidin glucósido, malvidin acetil glucósido.
Con la intensidad, gusto fresco, dulce, tánico y equilibrado y con la madurez, grado alcoholico, pH y ácido tartárico.

4. Conclusiones

A mayor concentración de compuestos del vino como el ácido gálico, ácido protocatéquico, la epicatequina, las procianidinas o flavonoles como el canferol, mayor es la contribución a la astringencia y el amargor.
Los ácidos cinámicos como el ácido cafeico, caftárico y cutárico son los que mayor contribución aportan al gusto vegetal de los vinos.
Las antocianidinas en general cuanto mayor sea su concentración, menores son los valores de acidez y gusto vegetal.
La copigmentación parece estar directamente relacionada con la astringencia y de manera inversa con la acidez y el gusto vegetal.
Cuanto mayor sean la tonalidad y el pH, mayor será la astringencia, el gusto amargo y vegetal. El contenido elevado de ácido tartárico se correlaciona con mayor acidez y más gusto vegetal.

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C.20. Comunicació 20. Pòster

Títol: Green mouthfeel wines have different phenolic profile.

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Congrés: 4th International Conference on Polyphenols and Health (ICPH 2009). Harrogate, UK 2009.

Green mouthfeel wines have different phenolic profile

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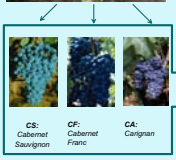
1. INTRODUCTION

In some climate conditions, minor compounds from grapes, secondary metabolites, do not evolve at the same rate than the major compound of grapes sugars and acids, they are still immature or *green* at grape maturity. Wines made from these *green* have an adequate alcohol concentration and acidity but the minor compounds give to the wine green sensory properties, characterized by excess of acididity and astringency compared to *soft* wines that have a light and finely textured astringency (1).

AIMS: The aim of this work was to determine differences between *green* and *soft* wines across to specific parameters of the wine and characterize some indicator responsible of this green mouthfeel properties.

2. Samples



Green wines

Soft wines

Total phenols (2)
Phenolic profile by HPLC-DAD (3)

Total polyphenols (2)

Acrodisc 13 mm CR PTFE 0.45 µm

1 mL

24 µL diluted sample

184 µL Milli-Q water
12 µL Folin-Ciocalteu reagent
& 30 µL sodium carbonate 20%

1 h in the dark

50 µL Milli-Q water

Absorbance at 765 nm in spectrophotometers UV/VIS

Phenolic profile by HPLC-DAD (3)

Acrodisc 13 mm CR PTFE 0.45 µm

10 µL

Flow: 4 mL/min

Zorbax ® Stable Bond C18 (30 mm x 4.6 mm)

time	face A	face B
0	100 %	0%
0,5	100 %	0%
2	98%	2%
8	92%	8%
15	85%	15%
18	77%	23%

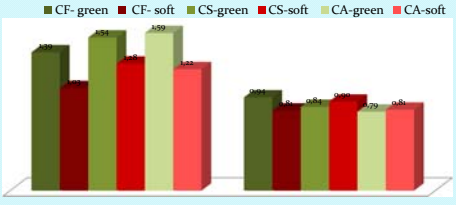
face A: Milli-Q water at 0.2% TFA

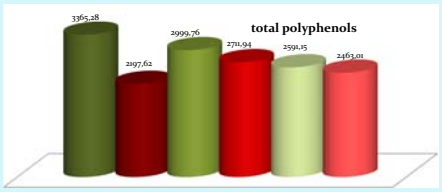
face B: acetonitril at 0.2% TFA

Hewlett-Packard (HP) 1050 (Palo Alto, CA) liquid chromatograph with a diode array detector HP 1050

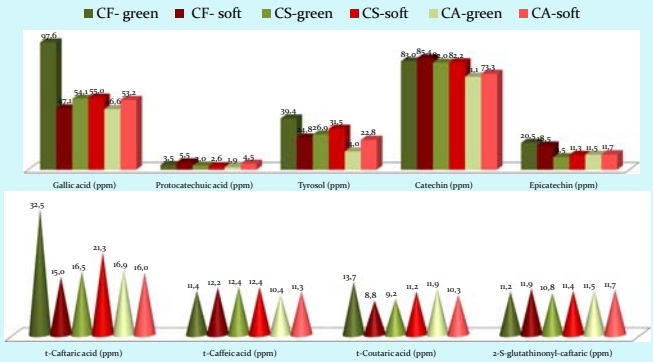
3. Results

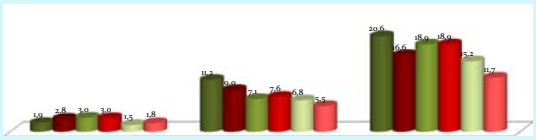
CS: Cabernet Sauvignon
CF: Cabernet Franc
CA: Carignan

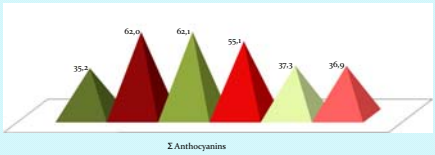


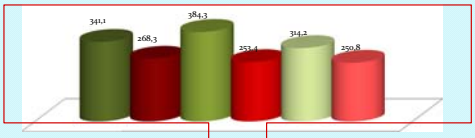


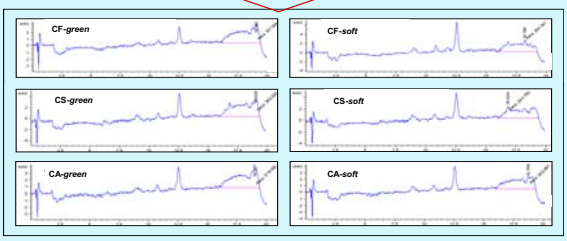
Mean (mg Gallic acid equivalent/L)











Co-pigmentation grade at 520 nm

4 CONCLUSIONS

The color intensity of the *green* "*green*" wines are higher than *"soft"* wines from the corresponding grapevines.

Differences in total phenolics contents were observed among the "*green*" and the *soft* wines.

"*Green*" wines have higher grade of co-pigmentation than *soft* wines. Moreover the polymerized anthocyanins at 520 nm may be a good parameter to differentiate green and soft mouthfeel wines, however more analysis with different are need

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