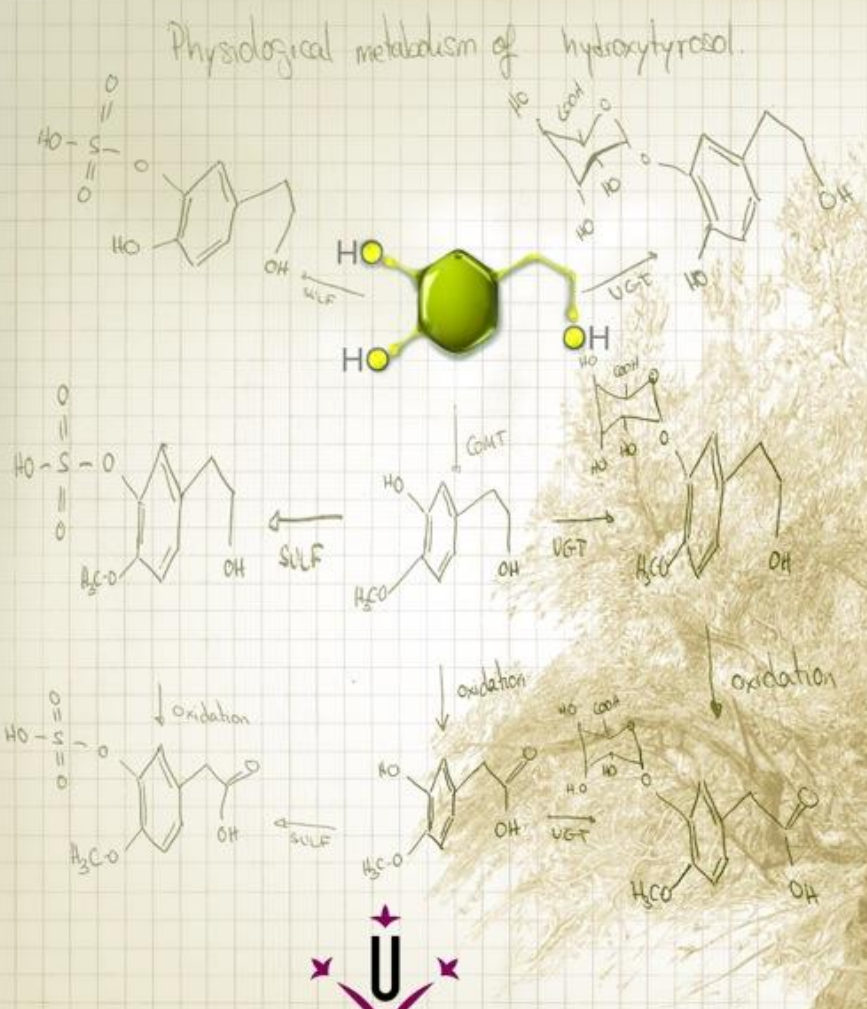
Advances in the knowledge of olive oil phenolic compounds. From biotransformations after their intake to their metabolic fate for exerting biological activities

Maria del Carmen López de las Hazas Mingo

http://hdl.handle.net/10803/402402



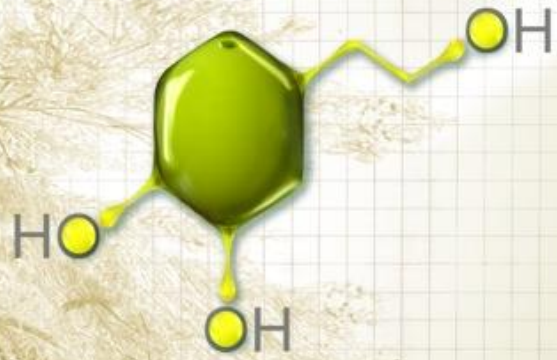
Universitat de Lleida
Escola Tècnica Superior d'Enginyeria Agrària
Departament de Tecnologia dels Aliments

Maria del Carmen
López de las Hazas
Mingo

ADVANCES IN THE KNOWLEDGE
OF OLIVE OIL PHENOLIC COMPOUNDS.
FROM BIOTRANSFORMATIONS AFTER
THEIR INTAKE TO THEIR METABOLICAL
FATE FOR EXERTING BIOLOGICAL ACTIVITIES

2017

ADVANCES IN THE KNOWLEDGE OF OLIVE OIL PHENOLIC COMPOUNDS. FROM BIOTRANSFORMATIONS AFTER THEIR INTAKE TO THEIR METABOLICAL FATE FOR EXERTING BIOLOGICAL ACTIVITIES



Maria del Carmen López de las Hazas Mingo
Doctoral Thesis

Universitat de Lleida

TESI DOCTORAL

Advances in the knowledge of olive oil phenolic compounds. From biotransformations after their intake to their metabolic fate for exerting biological activities

Maria del Carmen López de las Hazas Mingo

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Ciencia i tecnologia agrària i Agroalimentària

Director/a
Maria José Motilva Casado

Tutor/a
Carme Piñol Felis

2017

Doctoral Thesis presented by Maria del Carmen López de las Hazas Mingo to obtain a PhD degree from the University of Lleida. This Doctoral Thesis was supervised by María José Motilva Casado and Carme Piñol Felis. The present work was carried out in the Department of Food Technology's Antioxidants Research Group and comes under the "Agricultural and Food Science and Technology" doctoral programme.

This Doctoral Thesis is framed within the context of two different projects:

The MEFOPC project: *Metabolic fate of olive oil phenolic compounds in humans: Study of metabolic pathways and tissue distribution* (Ref. AGL2012-40144-C03-03).

The INCOMES project: *Guide for the substantiation of health claims in foods: Immune, cognitive functions and metabolic syndrome* (Collaborative project between Food Companies & Research Centres).

The PhD candidate received a doctoral grant from the University of Lleida (November 2013 – October 2016) and an internship grant from the University of Lleida.

The Language Institute at the University of Lleida awarded a grant (2017 call) for the language revision of this Doctoral Thesis.

PhD Candidate

Maria del Carmen López de las Hazas Mingo

Supervisor

María José Motilva Casado, PhD

Signature of approval

Co-supervisor

Carme Piñol Felis, PhD

Signature of approval

I want to try everything

I want to try even though I could fail

I won't give up, no I won't give in

Till I reach the end and then I'll start again

Sia Furler

ACKNOWLEDGMENTS

"*Que tu medicina sea tu alimento, y el alimento tu medicina*." La frase que despertó mi motivación por la ciencia, y con el paso del tiempo ha ido forjando mi camino. Ahora que se acaba esta etapa, puedo decir que he sido muy afortunada porque he contado con el mejor apoyo, tanto que resulta difícil resumir en pocas líneas la gratitud que siento.

En primer lugar, me gustaría agradecer a la Dra. María José Motilva el haberme brindado la oportunidad de haber realizado la tesis en el grupo de Antioxidants. Gracias por todo tu esfuerzo y trabajo dedicado, agradezco muchísimo todos los consejos, tu guía y disponibilidad bañada de tanta cercanía, ha sido una suerte tenerte. Gracias por contagiarme de tu pasión por la ciencia. Gracias a la Dra. Carme Piñol tu atención, confianza y todas las facilidades que me has dado para que las tareas del estabulario fuesen más sencillas.

Gracias a todo Antioxidant's lab team! Alba… No tinc palaures per descriure la teva aportació a la tesi. Merci per tota la teva ajuda! Has sido la pieza clave para el desarrollo de la tesis, compartir momentos tan buenos y ser la mejor maestra de Catalán del mundo!. Laura, ¡la mamá de esta tesis! no solo porque ésta continúa el camino que abriste con la tuya, sino porque no has faltado de estar ahí ni un minuto como guía y ayuda ¡¡¡GRACIAS!!!; Iziar, ¡ha sido un gustazo compartir laboratorio y experimentos contigo! Muchas gracias por tu cercanía, compartir tu sabiduría y tus millones de consejos; David, gracias ¡por tantos momentos de estabulario y laboratorio! Ha sido fantástico ¡Animo con la tesis!; MªPaz, muchísimas gracias por toda tu ayuda y soporte en el laboratorio, por dejarlo todo para ayudarme con las ASE averías! Además has sido la maestra que nos permitió crear nuestra propia cerveza artesana! (Mospez & Tremoló)… mi Juana, mi socia cervecera… porque hemos compartido miles de horas y recorrido juntas un largo camino y siempre has

estado ahí para ayudarme, escucharme y aconsejarme, por todo lo que he crecido a tu lado, por tantos momentos dentro y fuera del laboratorio ¡¡¡Cuánto te echo de menos!!!.

Gracias a la tropa Chimichurris, por ser mis lab mates y mi familia lleidatá, ¡Cuántos momentos imborrables regados con cervecitas y alimentados con pizzas, nachos y queso! Gracias en especial a Laila, que bueno haber compartido otros 3 años de camino juntas, ahora el destino ¿nos llevará de la mano? O esta vez nos llevará por otro lado? Lo bueno, es que todos estos años han sido una fuente magnifica de buenos momentos ¡¡Que sigan siendo!!; Joana, me siento orgullosa de conocer a una persona tan generosa y admirable, ¡¡gracias por tantos consejos!! ;) Y Sandra, mi dedo gatillo, por enseñarme a vivir de otra manera, ser tan buena compañera de aventuras y mejor amiga.

Gracias a todas personas que forman y formaron parte del departamento de TECAL, gracias por el día a día, por inmunerables risas a la hora de la comida, en especial a Manel… ¡¡¡San Manel!!!¡el Mago que arregla desde un roto hasta un descosido! Y Montse y Jordi, porque sobran las palabras ¡¡Mil gracias por todo vuestro apoyo!!

Gracias a toda la gente simpática con la que he coincidido en el IRB en especial a Ana por toda su ayuda con la histología.

Gracias a todo el Reus Team! Ursula, Sara, Laura y Rosa, ha sido un placer trabajar con vosotras, gracias por vuestra ayuda, cercanía e introducirme en el mundo mecanismos.

I would especially like to thank Claudia Nunes do Santos and all the members of the Molecular Nutrition and Health Laboratory at the *Instituto de Biologia Experimental e Tecnológica* and *Instituto de Tecnologia Química e Biológica* (ITQB/UNL) for their warm welcome during my 3-month intership. Thanks so much! I'm so gratefull

and I'll never forget these months with you! Thanks for all the good moments both inside and outside the lab! I miss you so much!!!

Gracias a Miri, Amparo y la familia gatuna Sheera, Pepper y… Manolo! Han sido 3 años mágicos de convivencia! Gracias por todos esos momentos hogareños tan fabulosos.

A mis amigas, las de siempre, con las que "se me cayeron los dientes" y he compartido tantos momentos. Espero seguir aprendiendo a vivir año tras año con vosotras ¡total… son 25 años compartidos ya! Vamos de cabeza a por los 50!;) A su vez, gracias a todos los que se incorporaron en el camino, "albucheños por el mundo"¡¡gracias familia!! por vuestra comprensión y apoyo, pese a que la distancia nos separe, mi corazón siempre recibe la energía positiva que irradiáis.

Gracias a todos los compañeros que tuve en el CIAL, en especial a Juanan, quien me enseñó a caminar científicamente hablando, gracias por despertar una de mis grandes pasiones, y por tantos momentos imborrables. También quiero agradecer a todos mis profesores de la UAM, especialmente a Guillermo y Susana, por la oportunidad que me dieron de empezar en el mundo de la investigación, y pusieron todo lo que estaba en su mano para enseñarme.

Gracias a mi familia Piruleteriana, porque, aunque estemos lejos, nos seguimos muy cerca.
Gracias a la García family, por todo su apoyo y ayuda durante este tiempo, en especial a Dani, por su magnífica contribución a la tesis diseñando moléculas en aceitunas.

Y llegando al hogar, gracias a mi familia, a mis hermanos y cuñados, por ser un gran ejemplo para mí, por ser "mis segundos papis", gracias tatita por cuidarme, entenderme, orientarme y ayudarme tanto. Gracias a mis sobrinitos, Pablo, Diego,

Irene, Marcos y Sara… me robasteis el corazón… y me recargáis de energía cada ratito que puedo disfrutar de vosotros. Y por último a los más importantes, mis padres, por apoyarme incondicionalmente, por todos los consejos tan sabios que me dais y por ayudarme a lograr todos mis sueños.

Y para concluir dar las gracias a la persona más importante de mi vida, GRACIAS Mario… No encuentro las palabras para describir tu presencia en mi vida. Gracias por las 10.500 horas que hemos pasado al teléfono para llenarme cada día de ilusión y alegría, por tener tantísima paciencia, por ayudarme a levantarme cuando he caído, por recorrer tantos km para pasar un rato juntos. Gracias por escucharme e intentar ponerte al día de lo que va mi trabajo e intentar aconsejarme siempre lo mejor posible sin duda, esta tesis también te pertenece!!!.

A mis padres,

A mis hermanos,

A Mario

This Doctoral Thesis is based on the following publications:

Publication I: M-C. López de las Hazas, L. Rubió, A. Kotronoulas, R. de la Torre, R. Solà, M-J. Motilva. Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats. Molecular Nutrition Food Research 59: 1395-1399 (2015).

Publication II: M-C. López de las Hazas, C. Piñol, A. Macià, M-P. Romero, A. Pedret, R. Solà, L. Rubió, M-J. Motilva. Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids. Journal of Functional Foods 22: 52-63 (2016)

Publication III: M-C. López de las Hazas, M-J. Motilva, C. Piñol, A. Macià. Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood. Talanta 159: 189-193 (2016).

Publication IV: U. Catalán*, M-C. López de las Hazas*, L. Rubió, S. Fernández-Castillejo, A. Pedret, R. de la Torre, M-J. Motilva, R. Solà. Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. Molecular Nutrition Food Research 59: 2523-2536 (2015)

Publication V: U. Catalán, L. Rubió, M-C. López de las Hazas, P. Herrero, P. Nadal, N. Canela, A. Pedret, M-J. Motilva. Hydroxytyrosol and its complex forms (secoiridoids) modulate aorta and heart proteome in healthy rats: Potential cardio-protective effects. Molecular Nutrition and Food Research 60:2114-2129 (2016).

Publication VI: M-C. López de las Hazas, C. Piñol, A. Macià, M-J. Motilva. Hydroxytyrosol and its colonic metabolites produce apoptosis and increase cell death in colon cancer cells. Journal of Agricultural and Food Chemistry Chemistry (2017

in press) (DOI: 10.1021/acs.jafc.6b04933).

Publication VII: M-C. López de las Hazas, J.I. Mosele, A. Macià, I.A. Ludwig, M-J. Motilva. Exploring the colonic metabolism of grape and strawberry anthocyanins fractions and their *in vitro* apoptotic effects in HT-29 colon cancer cells. Journal of Agricultural and Food Chemistry (2016 in press) (DOI: 10.1021/acs.jafc.6b04096).

Congress and conference contributions

Poster. 8th World Congress on Polyphenols Applications. Bioavailability and tissue disposition of hydroxytyrosol in rats: dose-response study. M-J. Motilva, L. Rubió, M-C. López De Las Hazas, A. Macià, A. Kotronoulas, N. Pizarro, P. Robledo, M. Farré, R. De La Torre. Lisbon (Portugal) (2014).

Poster. International Conference on Olive Oil. Current trends and new challenges in olive oil sector. Impacto de la estructura química de los precursores de hidroxitirosol sobre su biodisponibilidad y metabolismo en modelo de rata wistar. M-C. López de las Hazas; C. Piñol; A. Macià; M-P. Romero; L. Rubió; M-J. Motilva. Murcia (Spain) (2015).

Poster. XXVIIIth International Conference on Polyphenols. Hydroxytyrosol and their colonic metabolites produce apoptosis and increase cell death in colon cancer cells. M-C. López de las Hazas; C. Piñol; M-J. Motilva. Vienna (Austria) (2016).

Poster. XXVIIIth International Conference on Polyphenols. Colonic fate of anthocyanins and antiapoptotic effects of malvidin, pelargonidin and their microbial metabolites. J. Mosele, M-C. López de las Hazas; A. Macià; M-J. Motilva, I. Ludwig. Vienna (Austria) (2016).

ABSTRACT

Olive oil, the main source of fat in the Mediterranean diet. is associated with an improvement of the health status, because in its unsaponifiable fraction possesses an exclusive family of phenolic compounds (OOPCs) being hydroxytyrosol (HT) the most bioactive molecule with attributed pharmacological properties. Recently the European Food Safety Authority (EFSA) stablished a health claim stating that the properties of OOPCs countered the cardiovascular disease prevention. Since then, an ever-expanding range of products based on OOPCs has come onto the market, but there is little information about the dose effects and the form in which these compounds become more bioavailable.

In this Doctoral Thesis, our aim was to deepen the knowledge of HT metabolism and its biological activities. By the study of the relationship between the dose intake and its tissue uptake in rats, and also, the impact of the chemical structure of administration hydroxytyrosol or by its complex structures (oleuropein and secoiridoids) for its bioavailability in rats.

To deep in the knowledge of the contribution of hydroxytyrosol and its plasma metabolites in the prevention of cardiovascular diseases, these compounds have been tested in a cellular model of endothelial dysfunction (HAEC cells). To perform that, the plasma metabolites have been biologically synthetized by the exposition of hydroxytyrosol to differenciated Caco-2 cell line. In addition, we performed an *in vivo* study in healthy Wistar rats supplemented with HT and secoiridoids to evaluate antiatherosclerotic effect through the proteomic modifications of aorta and heart tissue depending on the diet.

Also, our goal was focus on study the metabolism and protective effects of the unabsorbed phenols and its catabolites formed in the gut as consequence of microbial metabolisms and the protective activity of these compounds against colon cancer.

Our results show that the administration of different doses of HT has a dose-response impact on its bioavailability, and also, the chemical structure affects to the bioavailability of the hydroxytyrosol plasma metabolites achieving higher bioavailabily the most complex forms. The study of the bioactivity of the mixture of phase-II metabolites of HT reveals that these compounds possess the same bioactivity as the HT. Also, the diet supplementation with HT and especially with secoiridoids modulates the proteome of aorta and heart tissue to improve the cardiac functions and decrease the atherosclerotic processes.

The colonic metabolites formed after the intake of phenolic products showed a protective role in the prevention of cancer development due to the induction of the pro-apoptotic activities in carcinogenic cell lines.

In conclusion, the HT is thoroughly metabolized in the digestive tract and also, it seems that the formed metabolites (both phase II and catabolites) possess the same bioactivity as the native molecule and contributing to maintain the health status.

RESUMEN

El aceite de oliva, como principal fuente de grasa de la dieta mediterránea se asocia con una mejora del estado de salud, ya que en su fracción insaponificable posee una familia exclusiva de compuestos fenólicos (OOPCs) siendo el hidroxitirosol (HT) la molécula más bioactiva, reconocida por sus propiedades farmacológicas. Recientemente, la Autoridad Europea de Seguridad Alimentaria (EFSA) hizo una alegación de salud afirmando las propiedades beneficiosas de los OOPCs en la prevención de enfermedades cardiovasculares.
Desde entonces, en el mercado existe una gama cada vez mayor de productos basados en los OOPCs, sin embargo, existe poca información sobre los efectos de la dosis y la forma en que estos compuestos se vuelven más biodisponibles.

En la presente tesis doctoral, nuestro objetivo fue profundizar en el conocimiento del metabolismo del HT y sus actividades biológicas. Por ello, se llevó a cabo el estudio de la relación entre la dosis de HT ingerida y la distribución de los metabolitos plasmáticos de hidroxitirosol en los tejidos en un modelo de rata Wistar. Además, se estudió el impacto en la biodisponibilidad de los metabolitos plasmáticos de HT en función de la estructura química ingerida bien hidroxitirosol o sus estructuras complejas (oleuropeína y secoiridoides).

El efecto protector contra enfermedades cardiovasculares del hidroxitirosol y de sus metabolitos plasmáticos fue testado en un modelo celular de disfunción endotelial (células HAEC). Para ello, los metabolitos plasmáticos se sintetizaron biológicamente mediante la exposición de HT a la línea celular Caco-2 diferenciada. A su vez, se realizó un experimento *in vivo* donde se estudió el proteoma de la aorta y corazón de ratas Wistar sanas tras la ingesta sostenida de HT y secoiridoides.

Por otro lado, estudiamos el efecto protector de los compuestos fenólicos no absorbidos y sus catabolitos formados en el intestino como consecuencia del metabolismo microbiano, así como, la actividad protectora de estos compuestos contra el cáncer de colon.

Nuestros resultados muestran que la administración de diferentes dosis de HT tiene un impacto dosis-respuesta sobre su biodisponibilidad. Otro factor importante en la biodisponibilidad de los compuestos es la estructura química del HT, cuanto más compleja es la estructura, mayor es su biodisponibilidad.

Tanto el HT como la mezcla de metabolitos de fase II ejercieron un efecto cardioprotector mediante la disminución de la expresión de moléculas de adhesión. Además, la suplementación dietética del HT, especialmente con secoiridoids modula el el proteoma de la aorta y el tejido cardíaco para mejorar las funciones cardíacas y disminuir los procesos ateroscleróticos.

Los metabolitos del colon formados después de la ingesta de productos fenólicos mostraron un papel protector en la prevención del desarrollo del cáncer debido a la inducción de las actividades pro-apoptóticas en las líneas celulares carcinógenas.

En conclusión, el hidroxitirosol es ampliamente metabolizado en el tracto digestivo, además, parece que tanto la molécula nativa como sus metabolitos de fase II y catabolitos contribuyen en el mantenimiento del estado de salud.

.

RESUM

L'oli d'oliva, com a principal font de greix de la dieta mediterrània s' associa amb una millora de l'estat de salut, ja que en la seva fracció insaponificable posseeix una família exclusiva de compostos fenòlics (OOPCs) essent l' hidroxitirosol (HT) la molècula més bioactiva, reconeguda per les seves propietats farmacològiques. Recentment, l'Autoritat Europea de Seguretat Alimentària (EFSA) va fer una al·legació de salut afirmant les propietats beneficioses dels OOPCs a la prevenció de malalties cardiovasculars.
A partir d' aquell moment, en el mercat trobem una gamma cada vegada més àmplia de productes basats en els OOPCs, tot i que hi ha poca informació sobre els efectes de la dosi i la forma en què aquests compostos són més biodisponibles.

En la Tesi Doctoral que es presenta, el nostre objectiu ha estat aprofundir en el coneixement del metabolisme del HT i les seves activitats biològiques. Per aquest motiu, s' ha estudiat la relació entre la dosi ingerida de HT i la distribució dels seus metabòlits plasmàtics així com en els teixits en un model de rata Wistar. A més a més, s'estudià l'impacte en la biodisponibilitat dels metabòlits plasmàtics de HT en funció de l'estructura química ingerida sigui a partir del mateix HT o a partir de les seves estructures complexes (l'oleuropeina i els secoiridoides).

Amb la finalitat d'estudiar l'efecte protector tant de l'hidroxitirosol com dels seus metabòlits plasmàtics contra el desenvolupament de malalties cardiovasculars, es van obtenir els metabòlits plasmàtics sintetitzats biològicament mitjançant l'exposició de HT a la línia cel·lular Caco-2 diferenciada. Tant el HT com seus metabòlits plasmàtics han estat provats en un model cel·lular de disfunció endotelial

(cèl·lules HAEC). A continuació es va estudiar el proteoma de l'aorta i cor de rates Wistar sanes després de la ingesta sostinguda del HT i secoiridoides.
Per altra banda, s'ha estudiat l'efecte protector dels compostos fenòlics no absorbits i els seus catabòlits formats a l'intestí com a conseqüència del metabolisme microbiològic així com també l'activitat protectora d'aquests compostos contra el càncer de colon.
Els nostres resultats mostren que l'administració de diferents dosis de HT té un impacte dosi-resposta sobre la seva biodisponibilitat. Un altre factor important en la biodisponibilitat dels compostos és l'estructura química de l'HT, com més complexa és l'estructura, s'ha observat més biodisponibilitat.

Tant el HT com la barreja de metabòlits de fase II van exercir efecte cardioprotector mitjançant la disminució de l'expressió de les molècules d'adhesió. A més a més, la suplementació dietètica del HT, especialment amb secoiridoides modula el proteoma de l'aorta i el teixit cardíac per millorar les funcions cardíaques i disminuir els processos ateroscleròtics.
Els metabòlits del colon formats després de la ingesta de productes fenòlics van mostrar un paper protector de la prevenció del desenvolupament del càncer a causa de l'inducció de les activitats pro-apoptòtiques en línies cel·lulars carcinogèniques.
En conclusió, l'hidroxitirosol és àmpliament metabolitzat en el tracte digestiu; i, sembla que tant la molècula nativa així com els seus metabòlits de fase II i catabòlits contribueixen en el manteniment adequat de l'estat de salut.

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALR	Aldehyde/Aldose reductase
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BAX	Protein regulator of apoptosis (pro-apoptotic)
BCL-2	Protein regulator of apoptosis (anti-apoptotic)
CDK	Cyclin-dependent kinase
COMT	Catechol-*O*-methyltransferases
COX-2	Cyclooxygenase 2
CVD	Cardiovascular diseases
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	3, 4-dihydroxyphenylacetaldehyde
EGFR	Epidermal growth factor receptor
FVOO	Functional virgin olive oil enriched with its own phenolic compounds
FVOOT	FVOO enriched with phenolic compounds from olive oil and thyme
GLT	Glutathione
GPx	Glutathione peroxidase
GST	Glutathione-S-transferase
HDL	High-density lipoprotein
HT	Hydroxytyrosol
HTN	Hypertension
HT-S	Hydroxytyrosol sulfate
HT-AC	Hydroxytyrosol acetate
HT-AC-S	Hydroxytyrosol acetate sulfate
HO-1	Heme oxygenase-1
HVAc	Homovanillic acid
HVAlc	Homovanillic alcohol
HVALC-S	Homovanillic alcohol sulfate
iKBα	Inhibitory protein of the complex NF-κB
iNOS	Nitric oxide synthase

JAK	Janus kinase-signal transducer
MAO	Monoaminoxidase
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde
MedDiet	Mediterranean Diet
MMPs	Enzymes matrix metalloproteinases
NF-κB	Nuclear transcription factor-kappa B
Nrf2	Nuclear factor-E2-related factor-2 transcription factor
OO	Olive Oil
OLE	Oleuropein
OOPCs	Olive Oil phenolic compounds
ox-LDL	Oxidized low-density lipoproteins
PBMC	Peripheral blood mononuclear cells
PCs	Phenolic compounds
PD	Parkinson's disease
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase/Akt pathway
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SEC	Secoiridoids
SULT	Sulfotransferase
TG	Triglycerides
TNF-α	Tumour necrosis factor
TYR	Tyrosol
UGTs	Uridine 5'-diphosphoglucuronosyl transferases
UPLC-MS/MS	Ultra-performance liquid chromatography coupled with tandem mass spectrometry
VOO	Virgin Olive Oil
LPS	Lipopolysaccharide

CONTENTS

INTRODUCTION…….……..….…..…..1

1. Dietary and endogenous sources of HT……5

1.1. Dietary sources of HT…….5

1.2. Endogenous sources of HT ……..…10

2. Metabolic fate of HT: from *in vitro* to *in vivo* approaches…….....…12

2.1. Absorption, distribution, metabolism and excretion of HT……..13

2.2. *In vitro* approaches to studying HT metabolism.....14

2.3. *In vivo* approaches to studying HT bioavailability and metabolism …….…….…….……..……..16

2.4. Colonic microbial metabolism of HT ……..……..……20

3. Potential pharmacological applications of HT……..…22

3.1. Preventive function of HT in cardiovascular diseases…….22

3.1.1. Human evidence of HT cardioprotective effects ……...23

3.1.2. Mechanistic studies of HT CVD cardioprotective effects……..32

3.1.2.1. Mechanistic studies in animal models …..……..……...32

3.1.2.2. Mechanistic studies in cell lines……..……..…..…..33

3.2. Role of HT in cancer prevention…….…....34

3.2.1. Human evidence of HT against cancer…….…….…….35

3.2.2. Mechanistic studies of HT against cancer……..…….…..36

3.2.2.1. Mechanistic studies in animal models…….…….36

3.2.2.2. Mechanistic studies in cells……..……...……..36

3.3. Role of HT in the prevention of neurological disorders ……..……41

3.3.1. Human evidence of HT neuroprotective effects……..…..…….43

3.3.2. Mechanistic studies of HT against neurological disorders …….44

3.3.2.1. Molecular mechanisms in the prevention of AD……..…45

3.3.2.2. Molecular mechanisms in the prevention of PD……47

3.3.2.3. Molecular mechanisms of HT in other cognitive disorders……..…48

4. Future perspectives…….…….…….....…...49

4.1. Exploring the effects of HT biological metabolites……49

4.2. Development of HT lipophilic derivates……50

5. Conclusions…….…….……...51

6. References…….…….…….…….…….…….…….…….……...53

HYPOTHESIS AND OBJECTIVES……..……....79

WORK PLAN…….…….……..……..85

RESULTS AND DISCUSSION…….……....……..89

Chapter 1: Metabolic fate of hydroxytyrosol and related compounds……....……..91

Publication 1: Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats…….…….…….93

Publication 2: Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids.……..……...109

Publication 3: Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood…….……...139

Chapter 2: Biological effects of hydroxytyrosol and its biological plasma metabolites.....……..……...153

Publication 4: Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. …….…….…….……155

Publication 5: Hydroxytyrosol and its complex forms (secoiridoids) modulate aorta and heart proteome in healthy rats: potential cardio-protective effects…….…….…….…….…….…….…….185

Chapter 3: Biotransformations of phenols into its microbial metabolites and its health effect in colon cancer cells…….…….……..……221

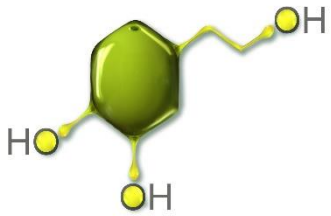
Publication 6: Hydroxytyrosol and its colonic metabolites produce apoptosis and increase cell death in colon cancer cells……..……223

Publication 7: Exploring the colonic metabolism of grape and strawberry anthocyanins fractions and their *in vitro* apoptotic effects in HT-29 colon cancer cells…….……....……..…….…….……249

GENERAL DISCUSSION…….……..…...…...281

CONCLUSIONS…….…….…….…….……..…...…..…307

Introduction



INTRODUCTION

Nowadays, life expectancy is increasing in developed countries. However, an aging population is associated with a high prevalence of chronic diseases such as cardiovascular diseases (CVD), cancer, neurodegenerative diseases, and other inflammatory syndromes (Niccoli & Partridge, 2012). Among others factors, unhealthy dietary habits, smoking and a sedentary lifestyle promote alterations in the organic system that contribute to an increased prevalence of these chronic disorders. Recent epidemiological evidence has indicated a convincing inverse association between the consumption of fruit, vegetables, nuts, olive oil, legumes and unprocessed cereals, in the frame of the so-called Mediterranean diet (MedDiet), and the incidence of chronic diseases such as coronary heart disease, neurological disorders and cancer (Carruba et al., 2016; Lipworth et al., 1997; Lourida et al., 2013; Niccoli & Partridge, 2012; Owen et al., 2000; Psaltopoulou et al., 2013; Psaltopoulou, et al., 2011; Sofi et al., 2010).

The MedDiet is characterized by consumption of foods rich in vitamins, minerals and phytochemicals such as carotenoids, alkaloids, organosulfur compounds, saponins, triterpenes, glycosides, phytosterols and phenolic compounds (PCs) (Morbidelli, 2016; Saura-Calixto & Goñi, 2009). As reviewed by Ostan et al. (2015), there is consistent evidence that these dietary compounds modulate multiple interconnected processes that play a major role in both carcinogenesis and inflammatory responses, including free radical production, nuclear transcription factor-kappa B (NF-κB) activation, the expression of inflammatory cytokines and the eicosanoid pathway. In particular, the MedDiet may have a positive impact on the so-called “inflammaging”, a chronic and low-level systemic inflammation, as well as on emerging topics, such as the maintenance of gut microbiota homeostasis and epigenetic modulation through specific microRNAs (Ostan et al., 2015). Among these bioactive compounds, a special focus must be placed on plant PCs, given that

many of the beneficial effects of the MedDiet derived from consuming high levels of fruit and vegetables have been attributed to these compounds (Ortega, 2006). PCs are secondary products of plant metabolism and are widely distributed in all plant-based food products. Their molecular structure is based on a phenol group linked to at least one hydroxyl group. The term polyphenols refers to a large (around 8,000) and heterogeneous group of compounds divided into different families based on differences in their chemical structure that determine their specific bioavailability and bioactivity (Manach et al., 2004). Of nutritional interest, their levels are much higher than all other known dietary antioxidants, about ten times more than vitamin C and 100-fold higher than vitamin E and carotenoids (Rodriguez-Mateos et al., 2014).

In Mediterranean countries, the main dietary source of PCs is coffee and fruit, but the most important differentiating factor with respect to other countries is the consumption of PCs from olives and VOO (Tresserra-Rimbau et al., 2013). VOO possess around 230 minor compounds present in the unsaponifiable fraction (1-2% of the total VOO composition), which can be distributed in different groups depending on their polarity. Particular attention has been paid to the nutraceutical properties of the hydrophilic PCs of VOO, which also contribute to a long oil shelf life and influence several organoleptic characteristics. Among all the PCs found in VOO, HT and its precursors oleuropein (OLE) and OLE aglycon, also known as secoiridoids (SEC) (El Riachy et al., 2011), represent the molecules of major interest for their pharmacological properties, and are among the most widely-researched natural antioxidant compounds. Most of the studies agree that SEC are not bioavailable when consumed in dietary doses, being rapidly hydrolyzed and resulting in the formation of HT. Therefore, attention on the biological effects is mainly focused on HT.

Taking into account that HT is the most representative phenol in VOO, this compound has attracted the interest of the food industry. HT and its precursors are

easily and cost-effectively recovered from olive oil pomace, a solid by-product from the VOO extraction process, adding value to this abundant material. In addition, the lack of bitterness of HT, compared to its precursor oleuropein, should facilitate its use in food formulations.

In this section, I first focus on the metabolic fate of HT, with special emphasis on the circulating metabolites detected in animal and human studies after consumption of HT and its precursors, the ones that can potentially exert the health benefits. I also discuss and summarize the most recent epidemiological relevance of HT in countering lifestyle-associated pathologies such as cancer and cardiovascular and neurodegenerative diseases. Finally, the newest perspectives on the mechanisms of action of HT involving in vitro and animal studies are also reviewed.

1. Dietary and endogenous sources of HT

1.1. Dietary sources of HT

HT is present in three of the traditional components of the MedDiet: table olives, VOO and wine (Fernández-Mar et al., 2012; Rodríguez-Morató et al., 2016). **Figure 1** shows a schematic representation of the pathway of the dietary sources of HT and its precursors.

Olive fruit or table olives are an important food for the general population in many Mediterranean countries and contain a wide range of PCs. Among the most important are: i) the phenolic alcohols HT and tyrosol (TYR), ii) their precursors: oleuropein (OLE), demethyloleuropein, ligstroside, and nuzhenide, and iii) verbascoside, which is a hydroxycinnamic acid derivative, which also contains an HT moiety in its molecular structure. The predominant PC in olives is OLE, and this is what gives them their characteristic bitter taste. However, the concentration of OLE can vary significantly between different olive cultivars, ranging from 3.6 to

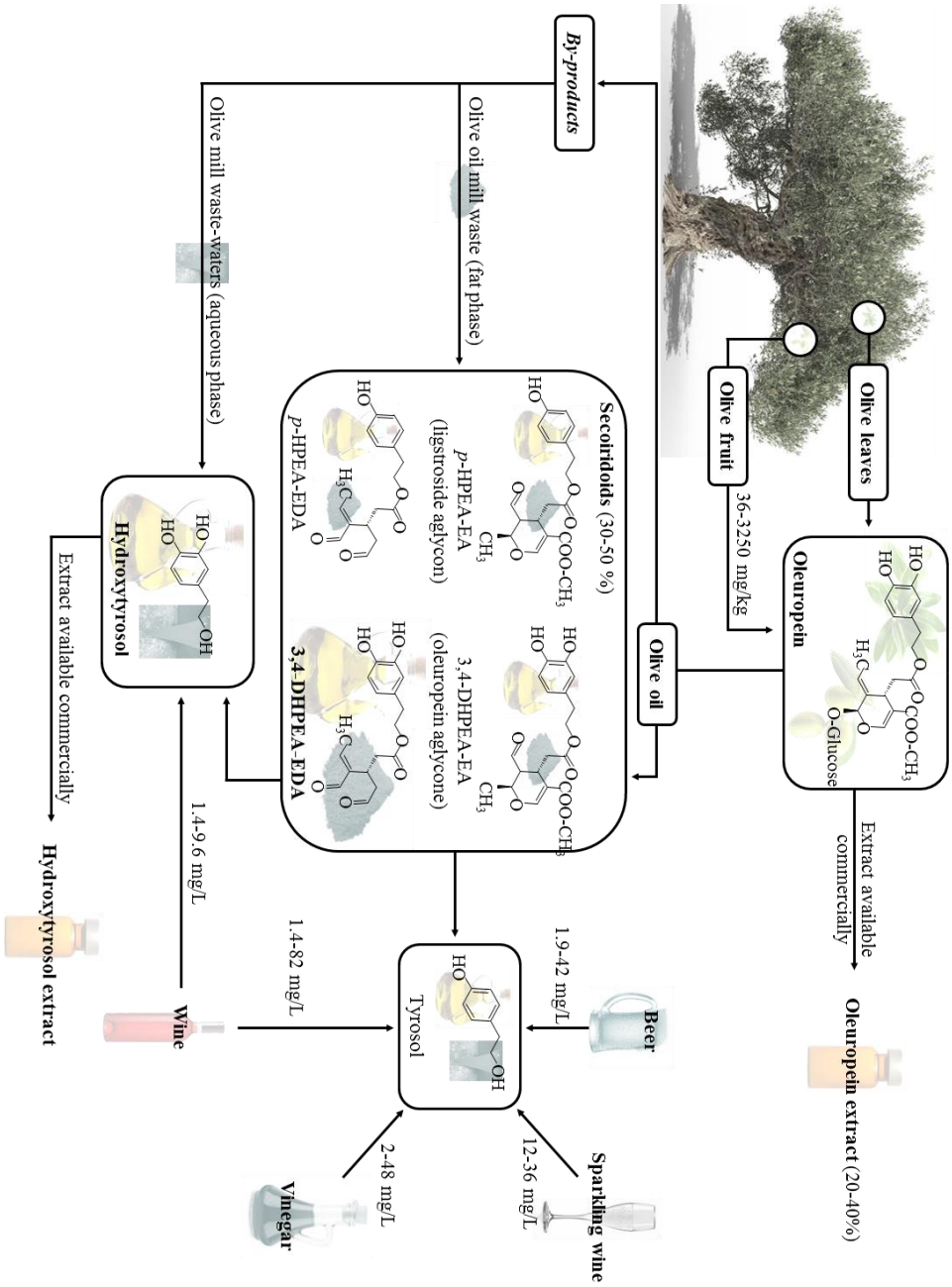


Figure 1. Sources and concentration of hydroxytyrosol and its precursors in different dietary sources.

325 mg OLE/100 g olive pulp in different Spanish, Portuguese, Greek and Italian olive cultivars (Bianco & Uccella, 2000; Romani et al., 1999).

During the VOO extraction process, the more complex phenolic structures in olive fruit, such as OLE and ligstroside, are hydrolyzed into aglycon structures (also commonly known as SEC), which constitute the main phenolic compounds in VOO. These PCs can be divided into two main groups: i) the OLE aglycones (3,4-DHPEA-EDA dialdehydic form of decarboxymethyl oleuropein aglycon and 3,4-DHPEA-EA oleuropein aglycon), in which HT is part of their molecular structure, and ii) the ligstroside aglycones (*p*-HPEA-EDA dialdehydic form of decarboxymethyl ligstroside aglycon and *p*-HPEA-EA ligstroside aglycon), in which TYR is part of their molecular structure (**Figure 1**). The concentration of phenolic compounds in VOO ranges from 60 to 1,200 mg/kg oil, with a percentage between 50-70% of HT and its precursors (Beltrán et al., 2007; Brenes et al., 2001). Besides that, hydroxytyrosol acetate (HT-AC) is present in low concentrations (31-94 mg/kg oil) in VOO (Fregapane et al., 2013).

The variability in VOO phenol concentration is related to the strong influence of different agronomical factors, such as olive variety, fruit maturity and the climatological and edaphological conditions. Other possible influences include the conditions of the oil extraction process, such as temperature and the malaxation time of the olive paste, the addition of water to the decanter or the time and storage conditions of VOO (Beltrán et al., 2005; Fregapane & Salvador, 2013; Fregapane et al., 2013). There are also large divergences in the literature regarding the concentration of PCs in VOO, mainly attributed to differences in analytical procedures arising from the lack of OLE and lisgtroside aglycon standards.

Apart from VOO as a dietary phenolic source of HT, in recent years, various dietary supplements or nutraceuticals prepared from different olive sources have been developed and are commercially available. Olive leaf extract is a supplement derived from olive leaves, with OLE (20-40%) being the main bioactive component.

Olive leaf extract is available in tablet or liquid form, and dried olive leaves can be used to make a beverage similar to tea. HT extracts, as their name indicates, are rich in HT, and are obtained from by-products of olive oil production, mainly by extraction or direct ultracentrifugation of the wastewater.

Apart from VOO as a dietary phenolic source of HT, in recent years, various dietary supplements or nutraceuticals prepared from different olive sources have been developed and are commercially available. Olive leaf extract is a supplement derived from olive leaves, with OLE (20-40%) being the main bioactive component. Olive leaf extract is available in tablet or liquid form, and dried olive leaves can be used to make a beverage similar to tea. HT extracts, as their name indicates, are rich in HT, and are obtained from by-products of olive oil production, mainly by extraction or direct ultracentrifugation of the wastewater.

Apart from these commercial olive phenol supplements, other extracts obtained from by-products of the olive oil extraction process have been reported in the literature (Araújo et al., 2015; Ghanbari et al., 2012; Obied et al., 2005). During the VOO extraction process, the native phenols in the olive fruit migrate into the fat phase or aqueous phase according to their lipophilic/hydrophilic nature. The phenolic extracts obtained from olive oil mill waste or pomace are rich in secoiridoid compounds, mainly 3,4-DHPEA-EDA (Araújo et al., 2015; Ghanbari et al., 2012; Obied et al., 2005; Suárez et al., 2009). On the other hand, the extracts obtained from the aqueous phase, which is also known as olive mill wastewater or vegetation waters, are rich in HT, the most hydrophilic phenol compound (Araújo et al., 2015; Ghanbari et al., 2012; Gómez-Caravaca et al., 2011; Obied et al., 2005) (**Figure 1**). These phenolic extracts obtained from olive oil by-products represent an opportunity to add value to the waste products that are an environmental problem in olive-producing countries.

Wine is another important dietary source of HT and TYR (Boselli et al., 2006; Di Tommaso et al., 1998), although the concentrations of these phenolic

alcohols are lower than those found in olive fruit and VOO. TYR and HT, the latter formed by hydroxylation of the aromatic ring of TYR, are considered secondary metabolites of tyrosine formed by yeasts during alcoholic fermentation. In 1998, Di Tommaso et al. (1998) described HT in Italian wines for the first time. These authors reported that the concentration of HT and TYR in red wines (3.61-4.8 mg/L) was higher than in white wines (1.42-2.34 mg/L). Similar TYR and HT concentrations were also observed in the analysis of different wines from Argentina, Brazil, Chile and Portugal, these concentrations being 5.9-9.6 mg/L in red wines, 1.6-2.7 mg/L in white wines, and 6 mg/L in rosé wines (Minussi et al., 2003). In other red wines, the concentration of TYR was found to be higher than HT, which could be explained by the different factors that affect yeast activity during wine fermentation, such as the sugar content or temperature (Bordiga et al., 2016). The TYR concentration was 20-40 mg/L in different Spanish wines (Motilva et al., 2016; Piñeiro et al., 2011), 17-62 mg/L in Italian wines (Bevilacqua et al., 2004; Dudley et al., 2008), and 38-82 mg/L in Hungarian and Canadian wines (Pour Nikfardjam & Pickering, 2008).

Apart from table olives, olive oil and wine, vinegar and other alcoholic beverages, such as beer and sparkling wine, have also been reported to be dietary sources of TYR (**Figure 1**). Wine vinegar is largely produced in Mediterranean countries and is usually used in food preparation, vinaigrettes, sauces, and other salad dressings. The concentration of TYR in vinegar from different Spanish wine varieties ranges from 0.23 to 4.79 mg/100 mL (García-Parrilla et al., 1997). TYR was the second most abundant phenolic compound after caftaric acid in sparkling wines. The TYR concentration was 12-36 mg/L (Chamkha et al., 2003) and 14 mg/L (Ibern-Gómez et al., 2000) in sparkling wine varieties from France (champagne) and from Catalonia (cava), respectively. Beer is also a source of TYR, and its concentration ranges from 1.9 to 42 mg/L (Dufour et al., 2002; Floridi, Montanari, Marconi, & Fantozzi, 2003).

1.1. Endogenous sources of HT

HT is naturally present in the brain as a metabolite of dopamine (de la Torre et al., 2006). Deamination of dopamine by monoaminoxidase (MAO) produces 3, 4-dihydroxyphenylacetaldehyde (DOPAL), an unstable and toxic oxidation product that can be easily oxidized by aldehyde dehydrogenase (ALDH) to the acid metabolite 3,4-dihydroxyphenylacetic acid (DOPAC).

In a lesser way, DOPAL is reduced to HT (also known as DOPET) by the aldehyde/aldose reductase (ALR) and HT can reversely return to DOPAL mediated by alcohol dehydrogenase (ADH). Furthermore, DOPAC can be transformed into HT by DOPAC reductase (Xu & Sim, 1995). DOPAC and HT are also substrates of catechol-O-methyltransferase (COMT) that transforms them into homovanillic acid (HVAc) and homovanillic alcohol (HVAlc) respectively (**Figure 2**).

The basal HT content in whole rat brain is at trace level (Gallardo et al., 2014; Goldstein et al., 2016b; Serra et al., 2012). Peng et al. found, in APP/PS1 mice, a brain basal HT concentration of 2 ng/g. However, after the dietary supplementation of 5 mg/kg/day, the HT brain concentration rose to 8.2 ng/g.

In addition, the HT concentration differs depending on the part of the brain. So, the HT concentration detected in the rostral parts and cerebellum is around 1 ng/g, in the frontal part 3 ng/g, and the highest concentration (6 ng/g) has been detected in the striatum area (Thiede & Kehr, 1981).

Some studies also suggest that the metabolization of ethanol might block ADH and ALDH, which could increase the circulating levels of HT in the blood (Pérez-Mañá et al., 2015) by transforming DOPAC into HT (Xu & Sim, 1995) or inducing the oxidation of DOPAL by ADH (Marchitti et al., 2007).

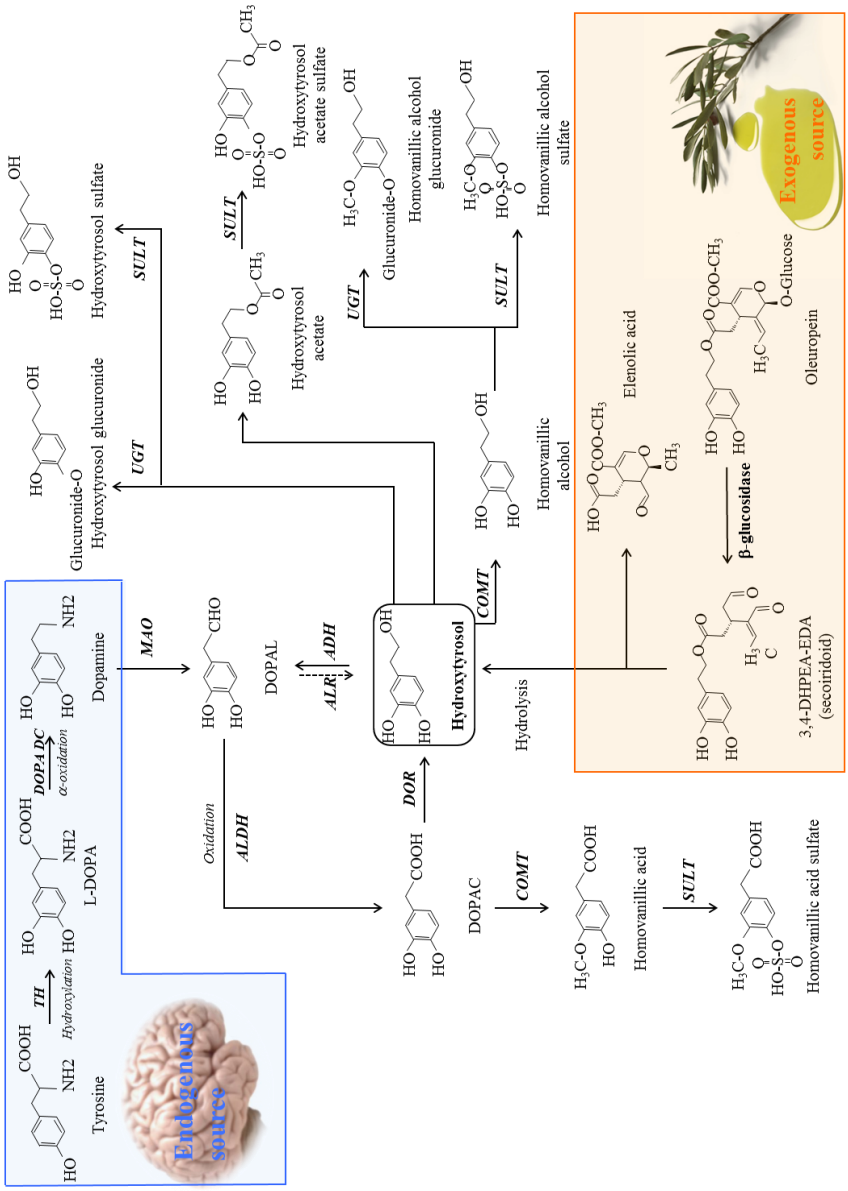


Figure 2. Endogenous and exogenous metabolic pathways of HT. TH: Tyrosine hydroxylase; DOPA DC: DOPA decarboxylase; MAO: Monoaminoxidase; ALDH: Aldehyde dehydrogenase; ALR: Aldehyde/aldose reductase; ADH: Alcohol dehydrogenase; DOR: DOPAC reductase; COMT: Catechol-O-Methyltransferase enzyme; SULT: Sulfotransferase enzyme; UGT: Glucuronosyl-transferase enzyme.

2. Metabolic fate of HT: From *in vitro* to *in vivo* approaches

One of the most important shortcomings in phenolic compound intervention studies stems from their investigation of the metabolism and bioavailability and the lack of standardized biomarkers of intake. In fact, the EFSA established in 2011 that "*in order to have the food constituent well characterized it is necessary to show that this constituent is bioavailable and provide a rationale of how the constituent reaches the target site*" (EFSA, 2011). Therefore, in order to evaluate the potential bioaccessibility and bioavailability of PCs, it is necessary to establish valid biomarkers of intake, through in vitro and *in vivo* experiments carried out before the evaluation of their efficacy.

Regarding the bioavailability of HT and its precursors, many studies have been conducted using in vitro, animal or human models. The use of culture cells treated with the duodenal digestion mixture (bioaccessible fraction) obtained after the gastrointestinal simulation of HT extracts can be also useful for predicting intestinal absorption and metabolism (Rubió et al., 2014a). In the *in vivo* studies, different doses and intake vehicles (olive oil, olive leaf extract, olive oil pomace extracts, pure compounds, etc.) providing HT in different molecular structures have been tested, and it has been observed that bioavailability can differ greatly. In this section, the absorption, distribution, metabolism and excretion of HT and the most important factors affecting its bioavailability will be discussed. Special emphasis will be placed on the circulating metabolites identified in the *in vivo* studies after the consumption of HT and its precursors, which are the ones that can reach the target tissues and exert potential health benefits. In recent years, increasing attention has also been paid to the metabolites formed by the intestinal microbiota, which supposes an increase in the variety of HT metabolites found in biofluids.

2.1. Absorption, distribution, metabolism and excretion of HT

The most common dietary source of HT is VOO, which is mainly present in its precursors oleuropein aglycone (3,4-DHPEA-EA) and the dialdehydic form of deacetoxy (3,4-DHPEA-EDA) also known as SEC (**Figure 1**). Most studies agree that these compounds are not bioavailable, being rapidly hydrolyzed and resulting in the formation of HT (Corona et al., 2006; Vissers et al., 2004). Therefore, at dietary doses, SEC are mainly hydrolyzed during gastrointestinal digestion, giving rise to the simple phenol structure of HT, which is further extensively metabolized before its absorption.

Firstly, SEC are hydrolyzed by the action of β-glucosidase enzymes and the acid conditions of the stomach, releasing HT into the digesta (Rubió et al., 2014a). Once in the small intestine, HT is extensively metabolized. The rate of absorption has been shown to vary depending on the composition of the food matrix through which HT is administered, observing that its stability under digestion conditions is higher after its administration as a natural component of olive oil than after its addition to a water vehicle or refined olive oil (Tuck & Hayball, 2002; Visioli et al., 2003). The poor bioavailability of HT observed in different studies related to the low or null concentration of circulating HT is due to the subsequent extensive first-pass I and II metabolism in the gut and liver. The enzymes involved in HT phase-I metabolism, mostly present in the intestinal wall, are non-microsomal alcohol and aldehyde dehydrogenases, both located in the cytosol, resulting in the metabolites of DOPAL and DOPAC. As indicated in section 2.2, these metabolites are non-specific as they are metabolites not only of dietary HT, but also of dopamine (**Figure 2**). On the other hand, the enzymes involved in HT phase-II reactions, mostly present in the liver, are sulfotransferases, uridine 5'-diphosphoglucuronosyl transferases (UGTs), COMT, resulting in the main HT metabolites detected in biological samples. It seems that under alkaline conditions in the human lumen, HT can also be a substrate of

acyltransferases to form HT-AC (Rubió, Macià, et al., 2012). For this reason, the circulating HT is in the form of its phase-I and II conjugated metabolites, rather than in the form of the free HT structure.

Hydroxytyrosol sulfate (HT-S) is the main circulating metabolite detected in rat plasma (D'Angelo et al., 2005; Serra et al., 2012), whereas in humans, HT-S together with hydroxytyrosol acetate sulfate (HT-AC-S) are the main metabolites detected in plasma after the consumption of HT or HT derivatives at normal dietary doses (Mateos et al., 2016; Rubió et al., 2012). Furthermore, the TYR absorbed could be interconverted into HT (Pérez-Mañá et al., 2015), as observed in liver microsomes, by the activity of CYP2A6 and CYP2D6 (Rodríguez-Morató et al., 2017). Blood circulation distributes the phenolic metabolites in a dose-dependent way throughout the organism, and they are then taken up in different tissues and organs (D'Angelo et al., 2001; Serra et al., 2012; Wu et al., 2009), and even in the brain, thus fulfilling the essential requirement of crossing the blood-brain barrier to be used as a neuroprotective agent.

Once HT and their metabolites have been absorbed, they may follow the biliary or urinary excretion routes. The biliary route redirects HT metabolites from the liver back into the duodenum, where they can be transformed and reabsorbed. Thus, this enterohepatic recycling may lead to a longer presence of HT and metabolites within the body. Another important portion of HT metabolites, mainly phase-II conjugates, is usually excreted in the urine (D'Angelo et al., 2001; Kotronoulas et al., 2013; Serra et al., 2012).

2.2. *In vitro* approaches to studying HT metabolism

The Caco-2 cell line is the most widely used cell culture in nutritional modelling for predicting the intestinal absorption and metabolism of phenolic compounds (e.g., the transport system – diffusion, endocytosis, transcytosis and

conjugations). The human hepatoma cell line (HepG2) and the primary hepatocytes are other widely used families of culture cells more suitable for evaluating hepatic biotransformations and are of great relevance in toxicological and pharmaceutical studies (Wilkening et al., 2003). Based on the interest of the metabolism and potential absorption of phenolic compounds, the use of Caco-2 and HepG2 cells, alone or as a double-layered co-culture, are the most recommended cell lines in the first phases of the functional food development (Rubió et al., 2014b). Prior to cell exposure, in vitro digestion models are also recommended, as the digested extracts are considered to be the bioaccessible fraction that can be subjected to a bioavailability study. After the treatment of the cells, the cell media (apical and basolateral) are recovered and the metabolites can be analyzed to determine metabolism and transport.

Very few *in vitro* experiments have been performed to study the cell uptake and metabolism of olive PCs. Rubió et al. (2014b) exposed the bioaccessible fraction, obtained from the *in vitro* digestion model of a SEC extract, to a co-culture of Caco-2 and HepG2. The results of this study showed that HT was almost completely converted into HT-S, HT-AC-S and homovanillic alcohol sulfate (HVAlc-S), with most appearing on the basolateral side, which indicates an extensive sulfation and methyl-sulfation during the first-pass metabolism in human epithelial cells. In a previous study by Soler et al. (2010), HT was exposed to differentiated Caco-2 cell monolayers, HVAlc being the main metabolite detected, with a low conversion that ranged from 10.7% to 18.6%, and a low proportion of sulfated and methyl-sulfated conjugates. The second pass of HT through the liver was also studied in HepG2 cells by Mateos et al. (2005). In this case only methylated and glucuronidated forms of HT were detected.

The polar character of HT hinders its solubility in lipid media, thus restricting its use as a food additive. Furthermore, its hydrophilic character impedes it from passing across the bilayer membrane at the intestinal level. In recent years,

various lipophilic compounds derived from HT have been synthesized to improve HT pharmacological activities and to develop new lipophilic antioxidants for food applications (de Bock et al., 2013). HT-AC is the most widely studied lipophilic derivative of HT, which can also be found naturally in VOO at low concentrations. Due to its lipophilic nature, HT-AC can cross the intestinal barrier easily (Mateos et al., 2011). Moreover, acyltransferase human activity leads to the metabolization of HT into HT-AC, so increasing the bioavailability of HT in humans (Rubió et al., 2012). Conversely, HT-AC can be partially hydrolyzed into free HT as seen by in vitro digestion (Pereira-Caro, 2012).

Other synthetic HT ether derivatives have shown an efficient absorption, displaying different intestinal transport rates according to their lipophilicity (butyl > propyl > ethyl > methyl) (Pereira-Caro et al., 2010). These compounds are poorly metabolized in intestinal Caco-2/TC7 monolayers where they are only conjugated into methyl forms (Pereira-Caro et al., 2010), whereas in HepG2 cells, they are metabolized into their methyl-glucuronide and glucuronide forms (Pereira-Caro et al., 2010b). These lipophilic HT derivatives have shown antioxidant and anti-inflammatory activity (Pereira-Caro et al., 2010). The alkyl length chain influences their bioactivity, it having been observed that short-to-medium length chains possess even higher antioxidant activity than HT, whereas long chains reduce their bioactivity (Grasso et al., 2007; Pereira-Caro et al., 2009).

2.3. *In vivo* approaches to studying HT bioavailability and metabolism

Several *in vivo* studies have been conducted to look into the bioavailability of HT and its precursors using different phenolic extracts or food ingredients, with significant differences being observed depending on the form of administration. **Table 1** shows the main metabolites detected in plasma depending on the model used

(animal or human), the HT source administered (SEC, OLE or free HT) and the dose.

In rats, phenolic extracts derived from olive products administered through an acute intake (180 mg SEC/kg rat weight) (Serra et al., 2012), the sulfate conjugates of HT (89 μM) and the native forms of OLE and HT were detected at higher concentrations in rat plasma (between 21 and 5.2 μM, respectively).

Although the absorption and metabolism of HT and its derivatives present in VOO have previously been reported in humans mostly through VOO intake (Rubió, Macià, et al., 2014a; Rubió, Valls, et al., 2012), HT biotransformation has also been recently studied after the administration of HT as an ingredient in a baked cereal food (Mateos et al., 2016). The sulfated derivatives of HT were the predominant metabolites observed, in addition to glucuronidated HT derivatives and other metabolites, such as DOPAC, HVAC and their glucuronide and sulfate conjugates, which are involved in dopamine biotransformation although in much lower amounts.

When HT is administered in the form of OLE, other metabolites were detected in plasma. The OLE-aglycone-glucuronide and an OLE-derivative were detected in human plasma after the intake of an olive leaf extract (García-Villalba et al., 2014) (**Table 1**).

The important differences observed in the HT bioavailability level and the plasma metabolic profiles between authors could also be attributed to the analytical approach applied and the number of metabolites monitored (in turn, very much dependent on the availability of reference standards). However, the predominant phenolic metabolites in plasma are always phase-II metabolites of HT, HT-S being the main metabolite in plasma whether VOO, SEC extracts, olive leaf extracts or enriched baked products are used. Therefore, it can be concluded that HT-S could be considered the most valid intake biomarker for HT and its derivatives.

Table 1. Comparison of the plasmatic metabolic profile observed after the *in vivo* administration of hydroxytyrosol in different molecular precursors (secoiridoids, oleuropein and free form) and different administration doses.

HT source	Model	Administration form	Intake dose	Plasma metabolites	Plasmatic concentration
Secoiridoids	Rat (Rubió et al. 2014)	Secoiridoid extract in aquous solution	34.5 mg secoiridoids (acute intake)	*Main*: HT-S *Minor*: HT-G, homovanillic alcohol sulphate	83 µM HT-S (C_{max})
	Rat (Serra et al 2012)	Secoiridoid extract in aquous solution	45 mg secoiridoids (acute intake)	*Main:* HT-S, tyrosol sulphate *Minor*: HT, HVACS, oleuropein derivative	89 µM HT-S (C_{max}) 53 µM Tyr-S (C_{max})
	Human (Rubió et al. 2014)	Phenol-enriched olive oil (25 mL)	8.49 mg of HT and secoiridoids (sustained intake 3w)	*Main*: HT-S, HT-AC-S, homovanillic alcohol sulphate *Minor:* homovanillic acid sulphate	~ 3 µM HT-S ~ 2.5 µM HT-AC-S
Oleuropein	Human (Garcia-Villalba et al.2014)	Oleuropein olive leaf extract (gelatin capsules)	100 mg oleuropein (acute intake)	*Main:* HT-S, HT-sulfoglucuronide, oleuropein aglycone glucuronide, homovanillic alcohol glucuronide, oleuropein derivative *Minor*: HT-G	(Not quantified)
	Human (Bock et al., 2013)	Oleuropein olive leaf extract (aquous solution)	51.1 mg oleuropein / 9.7 mg HT (acute intake)	*Main*: sulphated and glucuronidated conjugates of HT *Minor*: homovanillic acid	2.5 ng/mL OLE 148 ng/mL conjugated metabolites HT
Hydroxytyrosol	Rat perfused model (Corona 2006)	HT			
	Human (Mateos et al., 2016)	HT enriched-biscuits (30g)	5.25 mg HT (acute)	*Main:* HT-S and (DOPAC)-S, DOPAC, HVAC *Minor:* HT-G, DOPAC-G, HVAC-G and HVAC-S	~ 1µM HT-S (C_{max}) ~ 0.5 µM DOPAC-S(C_{max})

HT: Hydroxytyrosol; HT-S: Hydroxytyrosol Sulphate; HT-G: Hydroxytyrosol glucuronide; HT-AC: Hydroxytyrosol Acetate; HT-AC-S: Hydroxytyrosol Acetate Sulphate; DOPAC: 3,4-dihydroxyphenylacetic acid; DOPAC-S: 3,4-dihydroxyphenylacetic acid sulphate; DOPAC-G: 3,4-dihydroxyphenylacetic acid glucuronide; HVAC; Homovanillic acid; HVAC-S; Homovanillic acid sulfate; HVAC-G: Homovanillic acid glucuronide

2.4. Colonic microbial metabolism of HT

In recent years, the behaviour of PCs in the colon has attracted much attention as most PCs pass through the small intestine without being absorbed, encountering the gut microbiota and possibly resulting in an impact on the health of the human host. This has led to the development of a two-way mutual reaction between PCs and gut microbiota. First, non-digested PCs are biotransformed into their catabolic metabolites by gut microbiota, and this results in an increase in their bioavailability (Ozdal et al., 2016). Secondly, the PCs themselves modulate the composition of the gut microbial community, mostly through the inhibition of pathogenic bacteria and the stimulation of beneficial bacteria such as bifidobacteria acting as prebiotics (Parkar et al., 2013).

PCs can reach the colon in different ways: the non-absorbed food fraction, metabolites resulting from enterohepatic recirculation or as a consequence of the basolateral-apical transport of intestinal cells (Mosele et al., 2014). Therefore, the role of HT and its precursors in the large intestine has been under study recently. For a first assessment of gut metabolism and to characterize the microbial fermentation products of dietary polyphenols, many researchers opt for an *in vitro* fermentation model using faeces from humans or animals as the inoculum. Studies focused on the colonic metabolic pathways of HT and its precursors have shown that OLE submitted to an *in vitro* colonic model was transformed into its aglycone structure, elenolic acid, free HT and HT-AC (Corona et al., 2006b; Mosele et al., 2014). Individual fermentation of TYR and HT confirmed the low microbial metabolism of these compounds and their relative stability under *in vitro* colonic conditions (Mosele et al., 2014). An *in vivo* experiment following the administration of OLE to rats detected other related metabolites, such as HVAC, in the faeces (Lin et al., 2013).

The presence of colonic metabolites from olive PCs was also studied in human faecal samples obtained before and after the sustained intake of a daily dose of 25 mL of a phenol-enriched VOO (500 mg total phenols/kg oil) for 3 weeks. Of special interest was the significant increase in the concentration of free HT in the faeces after dietary supplementation with phenol-enriched VOO ($p < 0.05$) (Mosele et al., 2014).

Another hypothesis is that free HT could be present in the faeces as a result of the deconjugation of the phase-II metabolites by the action of the microbial enzymes, including β-glucuronidase and sulfatase (Selma et al., 2009). These biotransformations of PCs into their catabolic metabolites can increase their bioavailability and exert beneficial effects. For instance, simple phenolic acids derived from the colonic fermentation could modulate the composition of the microbiota by increasing beneficial populations and decreasing pathogenic bacteria. In addition, these phenolic acids can reach the peripheral circulation through the gastrointestinal barrier, where they can reach target tissues and exert beneficial effects (Ozdal et al., 2016).

Based on the intense colonic metabolism of dietary PCs, the products of the catabolism of HT and its precursors could be good candidates for novel preventive strategies and open a promising line of research into the preventive action of these compounds in colon and other bowel diseases. Nevertheless, more studies are needed to reinforce our knowledge about the relationship between these compounds and human intestinal health.

3. Potential pharmacological applications of HT

Currently, much information about the potential health effects of HT is available, and its properties as an antioxidant, anti-inflammatory, antiatherogenic, or antiplatelet are well documented, being as it is, the subject of different clinical studies. In fact, in recent years, several epidemiological studies have shown a special link between the consumption of VOO, the main dietary source of HT and HT derivatives, and a broad beneficial impact on health (Covas et al., 2006a; Estruch et al., 2013; Guasch-Ferré et al., 2014; Razquin et al., 2009). In terms of toxicity and safety, HT is considered a safe compound as it has been found to be non-genotoxic and non-mutagenic after being tested *in vivo* at high concentrations (Auñon-Calles et al., 2013; Auñon-Calles et al., 2013b). HT's lack of toxicity enables its use as a nutraceutical as a strategy to administer higher doses than those attainable through the diet. In fact, functional VOO enriched with its own phenolic compounds, olive leaf extracts or pure HT has been tested in human clinical trials.

In this section, I review the most recent advances (human and mechanistic studies using animal models) in the possible clinical use of HT and its precursors (OLE and SEC derivatives), mainly related to CVD, neurodegenerative diseases and cancer. Studies involving other minor VOO compounds or extracts that are not well characterized have not been included in this review.

3.1. Preventive function of HT in cardiovascular diseases

Atherosclerosis is a chronic hypercholesterolemia-driven inflammatory process, arising from the progressive accumulation of lipids in the intima layer of the aortic walls (Silvestre-Roig et al., 2014), and oxidative stress is considered a key issue in the development of atherosclerosis. Reactive oxygen species (ROS) are involved in the progression of endothelial damage as they cause progressive respiratory chain dysfunction and DNA damage, and induce the oxidation of low-

density lipoproteins (LDL). Plasmatic ox-LDLs increase the expression of adhesion molecules (Meisinger et al., 2005) and cause both endothelial dysfunction and atheromatous plate formation (Victor et al., 2009).

Diet antioxidants that can prevent lipid peroxidation, such as PCs, could play an important role in preventing the oxidative modification of LDL. In fact, most of the human interventional studies looking into the benefit of VOO intake on CVD have investigated the effect of PCs on the prevention of LDL oxidation. So, the strong antioxidant properties of HT could explain, at least in part, the cardioprotective effects of VOO.

As reviewed in this section, over the course of the last decade, in addition to the classical risk factors for CVD development, scientists have suggested that HT can downregulate pro-atherogenic genes in humans and positively modulate the vascular endothelial function, high density lipoprotein (HDL) functionality and thrombosis-associated factors, as well as other emergent risk factors of CVD.

3.1.1. Human evidence of HT cardioprotective effects

The cardioprotective effects of HT and its precursors have been thoroughly studied in healthy and high-risk subjects by postprandial or sustained diet interventions. These studies are compiled in **Table 2**.

The European Study of the Antioxidant Effects of Olive Oil and its Phenolic Compounds on lipid oxidation (EUROLIVE) was a human clinical trial designed to evaluate the effects of the phenolic content of olive oil on the plasma lipid profile and lipid oxidative damage (Cicero et al., 2008). The main results of the study showed that the systemic markers of lipid oxidation decreased inversely with the phenolic content of the OO administered (particularly LDL oxidation markers and HDL cholesterol levels). This clinical trial was one of the key reports that led the

European Food Safety Authority (EFSA) to release a claim about the benefits of daily ingestion of VOO rich in phenolic compounds. Based on the EUROLIVE results, it is considered that at least 5 mg of HT per 20 g of olive oil should be consumed daily in order to support the claim (EFSA, 2011). Other clinical trials have also shown that HT reduces plasmatic ox-LDL after the acute consumption of HT-enriched biscuits (Mateos et al., 2016) and phenol-enriched VOO (Valls et al., 2015). In these studies, the protection against LDL oxidation was attributed to an increase in HT metabolites in plasma.

Most recent studies have focused on HDL, reporting that the functional capacities of HDL lipoproteins could be more relevant than the mere quantity of HDL cholesterol. HT and its precursors have been shown to promote these HDL capacities, like the increase in the expression of cholesterol-efflux-related genes and HDL-subclass distribution and composition (Farràs et al., 2015).

The effect of olive oil PCs on the modulation of plasma lipids remains unclear. Some studies have demonstrated that the intake of olive oil PCs can reduce total cholesterol, LDL and triglycerides (TG) (Toledo et al., 2015), and increase plasma HDL (Farras et al., 2015). However, in other studies, they had no influence on plasmatic lipids (Valls et al., 2015).

Epidemiological studies have confirmed that adherence to the MedDiet, in which VOO is one of the main fat sources, is associated with an increase in serum markers of atheroma plaque stability (I-CAM and P-selectin) and a decrease in inflammatory biomarkers (C-reactive protein and interleukin-6), suggesting a protective role for olive oil PCs against ischemic heart disease (Li et al., 2014). The modulation of these CVD biomarkers seems to be in accordance with the improvement observed in ischemic reactive hyperaemia after the acute intake of a high-phenolic VOO (Ruano et al., 2005) and the improvement in blood pressure and oxidative biomarkers after a sustained consumption of polyphenol-rich VOOs (Moreno-Luna et al., 2012). Along similar lines, other human clinical trials have

Table 2. Protective effect of hydroxytyrosol and its precursors in cardiovascular diseases in human interventional studies.

Status	Sub-jects	Sex	Type of study	Duration	Supplemen-tation	CVD risk biomarkers	Reference
Healthy	13	-	Crossover, randomized, double-blind	Postpran-dial (Fasten conditions)	30 g biscuits enriched with 5.25 mg HT	↓ Postprandial ox-LDL	(Mateos et al., 2016)
	12		Randomized, controlled, double-blind, crossover trial	3 w 2w w/o	25 mL/day 3 OO with ≠ PCs content	↑Phenol in LDL ↓ ox-LDL	(Martín-Peláez et al., 2015)
	18			3 w 2w w/o	25mL 2 OO ≠phenol content	↓monocyte chemoattractant protein 1 ↓expression of proatherogenic genes in PBMC: CD40 ligand, IL-23α subunit p19, adrenergic β-2 receptor, oxidized LDL receptor 1, and IL-8 receptor-α	(Castaner et al., 2012)
	90	♂ ♀	Randomized, parallel, controlled clinical trial	3 m	MedDiet with VOO	↓ plasma oxidative ↓ plasma inflammation ↓ inflammation, oxidative stress and polymerase gene expression in PBMCs Modificate the lipid profile ↓ plasma IFN-α, F2α-isoprostanes, and s-P-selectin ↑ HDL-C in ♀; ↓ LDL-C in ♂	(Konstantinidou et al., 2010)
	98			Postpandrial	2 mL of olive mill wastewater	= plasma AOX capacity ↑ glutathione concentration	(Visioli et al., 2009)
	187		Follow a MedDiet	3 years	Follow the MedDiet	↑total AOX capacity ↓body weight	(Razquin et al., 2009)
	12	♂	Cross-over	Postprandial	40mL 3 OO with ≠ PCs content	↑ phenols in LDL = LDL ↓ ox-LDL Low increase of TGs, F2-isoprostanes, ox-	(Covas et al., 2006a)

						LDL, and Ab-oxLDL	
	200	♂	Randomized, crossover, controlled trial.	3 w 2w w/o	25 mL 3 OO with ≠ PCs content	↓ lipid cardiovascular risk factors ↑ glutathione aox status ↓ ox-LDL; ↑ HDL; ↓ TGs	(Covas et al., 2006b)
Non smoking, healthy	36	♂	Randomized controlled, double-blind, crossover design	3 w	25mL 2 OO ≠ phenol content	↓ oxidation markers ↓ ox-LDL, conjugated dienes, and hydroxy fatty acids	(de la Torre-Carbot et al., 2010)
High CVD risk	55	♂ ♀	Randomized	12 months	EVOO 50mL/d	↓VCAM, ICAM, P-Selectin ↓ blood pressure (both) ↓ LDL ↓ CRP &IL-6 ↓ IL-10/IL-10 ratio	(Casas et al., 2014)
High CVD risk	199	♂ ♀	Parallel group	Follow diet (frequency questionnaire)	VOO consumption vs others.	↓ atherosclerotic plates in carotid arteries. ↓ IMT	(Buil-Cosiales et al., 2008)
pre-HTN	60	♂	Randomised, double-blind, controlled, crossover trial	6 w before switching to the alternate arm after a 4-week w/O	olive leaf extract (136 mg OLE; 6 mg HT)	↓ Plasma total Cho; ↓LDL; ↓TG ↓IL-8 = Biomarkers of inflammation, vascular function and glucose metabolism = HTN	(Lockyer et al., 2016)
Pre-HTN no treat	13	7 ♂ 6♀	Randomized, controlled, double blind and crossover	postprandial	30 ml of VOO with (high/low olive phenols)	↓ ox-LDL ↑ABCA1, SR-B1, PPARBP, PPARα, PPARγ, PPARδ and CD36 genes expression	(Farràs et al., 2013)
HTN	24	♀	Double-blind, randomized,	4 months (2 months of each oil)	polyphenol-rich olive oil ~30 mg/day// OO w/o	↓ BP (both) serum asymmetric dimethylarginine ↓ox-LDL	(Moreno-Luna et al., 2012)

			crossover dietary-intervention		free PC	↓ CRP ↑plasma nitrites/nitrates ↑ ↑hyperemic area after ischemia	
	13	7 ♂ 6 ♀	Randomised, double blind, crossover trial.	Postprandial (after 5 h of consumption)	30 mL of FVOO	Endothelial function, ↑ IRH, ↓ ox-LDL, = BP ↓ Postprandial values for glucose, TG and PAI-I ↑ endothelium-dependent microvascular dilatation	(Valls et al., 2015)
Hypercholesterolemic	33	♂ ♀	Randomized, double-blind, crossover, controlled trial	3 w 2w w/o	25mL/day: VOO; FVOO, FVOOT.	decrease in LDL-C after FVOO = BP =BMI ↓ LDL-P, IDL-P, and total ApoB100 containing lipoproteins ↑ HDL particle size; ↓ VLDL particles vs VOO intervention specially after FVOO intervention ↓LDL particle size ↓ Lipoprotein insulin resistance index (LP-IR)	(Fernández-Castillejo et al., 2016)
	33		Randomized, double-blind, crossover, controlled trial	3w	25mL of: VOO, FVOO, FVOOT	FVOOT: ↑ HDL	(Farràs et al., 2015)
	21	5 ♂ 16 ♀	Randomized sequential crossover design	Postpran-dial Baseline and 2 h after intake	Breakfast with ≠ PCs reduced from 400 to 80 ppm	Biomarkers of compliance ↑FVIIa activity ↓ FVIIag activity ↓ PAI-1 activity	(Ruano et al., 2007)
	21	5 ♂ 16 ♀	Randomized sequential crossover design	Postpran-dial Baseline and 2 h after intake	Breakfast with ≠ PCs reduced from 400 to 80 ppm	↑ Endothelial function, ↑ IRH ↑ NO (x), Lower increase of LPO and ↓8-epi prostaglandin-$F_{2\alpha}$ = lipid parameters	(Ruano et al., 2005)

Hyper-lipidemic	30	19 ♂ 14 ♀	Randomized, crossover, controlled trial	3 w 2 w w/o	25mL of VOO. FVOO, FVOOT	↓ DNA damage ↑ SOD	(Romeu et al., 2016)
Coronary heart disease	40	♂	Controlled, crossover, randomized trial	3 w 2 w w/o	50mL 3 OO with ≠ PCs content	↓ systolic BP in HTN patient = diastolic = glucose = lipids and antibodies against ox-LDL	(Fitó et al., 2005)

Ischemic reactive hyperemia (IRH); plasminogen activator inhibitor-1 (PAI-1); factor VII antigen (FVIIag); activated factor VII (FVIIa) IMT: Intima-media Thickness; MedDiet: Mediterranean Diet; w: week; PC's; OLE: Oleuropein, HT: Hydroxytyrosol; ≠: different; ox-LDL: oxidized LDL, BP: Blood Pressure; HTN: artherial Hypertension; w/o: wash-out; VOO: Virgin Olive Oil (80 ppm); FVOO: functional Virgin Olive Oil (500 ppm); FVOOT: functional Virgin Olive Oil (500 ppm: 250 ppm and those of thyme (250 ppm); TGs: Triglicerides; SOD: Superoxide dismutase; PBMCs: peripheral blood mononuclear cells; CRP: C-reactive protein; ischemic reactive hyperemia (IRH).

shown that olive oil PCs improve the postprandial prothrombotic biomarkers (Ruano et al., 2007) and lipoprotein structure in hypercholesterolemic patients (Fernández-Castillejo et al., 2016).

Other human studies have also shown that HT and its precursors can protect DNA from oxidative damage by decreasing 8-hydroxy-2′-deoxyguanosine and can also stimulate the endogenous antioxidant system, with a significant increase in superoxide dismutase (SOD) in hyperlipidemic patients (Romeu et al., 2016). An improvement in glutathione (GLT) status was also observed in both the high cardiovascular risk population (Covas et al., 2006a) and healthy volunteers (Visioli et al., 2009). In one of these studies (Romeu et al., 2016), a clear parallelism emerged between the modulations of oxidative markers and PC metabolites observed in urine and in erythrocytes after phenol enriched VOO.

Regarding the effects of HT and its precursors on blood pressure (BP), controversial results have been found. Some studies support that consumption of VOO rich in PCs does not modulate BP (Covas et al., 2006a; Fernández-Castillejo et al., 2016; Lockyer et al., 2016), whereas other studies have observed a decrease in BP (Covas et al., 2006b; Fitó et al., 2005; Moreno-Luna et al., 2012). The mechanisms underlying this effect have been associated with direct antioxidant activity, an improvement in the endothelial function (Ramírez-Expósito et al., 2010) or the modulation of other antihypertensive molecules.

There is little information about the cardioprotective effects in humans after the intake of free HT. Mateos et al. recently published a crossover, randomized, double-blind and postprandial study on healthy individuals involving 30g biscuits enriched with 5.25 mg HT. Plasmatic ox-LDL was significantly reduced in comparison to the control group, but no effects were observed on the antioxidant enzymes (Mateos et al., 2016). Also, HT has been detected in LDL molecules after the administration of 2.5 mg/kg body weight of pure HT in healthy individuals (González-Santiago et al., 2010). In another randomized controlled study, the intake

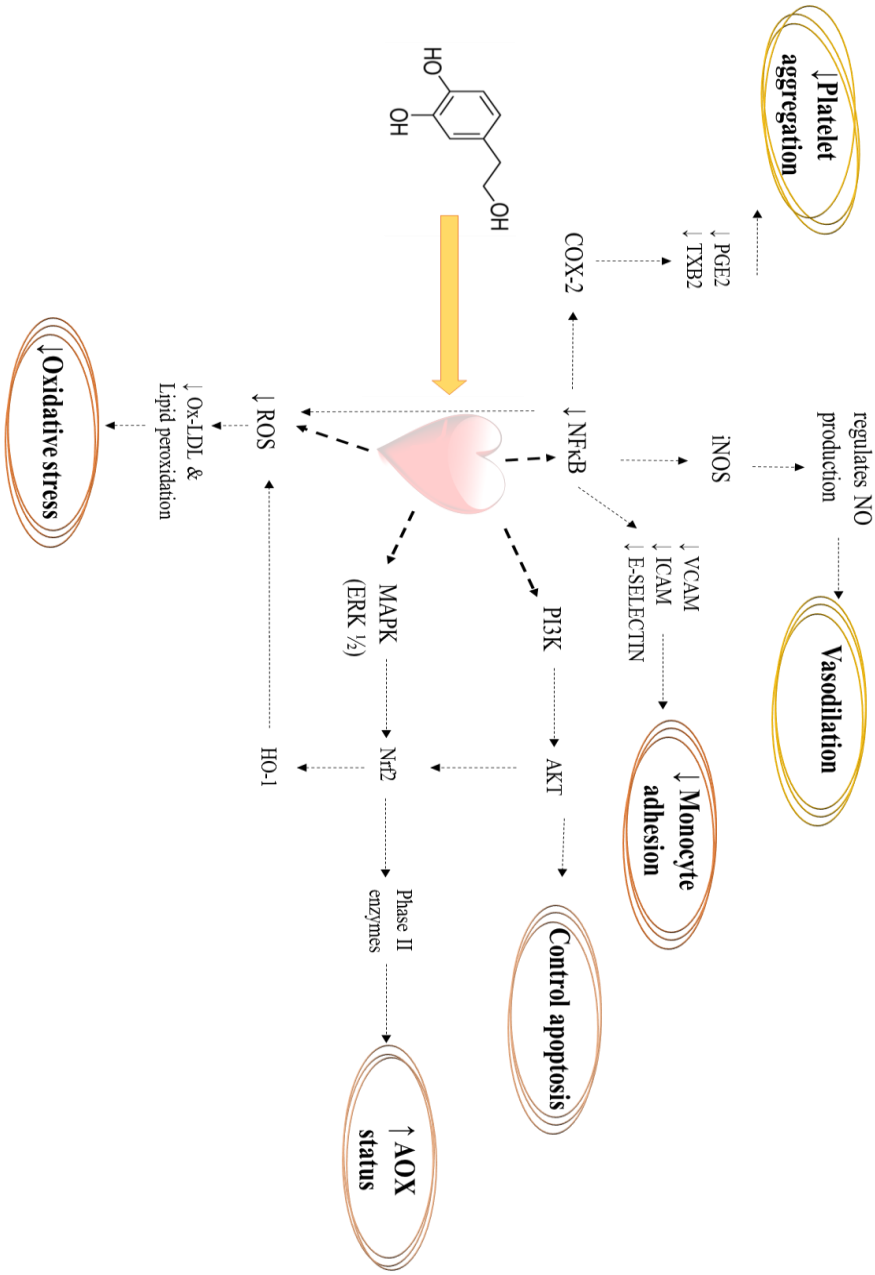


Figure 3. Proposed mechanisms by which HT is involved in the protection against cardiovascular diseases

Table 3: Protective molecular mechanisms of hydroxytyrosol against cardiovascular diseases in animal studies.

Status	Goal	Subjects	Type of study	Duration	Supplementation	CVD risk biomarkers	References
Healthy	Nutrigenomic effects cardiometabolic prevention by control of adipose tissue	14	Sustained	8 weeks 0.03gm% HT Group c vs ht	Adipocytes	Modulates GSH after oxidative stress ↓ Leptin Modulate 720 genes. GSH transferase, GSH peroxidase, GSH oxidoreductase activity ↓ intracellular GSSG-to-GSH ratio	(Giordano et al., 2014)
Healthy	Myocardial effects	100	Injection before reperfusion		20mg/kg rat weight	↓ myocardial infarction size ↓ MDA ↑Mitochondrial SOD activity ↓ apoptosis of cardiomyocites ↑ pakt and pGSK-3β	(Pei et al., 2016)

HT: Hydroxytyrosol; HT-AC: Hydroxytyrosol acetate; Gap junction alpha-1 protein: Gja1, ras-related C3 botulinum toxin substrate 1): Rac1; Hexokinase-2: HK2, Heat shock 70 kDa protein: Hspa1a; GSH: glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase; pAKT: akt phosphorilated

of HT pills for a week, with a concentration of 5-25 mg/day, reduced TXB_2, thus preventing thrombotic processes (Crespo et al., 2015).

Hohmann et al. (2015) recently performed a meta-analysis that evaluated eight crossover studies involving 355 subjects, comparing sustained ingestion of VOO with high vs. low PCs. They concluded that the administration of VOO enriched with its own PCs could reduce plasmatic ox-LDL and systolic BP. However, no significant impact on diastolic levels, plasmatic levels of total cholesterol, LDL or TG contents could be reported.

3.1.2. Mechanistic studies of HT CVD cardioprotective effects

Cellular and animal models offer an important tool for studying the involvement of HT in the prevention of atherosclerosis. The atheroprotective molecular mechanisms of HT are summarized in **Figure 3**, and the most recent advances are discussed in the following sections.

3.1.2.1. Mechanistic studies in animal models

In recent years, different animal studies have been conducted to identify the mechanisms underlying the cardioprotective effects of HT (**Table 3**). A study, supplementation with HT (20 mg/kg) in rats modulated the PI3K/Akt pathway and prevented the apoptosis of cardiomyocytes in a myocardial ischemia reperfusion model (Pei et al., 2016). Furthermore, dietary supplementation with HT for 8 weeks in C57/BL6 mice modulated the GLT metabolism and its associated enzymes, probably by the nuclear factor-E2-related factor-2 transcription factor (Nrf2) pathway, and reduced the leptin concentrations in adipose tissue, which contributed to reducing the obesity responsible for endothelial dysfunctions (Giordano et al., 2014).

3.1.2.2. Mechanistic studies in cell lines

Experiments performed in vascular cell lines have shown different mechanisms underlying HT functionality in improving the atherosclerotic state. HT has been shown to directly reduce the ROS in porcine pulmonary artery endothelial cells (VECs) and to increase catalase activity by the activation of the AMP-activated protein kinase (AMPK) and FOX03 protein signalling pathways (Zrelli et al., 2011). The activation of AMPK is required to attenuate the expression of the cell adhesion molecule ICAM-1, which is crucially involved in early atherogenesis.

HT has also shown cell protection against H_2O_2 cytotoxicity through the activation of the PI3K/Akt and ERK1/2 signalling pathways (Zrelli, et al., 2011b) and through the reduction of inducible nitric oxide synthase (iNOS) and ciclooxigenase-2 (COX-2) gene expression (Maiuri et al., 2005). It has been reported that HT prevents the release of metalloproteinases (MMP)-9 in peripheral blood mononuclear cells (PBMC) and in U937 monocytes, which directly participate in the process of arteriosclerotic plaque rupture. In the same study, HT also modulated the inflammatory response by activating the protein kinase C (PKC) and reducing prostaglandin E2 (PGE2) and COX-2 expression (Scoditti et al., 2014).

At physiological concentrations (1-10 µM), HT reduced the expression of proatherogenic adhesion molecules such as ICAM-1, VCAM-1 and E-selectin in human umbilical vascular endothelial cells (HUVEC) (Dell'Agli et al., 2006). Furthermore, HT reduced the mRNA expression of VCAM-1 by blocking the activation of the transcription factors NF-κB and AP-1 and decreased the monocyte cells stimulated by lipopolysaccharide (LPS) (Carluccio et al., 2003).

After vascular injury, HT (30-100 µM) induced apoptosis in smooth vascular muscle cells (VSMC) by increasing caspase-3 and caspase-9 and activated protein phosphatase 2 (PP2A) (Zrelli et al., 2011). The role of HT in inflammation treatment has been related to a decrease in TNFα and inhibition of COX-2, which decreases

PGE2 in both human PBMC and glioblastoma (U-87 MG) cells (Fuccelli et al., 2015; Lamy et al., 2016). LPS stimulation leads to the activation of the p38 Mitogen-activated protein kinase (MAPK) signalling cascade, induces the STAT1 transcription factor and consequently Toll like receptor-4 activation, initiating the inflammatory response in macrophages (Takeda et al., 2014). Treatment with HT in peritoneal macrophage obtained from BALB/c mice, and stimulated by LPS, suppressed the NO overproduction by decreasing iNOS gene expression through a mechanism independent from the NF-κB signalling pathway (Takeda et al., 2014). The control of these factors by HT and HT-AC finalizes in the inhibition of collagen-induced platelet aggregation (Correa et al., 2009). Altogether, it can exert synergic effects on the inhibition of platelet aggregation (Rubio-Senent et al., 2015).

At the cellular level, HT is a metal chelator and reduces the damage induced in cells. At a dose of 10–50 μM, HT prevented ROS generation and avoided the haemolysis and shape modification of red blood cells induced by Hg (Tagliafierro et al., 2015), and also avoided LDL oxidation induced by copper (Visioli et al., 1995).

In conclusion, a large number of *in vitro* studies provide evidence of the underlying mechanisms of HT in improving endothelial dysfunction as well as protecting against oxidation at the cellular level. However, more studies are needed to test the biological plasma circulating metabolites to elucidate fully the HT cardiopreventive mechanisms of action.

3.2. Role of HT in cancer prevention

The development and progression of tumour cells result from an imbalance between cell growth and death caused by the suppression of apoptosis, fast angiogenesis and replication of malignant cells. These cells evade the immune system and are insensitive to growth inhibitory signals. At the cellular level, the pesence of ROS drives the development of carcinogenesis by a direct reaction with

the DNA and the generation of hydroxyl radicals, and by inducing chronic inflammation. The inflammatory process leads to an upregulation of both the cell cycle and antiapoptotic genes, as well as the activation of the NF-κB pathway and hypoxia inducible factor 1α (HIF-1 α) increasing cell survival and proliferation (Ziello, Jovin, & Huang, 2007).

Knowledge about the antiproliferative and pro-apoptotic effects of HT and its precursors has increased in recent years and leads to the conclusion that these effects stem from different mechanisms depending on the cell type. The most recent advances in this field are reviewed in this section.

3.2.1. Human evidence of HT against cancer

There are a few human studies that have established an inverse relationship between MedDiet supplemented with VOO and the development of cancer (Carruba et al., 2016; López-Guarnido et al., 2015; Sofi et al., 2008). An observational study, which followed up the incidence of breast cancer over 3 years in 4,282 postmenopausal women (4,152 with no previous history of breast cancer), compared the effect of a VOO supplemented diet with a control diet. The results of this study revealed a reduction of 68% in the incidence breast cancer in women who received a diet supplemented with VOO (Toledo et al., 2015).

The tumoral healing process seems to be associated with different factors such as the nature of the tissue, the kind of tumour, the levels of estrogens, inter-individual variability and environmental factors (Kolonel et al., 2004). The goal of future studies will be to carry out long-term clinical trials with a high-risk population to follow the progression of tumours and verify the effectiveness of OOPCs in reducing the incidence of cancer.

3.2.2. Mechanistic studies of HT against cancer

Despite the scarcity of results about the protective effect of HT against the development of cancer in humans, there are some animal studies and broad cellular experiments that support the hypothesis of the involvement of HT in reducing the progression of cancer by different mechanisms (**Figure 4**). Some of these mechanisms are the direct antiproliferative (control of cell cycle progression) and chemotherapeutic properties related to the interaction of HT and its biological metabolites with genes, signalling pathways and enzymes to reduce inflammation and increase the pro-apoptotic signalling pathways.

3.2.2.1.Mechanistic studies in animal models

The most recent studies conducted in animals are summarized in **Table 4** and overall, HT has shown the ability to reduce the propagation of a tumour and to increase the apoptosis of carcinogenic cells (Granados-Principal et al., 2014; Li et al., 2014). One of the suggested molecular mechanisms is the suppression of the epidermal growth factor receptor (EGFR), which arrests the apoptotic process, controls inflammation and upregulates the pro-angiogenic signalling pathways (Li et al., 2014; Terzuoli et al., 2015). HT also modulates the expression of key genes involved in oncogenesis to reduce the tumour (Granados-Principal et al., 2011).

3.2.2.2. Mechanistic studies in cells

Despite the scarce *in vivo* studies evaluating the protective effect of HT against the progression of cancer, a large number of studies have tested HT in both carcinogenic and healthy cell lines from white blood cells and different tissues such as breast, prostate colorectal, urinary, bladder, brain, bile duct, gallbladder, skin and liver.

Traditionally, it is believed that HT is able to decrease the H_2O_2 cell content

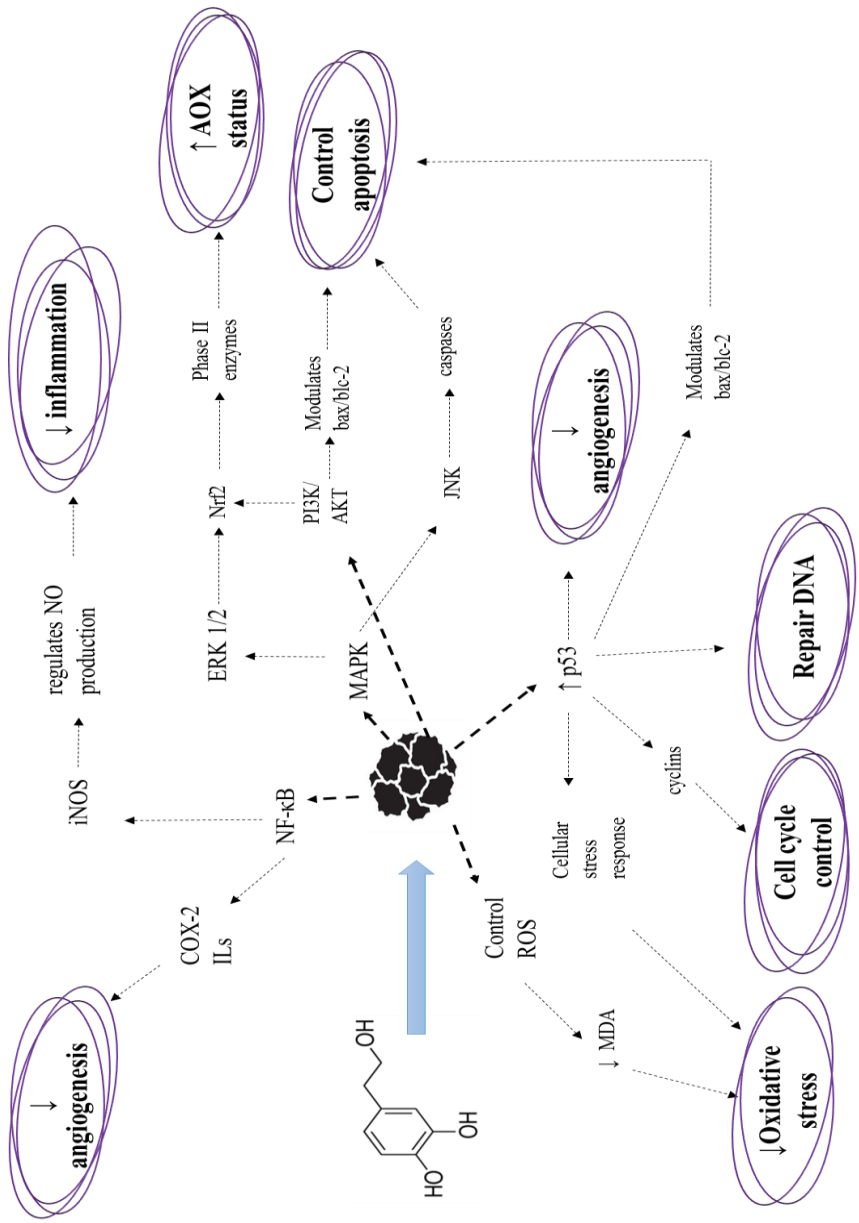


Figure 4. Proposed mechanisms by which HT is involved in protecting against the development of cancer

Table 4. Mechanisms of hydroxytyrosol and its precursors against cancer based on animal models.

Tumour	Animal	Sex	Duration	Supplementation	Improve of carcinogenic parameters	Authors
Cholangiocarcinoma	TFK-1 xenografts in nude mice Male nude BALB/c mice	♂	Intraperito-neal injection every day for 3 w	500 mg HT/kg/day	G2 cell cycle arrest ↑ Apoptosis→↓Bcl-2/Bax ratio = tissue & body weight ↓ protein expression levels for caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, cyclin B1 and p-cdc2 (Tyr15)	(Li et al., 2014)
Colon cancer xenografts	HT-29 TUMOUR inoculated	♀	14 days	10 mg HT/kg/day (i.p. injection)	↓ tumour growth ↓ cell proliferation ↓ EGFR expression ↑ EGFR phosphorylated at y1045	(Terzuoli et al., 2015)
experimental mammary tumours	Sprague-Dawley	♀ 10	5 days/w for 6 w	5 mg HT/kg/day	HT modulate genes involved in protein metabolism, cell proliferation, apoptosis and the Wnt signaling pathway, promoting a high expression of Sfrp4	(Granados-Principal et al., 2011)
Glioma	Wistar rats C6 glioma cell implantation	40 ♂	5 days	100 µg OLE or HT or mixture (Subcutaneous injection)	↓ tumor volume in HT group. No synergic effects with OLE were founded ↓ lipid peroxidation and protein oxidation levels ↓ glutathione reduced and specially oxidized ↑ glutathione peroxidase = lipids biomarkers	(Martínez-Martos et al., 2014)
Breast cancer indu-ced by single dose of 5mg of DMBA	Sprague-Dawley	167 ♀	Euthanized at 105 and 246days	VOO (17% of diet)	VOO → Modify the expression of metabolism genes Low tumor	(Moral et al., 2016)

W: week; HT: Hydroxytyrosol; EGFR: Epidermal growth factor receptor; DMBA: dimethylbenzanthracene

by its radical scavenging properties and to protect cells from DNA damage in both healthy (Fabiani et al., 2008; Warleta et al., 2011) and tumoral cells such as PBMC, colon cancer and melanoma cells (D'Angelo et al., 2005; Meisinger et al., 2005).

Other hypotheses sustain that HT does not affect the H_2O_2 concentration in the media, but could protect the cell against induced oxidative damage (Manna et al., 1997). In addition, HT downregulates the balance among the proteins that are regulators of BAX (antiapoptotic)/Bcl-2 (proapoptotic) apoptosis, so improving the mitochondrial function to reduce cell apoptosis (Chen et al., 2015; Granados-Principal et al., 2014). A contrary hypothesis suggests that PCs could exert "pro-oxidant" activity in cells through the increase in ROS levels in the media (Fabiani et al., 2012), and the excessive ROS content could be responsible for the cytotoxic and pro-apoptotic effects (León-González et al., 2015; Mileo et al., 2016).

One of the best-described anticarcinogenic mechanisms of HT is its ability to detain the cell cycle. Cyclins are the main factor responsible for control of the cell cycle. It has been found that HT induces cell cycle arrest. The main mechanisms of action described in different carcinogenic cell lines is the detention of the cell cycle in G2/M phase (Cárdeno et al., 2013; Corona et al., 2009; Goulas et al., 2009; Li et al., 2014; Rosignoli et al., 2016; Zhao et al., 2014), the decrease in cyclin B1 expression (Chimento et al., 2014; Corona et al., 2009; Li et al., 2014) and the increase in p21 content (Chimento et al., 2014). Other authors highlight that HT (50-100µM) induces the detention of the cell cycle in the G0/G1 stage in breast and leukaemia cancer cell lines (Bouallagui et al., 2011a; Fabiani et al., 2008; Han et al., 2009; Ragione et al., 2000) by activating the MAPK pathway, reducing the levels of cyclin D1 (Bouallagui et al., 2011b) and CDK 6 and increasing the levels of the CDK inhibitors p21(WAF1/Cip1), p27(Kip1) and cyclin D3 (Fabiani et al., 2008). There is no consensus about the specific point where HT produces the detention of the cell cycle, which is probably due to the wide variety of cell lines, kinds of tumour cells, conditions of the study, number of passages and cell mutations.

Angiogenesis is essential to provide growing tissues with oxygen and nutrients. HT has been shown to downregulate some growth factors normally overexpressed in different cancer cells, such as EGFR and TNF-α (Terzuoli et al., 2015), and PGE2 or COX-2 (Cárdeno et al., 2013; Guichard et al., 2006; Terzuoli et al., 2010, 2015; Ziello et al., 2007). HT also regulates the expression of PPARγ (the manager of cell proliferation and growth) to inhibit tumorigenesis (Cárdeno et al., 2013).

The involvement of HT in controlling apoptosis is strongly associated with the activation of PP2A (Guichard et al., 2006), which regulates many critical molecules in cancer treatment, including p53 and the Akt (Seshacharyulu et al., 2013). HT binds onto the ligand side of the G protein of carcinogenic cells, triggering the activation of the MAPK-ERK ½ pathway to inhibit cell growth (Goulas et al., 2009; Hoshino et al., 1999) through c-jun NH2 terminal kinase (JNK) transduction pathways. The JNK pathway induces Bcl-2 phosphorylation and activates Caspase-3 apoptosis (Della Ragione et al., 2002; Luo et al., 2013; Zou et al., 2012). Also, HT induces cellular apoptosis by increasing the BAX (protein regulator to decrease cell apoptosis) (Chimento et al., 2014; Corona et al., 2009; Li et al., 2014). The addition of HT (0.5-10μM) to the HepG2 cell line increased the expression of the Nrf2 pathway and therefore increased antioxidant and phase-II enzyme expression, such as glutathione peroxidase (GPx), GLT reductase and GLT S-transferase (GST) (Martín et al., 2010). The activation of the Nrf2 pathway with the consequent increase in the transcription of phase-II antioxidant enzymes was also found in human retinal pigment epithelial cells under oxidative stress (Zou et al., 2012).

Furthermore, HT reduced the expression of PI3K/Akt (Sun et al., 2014), which is involved in cell proliferation, transcription and cell migration, thus decreasing the risk of cell damage (Martín et al., 2010). However, the regulatory effect of HT on NF-κB is not clear. Some authors report that dietary supplementation with HT downregulates the NF-κB regulated gene products, whereas other studies

show that HT has no direct effect on NF-κB (Cardeno et al., 2013).

It has been demonstrated that HT has the capacity to reduce oxidative stress by upregulating numerous antioxidant proteins and enzymes, including heme oxygenase-1 (HO-1), glutaredoxin and GPx in keratinocytes (20 µM HT) (Rafehi et al., 2012). Furthermore, in PC3 prostate cells, HT (10µM) decreased the mRNA gene expression of GPx as well as reducing DNA oxidation (Quiles et al., 2002). However, higher doses of HT (80 µM) in PC3 and DU145 prostate cells induced superoxide enzymatic activity. This pro-oxidant response produces mitochondrial dysfunction, defects in autophagy and activation of MAPK (Luo et al., 2013). Although it seems difficult to reduce cell proliferation once the pathology is established (Quiles et al., 2002), what it is clear is that HT has been shown to protect healthy cells and thus can reduce the harmful effects of anticarcinogenic drugs (Corona et al., 2009) by regulating several genes.

3.3.Role of HT in the prevention of neurological disorders

Neuropathies are associated with age and are mainly caused by a disproportionate content of ROS in the brain. ROS drives protein aggregation, neuroinflammation, and promotes DNA damage by activating the cascade of reactions that lead to cell death and loss of brain structure (Niccoli et al., 2012). Many studies have shown the ability of HT and its biological metabolites to cross the blood-brain barrier (D'Angelo et al., 2001; Serra et al., 2012; Wu et al., 2009), where they could exhibit different mechanisms to reduce ROS concentration and restore neuronal function (**Figure 5**). Considering the molecular structure of HT (two hydroxyl groups), there is a leading hypothesis about its protective role as a radical scavenger in the brain (De La Cruz et al., 2015; Tuck et al., 2002).

HT has been associated with an improvement in patients suffering from Alzheimer's disease (AD) (Arunsundar et al., 2015; Peng et al., 2016), Parkinson's

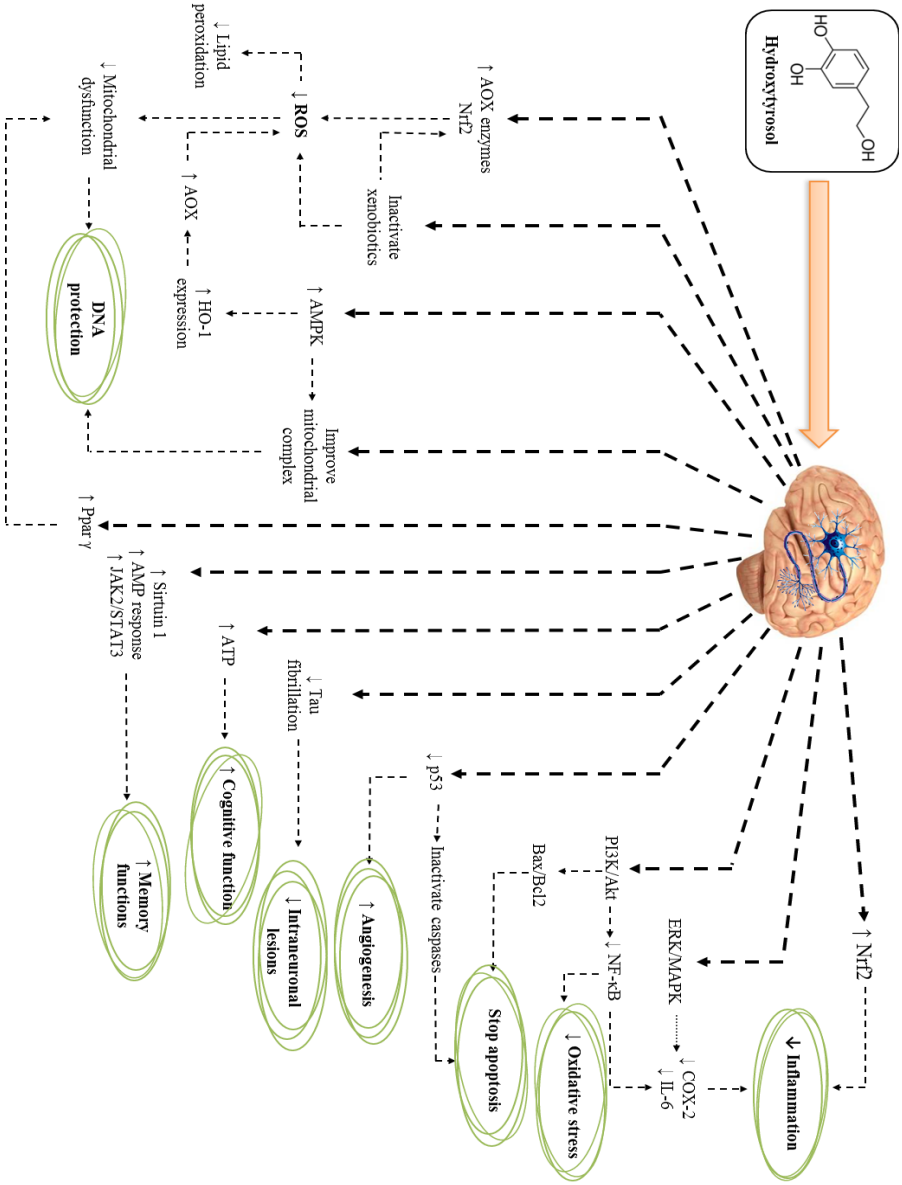


Figure 5. Proposed mechanisms by which HT is involved in the protection against neurological disorders

disease (PD) (Goldstein et al., 2016a), Huntington's disease (Tasset et al., 2011), cerebral ischemia (González-Correa et al., 2008), schizophrenia (Young et al., 2008), peripheral diabetic neuropathy (Ristagno et al., 2012), subarachnoid haemorrhage (Fu & Hu, 2016) and aging (Valls-Pedret et al., 2015). In this section, I review the most recent and relevant studies regarding the *in vivo* neuroprotective effects of HT and the potential underlying molecular mechanisms.

3.3.1. Human evidence of HT neuroprotective effects

There are few human intervention studies that assess the effect of HT and its derivatives on the improvement of cognitive functions (**Table 5**). Some of these studies have been conducted within the framework of a randomized clinical trial entitled *Prevención con Dieta Mediterránea* (PREDIMED). In a first sub-study within PREDIMED analyzing cognitive functions (Martínez-Lapiscina et al., 2013), those participants undergoing neuropsychological testing at the end of the trial reported improved cognition with the MedDiet supplemented with VOO or nuts, respectively, compared to the control diet. However, the lack of baseline evaluation precluded an assessment of changes over time. To address this issue, another sub-study within PREDIMED evaluated cognitive changes over time with repeated measurements using a battery of standard neuropsychological tests in a sub-cohort of cognitively healthy individuals (Valls-Pedret et al., 2015). The results from this study indicated that supplementation of the MedDiet with VOO improves the frontal function and overall cognition. Previous evidence, in the prospective Three-City Study (Berr et al., 2009), established a weak association between the increase in VOO intake and the decrease in the risk of cognitive decline during a 4-year follow-up. The hypothesis is that the beneficial effect of the MedDiet supplemented with VOO on cognition probably stems from the PCs that it provides. These compounds might counteract oxidative processes in the brain, controlling the early phases of

neurodegeneration. This hypothesis has been confirmed in animal and *in vitro* studies, which suggest that HT and its precursors can ameliorate neurological health by different mechanisms.

3.3.2. Mechanistic studies of HT against neurological disorders

Several animal (**Table 5**) and *in vitro* studies have demonstrated that HT can ameliorate neurological health by different mechanisms, including directly reducing ROS, increasing Nrf2 to boost the antioxidant defence or modulate specific genes involved in cognitive functions to reduce early apoptosis, and increasing angiogenesis, among others (**Figure 5**) Cytoprotective effects of HT have been observed in potentiate adrenal gland (PC12 cells), with a decrease in the DNA damage induced by H_2O_2 (Hashimoto et al., 2004; Young et al., 2008). Also, Schaffer et al. found that administration of HT after damage induced by NO and Fe^{2+} in PC12 cells produced an increase in the adenosine triphosphate (ATP) and the mitochondria transmembrane potential, followed by a decrease in the malondialdehyde (MDA) levels (Schaffer et al., 2010). These results are in accordance with a previous study conducted in dissociated mouse brain cells in which pre-incubation with HT reduced the cytotoxic effects exerted by metals (Schaffer et al., 2007). In another study, HT at 5 µM exerted neuroprotection against toxicity induced by methyl-mercury in IMR-32 human neuroblastoma cells, maintaining the concentration of GLT, SOD, GST and catalase activity (Mohan et al., 2015). Furthermore, HT reduced the p53 gene expression and downregulated the apoptotic signalling pathways of BAX, cytochrome C and caspase 3 and upregulated the metallothionein and Nrf2 pathways.

It has been also reported that HT at 20 µM in the SH-SY5Y neuroblastoma cell line prompted the expression of phase-II detoxifying enzymes and enhanced quinone oxidoreductase 1, GST, glutamate cysteine Ligase and HO-1, NAD(P)H

(Yu et al., 2016). HT (50-100µM) also reduced NF-κB activation in the neuroblastoma N2a cell line in response to Aß induced toxicity (St-Laurent-Thibault et al., 2011). HT and HT-AC exerted neuroprotective effects, reducing lactate dehydrogenase efflux in a model of hypoxia-reoxygenation with no previous damage (González-Correa et al., 2008).

3.3.2.1. Molecular mechanisms in the prevention of AD

AD is the most frequent neuropathy and is characterized by extracellular senile plaques with amyloid-beta (Aβ) peptide deposits and intracellular tau-protein aggregates that cause neuronal stress, loss of cholinergic neurons and disruption of cerebral neuronal circuits. All these events activate the inflammatory response in the brain (Querfurth & LaFerla, 2010; Salawu, Umar, & Olokoba, 2011). Some studies have been carried out in animal models to evaluate the involvement of HT in the control of AD. A study conducted by Peng et al. in an APP/PS1 mice model showed that supplementation with 5 mg/kg HT for 6 months reduced mitochondrial oxidation and normalized the mitochondrial complex II-IV activities by modulating antioxidant enzymatic status as well as improving cognitive functions and some inflammation biomarkers (Peng et al., 2016).

Supplementation with 10 mg/kg/day of HT for 14 days in C57BL6 mice with induced neurobehavioral dysfunction improved their memory functions by upregulating genes related with neuronal functions, such as sirtuin-1, and activated the survival cascade protein expression of PI3K/Akt1, ERK-MAPK/RSK2 and janus kinase-signal transducer and activator of transcription JAK2/STAT3 (Arunsundar et al., 2015). In addition, in an *E. Coli* model designed to mimic the aggregation of the TAU protein, the addition of OOPCs, including HT, prevented TAU fibrillation and decreased the intraneuronal and glial lesions (Daccache et al., 2011).

Table 5. Protective effect of hydroxytyrosol and its precursors in neurological-cognitive disorders in human intervention studies.

Goal	Status	Subject	Sex	Type of study	Duration	Supplementation	Improve of neurological parameters	Reference
Cognitive function	Risk CVD	447	♀ ♂	Parallel-group randomized	Median 4.1 years	EVOO (1L/w)	↑ frontal and global cognition	(Valls-Pedret et al., 2015)
Cognitive functions	Risk CVD	285	♀ ♂	Random	Measure of cognitive status after 6.5 years of nutrition intervention	EVOO (1L/w)	↑ Memory task ↓ MCI ↑ visual memories ↑ verbal fluency and visual memory	(Martínez-Lapiscina et al., 2013)
Cognitive function	Risk CVD	447	♀ ♂	Cross sectional			↑ immediate verbal memory ↓ recall	(Valls-Pedret et al., 2012)
Cognitive functions		6947	♀ ♂		4 years	Low, moderate, intensive OO use	↑ visual memories ↑ verbal fluency and visual memory	(Berr et al., 2009)
Stroke risk		7625	♀ ♂	Multicenter prospective cohort study	5.25 years (follow 6 years of incidence stroke)	Low, moderate, intensive OO use	↑ OO intake ↓ STROKE incidence	(Samieri et al., 2011)

[MCI] (mild cognitive impairment or dementia); OO olive oil; EVOO: Extra Virgin Olive oil

3.3.2.2 Molecular mechanisms in the prevention of PD

Several studies have reported that the presence of HT in the brain could have a positive impact on the reduction of the incidence of PD (Aiello et al., 2015; Goldstein et al., 2016a; Khalatbary, 2013; Yu et al., 2016). Studies conducted in a SH-SY5Y neuroblastoma cell line showed that an excess of dopamine or its derivative 6-hydroxydopamine are highly susceptible to oxidation and produce ROS, thus inducing cell death. However, supplementation with 90 µM of HT reduced this oxidation and increased the phase-II detoxifying enzymes, especially NAD(P)H quinone oxidoreductase 1 (NQO1), in a dose-dependent manner, as well as increasing GST and glutamate cysteine ligase which scavenged ROS and induced the HO-1 expression (Yu et al., 2016). Traditionally, it was believed that the inhibition of dopamine oxidation by MAO inhibitors reduced the levels of oxidation products (DOPAL and DOPAC), which protected against PD development (Goldstein et al., 2016a). However, recently it has been demonstrated that MAO inhibition induces autoxidation of dopamine, which releases 5-S-cysteinyl-dopamine, another toxic compound (Goldstein et al., 2016b). The addition of exogenous HT (10 µM) to culture media in PC12 cells reduced the synthesis of dopamine and the formation of its oxidation (Goldstein et al., 2016a). Contrary to these results, in a striatal brain microdialysate model, the administration of HT (20 mg/kg) enhanced the dopamine content (Gallardo et al., 2014). Pharmacological treatment employing inhibitors of COMT are being used as antiparkinsonians, increasing the levels of HT, which is more bioactive than HVAlc (Grasso et al., 2007; Rietjens et al., 2007). Some HT derivatives, such as nitrohydroxytyrosyl acetate and ethylnitrohydroxytyrosyl ether, also act as COMT inhibitors, and these results open new potential treatments for PD (Gallardo et al., 2014).

All these studies suggest that HT could contribute to the good maintenanc

Table 6. Mechanisms of hydroxytyrosol and its precursors against neurological-cognitive disorders in animal studies.

Goal	Animal	Sex	Time	Supplementation	Improve of parametres	Authors
Cytoprotective effects	mice	♀	12 days	100mg HT/kg/ bw	↑Mitochondrial membrane potential ↑ATP ↓ MDA	(Schaffer et al., 2007)
Cognitive function	APP/PS1 mice (AD model) C57BL/6J background	♀	6 months	5 mg/kg/day	= β-amyloid (Aβ) accumulation ↓ Mitochondrial dysfunction ↑protein expression of mitochondrial complex II, III, and IV. ↑ ATP content ↑ cognitive function ↑ SOD expression Restore GSH balance ↑ HO-1 expression Normalize p-JNK and reverts to normal values p-JNK/JNK, p-JNK/Actin, and p-ERK/Actin ↓ Caspase-1 protein expression ↓ Inflammatory biomarkers (NFkB) ↓ IL-18, IL-6 and COX-2expression ↓ p53 expression; ↓ Caspase-3	(Peng et al., 2016)
Cognitive function	C57BL/6 mice induced neuro-behavioral dysfunction with soluble oligomeric amyloid β_{1-42} plus ibotenic acid (oA42i)	30 ♂	14 days Intracerebroventricular injection	10 mg/kg/day	↑spatio-cognitive abilities ↑genes memory functions sirtuin-1, cyclic AMP response, CREB-target genes (BDNF, c-*Fos*, *Nurr1*, and *Egr1*) ↑disintegrin and metalloprotease 10 ↑ERK-MAPK/RSK2, ↑PI3K/Akt1 ↑ JAK2/STAT3 in hippocampal neurons. Restore Bcl-2/Bad levels & ↓ Caspase 9/3 cytochrome c, apoptotic protease activating factor-1	(Arunsundar et al., 2015)

Mitochon-drial function & oxidative stress	db/db mice C57BL/6J background (exhibited over obesity and hyper-glycaemia)		8 w	10 /50 mg/kg/day	↑expression of mitochondrial respiratory chain complexes I/II/IV. ↑ Activity of complex I. ↑ Nrf2; ↑ SOD ↑ HO-1 ↓ protein oxidation ↑ AMP-activated protein kinase (AMPK), sirtuin 1 and PPARγ coactivator-1α. ↑mRNA levels of Arc, NMDAR1 and NGF (neuronal survival)	(Zheng, Li, Xu, et al., 2015)
Prenatal stress & decline of cognitive functions	Rats exposed to restraint stress on days 14–20 of pregnancy.	♀		HT was given at doses of 10 and 50 mg/kg/day	↓Prenatal stress → ↓ injure ↑ Neurogenesis by ↑ proteins (BDNF, GAP43, synaptophysin, and glutamate receptors NMDAR1, NMDANR2A and NMDANR2B). ↑ Mitochondrial functions → Increase complex I, II, III, IV and V components ↑ Mitochondrial DNA ↑ phase II enzyme-related proteins, Nrf2 and HO-1. ↑ SOD ↑transcription factors FOXO1 and FOXO3	(Zheng, Li, Cao, et al., 2015)
Oxidative stress Model of HD	Wistar rats 3-nitropropionic acid (3NP) administered intraperitoneally at a dose of 20 mg/kg body weight	40 ♂	14 days orally	2.5 mg/kg body weight	↓ lipid peroxidation product levels blocks the GSH depletion ↑ Succinato DH	(Tasset et al., 2011)
Oxidative stress	Wistar rats sodium pentobarbital (0.1 ml/100 g, i.p.)	40 ♂	6 weeks	100mg HT/body weight	↓ bbb permeability↓ water contain ↑SOD, CAT GSH-PX; ↓ MDA ↑ AKT → > Apoptosis ↓NF-κB protein expression ↓ caspase 3 activity	(Fu & Hu, 2016)

HO-1: Haeme oxygenase 1; SOD: Superoxido dismutase; H.D. Huntington Disease; BBB: blood brain barrier;

of dopaminergic cells and homeostasis of dopamine in the brain, as well as reducing the levels of oxidation products to avoid cell damage and preventing the incidence of PD.

Numerous studies have demonstrated that patients with diabetes have an increased risk of developing AD compared to healthy individuals, and recent studies suggest that HT might be an effective agent for the prevention and treatment of diabetic complications, such as brain damage. The principal biological mechanisms that relate the progression of diabetes and AD are impaired insulin signalling and uncontrolled metabolism, which produce an overproduction of glycation products susceptible to be transformed into superoxides and stimulate the inflammatory pathways (Rani et al., 2016).

After 8 weeks of treatment with 10 and 50 mg HT/kg, in a db/db mice model of diabetes mellitus type 2, cognitive functions were improved by the activation of AMPK, sirtuin 1 and PPARγ coactivator-1α, which constitute an energy-sensing protein network known to improve mitochondrial functions and decrease oxidative stress responses (Zheng et al., 2015).

3.3.2.3 Molecular mechanisms of HT in other cognitive disorders

In a rat model simulating Huntington's disease (HD), the administration of 2.5 mg/kg body weight of HT for 14 days reduced lipid peroxides, increased GLT content and the levels of succinate (Tasset et al., 2011). Supplementation with 100 mg/kg/day of HT in a rat model of subarachnoid haemorrhage for 6 weeks produced the activation of Akt and a decrease in Caspase-3, NF-κB and MDA (Fu & Hu, 2016). In the same study, a reduction in the quantity of water in the brain and the permeability of the blood-brain barrier was observed.

4. Future perspectives

4.1. Exploring the effects of HT biological metabolites

The future perspectives on HT research will be to study the effect of the conjugate metabolites. Most *in vitro* studies are conducted with aglycone, but this form is hardly available *in vivo* due to extensive metabolism of the native molecule in the intestine and liver. Additionally, the plasmatic concentrations of HT and its metabolites of 0.1–25 μM (Rubió et al., 2012; Vissers et al., 2004) are really low compared to most of the pharmacological doses employed *in vitro*. At present, very few studies have tested the bioactivity of HT metabolites (Atzeri et al., 2016; Deiana et al., 2011; Giordano et al., 2015; Khymenets et al., 2010), but it seems probable that these are bioactive and responsible to a great extent for HT biological effects. A deconjugation process was observed in red blood cells after an HT exposure of 6 hours (Rubió, et al., 2014). Thus, the possibility of deconjugation at the cellular level in the target site, generating the native HT, represents an additional challenge when attributing a particular biological effect to HT metabolites.

The difficulty of synthesising phase-II metabolites has long prevented the study of their bioactivity. In recent years, different methods of obtaining phase-II HT metabolites by chemicals means (Atzeri et al., 2016; Gomes, Torres, Rodríguez-Borges, & Paiva-Martins, 2015) have been developed. The sulfate forms of HT and TYR showed an effect comparable to their precursors at preventing the oxidative damage by ox-LDL in Caco-2 cells (Atzeri et al., 2016). The glucuronide forms of HT neutralized endoplasmic reticulum oxidative stress in the HepG2 cell line that is related to the development of CVD (Giordano et al., 2015). In a recent study, a mixture of HT-3-Glu and HT-4-Glu protected against the induced haemolysis by H_2O_2 in red blood cells in a dose-dependent manner (Paiva-Martins et al., 2013). By contrast, in a porcine kidney epithelial cell line, co-incubation with HT-3-Glu and

HT-4-Glu did not inhibit oxidative damage and cell death induced by H_2O_2. However, pre-incubation with 10 µM of HT glucuronides protected against tubular membrane oxidative damage like native HT did, and also diminished the formation of MDA, fatty acid hydroperoxides and 7-ketocholesterol (Deiana et al., 2011).

4.2. Development of HT lipophilic derivates

Another promising field in HT research is the development of lipophilic HT derivatives in order to increase HT bioavailability. The lipophilic derivate HT-AC has been the most studied one, and several *in vitro* studies have shown its effect against oxidation, inflammation and carcinogenesis. HT-AC has been reported to exert higher antioxidant activity than HT (Gordon, Paiva-Martins, & Almeida, 2001; Lisete-Torres et al., 2012; Tasset et al., 2011). The antioxidant properties of HT-AC contributed to exerting neuroprotective effects in an *in vitro* model of hypoxia-reoxygenation brain slice (Tasset et al., 2011), and inhibited platelet aggregation in whole blood (Correa et al., 2009).

Rosillo et al. found that feeding DBA-1/J arthritic mice with HT-AC (0.05%) for 6 weeks reduced the inflammation by diminishing the pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6 and IL-17) and activated different signalling pathways, such as the JAK/STAT. It also reduced the MAPK and NF-κB pathways and upregulated the protein expression of Nrf2 and HO-1 (Rosillo et al., 2015). In murine peritoneal macrophages stimulated with LPS, 50 and 100 µM of HT-AC reduced the iNOS and COX-2 expression and prevented liberation of the complex iKBα, which binds with NF-κB and prevents its release (Aparicio-Soto et al., 2015).

It seems that HT-AC also contributes to reducing carcinogenesis, as seen in Caco-2 cells exposed to 10-30 µM HT-AC, where the arrest of the cell cycle was induced by upregulation of cyclin p21 and cyclin G2 and downregulation of cyclin B1. HT-AC also upregulated the apoptotic caspase-3, and the CYP1A1 and

UGT1A10 phase-II metabolizing enzymes, thus increasing carcinogen detoxification (Mateos et al., 2013). Furthermore, HT-AC (0-75 μM) increased glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes through the downregulation of PPARγ, C/EBPα, and SREBP-1c and their downstream target genes (GLUT4, CD36, and FAS) (Drira & Sakamoto, 2013). Another study reported that the presence of both HT and HT-AC exerted synergic effects on the inhibition of lipid oxidation (Drira & Sakamoto, 2013).

Short and medium lipophilic derivatives of HT have shown to prevent oxidative stress and DNA damage (R. Bernini, Merendino, Romani, & Velotti, 2013; Bouallagui et al., 2011a; Grasso et al., 2007), as well as preventing lipidic oxidation and GSH depletion (Guerrero et al., 2012). HT and α-lipoic ester induced cell cycle arrest in the G2/M phase in HT-29 cell (Roberta Bernini et al., 2011). HT-laurate in U937 monocytoid and in C2C12 myoblasts showed better antiapoptotic activity than HT after its induction by H_2O_2 (Burattini et al., 2013).

5. Conclusions

Currently, HT is the gold standard in studies related to VOO because of its antiatherogenic, cardioprotective, anti-inflammatory and chemopreventive activities on the one hand, and its capacity to inhibit the proliferation and growth of different types of tumour cells on the other. The results outlined in this review emphasize the potential role of HT in the prevention or delay of chronic diseases and offer new perspectives on HT pharma applications.

Besides its antioxidant and anti-inflammatory capacities, HT is able to modify gene expression coding in a protective mode for proteins participating in the cellular mechanisms involved in redox status, inflammation or apoptosis, among others. My results indicated show that several mechanisms underlying HT effects are common to several diseases. For instance, both inflammation and angiogenesis

are crucial in the development of atherosclerosis, cancer and neurodegenerative disorders, observing similar effects of HT in the regulation of pathways such as COX-2, matrix MMPs or NFκB. In other cases, the activity depends on the specific disease. For instance, modulation of the p53 gene expression is different in neurodegenerative disease (its expression is overregulated) and in a cancer situation (where its expression is inhibited). HT also promotes apoptosis in carcinogenic cells while, in neurodegenerative diseases, it reduces the overapoptosis that occurs. So, current evidence suggests that supplementation with HT is a feasible way of protecting the tissues against the occurrence of age-related disorders by several mechanisms.

Further studies are needed to identify translational studies of HT, its metabolites and its lipidic derivatives in order to elucidate the concentration needed to activate the molecular mechanisms and whether different compounds ingested through the diet or other compounds present in the cells may synergize, leading to the achievement of the concentrations required to obtain preventive and beneficial effects. Human intervention trials focusing on new outcomes related to proteomics and nutrigenomics are also needed to better clarify pathways/mechanisms by which HT and its precursors act on chronic disease risk factors.

In addition to better-defined human intervention studies aimed at assessing physiological endpoints linked to disease, further research is also required on the bioavailability of HT and its precursors, particularly with regard to the effects of food matrices on absorption and the influence of individual gut microbiota variability, age, gender and genotype on both absorption and metabolism. These studies are required in order to help determine the physiological metabolic forms responsible for activity in vivo.

6. REFERENCES

Aiello, A., Guccione, G. D., Accardi, G., & Caruso, C. (2015). What olive oil for healthy ageing? *Maturitas*.

Aparicio-Soto, M., Sánchez-Fidalgo, S., González-Benjumea, A., Maya, I., Fernández-Bolaños, J. G., & Alarcón-de-la-Lastra, C. (2015). Naturally Occurring Hydroxytyrosol Derivatives: Hydroxytyrosyl Acetate and 3,4-Dihydroxyphenylglycol Modulate Inflammatory Response in Murine Peritoneal Macrophages. Potential Utility as New Dietary Supplements. *Journal of Agricultural and Food Chemistry*, *63*(3), 836–846.

Araújo, M., Pimentel, F. B., Alves, R. C., & Oliveira, M. B. P. P. (2015, October 1). Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. Elsevier Ltd.

Arunsundar, M., Shanmugarajan, T. S., & Ravichandran, V. (2015). 3,4-Dihydroxyphenylethanol Attenuates Spatio-Cognitive Deficits in an Alzheimer's Disease Mouse Model: Modulation of the Molecular Signals in Neuronal Survival-Apoptotic Programs. *Neurotoxicity Research*, *27*(2), 143–155.

Atzeri, A., Lucas, R., Incani, A., Peñalver, P., Zafra-Gómez, A., Melis, M. P., … Torre, R. de la. (2016). Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells. *Food Funct.*, *7*(1), 337–346.

Auñon-Calles, D., Canut, L., & Visioli, F. (2013). Toxicological evaluation of pure hydroxytyrosol. *Food and Chemical Toxicology*, *55*, 498–504.

Auñon-Calles, D., Giordano, E., Bohnenberger, S., & Visioli, F. (2013). Hydroxytyrosol is not genotoxic in vitro. *Pharmacological Research*, *74*, 87–93.

Beltrán, G., Aguilera, M. P., Rio, C. Del, Sanchez, S., & Martinez, L. (2005). Influence of fruit ripening process on the natural antioxidant content of Hojiblanca virgin olive oils. *Food Chemistry*, *89*(2), 207–215.

Beltrán, G., Ruano, M. T., Jiménez, A., Uceda, M., & Aguilera, M. P. (2007). Evaluation of virgin olive oil bitterness by total phenol content analysis. *European Journal of Lipid Science and Technology*, *109*(3), 193–197.

Bernini, R., Crisante, F., Merendino, N., Molinari, R., Soldatelli, M. C., & Velotti, F. (2011). Synthesis of a novel ester of hydroxytyrosol and α-lipoic acid

exhibiting an antiproliferative effect on human colon cancer HT-29 cells. *European Journal of Medicinal Chemistry* (Vol. 46).

Bernini, R., Merendino, N., Romani, A., & Velotti, F. (2013). Naturally Occurring Hydroxytyrosol: Synthesis and Anticancer Potential. *Current Medicinal Chemistry*, *20*(5), 655–670.

Berr, C., Portet, F., Carriere, I., Akbaraly, T. N., Feart, C., Gourlet, V., … Ritchie, K. (2009). Olive Oil and Cognition: Results from the Three-City Study. *Dementia and Geriatric Cognitive Disorders*, *28*(4), 357–364.

Bevilacqua, L., Buiarelli, F., Coccioli, F., & Jasionowska, R. (2004). Identification of compounds in wine by HPLC-tandem mass spectrometry, *94*(9–10), 679–689.

Bianco, A., & Uccella, N. (2000). Biophenolic components of olives. *Food Research International*, *33*(6), 475–485.

Bordiga, M., Lorenzo, C., Pardo, F., Salinas, M. R., Travaglia, F., Arlorio, M., … Garde-Cerdán, T. (2016). Factors influencing the formation of histaminol, hydroxytyrosol, tyrosol, and tryptophol in wine: Temperature, alcoholic degree, and amino acids concentration, *197*, 1038–1045.

Boselli, E., Minardi, M., Giomo, A., & Frega, N. G. (2006). Phenolic composition and quality of white d.o.c. wines from Marche (Italy) (Vol. 563, pp. 93–100).

Bouallagui, Z., Bouaziz, M., Lassoued, S., Engasser, J. M., Ghoul, M., & Sayadi, S. (2011a). Hydroxytyrosol acyl esters: biosynthesis and activities. *Applied Biochemistry and Biotechnology*, *163*(5), 592–9.

Bouallagui, Z., Han, J., Isoda, H., & Sayadi, S. (2011b). Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association*, *49*(1), 179–84.

Brenes, M., García, A., García, P., & Garrido, A. (2001). Acid Hydrolysis of Secoiridoid Aglycons during Storage of Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*, *49*(11), 5609–5614.

Buil-Cosiales, P., Irimia, P., Berrade, N., Garcia-Arellano, A., Riverol, M., Murie-Fernández, M., … Serrano-Martínez, M. (2008). Carotid intima-media thickness is inversely associated with olive oil consumption. *Atherosclerosis*, *196*(2), 742–

8.

Burattini, S., Salucci, S., Baldassarri, V., Accorsi, A., Piatti, E., Madrona, A., … Falcieri, E. (2013). Anti-apoptotic activity of hydroxytyrosol and hydroxytyrosyl laurate. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association*, *55*, 248–56.

Cárdeno, A., Sánchez-Hidalgo, M., Rosillo, M. A., & Alarcón de la Lastra, C. (2013). Oleuropein, a secoiridoid derived from olive tree, inhibits the proliferation of human colorectal cancer cell through downregulation of HIF-1α. *Nutrition and Cancer*, *65*(1), 147–56.

Carluccio, M. A., Siculella, L., Ancora, M. A., Massaro, M., Scoditti, E., Storelli, C., … De Caterina, R. (2003). Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *23*(4), 622–9.

Carruba, G., Cocciadiferro, L., Di Cristina, A., Granata, O. M., Dolcemascolo, C., Campisi, I., … Traina, A. (2016). Nutrition, aging and cancer: lessons from dietary intervention studies. *Immunity & Ageing : I & A*, *13*(1), 13.

Casas, R., Sacanella, E., Urpí-Sardà, M., Chiva-Blanch, G., Ros, E., Martínez-González, M.-A., … Estruch, R. (2014). The effects of the mediterranean diet on biomarkers of vascular wall inflammation and plaque vulnerability in subjects with high risk for cardiovascular disease. A randomized trial. *PloS One*, *9*(6), e100084.

Castaner, O., Covas, M.-I., Khymenets, O., Nyyssonen, K., Konstantinidou, V., Zunft, H.-F., … Fito, M. (2012). Protection of LDL from oxidation by olive oil polyphenols is associated with a downregulation of CD40-ligand expression and its downstream products in vivo in humans. *American Journal of Clinical Nutrition*, *95*(5), 1238–1244.

Chamkha, M., Cathala, B., Cheynier, V., & Douillard, R. (2003). Phenolic composition of champagnes from chardonnay and pinot noir vintages, *51*(10), 3179–3184.

Chen, Y., Sun, L., Wang, Y., Zhao, X., Neumann, B., Held, M., … Jiang, W. (2015). A dual-fluorescent whole-well imaging approach for screening active compounds against doxorubicin-induced cardiotoxicity from natural products. *RSC Adv.*,

5(129), 106431–106438.

Chimento, A., Casaburi, I., Rosano, C., Avena, P., De Luca, A., Campana, C., … Sirianni, R. (2014). Oleuropein and hydroxytyrosol activate GPER/ GPR30-dependent pathways leading to apoptosis of ER-negative SKBR3 breast cancer cells. *Molecular Nutrition & Food Research*, *58*(3), 478–489.

Cicero, A. F. G., Nascetti, S., López-Sabater, M. C., Elosua, R., Salonen, J. T., Nyyssönen, K., … EUROLIVE Study Group. (2008). Changes in LDL fatty acid composition as a response to olive oil treatment are inversely related to lipid oxidative damage: The EUROLIVE study. *Journal of the American College of Nutrition*, *27*(2), 314–20.

Corona, G., Deiana, M., Incani, A., Vauzour, D., Dessì, M. A., & Spencer, J. P. E. (2009). Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. *Molecular Nutrition & Food Research*, *53*(7), 897–903.

Corona, G., Tzounis, X., Assunta Dessì, M., Deiana, M., Debnam, E. S., Visioli, F., & Spencer, J. P. E. (2006a). The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. *Free Radical Research*, *40*(6), 647–58.

Corona, G., Tzounis, X., Assunta Dessì, M., Deiana, M., Debnam, E. S., Visioli, F., & Spencer, J. P. E. (2006b). The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. *Free Radical Research*.

Correa, J. A. G., López-Villodres, J. A., Asensi, R., Espartero, J. L., Rodríguez-Gutiérez, G., & De La Cruz, J. P. (2009). Virgin olive oil polyphenol hydroxytyrosol acetate inhibits in vitro platelet aggregation in human whole blood: comparison with hydroxytyrosol and acetylsalicylic acid. *The British Journal of Nutrition*, *101*(8), 1157–64.

Covas, M.-I., Nyyssönen, K., Poulsen, H. E., Kaikkonen, J., Zunft, H.-J. F., Kiesewetter, H., … Marrugat, J. (2006a). The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Annals of Internal Medicine*, *145*(5), 333–41.

Covas, M.-I., de la Torre, K., Farré-Albaladejo, M., Kaikkonen, J., Fitó, M., López-Sabater, C., … de la Torre, R. (2006b). Postprandial LDL phenolic content and

LDL oxidation are modulated by olive oil phenolic compounds in humans. *Free Radical Biology & Medicine*, *40*(4), 608–16.

Crespo, M. C., Tomé-Carneiro, J., Burgos-Ramos, E., Loria Kohen, V., Espinosa, M. I., Herranz, J., & Visioli, F. (2015). One-week administration of hydroxytyrosol to humans does not activate Phase II enzymes. *Pharmacological Research*, *95*, 132–137.

D'Angelo, S., Ingrosso, D., Migliardi, V., Sorrentino, A., Donnarumma, G., Baroni, A., … Galletti, P. (2005). Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. *Free Radical Biology & Medicine*, *38*(7), 908–19.

D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O., Morrica, P., Capasso, G., … Zappia, V. (2001). Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *29*(11), 1492–8.

Daccache, A., Lion, C., Sibille, N., Gerard, M., Slomianny, C., Lippens, G., & Cotelle, P. (2011). Oleuropein and derivatives from olives as Tau aggregation inhibitors. *Neurochemistry International*, *58*(6), 700–707.

de Bock, M., Thorstensen, E. B., Derraik, J. G. B., Henderson, H. V., Hofman, P. L., & Cutfield, W. S. (2013). Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (Olea europaea L.) leaf extract, *57*(11), 2079–2085.

De La Cruz, J. P., Ruiz-Moreno, M. I., Guerrero, A., Reyes, J. J., Benitez-Guerrero, A., Espartero, J. L., & González-Correa, J. A. (2015). Differences in the Neuroprotective Effect of Orally Administered Virgin Olive Oil (*Olea europaea*) Polyphenols Tyrosol and Hydroxytyrosol in Rats. *Journal of Agricultural and Food Chemistry*, *63*(25), 5957–5963.

de la Torre-Carbot, K., Chávez-Servín, J. L., Jaúregui, O., Castellote, A. I., Lamuela-Raventós, R. M., Nurmi, T., … López-Sabater, M. C. (2010). Elevated circulating LDL phenol levels in men who consumed virgin rather than refined olive oil are associated with less oxidation of plasma LDL. *The Journal of Nutrition*, *140*(3), 501–8.

De la Torre, R., Covas, M. I., Pujadas, M. A., Fitó, M., & Farré, M. (2006). Is dopamine behind the health benefits of red wine? *European Journal of Nutrition*,

45(5), 307–10.

Deiana, M., Incani, A., Rosa, A., Atzeri, A., Loru, D., Cabboi, B., … Assunta Dessì, M. (2011). Hydroxytyrosol glucuronides protect renal tubular epithelial cells against H(2)O(2) induced oxidative damage. *Chemico-Biological Interactions, 193*(3), 232–9.

Dell'Agli, M., Fagnani, R., Mitro, N., Scurati, S., Masciadri, M., Mussoni, L., … Caruso, D. (2006). Minor components of olive oil modulate proatherogenic adhesion molecules involved in endothelial activation. *Journal of Agricultural and Food Chemistry, 54*(9), 3259–64.

Della Ragione, F., Cucciolla, V., Criniti, V., Indaco, S., Borriello, A., & Zappia, V. (2002). Antioxidants induce different phenotypes by a distinct modulation of signal transduction. *FEBS Letters, 532*(3), 289–94.

Di Tommaso, D., Calabrese, R., & Rotilio, D. (1998). Identification and quantitation of hydroxytyrosol in Italian wines, *21*(10), 549–553.

Drira, R., & Sakamoto, K. (2013). Modulation of adipogenesis, lipolysis and glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes by hydroxytyrosol acetate: a comparative study. *Biochemical and Biophysical Research Communications, 440*(4), 576–81.

Dudley, J. I., Lekli, I., Mukherjee, S., Das, M., Bertelli, A. A. A., & Das, D. K. (2008). Does white wine qualify for French paradox? Comparison of the cardioprotective effects of red and white wines and their constituents: Resveratrol, tyrosol, and hydroxytyrosol, *56*(20), 9362–9373.

Dufour, J. P., Wierda, R., Leus, M., Lissens, G., Delvaux, F., Derdelinckx, G., & Larsen, D. (2002). Quantitative analysis of beer aromatic alcohols using stable isotope dilution assay (Vol. 60, pp. 88–96).

El Riachy, M., Priego-Capote, F., León, L., Rallo, L., & Luque de Castro, M. D. (2011). Hydrophilic antioxidants of virgin olive oil. Part 2: Biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors. *European Journal of Lipid Science and Technology, 113*(6), 692–707.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., … Martínez-González, M. A. (2013). Primary Prevention of Cardiovascular Disease

with a Mediterranean Diet. *New England Journal of Medicine*, *368*(14), 1279–1290.

Fabiani, R., Rosignoli, P., De Bartolomeo, A., Fuccelli, R., & Morozzi, G. (2008). Inhibition of cell cycle progression by hydroxytyrosol is associated with upregulation of cyclin-dependent protein kinase inhibitors p21(WAF1/Cip1) and p27(Kip1) and with induction of differentiation in HL60 cells. *The Journal of Nutrition*, *138*(1), 42–8.

Fabiani, R., Sepporta, M. V., Rosignoli, P., De Bartolomeo, A., Crescimanno, M., & Morozzi, G. (2012). Anti-proliferative and pro-apoptotic activities of hydroxytyrosol on different tumour cells: the role of extracellular production of hydrogen peroxide. *European Journal of Nutrition*, *51*(4), 455–464.

Farràs, M., Castañer, O., Martín-Peláez, S., Hernáez, Á., Schröder, H., Subirana, I., … Fitó, M. (2015). Complementary phenol-enriched olive oil improves HDL characteristics in hypercholesterolemic subjects. A randomized, double-blind, crossover, controlled trial. The VOHF study. *Molecular Nutrition & Food Research*, *59*(9), 1758–70.

Farràs, M., Valls, R. M., Fernández-Castillejo, S., Giralt, M., Solà, R., Subirana, I., … Fitó, M. (2013). Olive oil polyphenols enhance the expression of cholesterol efflux related genes in vivo in humans. A randomized controlled trial. *The Journal of Nutritional Biochemistry*, *24*(7), 1334–1339.

Fernández-Castillejo, S., Valls, R.-M., Castañer, O., Rubió, L., Catalán, Ú., Pedret, A., … Solà, R. (2016). Polyphenol rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial. *Molecular Nutrition & Food Research*, *60*(7), 1544–1554.

Fernández-Mar, M. I., Mateos, R., García-Parrilla, M. C., Puertas, B., & Cantos-Villar, E. (2012, February 15). Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review.

Fitó, M., Cladellas, M., de la Torre, R., Martí, J., Alcántara, M., Pujadas-Bastardes, M., … Covas, M. I. (2005). Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: a randomized, crossover, controlled, clinical trial. *Atherosclerosis*, *181*(1), 149–158.

Floridi, S., Montanari, L., Marconi, O., & Fantozzi, P. (2003). Determination of free phenolic acids in wort and beer by coulometric array detection, *51*(6), 1548–

1554.

Fregapane, G., Gómez-Rico, A., Inarejos, A. M., & Salvador, M. D. (2013). Relevance of minor components stability in commercial olive oil quality during the market period. *European Journal of Lipid Science and Technology*, *115*(5), 541–548.

Fregapane, G., & Salvador, M. D. (2013). Production of superior quality extra virgin olive oil modulating the content and profile of its minor components. *Food Research International*, *54*(2), 1907–1914.

Fu, P., & Hu, Q. (2016). 3,4-Dihydroxyphenylethanol alleviates early brain injury by modulating oxidative stress and Akt and nuclear factor-κB pathways in a rat model of subarachnoid hemorrhage. *Experimental and Therapeutic Medicine*, *11*(5), 1999–2004.

Fuccelli, R., Fabiani, R., Sepporta, M. V., & Rosignoli, P. (2015). The hydroxytyrosol-dependent increase of TNF-α in LPS-activated human monocytes is mediated by PGE2 and adenylate cyclase activation. *Toxicology in Vitro*, *29*(5), 933–937.

Gallardo, E., Madrona, A., Palma-Valdés, R., Trujillo, M., Espartero, J. L., Santiago, M., … Torre, R. D. la. (2014). The effect of hydroxytyrosol and its nitroderivatives on catechol-O-methyl transferase activity in rat striatal tissue. *RSC Adv.*, *4*(105), 61086–61091.

Gallardo, E., Palma-Valdés, R., Espartero, J. L., & Santiago, M. (2014). In vivo striatal measurement of hydroxytyrosol, and its metabolite (homovanillic alcohol), compared with its derivative nitrohydroxytyrosol. *Neuroscience Letters*, *579*, 173–6.

García-Parrilla, M. C., González, G. A., Heredia, F. J., & Troncoso, A. M. (1997). Differentiation of Wine Vinegars Based on Phenolic Composition, *45*(9), 3487–3492.

García-Villalba, R., Larrosa, M., Possemiers, S., Tomás-Barberán, F. A., & Espín, J. C. (2014). Bioavailability of phenolics from an oleuropein-rich olive (Olea europaea) leaf extract and its acute effect on plasma antioxidant status: Comparison between pre- and postmenopausal women, *53*(4), 1015–1027.

Ghanbari, R., Anwar, F., Alkharfy, K. M., Gilani, A. H., & Saari, N. (2012, March).

Valuable nutrients and functional bioactives in different parts of olive (Olea europaea L.)-A review.

Giordano, E., Dangles, O., Rakotomanomana, N., Baracchini, S., Visioli, F., Visioli, F., … Visioli, F. (2015). 3-O-Hydroxytyrosol glucuronide and 4-O-hydroxytyrosol glucuronide reduce endoplasmic reticulum stress in vitro. *Food Funct.*, *6*(10), 3275–3281.

Giordano, E., Dávalos, A., & Visioli, F. (2014). Chronic hydroxytyrosol feeding modulates glutathione-mediated oxido-reduction pathways in adipose tissue: A nutrigenomic study. *Nutrition, Metabolism and Cardiovascular Diseases*, *24*(10), 1144–1150.

Goldstein, D. S., Jinsmaa, Y., Sullivan, P., Holmes, C., Kopin, I. J., & Sharabi, Y. (2016a). 3,4-Dihydroxyphenylethanol (Hydroxytyrosol) Mitigates the Increase in Spontaneous Oxidation of Dopamine During Monoamine Oxidase Inhibition in PC12 Cells. *Neurochemical Research*, 1–6.

Goldstein, D. S., Jinsmaa, Y., Sullivan, P., Holmes, C., Kopin, I. J., & Sharabi, Y. (2016b). Comparison of Monoamine Oxidase Inhibitors in Decreasing Production of the Autotoxic Dopamine Metabolite 3,4-Dihydroxyphenylacetaldehyde in PC12 Cells. *The Journal of Pharmacology and Experimental Therapeutics*, *356*(2), 483–92.

Gomes, V. P. M., Torres, C., Rodríguez-Borges, J. E., & Paiva-Martins, F. (2015). A Convenient Synthesis of Hydroxytyrosol Monosulfate Metabolites. *Journal of Agricultural and Food Chemistry*, *63*(43), 9565–9571.

Gómez-Caravaca, A. M., Cerretani, L., Segura-Carretero, A., Fernández-Gutiérrez, A., Lercker, G., & Gallina Toschi, T. (2011). Qualitative phenolic profile (HPLCDAD-MS) from olive oil mill waste waters at different states of storage and evaluation of hydrolysis process as a pretreatment to recover their antioxidants, *13*(1), 22–30.

González-Correa, J. A., Navas, M. D., Lopez-Villodres, J. A., Trujillo, M., Espartero, J. L., & De La Cruz, J. P. (2008). *Neuroprotective effect of hydroxytyrosol and hydroxytyrosol acetate in rat brain slices subjected to hypoxia–reoxygenation*. *Neuroscience Letters* (Vol. 446).

González-Santiago, M., Fonollá, J., & Lopez-Huertas, E. (2010). Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from

olive oil, and evidence for its transient association with low-density lipoproteins. *Pharmacological Research*, *61*(4), 364–370.

Gordon, M. H., Paiva-Martins, F., & Almeida, M. (2001). Antioxidant Activity of Hydroxytyrosol Acetate Compared with That of Other Olive Oil Polyphenols. *Journal of Agricultural and Food Chemistry*, *49*(5), 2480–2485.

Goulas, V., Exarchou, V., Troganis, A. N., Psomiadou, E., Fotsis, T., Briasoulis, E., & Gerothanassis, I. P. (2009). Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. *Molecular Nutrition & Food Research*, *53*(5), 600–608.

Granados-Principal, S., El-Azem, N., Pamplona, R., Ramirez-Tortosa, C., Pulido-Moran, M., Vera-Ramirez, L., … Ramirez-Tortosa, M. (2014). Hydroxytyrosol ameliorates oxidative stress and mitochondrial dysfunction in doxorubicin-induced cardiotoxicity in rats with breast cancer. *Biochemical Pharmacology*, *90*(1), 25–33.

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C., Camacho-Corencia, P., Sanchez-Rovira, P., Vera-Ramirez, L., & Ramirez-Tortosa, M. C. (2011). Hydroxytyrosol inhibits growth and cell proliferation and promotes high expression of sfrp4 in rat mammary tumours. *Molecular Nutrition & Food Research*, *55 Suppl 1*, S117-26.

Grasso, S., Siracusa, L., Spatafora, C., Renis, M., & Tringali, C. (2007). Hydroxytyrosol lipophilic analogues: Enzymatic synthesis, radical scavenging activity and DNA oxidative damage protection. *Bioorganic Chemistry*, *35*(2), 137–152.

Guasch-Ferré, M., Hu, F. B., Martínez-González, M. A., Fitó, M., Bulló, M., Estruch, R., … Salas-Salvadó, J. (2014). Olive oil intake and risk of cardiovascular disease and mortality in the PREDIMED Study. *BMC Medicine*, *12*(1), 78.

Guerrero, A., De La Cruz, J. P., Muñoz-Marín, J., López-Villodres, J. A., Madrona, A., Espartero, J. L., & González-Correa, J. A. (2012). Neuroprotective effect of alkyl hydroxytyrosyl ethers in rat brain slices subjected to a hypoxia-reoxygenation model. *Food Chemistry*, *134*(4), 2176–2183.

Guichard, C., Pedruzzi, E., Fay, M., Marie, J.-C., Braut-Boucher, F., Daniel, F., … Ogier-Denis, E. (2006). Dihydroxyphenylethanol induces apoptosis by activating

serine/threonine protein phosphatase PP2A and promotes the endoplasmic reticulum stress response in human colon carcinoma cells. *Carcinogenesis, 27*(9), 1812–27.

Han, J., Talorete, T. P. N., Yamada, P., & Isoda, H. (2009). Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. *Cytotechnology, 59*(1), 45–53.

Hashimoto, T., Ibi, M., Matsuno, K., Nakashima, S., Tanigawa, T., Yoshikawa, T., & Yabe-Nishimura, C. (2004). An endogenous metabolite of dopamine, 3,4-dihydroxyphenylethanol, acts as a unique cytoprotective agent against oxidative stress-induced injury. *Free Radical Biology and Medicine, 36*(5), 555–564.

Hohmann, C. D., Cramer, H., Michalsen, A., Kessler, C., Steckhan, N., Choi, K., & Dobos, G. (2015). Effects of high phenolic olive oil on cardiovascular risk factors: A systematic review and meta-analysis. *Phytomedicine, 22*(6), 631–640.

Hoshino, R., Chatani, Y., Yamori, T., Tsuruo, T., Oka, H., Yoshida, O., … Kohno, M. (1999). Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene, 18*(3), 813–22.

Ibern-Gómez, M., Andrés-Lacueva, C., Lamuela-Raventós, R. M., Buxaderas, S., Singleton, V. L., & De La Torre-Boronat, M. C. (2000). Browning of cava (sparkling wine) during aging in contact with lees due to the phenolic composition, *51*(1), 29–36.

Khalatbary, A. R. (2013). Olive oil phenols and neuroprotection. *Nutritional Neuroscience, 16*(6), 243–249.

Khymenets, O., Fitó, M., Touriño, S., Muñoz-Aguayo, D., Pujadas, M., Torres, J. L., … de la Torre, R. (2010). Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 38*(9), 1417–21.

Kim, J.-H., Song, J., & Park, K. W. (2015). The multifaceted factor peroxisome proliferator-activated receptor γ (PPARγ) in metabolism, immunity, and cancer. *Archives of Pharmacal Research, 38*(3), 302–12.

Kolonel, L. N., Altshuler, D., & Henderson, B. E. (2004). The multiethnic cohort study: exploring genes, lifestyle and cancer risk. *Nature Reviews Cancer, 4*(7),

519–527.

Konstantinidou, V., Covas, M.-I., Muñoz-Aguayo, D., Khymenets, O., de la Torre, R., Saez, G., … Fito, M. (2010). In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *24*(7), 2546–57.

Kotronoulas, A., Pizarro, N., Serra, A., Robledo, P., Joglar, J., Rubió, L., … de la Torre, R. (2013). Dose-dependent metabolic disposition of hydroxytyrosol and formation of mercapturates in rats. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society*, *77*, 47–56.

Lamy, S., Ben Saad, A., Zgheib, A., & Annabi, B. (2016). Olive oil compounds inhibit the paracrine regulation of TNF-??-induced endothelial cell migration through reduced glioblastoma cell cyclooxygenase-2 expression. *Journal of Nutritional Biochemistry*.

Ledee, D., Smith, L., Bruce, M., Kajimoto, M., Isern, N., Portman, M. A., & Olson, A. K. (2015). c-Myc Alters Substrate Utilization and O-GlcNAc Protein Posttranslational Modifications without Altering Cardiac Function during Early Aortic Constriction. *PloS One*, *10*(8), e0135262.

León-González, A. J., Auger, C., & Schini-Kerth, V. B. (2015). Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. *Biochemical Pharmacology*.

Li, S., Han, Z., Ma, Y., Song, R., Pei, T., Zheng, T., … Liu, L. (2014). Hydroxytyrosol inhibits cholangiocarcinoma tumor growth: An in vivo and in vitro study. *Oncology Reports*, *31*(1), 145–152.

Lin, P., Qian, W., Wang, X., Cao, L., Li, S., & Qian, T. (2013). The biotransformation of oleuropein in rats. *Biomedical Chromatography*.

Lipworth, L., Martínez, M. E., Angell, J., Hsieh, C.-C., & Trichopoulos, D. (1997). Olive Oil and Human Cancer: An Assessment of the Evidence. *Preventive Medicine*, *26*(2), 181–190.

Lisete-Torres, P., Losada-Barreiro, S., Albuquerque, H., Sánchez-Paz, V., Paiva-Martins, F., & Bravo-Díaz, C. (2012). Distribution of hydroxytyrosol and hydroxytyrosol acetate in olive oil emulsions and their antioxidant efficiency.

Journal of Agricultural and Food Chemistry, 60(29), 7318–25.

Lockyer, S., Rowland, I., Spencer, J. P. E., Yaqoob, P., & Stonehouse, W. (2016). Impact of phenolic-rich olive leaf extract on blood pressure, plasma lipids and inflammatory markers: a randomised controlled trial. *European Journal of Nutrition*, 1–12.

López-Guarnido, O., Álvarez-Cubero, M. J., Saiz, M., Lozano, D., Rodrigo, L., Pascual, M., … Rivas, A. (2015). Mediterranean diet adherence and prostate cancer risk. *Nutrición Hospitalaria, 31*(3), 1012–9.

Lourida, I., Soni, M., Thompson-Coon, J., Purandare, N., Lang, I. A., Ukoumunne, O. C., & Llewellyn, D. J. (2013). Mediterranean diet, cognitive function, and dementia: a systematic review. *Epidemiology (Cambridge, Mass.), 24*(4), 479–89.

Luo, C., Li, Y., Wang, H., Cui, Y., Feng, Z., Li, H., … Liu, J. (2013). Hydroxytyrosol Promotes Superoxide Production and Defects in Autophagy Leading to Anti-proliferation and Apoptosis on Human Prostate Cancer Cells. *Current Cancer Drug Targets, 13*(6), 625–639.

Maiuri, M. C., De Stefano, D., Di Meglio, P., Irace, C., Savarese, M., Sacchi, R., … Carnuccio, R. (2005). Hydroxytyrosol, a phenolic compound from virgin olive oil, prevents macrophage activation. *Naunyn-Schmiedeberg's Archives of Pharmacology, 371*(6), 457–65.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition, 79*(5), 727–47.

Manna, C., Galletti, P., Cucciolla, V., Moltedo, O., Leone, A., & Zappia, V. (1997). The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *The Journal of Nutrition, 127*(2), 286–92.

Marchitti, S. A., Deitrich, R. A., & Vasiliou, V. (2007). Neurotoxicity and Metabolism of the Catecholamine-Derived 3,4-Dihydroxyphenylacetaldehyde and 3,4-Dihydroxyphenylglycolaldehyde: The Role of Aldehyde Dehydrogenase. *Pharmacological Reviews, 59*(2).

Martín-Peláez, S., Mosele, J. I., Pizarro, N., Farràs, M., de la Torre, R., Subirana, I.,

… Fitó, M. (2015). Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: implications of human gut microbiota. *European Journal of Nutrition*, 1–13.

Martín, M. A., Ramos, S., Granado-Serrano, A. B., Rodríguez-Ramiro, I., Trujillo, M., Bravo, L., & Goya, L. (2010). Hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation via extracellular regulated kinases and phosphatidylinositol-3-kinase/protein kinase B pathways in HepG2 cells. *Molecular Nutrition & Food Research*, *54*(7), 956–966.

Martínez-Lapiscina, E. H., Clavero, P., Toledo, E., San Julián, B., Sanchez-Tainta, A., Corella, D., … Martínez-Gonzalez, M. Á. (2013). Virgin olive oil supplementation and long-term cognition: the PREDIMED-NAVARRA randomized, trial. *The Journal of Nutrition, Health & Aging*, *17*(6), 544–52.

Martínez-Martos, J. M., Mayas, M. D., Carrera, P., Arias de Saavedra, J. M., Sánchez-Agesta, R., Arrazola, M., & Ramírez-Expósito, M. J. (2014). Phenolic compounds oleuropein and hydroxytyrosol exert differential effects on glioma development via antioxidant defense systems. *Journal of Functional Foods*, *11*, 221–234.

Mateos, R., Goya, L., & Bravo, L. (2005). Metabolism of the olive oil phenols hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate by human hepatoma HepG2 cells. *Journal of Agricultural and Food Chemistry*, *53*(26), 9897–905.

Mateos, R., Martínez-López, S., Baeza Arévalo, G., Amigo-Benavent, M., Sarriá, B., & Bravo-Clemente, L. (2016). Hydroxytyrosol in functional hydroxytyrosol-enriched biscuits is highly bioavailable and decreases oxidised low density lipoprotein levels in humans. *Food Chemistry*, *205*, 248–56.

Mateos, R., Pereira-Caro, G., Bacon, J. R., Bongaerts, R., Sarriá, B., Bravo, L., & Kroon, P. A. (2013). Anticancer Activity of Olive Oil Hydroxytyrosyl Acetate in Human Adenocarcinoma Caco-2 Cells. *Journal of Agricultural and Food Chemistry*, *61*(13), 3264–3269.

Mateos, R., Pereira-Caro, G., Saha, S., Cert, R., Redondo-Horcajo, M., Bravo, L., & Kroon, P. A. (2011). Acetylation of hydroxytyrosol enhances its transport across differentiated Caco-2 cell monolayers. *Food Chemistry*, *125*(3), 865–872.

Meisinger, C., Baumert, J., Khuseyinova, N., Loewel, H., & Koenig, W. (2005). Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary

heart disease events in apparently healthy, middle-aged men from the general population. *Circulation*, *112*(5), 651–7.

Mileo, A. M., Miccadei, S., Mileo, A. M., & Miccadei, S. (2016). Polyphenols as Modulator of Oxidative Stress in Cancer Disease: New Therapeutic Strategies. *Oxidative Medicine and Cellular Longevity*, *2016*, 1–17.

Minussi, R. C., Rossi, M., Bologna, L., Cordi, L., Rotilio, D., Pastore, G. M., & Durán, N. (2003). Phenolic compounds and total antioxidant potential of commercial wines, *82*(3), 409–416.

Mohan, V., Das, S., & Rao, S. B. S. (2015, October). Hydroxytyrosol, a dietary phenolic compound forestalls the toxic effects of methylmercury-induced toxicity in IMR-32 human neuroblastoma cells. *Environmental Toxicology*, pp. 1264–1275.

Moral, R., Escrich, R., Solanas, M., Vela, E., Ruiz de Villa, M. C., & Escrich, E. (2016). Diets high in corn oil or extra-virgin olive oil differentially modify the gene expression profile of the mammary gland and influence experimental breast cancer susceptibility. *European Journal of Nutrition*, *55*(4), 1397–1409.

Morbidelli, L. (2016). Polyphenol-based nutraceuticals for the control of angiogenesis: Analysis of the critical issues for human use. *Pharmacological Research*, *111*, 384–393.

Moreno-Luna, R., Muñoz-Hernandez, R., Miranda, M. L., Costa, A. F., Jimenez-Jimenez, L., Vallejo-Vaz, A. J., … Stiefel, P. (2012). Olive Oil Polyphenols Decrease Blood Pressure and Improve Endothelial Function in Young Women with Mild Hypertension. *American Journal of Hypertension*.

Mosele, J. I., Martín-Peláez, S., Macià, A., Farràs, M., Valls, R. M., Catalán, Ú., & Motilva, M. J. (2014). Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches. *Molecular Nutrition and Food Research*.

Motilva, M. J., Macià, A., Romero, M. P., Rubió, L., Mercader, M., & González-Ferrero, C. (2016). Human bioavailability and metabolism of phenolic compounds from red wine enriched with free or nano-encapsulated phenolic extract, *25*, 80–93.

Niccoli, T., & Partridge, L. (2012). Ageing as a Risk Factor for Disease. *Current*

Biology, *22*(17), R741–R752.

Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., Robards, K., & Stockmann, R. (2005, February 23). Bioactivity and analysis of biophenols recovered from olive mill waste.

Ortega, R. M. (2006). Importance of functional foods in the Mediterranean diet. *Public Health Nutrition*, *9*(8A), 1136–40.

Ostan, R., Lanzarini, C., Pini, E., Scurti, M., Vianello, D., Bertarelli, C., … Santoro, A. (2015). Inflammaging and cancer: a challenge for the Mediterranean diet. *Nutrients*, *7*(4), 2589–621.

Owen, R. ., Giacosa, A., Hull, W. ., Haubner, R., Spiegelhalder, B., & Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer*, *36*(10), 1235–1247.

Owens, G. K., Kumar, M. S., & Wamhoff, B. R. (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiological Reviews*, *84*(3), 767–801.

Ozdal, T., Sela, D. A., Xiao, J., Boyacioglu, D., Chen, F., & Capanoglu, E. (2016). The Reciprocal Interactions between Polyphenols and Gut Microbiota and Effects on Bioaccessibility. *Nutrients*, *8*(2), 78.

Paiva-Martins, F., Silva, A., Almeida, V., Carvalheira, M., Serra, C., Rodrígues-Borges, J. E., … Santos-Silva, A. (2013). Protective Activity of Hydroxytyrosol Metabolites on Erythrocyte Oxidative-Induced Hemolysis. *Journal of Agricultural and Food Chemistry*, *61*(27), 6636–6642.

Parkar, S. G., Trower, T. M., & Stevenson, D. E. (2013). Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*.

Paulo, J. A., McAllister, F. E., Everley, R. A., Beausoleil, S. A., Banks, A. S., & Gygi, S. P. (2015). Effects of MEK inhibitors GSK1120212 and PD0325901 in vivo using 10-plex quantitative proteomics and phosphoproteomics. *Proteomics*, *15*(2–3), 462–73.

Pei, Y., Chen, J., Xie, L., Cai, X., Yang, R.-H., Wang, X., … Gong, J. (2016). Hydroxytyrosol Protects against Myocardial Ischemia/Reperfusion Injury through a PI3K/Akt-Dependent Mechanism. *Mediators of Inflammation*, *2016*, 1–9.

Peng, Y., Hou, C., Yang, Z., Li, C., Jia, L., Liu, J., … Liu, J. (2016). Hydroxytyrosol mildly improve cognitive function independent of APP processing in APP/PS1 mice. *Molecular Nutrition & Food Research*.

Pereira-Caro G, Sarriá B, Madrona A, Espartero JL, Escuderos ME, Bravo L, et al. Digestive stability of hydroxytyrosol, hydroxytyrosyl acetate and alkyl hydroxytyrosyl ethers. Int J Food Sci Nutr. Taylor & Francis; 2012;63(6):703–7.

Pereira-Caro, G., Bravo, L., Madrona, A., Espartero, J. L., & Mateos, R. (2010). Uptake and Metabolism of New Synthetic Lipophilic Derivatives, Hydroxytyrosyl Ethers, by Human Hepatoma HepG2 Cells. *Journal of Agricultural and Food Chemistry*, *58*(2), 798–806.

Pereira-Caro, G., Madrona, A., Bravo, L., Espartero, J. L., Alcudia, F., Cert, A., & Mateos, R. (2009). Antioxidant activity evaluation of alkyl hydroxytyrosyl ethers, a new class of hydroxytyrosol derivatives. *Food Chemistry*, *115*(1), 86–91.

Pereira-Caro, G., Mateos, R., Saha, S., Madrona, A., Espartero, J. L., Bravo, L., & Kroon, P. A. (2010). Transepithelial Transport and Metabolism of New Lipophilic Ether Derivatives of Hydroxytyrosol by Enterocyte-like Caco-2/TC7 Cells. *Journal of Agricultural and Food Chemistry*, *58*(21), 11501–11509.

Pérez-Mañá, C., Farré, M., Pujadas, M., Mustata, C., Menoyo, E., Pastor, A., … de la Torre, R. (2015). Ethanol induces hydroxytyrosol formation in humans. *Pharmacological Research*, *95*, 27–33.

Piñeiro, Z., Cantos-Villar, E., Palma, M., & Puertas, B. (2011). Direct liquid chromatography method for the simultaneous quantification of hydroxytyrosol and tyrosol in red wines, *59*(21), 11683–11689.

Pour Nikfardjam, M. S., & Pickering, G. J. (2008). Influence of variety and commercial yeast preparation on red wine made from autochthonous Hungarian and Canadian grapes. Part I: Phenolic composition, *227*(4), 1077–1083.

Psaltopoulou, T., Kosti, R. I., Haidopoulos, D., Dimopoulos, M., & Panagiotakos, D. B. (2011). Olive oil intake is inversely related to cancer prevalence: a systematic review and a meta-analysis of 13,800 patients and 23,340 controls in 19 observational studies. *Lipids in Health and Disease*, *10*, 127.

Psaltopoulou, T., Sergentanis, T. N., Panagiotakos, D. B., Sergentanis, I. N., Kosti,

R., & Scarmeas, N. (2013). Mediterranean diet, stroke, cognitive impairment, and depression: A meta-analysis. *Annals of Neurology, 74*(4), 580–91.

Querfurth, H. W., & LaFerla, F. M. (2010). Alzheimer's Disease. *New England Journal of Medicine, 362*(4), 329–344.

Quiles, J. L., Farquharson, A. J., Simpson, D. K., Grant, I., Wahle, K. W. J., Arai, M., … Caruso, D. (2002). Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. *British Journal of Nutrition, 88*(3), 225.

Rafehi, H., Smith, A. J., Balcerczyk, A., Ziemann, M., Ooi, J., Loveridge, S. J., … Karagiannis, T. C. (2012). Investigation into the biological properties of the olive polyphenol, hydroxytyrosol: mechanistic insights by genome-wide mRNA-Seq analysis. *Genes & Nutrition, 7*(2), 343–55.

Ragione, F. Della, Cucciolla, V., Borriello, A., Pietra, V. Della, Pontoni, G., Racioppi, L., … Zappia, V. (2000). Hydroxytyrosol, a Natural Molecule Occurring in Olive Oil, Induces Cytochrome c-Dependent Apoptosis. *Biochemical and Biophysical Research Communications, 278*(3), 733–739.

Ramírez-Expósito, M. J., Carrera, M. P., & Martínez-Martos, J. M. (2010). Chapter 91 – Angiotensinase Activity and Olive Oil Supplementation. In *Olives and Olive Oil in Health and Disease Prevention* (pp. 853–862).

Rani, V., Deshmukh, R., Jaswal, P., Kumar, P., & Bariwal, J. (2016). Alzheimer's disease: Is this a brain specific diabetic condition? *Physiology & Behavior, 164*(Pt A), 259–67.

Razquin, C., Martinez, J. a, Martinez-Gonzalez, M. a, Mitjavila, M. T., Estruch, R., & Marti, a. (2009). A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain. *European Journal of Clinical Nutrition, 63*(12), 1387–93.

Rietjens, S. J., Bast, A., & Haenen, G. R. M. M. (2007). New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. *Journal of Agricultural and Food Chemistry, 55*(18), 7609–14.

Ristagno, G., Fumagalli, F., Porretta-Serapiglia, C., Orrù, A., Cassina, C., Pesaresi, M., … Bianchi, R. (2012). Hydroxytyrosol Attenuates Peripheral Neuropathy in Streptozotocin-Induced Diabetes in Rats. *Journal of Agricultural and Food Chemistry, 60*(23), 5859–5865.

Rodriguez-Mateos, A., Vauzour, D., Krueger, C. G., Shanmuganayagam, D., Reed, J., Calani, L., … Crozier, A. (2014). Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. *Archives of Toxicology, 88*(10), 1803–53.

Rodríguez-Morató, J., Boronat, A., Kotronoulas, A., Pujadas, M., Pastor, A., Olesti, E., … de la Torre, R. (2016, April 2). Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. Taylor and Francis Ltd.

Rodríguez-Morató, J., Robledo, P., Tanner, J.-A., Boronat, A., Pérez-Mañá, C., Oliver Chen, C.-Y., … de la Torre, R. (2017). CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol. *Food Chemistry, 217*, 716–725.

Romani, A., Mulinacci, N., Pinelli, P., Vincieri, F. F., & Cimato, A. (1999). Polyphenolic Content in Five Tuscany Cultivars of *Olea europaea* L. *Journal of Agricultural and Food Chemistry, 47*(3), 964–967.

Romeu, M., Rubió, L., Sánchez-Martos, V., Castañer, O., de la Torre, R., Valls, R. M., … Giralt, M. (2016). Virgin Olive Oil Enriched with Its Own Phenols or Complemented with Thyme Phenols Improves DNA Protection against Oxidation and Antioxidant Enzyme Activity in Hyperlipidemic Subjects. *Journal of Agricultural and Food Chemistry, 64*(9), 1879–1888.

Rosignoli, P., Fuccelli, R., Sepporta, M. V., Fabiani, R., Martínez-González, M. A., Dominguez, L. J., … Montedoro, G. F. (2016). In vitro chemo-preventive activities of hydroxytyrosol: the main phenolic compound present in extra-virgin olive oil. *Food Funct., 7*(1), 301–307.

Rosillo, M. A., Sánchez-Hidalgo, M., González-Benjumea, A., Fernández-Bolaños, J. G., Lubberts, E., & Alarcón-de-la-Lastra, C. (2015). Preventive effects of dietary hydroxytyrosol acetate, an extra virgin olive oil polyphenol in murine collagen-induced arthritis. *Molecular Nutrition & Food Research, 59*(12), 2537–46.

Ruano, J., Lopez-Miranda, J., de la Torre, R., Delgado-Lista, J., Fernandez, J., Caballero, J., … Perez-Jimenez, F. (2007). Intake of phenol-rich virgin olive oil improves the postprandial prothrombotic profile in hypercholesterolemic patients. *Am J Clin Nutr, 86*(2), 341–346.

Ruano, J., Lopez-Miranda, J., Fuentes, F., Moreno, J. A., Bellido, C., Perez-

Martinez, P., … Pérez Jiménez, F. (2005). Phenolic content of virgin olive oil improves ischemic reactive hyperemia in hypercholesterolemic patients. *Journal of the American College of Cardiology*, *46*(10), 1864–8.

Rubio-Senent, F., de Roos, B., Duthie, G., Fernández-Bolaños, J., & Rodríguez-Gutiérrez, G. (2015). Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E-deficient rats. *European Journal of Nutrition*, *54*(8), 1287–1295.

Rubió, L., Macià, A., Castell-Auví, A., Pinent, M., Blay, M. T., Ardévol, A., … Motilva, M.-J. (2014a). Effect of the co-occurring olive oil and thyme extracts on the phenolic bioaccesibility and bioavailability assessed by in vitro digestion and cell models. *Food Chemistry*, *149*, 277–284.

Rubió, L., Macià, A., Castell-Auví, A., Pinent, M., Blay, M. T., Ardévol, A., … Motilva, M. J. (2014b). Effect of the co-occurring olive oil and thyme extracts on the phenolic bioaccesibility and bioavailability assessed by in vitro digestion and cell models. *Food Chemistry*.

Rubió, L., Macià, A., Valls, R. M., Pedret, A., Romero, M.-P., Solà, R., & Motilva, M.-J. (2012). A new hydroxytyrosol metabolite identified in human plasma: hydroxytyrosol acetate sulphate. *Food Chemistry*, *134*(2), 1132–6.

Rubió, L., Serra, A., Macià, A., Piñol, C., Romero, M.-P., & Motilva, M.-J. (2014c). *In vivo distribution and deconjugation of hydroxytyrosol phase II metabolites in red blood cells: A potential new target for hydroxytyrosol*. *Journal of Functional Foods* (Vol. 10).

Rubió, L., Valls, R.-M., Macià, A., Pedret, A., Giralt, M., Romero, M.-P., … Motilva, M.-J. (2012). Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites. *Food Chemistry*, *135*(4), 2922–9.

Salawu, F., Umar, J., & Olokoba, A. (2011). Alzheimer′s disease: A review of recent developments. *Annals of African Medicine*, *10*(2), 73.

Samieri, C., Féart, C., Proust-Lima, C., Peuchant, E., Tzourio, C., Stapf, C., … Barberger-Gateau, P. (2011). Olive oil consumption, plasma oleic acid, and stroke incidence: the Three-City Study. *Neurology*, *77*(5), 418–25.

Saura-Calixto, F., & Goñi, I. (2009). Definition of the Mediterranean diet based on bioactive compounds. *Critical Reviews in Food Science and Nutrition*, *49*(2),

145–52.

Schaffer, S., Müller, W. E., & Eckert, G. P. (2010). Cytoprotective effects of olive mill wastewater extract and its main constituent hydroxytyrosol in PC12 cells. *Pharmacological Research, 62*(4), 322–327.

Schaffer, S., Podstawa, M., Visioli, F., Bogani, P., Müller, W. E., & Eckert, G. P. (2007). Hydroxytyrosol-Rich Olive Mill Wastewater Extract Protects Brain Cells in Vitro and ex Vivo. *Journal of Agricultural and Food Chemistry, 55*(13), 5043–5049.

Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781), "anti-inflammatory properties" (ID 1882), "contributes to the upper respiratory tract health" (ID 3468), "can help to maintain a normal function of gastrointestinal tract" (3779), and "contributes to body defences against external agents" (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal, 9*(4), 2033.

Scoditti, E., Nestola, A., Massaro, M., Calabriso, N., Storelli, C., De Caterina, R., & Carluccio, M. A. (2014). Hydroxytyrosol suppresses MMP-9 and COX-2 activity and expression in activated human monocytes via PKCα and PKCβ1 inhibition. *Atherosclerosis, 232*(1), 17–24.

Selma, M. V, Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. *Journal of Agricultural and Food Chemistry, 57*(15), 6485–501

Serra, A., Rubió, L., Borràs, X., Macià, A., Romero, M.-P., & Motilva, M.-J. (2012). Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. *Molecular Nutrition & Food Research, 56*(3), 486–96.

Seshacharyulu, P., Pandey, P., Datta, K., & Batra, S. K. (2013). Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Letters, 335*(1), 9–18.

Silvestre-Roig, C., de Winther, M. P., Weber, C., Daemen, M. J., Lutgens, E., & Soehnlein, O. (2014). Atherosclerotic plaque destabilization: mechanisms, models, and therapeutic strategies. *Circulation Research, 114*(1), 214–26.

Sofi, F., Abbate, R., Gensini, G. F., & Casini, A. (2010). Accruing evidence on

benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. *The American Journal of Clinical Nutrition*, *92*(5), 1189–96.

Sofi, F., Cesari, F., Abbate, R., Gensini, G. F., & Casini, A. (2008). Adherence to Mediterranean diet and health status: meta-analysis. *BMJ (Clinical Research Ed.)*, *337*(sep11_2), a1344.

Soler, A., Romero, M. P., Macià, A., Saha, S., Furniss, C. S. M., Kroon, P. A., & Motilva, M. J. (2010). Digestion stability and evaluation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. *Food Chemistry*.

St-Laurent-Thibault, C., Arseneault, M., Longpre, F., & Ramassamy, C. (2011). Tyrosol and Hydroxytyrosol Two Main Components of Olive Oil, Protect N2a Cells Against Amyloid-β-Induced Toxicity. Involvement of the NF-κB Signaling. *Current Alzheimer Research*, *8*(5), 543–551.

Suárez, M., Romero, M. P., Ramo, T., Macià, A., & Motilva, M. J. (2009). Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants, *57*(4), 1463–1472.

Sun, L., Luo, C., & Liu, J. (2014). Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation. *Food & Function*, *5*(8), 1909–14.

Tagliafierro, L., Officioso, A., Sorbo, S., Basile, A., & Manna, C. (2015). The protective role of olive oil hydroxytyrosol against oxidative alterations induced by mercury in human erythrocytes. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association*, *82*, 59–63.

Takeda, Y., Bui, V. N., Iwasaki, K., Kobayashi, T., Ogawa, H., & Imai, K. (2014). *Influence of olive-derived hydroxytyrosol on the toll-like receptor 4-dependent inflammatory response of mouse peritoneal macrophages*. *Biochemical and Biophysical Research Communications* (Vol. 446).

Tasset, I., Pontes, A. J., Hinojosa, A. J., de la Torre, R., & Túnez, I. (2011). Olive oil reduces oxidative damage in a 3-nitropropionic acid-induced Huntington's disease-like rat model. *Nutritional Neuroscience*, *14*(3), 106–111.

Terzuoli, E., Donnini, S., Giachetti, A., Iñiguez, M. A., Fresno, M., Melillo, G., &

Ziche, M. (2010). Inhibition of hypoxia inducible factor-1alpha by dihydroxyphenylethanol, a product from olive oil, blocks microsomal prostaglandin-E synthase-1/vascular endothelial growth factor expression and reduces tumor angiogenesis. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(16), 4207–16.

Terzuoli, E., Giachetti, A., Ziche, M., & Donnini, S. (2015). Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation. *Molecular Nutrition & Food Research*.

Thiede, H.-M., & Kehr, W. (1981). Catecholamine metabolism in rat brain. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *318*(1), 29–35.

Toledo, E., Salas-Salvadó, J., Donat-Vargas, C., Buil-Cosiales, P., Estruch, R., Ros, E., … E, R. (2015). Mediterranean Diet and Invasive Breast Cancer Risk Among Women at High Cardiovascular Risk in the PREDIMED Trial. *JAMA Internal Medicine*, *175*(11), 1752.

Tresserra-Rimbau, A., Medina-Remón, A., Pérez-Jiménez, J., Martínez-González, M. A., Covas, M. I., Corella, D., … Lamuela-Raventós, R. M. (2013). Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk: the PREDIMED study. *Nutrition, Metabolism, and Cardiovascular Diseases : NMCD*, *23*(10), 953–9.

Tuck, K. L., & Hayball, P. J. (2002). Major phenolic compounds in olive oil: metabolism and health effects. *The Journal of Nutritional Biochemistry*, *13*(11), 636–644.

Tuck, K. L., Hayball, P. J., & Stupans, I. (2002). Structural Characterization of the Metabolites of Hydroxytyrosol, the Principal Phenolic Component in Olive Oil, in Rats. *Journal of Agricultural and Food Chemistry*, *50*(8), 2404–2409.

Valls-Pedret, C., Lamuela-Raventós, R. M., Medina-Remón, A., Quintana, M., Corella, D., Pintó, X., … Ros, E. (2012). Polyphenol-Rich Foods in the Mediterranean Diet are Associated with Better Cognitive Function in Elderly Subjects at High Cardiovascular Risk. *Journal of Alzheimer's Disease*, *29*(4), 773–782.

Valls-Pedret, C., Sala-Vila, A., Serra-Mir, M., Corella, D., de la Torre, R., Martínez-González, M. Á., … AH, A. (2015). Mediterranean Diet and Age-Related Cognitive Decline. *JAMA Internal Medicine*, *175*(7), 1094.

Valls, R.-M., Farràs, M., Suárez, M., Fernández-Castillejo, S., Fitó, M., Konstantinidou, V., … Solà, R. (2015). Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomised controlled trial. *Food Chemistry*, *167*, 30–35.

van Amerongen, M. J., Diehl, F., Novoyatleva, T., Patra, C., & Engel, F. B. (2010). E2F4 is required for cardiomyocyte proliferation. *Cardiovascular Research*, *86*(1), 92–102.

Victor, V. M., Apostolova, N., Herance, R., Hernandez-Mijares, A., & Rocha, M. (2009). Oxidative stress and mitochondrial dysfunction in atherosclerosis: mitochondria-targeted antioxidants as potential therapy. *Current Medicinal Chemistry*, *16*(35), 4654–67.

Visioli, F., Bellomo, G., Montedoro, G., & Galli, C. (1995). Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis*, *117*(1), 25–32.

Visioli, F., Galli, C., Grande, S., Colonnelli, K., Patelli, C., Galli, G., & Caruso, D. (2003). Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. *The Journal of Nutrition*, *133*(8), 2612–5.

Visioli, F., Wolfram, R., Richard, D., Abdullah, M. I. C. B., & Crea, R. (2009). Olive phenolics increase glutathione levels in healthy volunteers. *Journal of Agricultural and Food Chemistry*, *57*(5), 1793–6.

Vissers, M. N., Zock, P. L., & Katan, M. B. (2004). Bioavailability and antioxidant effects of olive oil phenols in humans: a review. *European Journal of Clinical Nutrition*, *58*(6), 955–65.

Warleta, F., Quesada, C. S., Campos, M., Allouche, Y., Beltrán, G., & Gaforio, J. J. (2011). Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells. *Nutrients*, *3*(12), 839–857.

Wilkening, S., Stahl, F., & Bader, A. (2003). Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties, *31*(8), 1035–1042.

Wiśniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, *6*(5), 359–62.

Wu, Y.-T., Lin, L.-C., & Tsai, T.-H. (2009). Measurement of free hydroxytyrosol in

microdialysates from blood and brain of anesthetized rats by liquid chromatography with fluorescence detection. *Journal of Chromatography A*, *1216*(16), 3501–3507.

Xu, C. L., & Sim, M. K. (1995). *Reduction of dihydroxyphenylacetic acid by a novel enzyme in the rat brain. Biochemical Pharmacology* (Vol. 50). Elsevier.

Young, J., Wahle, K. W. J., & Boyle, S. P. (2008). Cytoprotective effects of phenolic antioxidants and essential fatty acids in human blood monocyte and neuroblastoma cell lines: Surrogates for neurological damage in vivo. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *78*(1), 45–59.

Yu, G., Deng, A., Tang, W., Ma, J., Yuan, C., & Ma, J. (2016). Hydroxytyrosol induces phase II detoxifying enzyme expression and effectively protects dopaminergic cells against dopamine- and 6-hydroxydopamine induced cytotoxicity. *Neurochemistry International*, *96*, 113–120.

Yue, Q.-X., Xie, F.-B., Song, X.-Y., Wu, W.-Y., Jiang, B.-H., Guan, S.-H., … Guo, D.-A. (2012). Proteomic studies on protective effects of salvianolic acids, notoginsengnosides and combination of salvianolic acids and notoginsengnosides against cardiac ischemic-reperfusion injury. *Journal of Ethnopharmacology*, *141*(2), 659–67.

Zhao, B., Ma, Y., Xu, Z., Wang, J., Wang, F., Wang, D., … Jiang, H. (2014). Hydroxytyrosol, a natural molecule from olive oil, suppresses the growth of human hepatocellular carcinoma cells via inactivating AKT and nuclear factor-kappa B pathways. *Cancer Letters*, *347*(1), 79–87.

Zheng, A., Li, H., Xu, J., Cao, K., Li, H., Pu, W., … Hayball, P. J. (2015). Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of db/db mice: role of AMP-activated protein kinase activation. *British Journal of Nutrition*, *113*(11), 1667–1676.

Ziello, J. E., Jovin, I. S., & Huang, Y. (2007). Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *The Yale Journal of Biology and Medicine*, *80*(2), 51–60.

Zou, X., Feng, Z., Li, Y., Wang, Y., Wertz, K., Weber, P., … Liu, J. (2012). Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: activation of Nrf2 and JNK-p62/SQSTM1 pathways. *The Journal of Nutritional Biochemistry*, *23*(8),

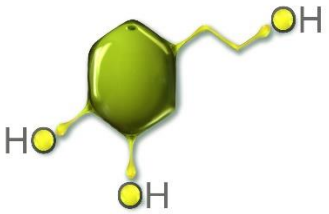
994–1006.

Zrelli, H., Matsuka, M., Araki, M., Zarrouk, M., & Miyazaki, H. (2011). Hydroxytyrosol induces vascular smooth muscle cells apoptosis through no production and PP2A activation with subsequent inactivation of akt. *Planta Medica*.

Zrelli, H., Matsuoka, M., Kitazaki, S., Araki, M., Kusunoki, M., Zarrouk, M., & Miyazaki, H. (2011). Hydroxytyrosol Induces Proliferation and Cytoprotection against Oxidative Injury in Vascular Endothelial Cells: Role of Nrf2 Activation and HO-1 Induction. *Journal of Agricultural and Food Chemistry*, *59*(9), 4473–4482.

Zrelli, H., Matsuoka, M., Kitazaki, S., Zarrouk, M., & Miyazaki, H. (2011). Hydroxytyrosol reduces intracellular reactive oxygen species levels in vascular endothelial cells by upregulating catalase expression through the AMPK–FOXO3a pathway. *European Journal of Pharmacology*, *660*(2), 275–282.

Hypotheses, aim and objectives



Mediterranean countries have low rates of chronic disorders, a situation that is favoured by its traditional dietary habits. The Mediterranean diet (MedDiet) is characterized by the intake of products containing a high content of polyphenols. These compounds are associated with a reduction in the risk of developing certain diseases. Virgin Olive Oil (VOO), the main source of lipids in MedDiet, contains an exclusive phenolic family, with hydroxytyrosol being the main phenolic compound. In the references, there is a considerable number of studies supporting the beneficial effects of HT for maintaining health status and decreasing the ratio of cardiovascular diseases and cancer, among others.

Based on the health effects of olive oil phenolic compounds (OOPCs), which have been proven by *in vitro*, *in vivo* and epidemiological studies, the European Food Safety Authority (EFSA) made a health claim about the protective effects of OOPCs. Since then, an ever-expanding range of products based on HT and other OOPCs has come onto the market. Although the consumption of these widely marketed products seem to be safe, the metabolism and bioavailability of the different OOPCs is not yet well understood, nor is the dose effect on phenolic biotransformation.

Also, the molecular form in which phenolic compounds reach the circulation and are deposited in the target tissues is an important parameter in order to study the behaviour of phenolic compounds at cellular level as well as the molecular mechanism of action.

Furthermore, it is important to highlight that not all the phenolic compounds ingested are absorbed; this mostly depends on the precursor and dose administered. These unabsorbed forms are susceptible to being transformed into their catabolites by microbiota in the small and large intestines and caecum. These formed compounds may possess different activities from their precursors.

Within the context of the interest in the beneficial properties associated with the intake of VOO compounds, the **aim** of this Doctoral Thesis is to study the metabolic fate of HT and its derived compounds (oleuropein and secoiridoids) from the perspective of the biotransformation, bioavailability and bioactivity of plasmatic and microbial metabolites.

Various specific objectives were defined in order to achieve that aim. The structure of the Objectives section is organized in accordance with the different chapters of the Doctoral Thesis:

1. Metabolic fate of hydroxytyrosol and related compounds

I. To establish the pharmacokinetic response and the tissue deposition of hydroxytyrosol plasmatic metabolites depending on the dose administered and the gender effect.

II. To assess, through the sustained intake of hydroxytyrosol and its derivates (oleuropein and secoiridoids) the most bioavailable olive oil phenolic compound (OOPC) and characterize the metabolic effect (plasmatic and microbial metabolism).

III. To develop and validate a rapid, simple, selective and sensitive analytical method to use in bioavailability studies for the determination of plasmatic metabolites derived from OOPCs by simple blood sampling and pre-treatment of blood using dried blood spot cards and direct analysis using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS).

2. Biological effects of hydroxytyrosol and its plasmatic metabolites

IV. To prepare a similar profile of plasmatic metabolites derived from OOPC intake using Caco-2 cell lines, and to evaluate its bioactivity in endothelial cells compared to the native hydroxytyrosol in order to assess its cardioprotective activities.

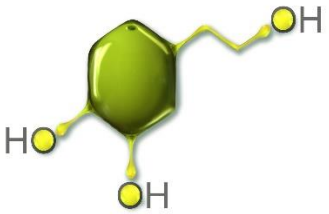
V. To study the molecular mechanism of action of hydroxytyrosol and its plasmatic metabolites by looking into the cellular protein expression of the heart and aorta.

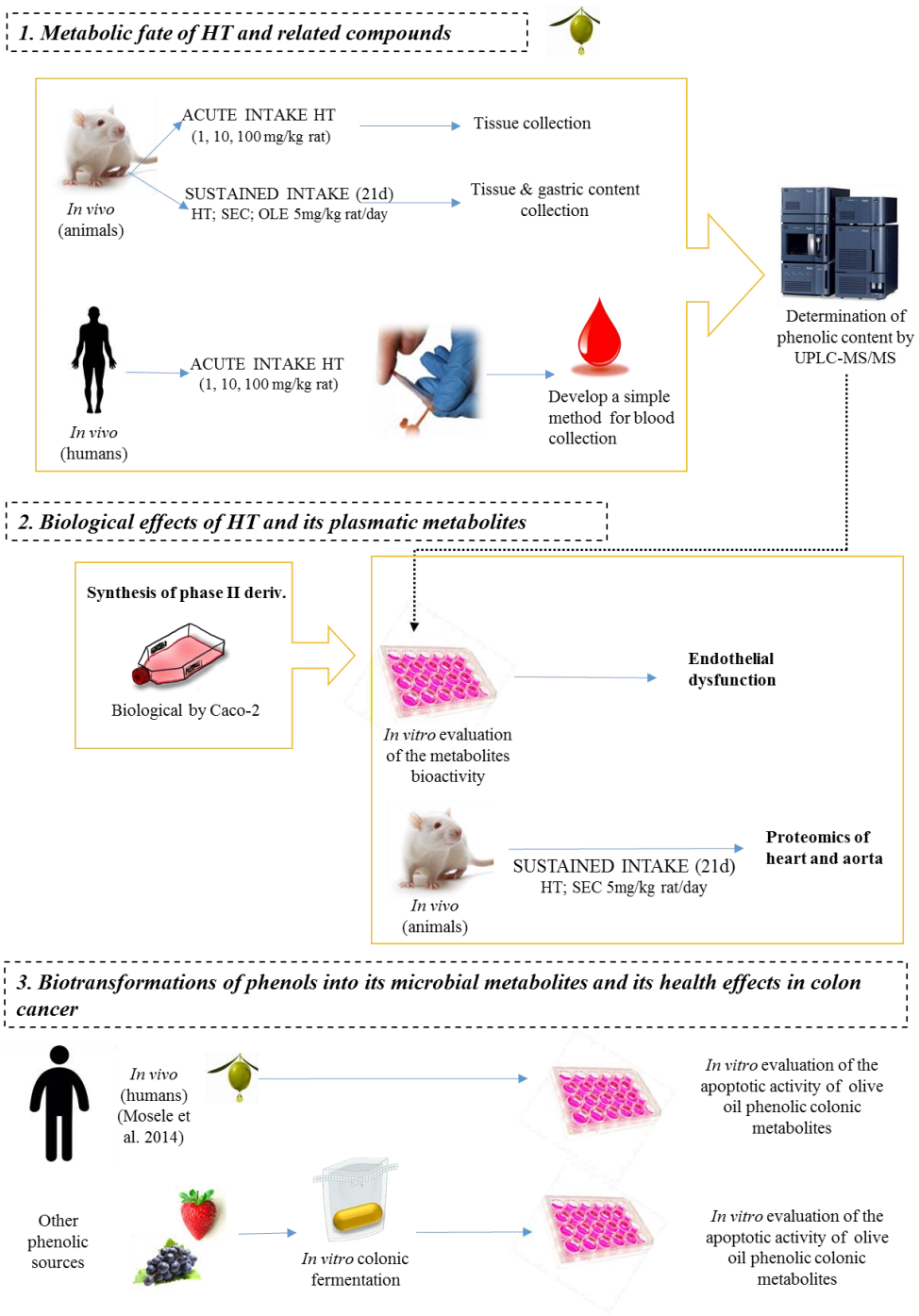
3. Biotransformations of phenols into their microbial metabolites and their health effect in colon cancer cells

VI. To study the apoptotic activity of the individual colonic metabolites found in human faeces after the sustained intake of olive oil enriched with its own phenolic compounds, in an *in vitro* model of colon cancer.

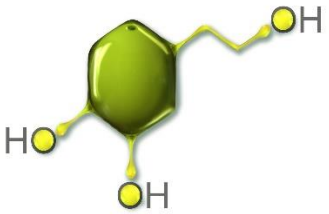
VII. To study the *in vitro* colonic metabolism derived from anthocyanin consumption and the consequent evaluation of the apoptosis of each compound in an *in vitro* model of colon cancer

Work plan




1. Metabolic fate of HT and related compounds
ACUTE INTAKE HT
(1, 10, 100 mg/kg rat)
Tissue collection
SUSTAINED INTAKE (21d)
HT; SEC; OLE 5mg/kg rat/day
Tissue & gastric content collection
In vivo
(animals)
ACUTE INTAKE HT
(1, 10, 100 mg/kg rat)
Develop a simple method for blood collection
In vivo
(humans)
Determination of phenolic content by UPLC-MS/MS
2. Biological effects of HT and its plasmatic metabolites
Synthesis of phase II deriv.
Biological by Caco-2
In vitro evaluation of the metabolites bioactivity
Endothelial dysfunction
SUSTAINED INTAKE (21d)
HT; SEC 5mg/kg rat/day
Proteomics of heart and aorta
In vivo
(animals)
3. Biotransformations of phenols into its microbial metabolites and its health effects in colon cancer
In vivo
(humans)
(Mosele et al. 2014)
In vitro evaluation of the apoptotic activity of olive oil phenolic colonic metabolites
Other phenolic sources
In vitro colonic fermentation
In vitro evaluation of the apoptotic activity of olive oil phenolic colonic metabolites

Results and discussion



Chapter 1

Metabolic fate of hydroxytyrosol and related compounds

Publication 1:

Molecular Nutrition Food Research 59: 1395-1399 (2015)

Publication 2:

Journal of Functional Foods 22: 52-63 (2016)

Publication 3:

Talanta 159: 189-193 (2016)

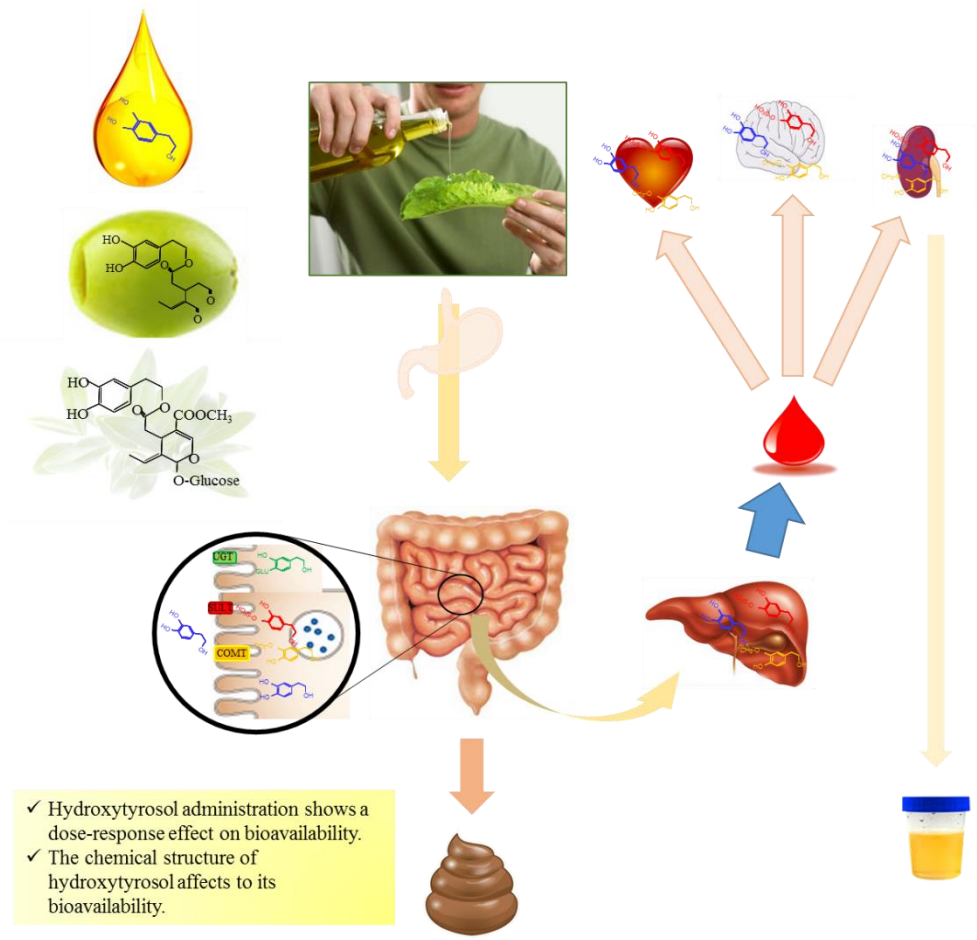


Figure 6: Experimental design and summatory of the main results of the chapter

Publication 1:

Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats.

Molecular Nutrition Food Research 59: 1395-1399 (2015)

FOOD & FUNCTION

DOSE EFFECT ON THE UPTAKE AND ACCUMULATION OF HYDROXYTYROSOL AND ITS METABOLITES IN TARGET TISSUES IN RATS

Maria-Carmen López de las Hazas[1], Laura Rubió[1], Aristotelis Kotronoulas[2,3], Rafael de la Torre[2,4], Rosa Solà[5] and Maria-José Motilva[1]

[1] Food Technology Department, Agrotecnio Research Center, Escuela Técnica Superior de Ingeniería Agraria, University of Lleida, Lleida, Spain
[2] Human Pharmacology and Clinical Neurociences Research Group, IMIM-Institut, Hospital del Mar d'Investigaciones Mèdiques, Barcelona, Spain
[3] Pompeu Fabra University (Group, REGICOR Study Group), IMIM-Research Institute Hospital del Mar, Barcelona, Spain
[4] Cardiovascular Risk and Nutrition Research Group, REGICOR Study Group, IMIM-Research Institute Hospital del Mar, Barcelona, Spain
[5] Unitat de Recerca en Lípids i Arteriosclerosis, CIBERDEM, St. Joan de Reus University Hospital, IISPV, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain

Abstract

Hydroxytyrosol (HT) is the most prominent phenolic compound of virgin olive oil and due to its scientifically validated biological activities it is entering to the market as a potentially useful supplement for cardiovascular disease prevention. The aim of the present study was to investigate the relationship between the HT dose intake and its tissue uptake in rats, and thus, providing complementary information in relation to the target-dose relationship. Rats were given a refined olive oil enriched with HT at different doses (1, 10, and 100 mg/kg) and they were sacrificed after 5 h to ensure the cell tissue uptake of HT and its metabolites. Plasma samples and different organs as liver, kidney, heart and brain were obtained, and HT metabolites were analyzed by UPLC-MS/MS. The results showed that HT and its metabolites could be accumulated in a dose-dependent manner basically in the liver, kidney, and brain and were detected in these tissues even at nutritionally relevant human doses. The detection of free HT in liver and kidney was noteworthy. To date, this appears to be the only biologically active form, and thus, it provides relevant information for optimizing the potential applications of HT to prevent certain hepatic and renal diseases. In recent years, HT and its derivatives have led to a great interest from the virgin olive oil producers and manufacturers of nutraceutical supplements. The increasing interest in HT is mainly due to the European Food Safety Agency (EFSA) Panel on Dietetic Products, Nutrition, and Allergies (NDA) scientific opinion that established a cause-and-effect relationship between the consumption of olive oil polyphenols and protection of LDL particles from oxidative damage [1]. Based on this positive opinion, the health claim *"Olive oil polyphenols contribute to the protection of blood*

lipids from oxidative stress" was included in the list of health claims [2], being the only authorized health claim in the European Union regarding polyphenols and health.

Keywords: Biodistribution; Dose-uptake; Hydroxytyrosol; Phenol metabolites; Tissue disposition

INTRODUCTION

In relation to the mechanisms by which olive phenolic compounds can exercise their cardioprotective activity it is becoming more evident that polyphenols exert their cellular protection by interacting with intracellular signaling pathways involved in pathological processes [3]. After absorption, plasma proteins, and also specific target organs in the human body can be targets of the phenolic metabolites [4]. Therefore, along with the effort to elucidate molecular targets of HT, knowledge about its organ distribution and tissue uptake may increase comprehension of its beneficial effects on health. For this, it is necessary to assess if the HT levels detected in tissues are relevant and sufficiently compatible with those that have been reported as in vitro modulators of biological functions. In relation to the metabolism and tissue uptake of HT, previous studies have been performed in rats, either using intravenously injected [14 C] HT [5] or by oral administration of an olive phenol extract [6]. Although the pharmacokinetic response and tissue distribution of HT was thoroughly investigated in these aforementioned studies, no information is available in the literature regarding the dose-dependent uptake of HT and its main metabolites into target tissues.

In this context, the present study aimed to investigate the relationship between the HT dose intake and tissue uptake in rats, and thus, providing complementary information in relation to the target/dose relationship. For this purpose, liver, kidney, brain, and heart were collected after the administration of three different doses: 1 mg HT/kg rat weight (HT-1) compatible with human dietary habits [7], and two higher doses (10 mg/kg rat weight: HT-10 and 100 mg/kg rat weight: HT-100) that could mimic medium and high exposures of HT through dietary supplements. To ensure efficient cell tissue uptake, the time point of 5 h was selected, which allowed us to assess the complete tissue disposition, especially for the higher dose (100 mg HT/kg rat weight). Detailed materials and methods are contained in the **Supporting Information**.

Results of the plasma analysis showed that free HT and its metabolites were detected and were measurable after the three administered doses (**Table 1**), except for the glucuronide conjugates, which were only detected at the highest doses (HT-10 and HT-100). Plasmatic phenolic levels reached after HT-1 and HT-10 doses resembled to those obtained in human plasma after consumption of phenol-enriched olive oils [8]. In the control plasma (vehicle), none of these compounds were detected, which validates them as

products of HT metabolism. Sulfation was the most relevant conjugation pathway at the three administered doses compared with the glucuronide conjugates. It is important to highlight that the free form of HT was detected in the plasma after the administration of all three doses and that the recovery of free HT appeared to rise in a dose-dependent fashion (from 0.05 µmol/L HT-1 to 12.9 µmol/L HT-100) as with the metabolites (p trend < 0.001).

The dose effect on the tissue uptake of HT was first studied on two metabolic tissues (liver, kidney) obtained 5 h after the intake of the vehicle (refined olive oil) and the different doses of HT (**Table 2**). In general, the nature of the HT metabolites was similar to the plasma and a significant dose-dependent uptake was observed for all metabolites studied (p trend < 0.001) except for homovanillic alcohol (HVAlc) in the kidney (**Table 2**). Additionally, significant differences were observed for the metabolites quantified at all HT doses compared to the vehicle group (p < 0.001). Interestingly, the free active form of HT was detected in both liver and kidney. If we compare our results with those that have been reported as in vitro modulators of biological functions, the concentrations of HT tested in in vitro cell lines showed significant effects in the range of 5–10 µM in the case of kidney [9] and in the range of 1−5 µM for hepatic cell lines [10], which were similar than those concentrations detected in plasma after HT-100 dose and much higher than those detected after the lowest administered doses (**Table 1**). Despite that, recent in vivo studies have reported that HT possesses hepatoprotective effects at lower administered doses (1–10 mg HT/kg) [11, 12]. So it can be hypothesized that apart from the active free active HT, its conjugated forms that were also detected in liver tissue may provide a pool for local or systemic regeneration of HT in vivo. HT metabolites, which might also show biological activities, probably explain the modulation of other pathways/mechanisms from those previously reported related to direct antioxidant/scavenging mechanisms [9, 13]. Indeed, the most recent hypothesis regarding the mechanism of action of polyphenols is that they can exert protective effects modulating signal transduction, cell signaling, gene expression, and cellular communication in several pathways [14].

Besides the metabolic tissues, two target tissues (brain and heart) were studied showing a lower deposition of HT metabolites (**Table 3**). Regarding the brain, some of the HT metabolites were also detected in the vehicle group suggesting that the low concentrations detected could be produced endogenously from dopamine metabolism [15]. It is remarkable, however, that at the highest dose (HT-100), hydroxytyrosol sulfate (HT-S), homovanillic acid (HVAc), homovanillic acid sulfate (HVAc-S), and homovanillic alcohol sulfate (HVAlc-S) presented significant increases compared to the other administered doses, indicating that only at higher doses some metabolites from the HT intake could cross the blood–brain barrier. In fact, dose-dependent accumulation of HT-S and HVAlc-S was

Table 1. Concentration (µmol/L plasma) of HT and its metabolites in rat plasma after 5 h of the administration of different doses of HT and the vehicle (refined oil)

Compound (µmol/L)	Vehicle	HT-1	HT-10	HT-100		
				Mean value	Male	Female
HT	n.d.	0.05 ±0.03 [a]	0.99 ± 0.66 [a]	12.87 ± 5.05 [b]	8.38 ± 4.10	17.36 ± 8.40
HT-S	n.d.	0.12 ± 0.05 [a]	1.28 ± 0.85 [a]	16.49 ± 6.40 [b]	9.82 ± 3.82	22.69 ± 8.40
HT 4'-Gluc	n.d.	n.d.	0.18 ± 0.12 [a]	1.87 ± 1.03 [b]	1.77 ± 1.13	1.93 ± 0.86
HT 3'-Gluc	n.d.	n.d.	0.11 ± 0.10 [a]	1.18 ± 0.69 [b]	0.91 ± 0.49	1.50 ± 0.68
HVAc	n.d.	0.05 ± 0.09 [a]	0.53 ± 0.37 [a]	7.65 ± 2.86 [b]	2.87 ± 0.63	11.21 ± 5.35
HVAc-S	n.d.	0.28 ± 0.15 [a]	2.17 ± 1.08 [a]	14.94 ± 5.42 [b]	13.30 ± 5.01	16.59 ± 5.83
HVAlc	n.d.	0.12 ± 0.09 [a]	0.14 ± 0.10 [a]	0.18 ± 0.16 [a]	0.09 ± 0.09	0.25 ± 0.17
HVAlc-S	n.d.	0.09 ± 0.03 [a]	0.38 ± 0.16 [b]	2.21 ± 1.09 [c]	2.32 ± 0.65	2.16 ± 1.26
HVAlc-Gluc	n.d.	n.d.	0.12 ± 0.06 [a]	0.57 ± 0.32 [b]	0.74 ± 0.43	0.48 ± 0.18

Effect of gender in plasma concentration at HT-100 dose. HT, hydroxytyrosol; HT-S, hydroxytyrosol-sulfate; HT 4-Gluc, hydroxytyrosol 4' glucuronide; HT 3-Gluc, hydroxytyrosol 3' glucuronide; HVAc, homovanillic acid; HVAc-S, homovanillic acid-sulfate; HVAlc, homovanillic alcohol; HVAlc-S, homovanillic alcohol-sulfate; HVAlc- Gluc, homovanillic alcohol-glucuronide. Values are mean ± SD (n = 8). Values male and female are mean ± SD (n = 4). Different superscript letters denote significant differences between doses in the same row ($p < 0.001$). * Means significant differences between genders in the same row of HT-100 ($p < 0.05$). n.d., not detected.

observed in the brain (p trend < 0.001) (**Table 3**). A previous study reported that the administration of 100mg/kg of HT in mice enhanced cytoprotection and the resistance of dissociated brain cells to oxidative stress [16]. Thus, it could be hypothesized that the accumulation of these metabolites in the brain after a pharmacological dose of HT could exert a neuroprotective activity in the central nervous system. In the present study, the free form of HT was not detected in the brain, in contrast to a previous study, in which 100 mg/kg of HT was administered through the femoral vein [17] and considerable amounts of free HT were detected in the brain. Compared with the oral route of our study, intravenous administration could explain the higher exposure of HT to the brain tissue and its detection in its free form.

In contrast to other tissues, HT metabolites were not detected in the heart in HT-1 and HT-10 groups (**Table 3**), being HT-S the main metabolite quantified after the HT-100. Unlike in the liver and kidney, free HT

was not detected in the heart. However, free HT and its metabolites were detected in plasma even at the nutritionally relevant dose (**Table 1**), which could explain the cardioprotective effects of virgin olive oil phenols previously described at different circulation targets. Reported in vivo studies indicate that HT can reduce endothelial activation [18], inhibit platelet aggregation [19], and reduce the plasma-reduced homocysteine concentration [20], effects that have been associated with cardiovascular protection and could be related to the presence of circulating HT and its metabolites rather than an accumulation in the heart.

Interestingly, in samples obtained from HT-100 group, a significant gender effect was observed in the plasma (**Table 1**), liver and kidney (**Table 2**). Plasmatic concentrations of free HT and its main metabolites HT-S and HVAc were significantly higher ($p < 0.05$) in females (**Table 1**). Similarly, the concentrations of free HT and metabolites (HT-S, HVAc, HVAc-S, and HVAlc-S) were significantly higher ($p < 0.05$) in the liver from females, this being the tissue where the gender effect was more striking (**Table 2**). No gender effect was observed in the uptake of HT metabolites in the brain and heart (data not shown). Our results are in line with previous studies in which genistein and daidzein, two major isoflavones in soy, also presented an enhanced oral bioavailability in females than in males [21]. The main reason that could explain these differences seems to be the different expression profiles of metabolizing enzymes and efflux transporters in the main metabolic disposition organs, such as the intestine, liver, and than in males [21].

The main reason that could explain these differences seems to be the different expression profiles of metabolizing enzymes and efflux transporters in the main metabolic disposition organs, such as the intestine, liver, and kidney [22]. In other studies, pulsatile versus continuous **Table 2.** Concentration (nmol/g fresh tissue) of HT and its metabolites in rat metabolic tissues (liver and kidney) after 5 hours of the administration of different doses of HT. Effect of sex in the tissue concentration of HT-100 dose. release of growth hormone in male and female rats, respectively, has been suggested as a major reason for the sex-dependent differences in the expression profiles of the hepatic phase I metabolizing enzymes [23].

Summarizing, our study showed that HT and its metabolites could be accumulated in a dose-dependent manner basically in the liver, kidney and brain and were detected in these tissues even at nutritionally relevant human dose, a dose that was not previously studied in tissue disposition. The detection of free HT in liver and kidney was noteworthy.

To date, this appears to be the only biologically active form, and thus, it provides relevant information for optimizing the potential applications of HT to prevent certain hepatic and renal diseases. However, the detection of greater

Table 2. Concentration (nmol/g fresh tissue) of HT and its metabolites in rat metabolic tissues (liver and kidney) after 5 hours of the administration of different doses of HT. Effect of sex in the tissue concentration of HT-100 dose.

Compound (nmol/g fresh tissue)	LIVER					
	Vehicle	HT-1	HT-10	HT-100		
				Mean value	Male	Female
HT	n.d.	0.1 ± 0.01 [a]	1.11 ± 0.59 [a]	17.54 ± 12.96 [b]	7.66 ± 1.68	27.42 ± 11.51
HT-S	n.d.	0.02 ± 0.01 [a]	1.26 ± 0.70 [a]	27.42 ± 26.53 [b]	8.76 ± 1.84	46.09 ± 26.62
HT 4-Gluc	n.d.	n.d.	n.d.	0.21 ± 0.09	0.16 ± 0.12	0.26 ± 0.03
HT 3-Gluc	n.d.	n.d.	n.d.	0.22 ± 0.14	0.14 ± 0.07	0.31 ± 0.14
HVAc	n.d.	n.d.	0.03 ± 0.05 [a]	2.67 ± 3.20 [b]	0.50 ± 0.17	4.85 ± 3.46
HVAc-S	n.d.	0.04 ± 0.02	0.56 ± 0.33 [a]	6.71 ± 3.28 [b]	4.92 ± 3.65	8.49 ± 1.63
HVAlc	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HVAlc-S	n.d.	n.d.	0.32 ± 0.13 [a]	5.23 ± 5.28 [b]	1.80 ± 0.27	8.66 ± 5.74
HVAlc-Gluc	n.d.	n.d.	n.d.	0.04 ± 0.03	0.02 ± 0.01	0,07 ± 0.02

Compound (nmol/g fresh tissue)	KIDNEY					
	Vehicle	HT-1	HT-10	HT-100		
				Mean value	Male	Female
HT	n.d.	n.d.	2.73 ± 2.33 [a]	33.95 ± 10.31 [b]	26.10 ± 3.28	41.80 ± 10.35
HT-S	0.14 ± 0.16 [a]	0.48 ± 0.1 [a]	10.08 ± 5.74 [b]	82.85 ± 38.98 [c]	78.88 ± 5.70	86.81 ± 29.76
HT 4-Gluc	n.d.	0.02 ± 0.05 [a]	0.25 ± 0.12 [b]	0.89 ± 0.39 [c]	0.88 ± 0.09	1.09 ± 0.10
HT 3-Gluc	n.d.	0.02 ± 0.05 [a]	0.25 ± 0.12 [b]	0.99 ± 0.45 [c]	0.94 ± 0.07	1.04 ± 0.31
HVAc	0.26 ± 0.17 [a]	0.33 ± 0.13 [a]	1.26 ± 0.76 [b]	7.90 ± 2.25 [c]	6.73 ± 2.89	9.07 ± 1.65
HVAc-S	1.22 ± 0.51 [a]	0.98 ± 0.31 [a]	2.32 ± 0.74 [b]	6.90 ± 1.33 [c]	8.49 ± 2.98	5.30 ± 0.05
HVAlc	0.23 ± 0.27 [a]	0.21 ± 0.22 [a]	0.46 ± 0.17 [b]	0.30 ± 0.30 [a]	0.20 ± 0.23	0.40 ± 0.01
HVAlc-S	0.26 ± 0.02 [a]	0.45 ± 0.09 [a]	3.23 ± 1.38 [b]	20.07 ± 4.98 [c]	19.31 ± 4.63	21.88 ± 12.71
HVAlc-Gluc	n.d.	0.14 ± 0.03 [a]	0.22 ± 0.03 [b]	0.39 ± 0.06 [c]	0.37 ± 0.10	0.41 ± 0.07

Effect of gender in the tissue disposition at HT-100 dose. HT, hydroxytyrosol; HT-S, hydroxytyrosol-sulfate; HT 4-Gluc, hydroxytyrosol 4' glucuronide; HT 3-Gluc, hydroxytyrosol 3' glucuronide; HVAc, homovanillic acid; HVAc-S, homovanillic acid-sulfate; HVAlc, homovanillic alcohol; HVAlc-S, homovanillic alcohol-sulfate; HVAlc- Gluc, homovanillic alcohol-glucuronide. Values are mean ± SD (n = 8). Values male and female are mean ± SD (n = 4). Different superscript letters denote significant differences between doses in the same row ($p < 0.001$). *Means significant differences between genders in the same row of HT-100 ($p < 0.05$). n.d., not detected.

amounts of HT metabolites in tissues, especifically the sulfate conjugates, indicates that the bioactivities of these metabolites are also worthy of future research in order to understand the clinical implications of olive oil phenolics. The obtained results regarding the target-dose relationship of HT and its metabolites, together with the literature data on the biological effects, allow increasing our understanding of the health beneficial effects of HT.

This study was supported by the Spanish Ministry of Education and Science (AGL2012-40144-C03-03, AGL2012-40144- C03-02 and AGL2012-40144-C03-

01 projects), by the University of Lleida through the M.C. López de las Hazas and L. Rubió grant. CIBERobn is an iniciative of Instituto de Salud Carlos III (ISCIII). The authors are grateful for the generous gift of hydroxytyrosol from Seprox Biotech, Madrid, Spain.

The authors have declared no conflict of interest

Table 3. Concentration (nmol/g fresh tissue) of HT and its metabolites in rat target tissues (heart and brain) after 5 hours of the administration of different doses of HT.

Compound	**BRAIN**			
(nmol/g fresh tissue)	**Vehicle**	**HT-1**	**HT-10**	**HT-100**
HT	n.d.	n.d.	n.d.	n.d.
HT-S	0.26 ± 0.00 [a]	0.32 ± 0.05 [a]	0.35 ± 0.10 [a]	1.26 ± 0.72 [b]
HT 4-Gluc	n.d.	n.d.	n.d.	0.14 ± 0.03
HT 3-Gluc	n.d.	n.d.	n.d.	0.15 ± 0.02
HVAc	0.34 ± 0.01 [a]	0.39 ± 0.04 [a]	0.32 ± 0.02 [a]	0.40 ± 0.05 [b]
HVAc-S	0.50 ± 0.03 [a]	0.72 ± 0.10 [b]	0.49 ± 0.08 [a]	0.58 ± 0.12 [b]
HVAlc	n.d	n.d.	n.d.	n.d.
HVAlc-S	0.14 ± 0.08 [a]	0.22 ± 0.01 [a]	0.41 ± 0.12 [b]	2.58 ± 1.26 [c]
HVAlc-Gluc	n.d.	n.d.	n.d.	n.d.
	HEART			
	Vehicle	**HT-1**	**HT-10**	**HT-100**
HT	n.d.	n.d.	n.d.	n.d
HT-S	n.d.	n.d.	n.d.	2.73 ± 2.19
HT 4-Gluc	n.d.	n.d.	n.d.	0.02 ± 0.02
HT 3-Gluc	n.d.	n.d.	n.d	n.d
HVAc	n.d.	n.d.	n.d.	0.08 ± 0.10
HVAc-S	n.d.	n.d.	n.d.	0.19 ± 0.22
HVAlc	n.d.	n.d.	n.d.	n.d
HVAlc-S	n.d.	n.d.	n.d.	0.42 ± 0.33
HVAlc-Gluc	n.d.	n.d.	n.d.	n.d

HT: hydroxytyrosol; HT-S: hydroxytyrosol-sulfate; HT 4-Gluc: hydroxytyrosol 4' glucuronide; HT 3-Gluc: hydroxytyrosol 3' glucuronide; HVAc: homovainillic acid; HVAc-S: homovainillic acid-sulfate; HVAlc: homovainillic alcohol; HVAlc-S: homovainillic alcohol-sulfate; HVAlc-Gluc: homovainillic alcohol-glucuronide. Values are mean ± SD (n=8). abc mean significant differences between doses in the same row ($p < 0.001$); n.d. not detected.

REFERENCES

[1] EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), Scientific opinion on the substantiation of health claims related to polyphenols in olive. Pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA J. 2011, 9, 2033.

[2] Commission Regulation (EU) 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. O.J. 2012, L136/1.

[3] Konstantinidou, V., Covas, M. I., Muñoz-Aguayo, D., Khymenets, O. et al., In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. FASEB J. 2010, 24, 2546–2557.

[4] Rohn, S., Possibilities and limitations in the analysis of covalent interactions between phenolic compounds and proteins. Food Res. Int. 2014, 65, 13–19.

[5] D'Angelo, E., Manna, C., Migliardi, V., Mazzoni, O. et al., Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. Drug. Metabol. Dis. 2001, 29, 1492–1498.

[6] Serra, A., Rubió, L., Borràs, X., Macià, A. et al., Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. Mol Nutr. Food Res. 2012, 56, 486–496.

[7] Kotronoulas, A., Pizarro, N., Serra, A., Robledo, P. et al., Dose-dependent metabolic disposition of hydroxy-tyrosol and formation of mercapturates in rats. Pharmacol. Res. 2013, 77, 47–56.

[8] Rubió, L., Valls, R. M., Macià, A., Pedret, A. et al., Impact of olive oil phenolic concentra-tion on human plasmatic phenolic meta-bolites. Food Chem. 2012, 135, 2922–2929.

[9] Deiana, M., Incani, A., Rosa, A., Atzeri, A. et al., Hydroxytyrosol glucuronides protect renal tubular epithelial cells against H(2)O(2) induced oxidative damage. Chem. Biol. Interact 2011, 193, 232–239.

[10] Giordano, E., Davalos, A., Nicod, N., Visioli, F., Hydroxytyrosol attenuates tunicamycin induced endoplasmic reticulum stress in human hepatocarcinoma cells. Mol. Nutr. Food Res. 2014, 58, 954–962.

[11] Pan, S., Liu, L., Pan, H., Ma, Y. et al., Protective effects of hydroxytyrosol on liver ischemia/ reperfusion injury in mice. Mol. Nutr. Food Res. 2013, 57, 1218–1227

[12] Poudyal, H., Campbell, F., Brown, L. Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in high carbohydrate high fat-fed rats J. Nutr. 2010 140, 946–953.

[13] Khymenets, O., Fitó, M., Tourino, S., Munoz-Aguayo, D. et al. Antioxidant

activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. Drug Metab. Dispos. 2010, 38, 1417–1421.

[14] Kang, N. J., Shin, S. H., Lee, H. J., Lee, K. W., Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis. Pharmacol. Ther. 2011, 130, 310–324.

[15] Gallardo, E., Madrona, A., Palma-Valdés, R., Trujillo, M. et al., The effect of hydroxytyrosol and its nitroderivatives on catechol-O-methyltransferase activity in rat striatal tissue. RSC Adv. 2014, 4, 61086–61091.

[16] Schaffer, S., Podstawa, M., Visioli, F., Bogani, P. et al., Hydroxytyrosol-rich olive mill wastewater extract protects brain cells in vitro and ex vivo. J. Agric. Food Chem. 2007, 55, 5043–5049.

[17] Wu, Y. T., Lin, L. C, Tsai, T. H., Measurement of free hydroxytyrosol in microdialysates from blood and brain of anesthetized rats by liquid chromatography with fluorescence detection. J. Chromatogr. A. 2009, 1216, 3501–3507.

[18] Carluccio, M. A., Siculella, L., Ancora, M. A., Massaro, M. et al., Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: Antiatherogenic properties of Mediterranean diet phyto-chemicals. Arterioscl. Thromb. Vasc. Biol. 2003, 23, 622–629.

[19] Visioli, F., Caruso, D., Virgin olive oil study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. Eur. J. Clin. Nutr. 2005, 44, 121–127.

[20] Priora, R., Summa, D., Administra-tion of minor polar compound-enriched extra virgin olive oil decreases platelet aggregation and the plasma concentration of reduced homocysteine in rats. J. Nutr. 2008, 138, 36–41.

[21] Kulkarni, K. H., Yang, Z., Niu, T., Hu, M., Effects of estrogen and estrus cycle on pharmacokinetics, absorption, and disposition of genistein in female Sprague-Dawley rats. J. Agric. Food Chem. 2012, 60, 7949–7956.

[22] Buckley, D. B., Klaassen, C. D., Mechanism of gender-divergent UDP-glucuronosyl-transferase mRNA expression in mouse liver and kidney. Drug Metab. Dispos. 2009, 37, 834–840.

[23] Waxman, D. J., Holloway, M. G., Sex differences in the expression of hepatic drug metabolizing enzymes. Mol. Pharmacol. 2009, 76, 215–228.

SUPPORTING INFORMATION

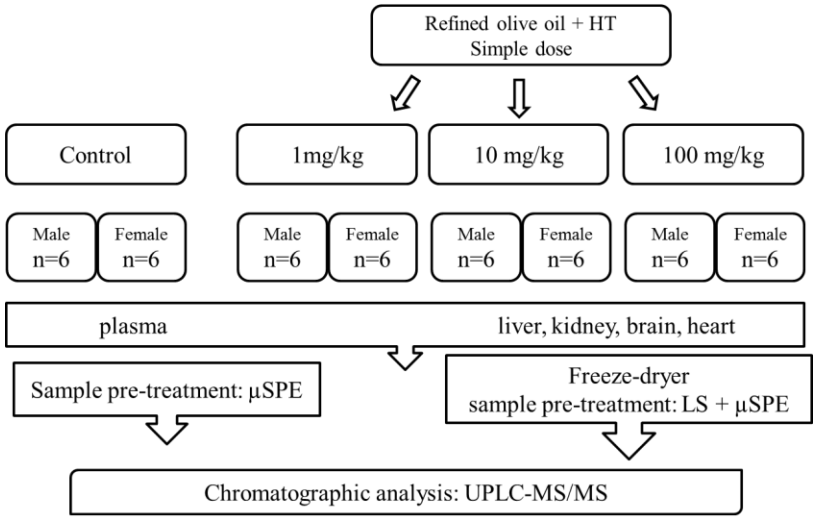


Figure S1. Scheme of study protocol.

1. MATERIALS AND METHODS

1.1. Chemicals and reagents

Hydroxytyrosol (Seprox Biotech, Madrid, Spain), homovanillic acid (Fluka Co., Steinheim, Switzerland) and catechol as the internal standard (IS) (Sigma-Aldrich, St. Louis, MO, USA) were of commercial origin. The hydroxytyrosol metabolites, hydroxytyrosol-3'-O-glucuronide (purity, 97.8%), hydroxytyrosol-4'-O-glucuronide (purity, 96.4%), and homovanillic alcohol-4-O-glucuronide (purity, 99.3%) were synthesized according to the method reported by Khymenets et al. [1]. The hydroxytyrosol-3-O-sulphate was custom synthesized by Toronto Research Chemicals Inc (Toronto, Ontario, Canada), and its purity was 98.4%. L(+)-Ascorbic acid (reagent grade), methanol (HPLC-grade), and acetonitrile (HPLC-grade) were purchased from Scharlab (Barcelona, Spain). Ortophosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system(Millipore Corp., Medford, MA, USA).

1.2. Experiments in animal models

Male and female Sprague-Dawley rats weighing 259.4 ± 21.5 g and 227.1 ± 9.8 g, respectively, were used in the experiments. The animals were housed two per cage in a temperature (21 ± 1ºC) and humidity (55 ± 10%) controlled room with a 12-h light/dark cycle (lights on at 08:00 h). The experiments were performed during the light period. Food and water were available *ad libitum* in both the home and metabolic cages. The animal procedures

were conducted in accordance with the guidelines of the European Community Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA - Universitat de Lleida, reference 7675).

In all the experiments, HT was suspended in refined olive oil (vehicle) and administered at a volume of 0.3 mL per 100 g of body weight by intragastric gavage. Different groups of male (n = 23) and female (n = 23) rats were treated with a single dose of 1 mg/kg (HT-1) (n = 5 male and n = 6 female), 10 mg/kg (HT-10) (n = 6 male and n = 5 female) and 100 mg/kg (HT-100) (n = 6 male and n = 6 female) of HT or vehicle (n = 6 male and n = 6 female) (**Supporting Information Figure S1**). In all cases, the HT dose was adjusted to the rat weight. On treatment days, the rats received HT or vehicle and were immediately placed in the cages for 5 h. At the end of the experiment, the rats were anaesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg), injected intraperitoneally at 0.1 mL per 100 g of body weight. Blood was collected by intracardiac puncture in heparinized tubes and plasma samples were obtained by centrifugation (2000 × g, 30 min at 4 ºC). Different tissues (kidney, liver, brain and heart) were excised from the rats, frozen in liquid N_2 immediately after collection, stored at −80 ºC and freeze-dried for phenolic analysis.

1.3. Quantification of HT and its metabolites in rat plasma and tissues

The tissues were sequentially pretreated with a combination of liquid-solid extraction (LSE) [2], and micro elution solid-phase extraction (µSPE). Briefly, for the analysis of LSE, 60 mg of freeze-dried tissues were mixed with 50 µL of ascorbic acid (1%), 100 µL of phosphoric acid (4%), and 50 µL of catechol as internal standard solution. Each sample was treated four times with 400 µL of extraction solution (water/methanol/phosphoric acid 4%; 94:4.5:1.5, v/v/v). Then, the samples were sonicated (S-150D Digital SonifierR Cell Disruptor, Branson, Ultrasonidos S.A.E., Barcelona, Spain) for 30 s maintaining the sample in an ice water bath, and then centrifuged for 15 min at 8784 g at 20 ºC (Hettich Universal 320-R, Tuttlingen, Germany). The supernatants were collected, and an aliquot of the extract was pre-treated by µSPE (OASIS HLB µElution Plates 30 mm, Waters, Milford, MA, USA) in order to clean up the sample before the chromatographic analysis. Briefly, micro-cartridges were conditioned sequentially by using 250 µL of methanol and acidified Milli-Q water. Then, 350 µL of the tissue extract and 350 µL of phosphoric acid (4%) were loaded, and the loaded plates were washed with 200 µL of Milli-Q water and 200 µL of methanol 5%. Finally, the elution of the retained phenolic compounds was done with 100 µL of methanol. The plasma samples were pretreated by µSPE following the same method described above.

The analysis of HT and its metabolites in the plasma and tissues was performed using a UPLC coupled to tandem MS (MS/MS). The chromatographic system is an AcQuity Ultra-Performance TM liquid chromatography and tandem MS from Waters (Milford MA, USA), as reported in our previous study [2]. The column was an Acquity UPLC™ BEH C18 (100 x 2.1 mm i.d., 1.7 µm), also from Waters (Milford MA, USA). The mobile phase was 0.2% acetic acid as solvent A and acetonitrile as solvent B, and the flow-rate was 0.4 mL/min. The

UPLC system was coupled to a PDA detector, AcQuity UPLC™ and a triple quadrupole detector (TQD™) mass spectrometer (Waters). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode. The ionization source parameters were the reported in our previous study (Ref Suárez 2009). HT and its generated metabolites were quantified by using the selected reaction monitoring (SRM) mode. Two transitions were acquired and the most sensitive was used for quantification and the second one for identification purposes. **Supporting Information Table S1** shows the MS/MS transitions for quantification and confirmation as well as the cone voltage and collision energy values for HT and its metabolites.

1.4. Statistical analysis

The differences between HT doses for concentrations of HT and its metabolites in plasma and in the studied tissues were evaluated using a one-way ANOVA, followed by the LSD post-hoc test. Differences were considered statistically significant at $p<0.001$ for dose response analysis, and $p<0.05$ for gender analysis. The linear trend was found using a univariate analysis of the general lineal model (p *trend*<0.001). All analyses were performed by SPSS Statistics 20 software, IBM 2011.

REFERENCES

[1] Khymenets, O., Joglar, J., Clapés, P., Parella, T., Covas, M-I., de la Torre, R. Biocatalyzed synthesis and structural characterization of monoglucuronides of hydroxytyrosol, tyrosol, homovanillic alcohol, and 3-(4'-hydroxyphenyl) propanol. *Advanced Synth. Catal.* 2006, *348*, 2155-2162.

[2] Serra, A., Rubió, L., Borràs, X., Macià, A., et al., Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. *Mol Nutr. Food Res.* 2012, *56*, 486–496.

Table S1. Selected Reaction Monitoring (SRM) conditions for the analysis of hydroxytyrosol and its metabolites by UPLC-MS/MS.

Phenolic compounds	Molecular weight (g/mol)	SRM (quantification)	Cone voltage (V)	Collision energy (eV)	SRM (identification)	Cone voltage (V)	Collision energy (eV)
Catechol (IS)	110	108.9 > 90.9	40	15	- - -		
Hydroxytyrosol	154	153 > 123	35	10	153 > 95	35	25
Hydroxytyrosol glucuronide	330	329 > 153	40	20	329 > 123	40	25
Hydroxytyrosol sulphate	234	233 > 153	40	15	233 > 123	40	20
Homovanillic alcohol sulphate	248	247 > 167	40	15	247 > 152	40	25
Homovanillic alcohol glucuronide	344	343 > 167	40	20	343 > 152	40	35
Homovanillic acid sulphate	262	261 > 181	40	15	261 > 137	40	25
Homovanillic acid glucuronide	358	357 > 181	40	20	357 > 137	40	25
Hydroxytyrosol acetate sulphate	275	275 > 195	35	15	195 > 135	35	10

Publication 2

Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids.

Journal of Functional Foods 22: 52-63 (2016)

Available online at www.sciencedirect.com

ScienceDirect

journal homepage:
www.elsevier.com/locate/jff

DIFFERENTIAL ABSORPTION AND METABOLISM OF HYDROXYTYROSOL AND ITS PRECURSORS OLEUROPEIN AND SECOIRIDOIDS

María-Carmen López de las Hazas[a], Carme Piñol[b], Alba Macià[a], María-Paz Romero[a], Anna Pedret[c], Rosa Solà[c], Laura Rubió[c, *], Maria-José Motilva[a].

[a] Food Technology Department, Agrotecnio Research Center, Escola Tècnica Superior d'Enginyeria Agrària, University of Lleida, Avda/Alcalde Rovira Roure 191, 25198 Lleida, Spain

[b] Department of Medicine, Facultat de Medicina, University of Lleida, Avda/Alcalde Rovira Roure 80, 25198 Lleida, Spain

[c] Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomedica en Red de Diabetes y Enfermedades Metabolicas Asociadas (CIBERDEM), University Hospital Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Sant Llorenç, 21, 43201 Reus, Spain

Abstract

This study investigated and compared the absorption, metabolism, and subsequently, the tissue distribution and excretion of hydroxytyrosol (HT) administered either in its free form or through its naturally occurring esterified precursors, namely oleuropein (OLE) and its aglycone forms known as secoiridoids (SEC). Here, rats were fed a diet supplemented with the equivalent of 5 mg phenol/kg/day for 21 days and the HT metabolites in the gastrointestinal digesta (stomach, small intestine and caecum), plasma, urine and metabolic tissues (liver and kidney) were analysed. Compared to HT and SEC, OLE showed greater stability during digestion, and, consequently, the bioavailability based on the urine excretion of HT metabolites was higher. OLE, as a glycoside molecule, reached the colon unaltered generating more diverse microbial metabolites. In terms of bioavailability, findings suggest that OLE might be the most suitable precursor of HT for incorporation into foods or nutraceutical formulations.

Keywords: Hydroxytyrosol; Microbial catabolism; Metabolic pathways; Olive oil, Phenolic compounds

1. INTRODUCTION

In recent years, numerous epidemiological and dietary intervention studies have demonstrated that plant-food consumption, attributed to bioactive chemical compounds, is directly associated with a lower risk of suffering chronic disease

(Sikand, Kris-Etherton, & Boulos, 2015). The growing interest in these phyto-chemicals has enhanced the development and marketing of food supplements and nutraceuticals, which have become the fastest growing segments of the food industry. Over the last decade, different engineering technologies (such as high pressure, supercritical fluid and molecular distillation) have been applied to extract components from raw materials or waste products and optimize their form and chemical structure to make them suitable for inclusion in new food products (Herrero, del Pilar Sánchez-Camargo, Cifuentes, & Ibáñez, 2015).

Specifically, an important expansion of the nutraceutical market has been observed with olive products due to the health benefits associated with their polyphenols (de Bock et al., 2013). The evidence of the protective effect of virgin olive oil (VOO) polyphenols for cardiovascular diseases (CVD) has been strengthened by the approved health claim based on the scientific report of the Panel on Dietetic Products, Nutrition and Allergies of EFSA (European Food Safety Authority) (COMMISSION REGULATION (EU) N° 432/2012 of 16 May 2012):

> Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil.

The most abundant polyphenols in olive fruit are oleuropein (OLE), demethyl-oleuropein, ligstroside and nüzhenide (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011). During mechanical extraction of VOO, hydrolysis reactions take place due to the activity of endogenous β-glucosidases, and aglycone derivatives known as secoiridoids (SEC) are released. From a chemical standpoint, SEC are characterized by the presence of elenolic acid (EA) or some of its derivatives, and hydroxytyrosol (HT) or tyrosol (TYR) in their molecule. The most abundant SEC in VOO are the dialdehydic form of decarboxymethyl EA linked to HT termed 3,4-DHPEA-EDA and the isomer of oleuropein aglycone (3,4-DHPEA-EA).

Based on the potential benefits of HT and its derivatives, different extracts or ingredients are available on the market. These are mainly obtained from the olive leaf, olive oil by-products (pomace) or waste-water, and the chemical nature of the phenolic compounds varies with the source. Generally, the extracts obtained from olive leaves are rich in OLE, the pomace yield extracts are rich in SEC and the waste-water gives extracts rich in HT. In this context, phenolic extracts derived from olive products can provide HT in different chemical structures, which could influence its uptake and metabolism. In the case of flavanols, it has been shown that their stereochemical configuration has a profound influence on their uptake and metabolism and their bioefficacy (Ottaviani et al., 2011). Similarly, the biological activity of the equol, a metabolite produced in vivo from the soy

phytoestrogen daidzein by the action of gut microflora, depends on the enantiomers R-equol and S-equol (Muthyala et al., 2004).

Considering the wide range of phenolic extracts derived from olive products available on the market, this study was designed to study differences in the absorption and metabolism of HT either in its free form or through its natural occurring esterified precursors: oleuropein (OLE) and its aglycone forms known as secoiridoids (SEC). For this proposal, rats were fed a diet supplemented with the equivalent of 5 mg phenol/kg rat weight for 21 days. The metabolism in the gastro-intestinal tract (GIT) was studied through the determination of the phenolic compounds and their metabolites in the contents (digesta) of the stomach, small intestine and caecal and in the faeces. Plasma, 24 h-urine and the metabolic tissues of liver and kidney were also analysed.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Hydroxytyrosol (HT) was provided by Seprox Biotech (Madrid, Spain), oleuropein by Extrasynthese (Genay, France), and homovanillic acid by Fluka Co. (Buchs, Switzerland). Catechol, p-hydroxyphenyl-acetic acid, 3,4-dihydroxyphenyl-acetic acid, 3-(4-hydroxyphenyl)propionic acid and hippuric acid were from Sigma-Aldrich (St. Louis, MO, USA). Ortophosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Hydroxytyrosol-3′-O-glucuronide (97.8% of purity), hydroxyl-tyrosol-4′-O-glucuronide (96.4% of purity), and homovanillic alcohol-4′-O-glucuronide (99.3% of purity) were synthe-sized according to the method reported by (Khymenets et al., 2006). Hydroxytyrosol-3-O-sulphate was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Methanol and acetonitrile (HPLC-grade) were purchased from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Medford, MA, USA).

2.2. Secoiridoid extract (SEC)

Secoiridoid extract was obtained from olive pomace by Pressurized Liquid Extraction (PLE), using an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA, USA) based on our previously described method (Suárez, Romero, Ramo, Macià, & Motilva, 2009). Briefly, 10 g of freeze-dried olive-cake were mixed with 5 g of diatomaceous earth. The best extraction of phenolic compounds was obtained with these conditions: ethanol/water (80:20, v/v) solvent at 80 °C, 60% setting volume and two static cycles of 5 min in each extraction. Finally, the sample was purged with nitrogen (≥99.99% purity, Alphagaz, Madrid, Spain). After that, the ethanol was rotary evaporated until its elimination (Buchi Rotavapor, New Castle, DE, USA). Aqueous extract was freeze-dried and stored at −80 °C in N2 atmosphere until use. The extract was mainly composed of 3,4-DHPEA-EDA (85%) and contained

minor proportions of free HT and other secoiridoids providing HT such as 3,4-DHPEA-EA (**Table S1** Supplementary Material). To calculate the administered dose of 5 mg/kg weight of SEC, only 3,4-DHPEA-EDA was considered as it is the main secoiridoid derivative providing HT.

2.3. Animals and experimental procedure

Thirty-two male and female Wistar rats weighing between 300 and 350 g were obtained from Charles River Laboratories (Barcelona, Spain). They were separated into four groups of 8 rats in each group (4 males, 4 females). Group A: Control, group B: hydroxytyrosol (HT), group C: secoiridoids (SEC) and group D: oleuropein (OLE) (**Fig. 1**). During the 21-day experiment, the animals were housed two per cage at a temperature- (21 ± 1 °C) and humidity controlled (55 ± 10%) room with a 12-h light/ dark cycle. Food and water were available ad libitum. The animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 10-06/14, 31st July 2014).

For the supplemented diets, commercial feed pellets (Harlan Laboratories, Madison, WI, USA) were crushed in an industrial mill and mixed with Milli-Q

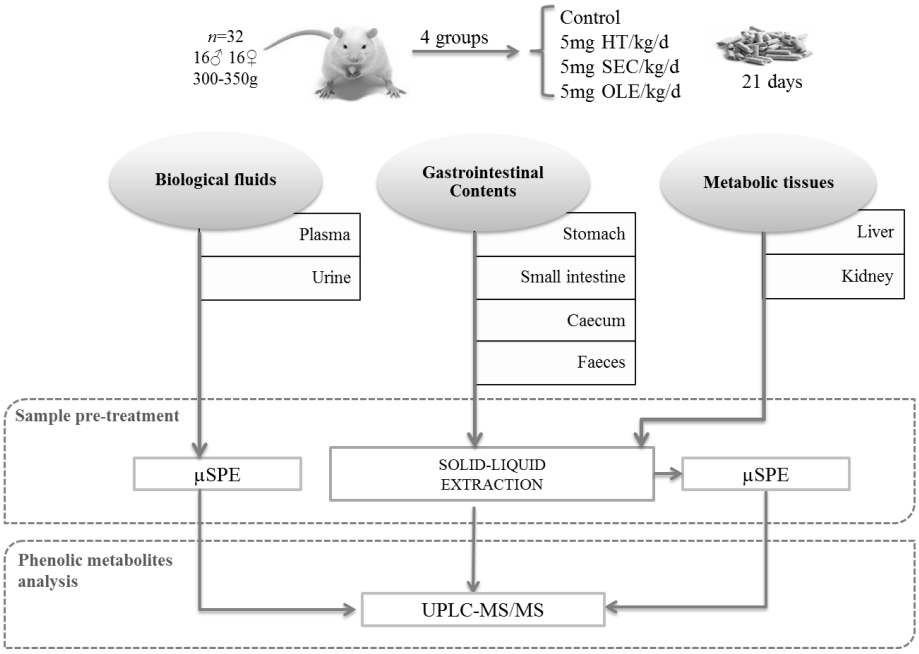


Fig. 1 – Experimental design of the study. µSPE, microelution solid-phase extraction; UPLC-MS/MS, Ultra-Performance liquid chromatography coupled to tandem MS.

water containing the equivalent of 5 mg of OLE, SEC or HT/kg rat weight in 16 g of crushed pellet (average daily consumption of each rat), respectively. New pellets were prepared and freeze-dried. Food and animals were weighed every 2 days to adjust the weekly dose of phenolic compound to 5 mg/kg rat weight/day. On the first day before treatment (day 0) and after treatment (day 21), the rats were caged in metabolic gavage for 24 hours to collect urine and faeces. The rats were sacrificed by intracardiac puncture after isoflurane anaesthesia (IsoFlo, Veterinarian Esteve, Bologna, Italy). Blood was collected in EDTA tubes and plasma samples were obtained by centrifugation (3000 × g, 10 min at 4 °C) and stored at −80 °C until analysis. After blood collection, the rats were perfused with an isotonic solution of sodium chloride (NaCl) 0.9% to remove the remaining blood irrigating the tissues and their livers and kidneys were excised. Additionally, the digestive tract was excised and the contents of stomach, small intestine and caecum were obtained to analyse the phenolic metabolites, expressed as nmols/g digesta. The small intestine content consisted of a pool of duodenum, jejunum and ileum contents. All samples were stored at −80 °C and freeze-dried for phenolic extraction and chromatographic analysis (**Fig. 1**).

2.4. Sample pre-treatment for phenolic metabolites analysis

Plasma and urine samples were processed using OASIS hydrophilic–lipophilic balance (HLB) µElution plates 30 µm (Waters, Milford, MA, USA). Pre-treatment of plasma was performed according to our method developed previously (Suárez et al., 2009). Briefly, 350 µL of plasma were mixed with 300 µL of phosphoric acid (4%) and 50 µL of catechol as the internal standard (IS). Then, the mixture was centrifuged and the supernatant was treated by microelution solid-phase extraction (µSPE). The cartridges were conditioned sequentially by using 250 µL of methanol and acidified Milli-Q water (Milli-Q water at pH 2 with diluted hydrochloric acid). After loading the plasma mixture, the plates were washed with 200 µL of Milli-Q water and 200 µL of 5% methanol. The retained phenolic compounds were then eluted with 2 × 50 µL of methanol and directly injected into the UPLC-MS/MS system. For the analysis of the urine samples, the cartridges were conditioned as described previously (Serra et al., 2013). Briefly, 100 µL of urine were mixed with 50 µL of phosphoric acid (4%) and 50 µL of catechol as IS, and this solution was loaded into the µSPE. The retained phenolic compounds were then eluted with 2 × 50 µL of methanol and injected into the UPLC-MS/MS system.

For the analysis of the GIT contents (stomach, small intestine and caecum) and faeces, 100 mg of freeze-dried sample was suspended in 1 mL of methanol/water (1:1, v/v). Each sample was shaken in a vortex (Multi vortex, VWR, Franklin, MA, USA) for 10 min. After that, the sample was centrifuged at 8784 × g for 10 min and supernatant was collected and centrifuged

under the same conditions. Finally, the supernatant was filtered with 0.22 µm syringe filter and transferred into chromatographic vials until the chromatographic analysis.

The kidney and liver samples were sequentially pretreated with a combination of liquid–solid extraction (LSE) combined with µSPE (Serra et al., 2012). Briefly, for the analysis of LSE, 60 mg of freeze-dried sample was mixed with 50 µL of ascorbic acid (1%), 100 µL of phosphoric acid (4%), and 50 µL of catechol. The samples were treated four times with 400 mL of water/methanol/phosphoric acid 4% (94:4.5:1.5, v/v/ v). In each extraction, the sample was sonicated for 30 s maintaining the sample in ice to avoid heating and then centrifuged. The supernatants were collected, and an aliquot of 350 µL was treated with µSPE following the same method described above for the plasma samples.

2.5. Chromatographic analysis

The phenolic compounds were analysed by Acquity Ultra-Performance™ liquid chromatography coupled to tandem MS as the detector system (UPLC-MS/MS) from Waters (Milford, MA, USA), as reported in our previous study (Serra et al., 2012). The column was an Acquity UPLC™ HSS T3 from Waters (100 mm, 2.1 mm i.d., 1.8 µm particle size). The mobile phase was 0.2% (v/v) acetic acid as solvent A and methanol as solvent B, with a flow rate of 0.4 mL/min. The gradient was performed as follows: 0–6 min, 3–15% B; 6–14 min, 15–70% B; 14–17 min, 70–00% B; 17–18 min, 100–3% B; 18–20 min, 3% B isocratic. The injection volume was 2.5 µL. The UPLC system was coupled to a PDA detector, AcQuity UPLC™ and a triple quadrupole detector (TQD™) mass spectrometer (Waters). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode, and the data were collected in the selected reaction monitoring (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h and desolvation gas flow rate, 800 L/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as the nebulizing and collision gases, respectively. The SRM transitions and the individual cone voltages and collision energies were optimized for each analyte by injection of each standard compound into a mixture of methanol/water (1:1, v/v) at a concentration of 10 mg/L. Two SRM transitions were studied to find the most abundant product ions, selecting the most sensitive transition for quantification and the second one for identification purposes. When standard phenolic compound were not available, the SRM parameters were selected by analysing the real sample in fullscan mode in MS and daughter-scan mode in tandem MS. Table S2 of Supplementary Material shows the tandem MS (MS/ MS) transitions for quantification and confirmation, as well as cone voltage and collision energy values optimized for each phenolic compound. Table S2 also shows the commercial standards used to quantify each phenolic compound. When standard

compounds were not available, the phenolic compound was tentatively quantified with its aglycone or with a phenolic compound with similar chemical structure.

2.6. Instrumental quality parameters

The instrumental quality parameters of the developed method, such as linearity, calibration curve, reproducibility, accuracy, detection limits (LODs) and quantification limits (LOQs) as well as extraction recovery and matrix effect were determined with a serial dilution of blank biological sample (plasma, urine, faeces, tissues and intestinal contents) with the standard phenolic compounds. The determination and calculation of these quality parameters are reported in our previous study (Suárez et al., 2009). The precision of the methods and accuracy were determined at three concentration levels, but only one concentration level is shown. The calibration curves (based on peak abundance) were plotted using $y = a + bx$, where y is the peak abundance ratio (analyte/internal standard) and x is the concentration ratio (analyte/internal standard). The internal standard (IS) used was catechol. For the other tissues (stomach, caecum, intestine and faeces), the calibration curves were plotted without IS, since catechol could be a metabolite.

2.7. Statistical analysis

Differences between diet groups were evaluated using a one-way ANOVA, followed by the LSD post-hoc test. Differences were considered statistically significant at $p < 0.05$. All analyses were performed with the Statgraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

3.1. Validation of the analytical procedure

Blank plasma, urine, faeces, intestinal contents and tissue samples with different phenolic concentration were analysed by sample pre-treatment and UPLC-MS/MS to determine the linearity, calibration curve, precision, accuracy, LOD and LOQ. The obtained results are shown in **Table S3** of the **Supplementary Material**.

The precision of the method for all the biological fluids and tissues was below 15%. The accuracy of the method for the different samples was between 95 and 105%. The extraction recovery (%R) and matrix effect (%ME) of the phenolic compounds in the different biological samples were higher than 75% and lower than 18%, respectively.

Once the instrumental quality parameters of the developed methods were studied, these were applied for the determination of hydroxytyrosol, oleuropein and their generated metabolites in the different biological samples

3.1. Phase I and II metabolism of HT, SEC and OLE in the gastrointestinal tract

Table 1 shows the phase I and II phenolic metabolite concentrations (nmols/g

digesta) detected in the GIT contents (stomach, small intestine and caecum) and faeces after the diet supplementation (21 days) with 5 mg/kg weight rat/day of HT, SEC and OLE. The corresponding proposed pathways for phase I and II metabolism of the three HT precursors are shown in **Fig. 2**.

At the gastric level, the SEC group underwent a complete degradation of the main secoiridoid 3,4-DHPEA-EDA (Table 1), which was hydrolysed into HT and EA and further metabolized (**Fig. 2**). OLE underwent a similar process in the stomach but a remaining amount of the native form was detected, indicating that OLE molecule was more resistant to the gastric acidic hydrolysis than 3,4-DHPEA-EDA. Accordingly, previous studies have shown that OLE is stable in human gastric juice (Corona et al., 2006; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002) and 3,4-DHPEA-EDA has been shown to be more sensitive to temperature, pH and enzyme activity compared with the glycoside form of OLE (Briante et al., 2002). In stomach, both the administered HT and the resulting HT from the hydrolysis of OLE and SEC underwent direct sulphation (hydroxytyrosol sulphate, Sulf) or methylation (homovanillic alcohol, HVAlc) and a further oxidation process (homovanillic acid, HVAc) followed by sulphation (HVAc-Sulf and HVAlc- Sulf) with a remaining amount of free HT and observing very similar metabolic profiles among the three HT precursors (**Table 1** and **Fig. 2**). Very few studies have analysed the in vivo metabolic capacity of the gastric mucosa to conjugate phenolic compounds, however, the presence of SULT1C2 isoform have been described in the stomach tissue (Nimmagadda, Cherala, & Ghatta, 2006). In a previous study by Zhao, Egashira, and Sanada (2004) they found that ferulic acid did not undergo any metabolic change in rat stomachs. In contrast, our results indicated that HT can suffer phase II metabolism at the gastric level of digestion, sulphation being the main conjugation process.

The detection of higher concentrations of phase I and II metabolites in the small intestine contents after the administration of the three precursors revealed an intense enzymatic activity in the intestinal epithelium with an efflux transport of the metabolites into the gut lumen, a phenomenon that has already been described for olive oil phenolic compounds (D'Angelo et al., 2001; Manna et al., 2000). It is worth noting that HVAc and its conjugated forms, HVAc-Sulf and HVAc-Glu, were the metabolites detected at the highest concentrations in the intestinal lumen in all groups including the control group (**Table 1**). HVAc is the major molecular species deriving from endogenous dopamine metabolism through the action of monoamine oxidase and catechol-O-methyltransferase in the central nervous system, which indicates that HVAc and its conjugated forms detected in the present study might be mainly of endogenous origin (D'Angelo et al., 2001). During digestion in the small intestine, part of the native form of OLE remained unaltered without being deglycosylated, resisting the action of

lactase phlorizin hydrolase (LPH), an enzyme found in the lumen of the brush border of the small intestine with β-glucosidase activity towards glycoside compounds such as flavonoids (Del Rio et al., 2013). Compared to HT and SEC groups, OLE also presented in the small intestine significantly higher concentrations of some phase II metabolites such as HVAc-Sulf and hydroxytyrosol acetate sulphate (HTAc-Sulf) (Table 2). These results could indicate that OLE could suffer less

Table 1 – Phase I and II metabolites' concentration (nmols/g digesta) in the stomach and intestinal (gut and caecum) contents and faeces obtained after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as the mean ± SEM.

Phenolic compound	Control	HT	SEC	OLE
Stomach content (nmols/g digesta)				
Hydroxytyrosol	n.d.	12.0 ± 2.69^{b}	8.75 ± 1.95^{b}	3.64 ± 0.79^{a}
Hydroxytyrosol sulphate	0.16 ± 0.13^{a}	3.28 ± 1.17^{b}	10.5 ± 10.2^{b}	3.45 ± 1.47^{b}
Homovanillic acid	1.67 ± 0.87^{a}	9.13 ± 1.74^{bc}	4.32 ± 1.95^{ab}	12.2 ± 4.12^{c}
Homovanillic acid	n.d.	0.17 ± 0.14^{a}	4.03 ± 2.66^{b}	0.55 ± 0.25^{a}
Homovanillic alcohol	n.d.	0.45 ± 0.29^{a}	4.74 ± 3.34^{b}	0.75 ± 0.22^{a}
Elenolic acid sulphate	–	–	6.99 ± 2.55^{b}	4.02 ± 1.25^{a}
Oleuropein	–	–	n.d.	4.14 ± 2.31
Small intestine (nmol/g digesta)				
Hydroxytyrosol sulphate	8.03 ± 1.53^{a}	30.5 ± 5.75^{b}	31.1 ± 12.6^{b}	29.6 ± 6.22^{b}
Hydroxytyrosol	n.d.	1.44 ± 0.91^{a}	2.99 ± 0.53^{b}	2.77 ± 0.58^{b}
Hydroxytyrosol acetate	28.7 ± 8.63^{a}	27.0 ± 7.36^{a}	32.1 ± 6.5^{a}	56.3 ± 13.0^{b}
Homovanillic acid	48.3 ± 17.6^{a}	36.6 ± 11.3^{a}	50.8 ± 12.1^{ab}	76.8 ± 14.4^{b}
Homovanillic alcohol	16.0 ± 5.19^{ab}	14.8 ± 2.67^{a}	27.4 ± 7.74^{bc}	32.9 ± 7.17^{c}
Oleuropein	–	–	n.d.	6.95 ± 3.34
Caecum content (nmols/g digesta)				
Hydroxytyrosol	0.36 ± 0.24^{a}	6.02 ± 1.32^{b}	0.04 ± 0.00^{a}	5.57 ± 0.65^{b}
Hydroxytyrosol sulphate	n.d.	0.52 ± 0.52^{a}	n.d.	3.12 ± 2.25^{b}
Elenolic acid sulphate	–	–	0.02 ± 0.01^{a}	9.38 ± 4.59^{b}
Faeces (nmols/g)				
Hydroxytyrosol	n.d.	6.20 ± 1.35^{c}	0.35 ± 0.01^{a}	3.12 ± 0.35^{b}
Hydroxytyrosol sulphate	n.d.	1.44 ± 0.78	0.01 ± 0.01	0.01 ± 0.01

n.d.: not detected.
$^{a–c}$ Indicate the levels which contain a significant difference at the 95.0% confidence level compared with the others in the same row.

degradation thanHT and SEC under alkaline intestinal conditions and therefore, it could have been more exposed to phase II metabolism. In faeces, the intact form of OLE was not detected, in contrast to a previous study in which OLE, HVAc, EA and OLE aglycone were detected in rat faeces collected over 24 h after an acute oral dose of OLE (Lin et al., 2013). This discrepancy could be explained by the differences in the administered dose, the dose administered in the present work being much lower (5 mg/kg vs 100 mg/kg). The free form of HT was detected in both the caecum content and the faeces, mainly in HT and OLE groups. In our previous work, free HT was also detected in human faeces after a sustained intake of an olive oil enriched with its own phenolic compounds (Mosele et al., 2014). Therefore, the present results confirm that the free form of HT is able to reach the large intestine, which could appear as a result of the deconjugation of the phase II metabolites by the action of the microbial enzymes, including β-glucuronidase and sulphatase (Selma, Espin, & Tomas-Barberan, 2009; Valdés et al., 2015).

3.2. Microbial metabolism of phenolics in the intestinal tract

The results showed that the microbial metabolism of HT, OLE and SEC started in the small intestine and was intensified in the lower part of the gut (caecum), detecting metabolites derived from oxidation, dehydroxylation, decarboxylation (α-oxidation) and carboxylation reactions along the GIT (**Table 2, Figs 3–5**). The microbial content of the GIT changes along its length, starting with a low number of microbes in the stomach, and increasing in the jejunum and the ileum, which are colonized by aerobe or facultative anaerobes. The highest concentrations are reached in the large intestine where the main microorganisms are anaerobes (Tiihonen, Ouwehand, & Rautonen, 2010; Zhao et al., 2015), which is in accordance with the amount of catabolites detected in caecum in the present study. Therefore, differences observed in the colonic metabolites can be attributed to the diverse microbiota populations that cause differences in the metabolite complexity in different sections of the digestive tract (Ouwehand & Vesterlund, 2003). The results of this study demonstrated that each administered HT precursor underwent different catabolic transformations with more or less intensity depending on the molecular structure of the precursor. In the case of the free form of HT, we observed that the predominant microbial metabolic transformation was the oxidation, converting the primary alcohol into dihydroxyphenylacetic acid (diHPAAc), followed by rapid and subsequent dehydroxylation giving rise to hydroxyphenylacetic acid (HPAAc), which was the most abundant microbial metabolite after HT treatment (**Table 2** and **Fig. 3**). HPAAc could be further carboxylated leading to the formation of hydroxyphenylpropionic acid (HPPAc) (**Fig. 3**), being the second most abundant microbial metabolite after HT intake (**Table 2**). The microbial origin and formation of these metabolites were demonstrated in our

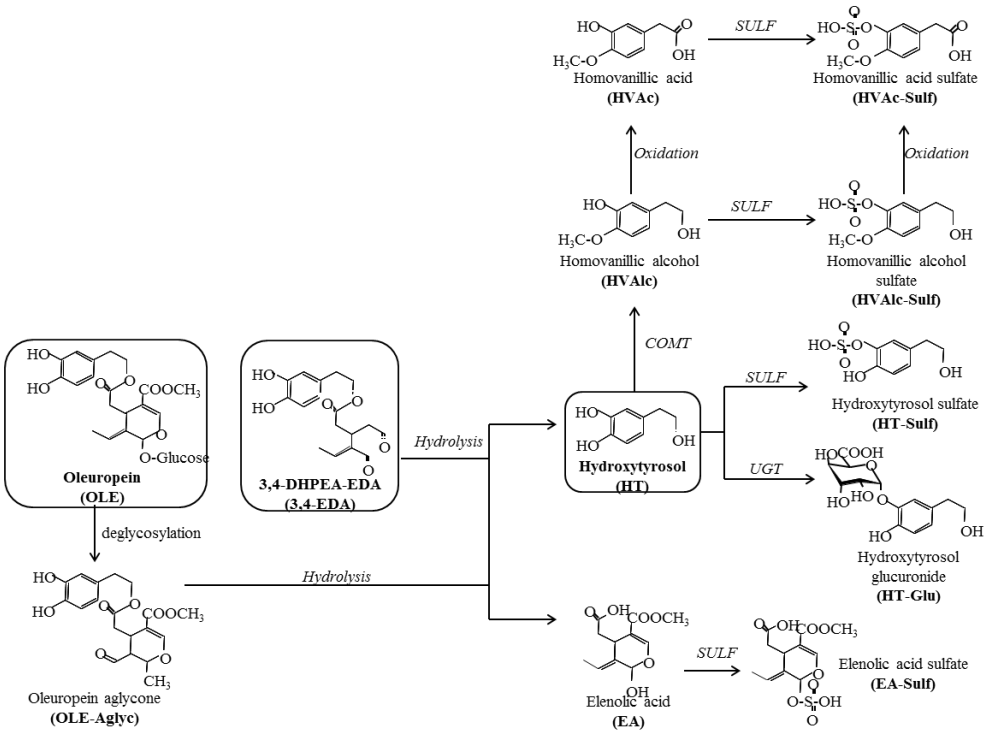


Fig. 2– Phase I and II metabolism pathways of HT, OLE and SEC. COMT: catechol-O-methyltransferase enzyme; SULF: sulphotransferase enzyme; UGT: glucuronosyl-transferase enzyme.

previous in vitro fermentation study with free HT (Mosele et al., 2014). When SEC was administered to rats, the microbial metabolic profile in GIT contents was similar to the HT group, lower concentrations (**Fig. 4**). On the other hand, OLE was characterized to undergo the same transformations as described in HT and SEC but in less intensity, and in addition, it suffered other kinds of microbial transformations. So, apart from the loss of a hydroxyl group, di- and mono-phenylacetic acids were also decarboxylated (α-oxidation) into phenolic compounds with lower molecular weights, including protocatechuic acid (PCAc) and hydroxybenzoic acid (HBZAc) (**Fig. 5**). HBZAc was predominately transformed to its glycine conjugate termed hydroxyhippuric acid (HHiPAc), which could suffer a further dehydroxylation and generate hippuric acid (HiPAc), a microbial transformation previously reported (Beyoglu & Idle, 2012). Consequently, of the three groups, OLE showed the most diverse microbial transformations and led to the formation of fermentation products with lower molecular weight (**Table 2**).

A significant higher concentration of diHPPAc was also observed after OLE compared to SEC and HT, which could be in great part due to the hydrolysis of OLE-aglycone into HTAc with a subsequent de-

Table 2 – Concentration (nmols/g digesta) of the microbial phenolic metabolites detected in the intestinal (gut and caecum) contents, and faeces obtained after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as mean ± SEM.

Phenolic metabolite	Control	HT	SEC	OLE
Small intestine (nmols/g digesta)				
Hydroxyphenylpropionic acid	9.29 ± 1.22[a]	16.7 ± 1.22[b]	11.8 ± 1.60[a]	12.1 ± 1.33[a]
Hydroxyphenylpropionic acid	2.57 ± 1.74[a]	16.2 ± 6.15[b]	15.4 ± 6.18[b]	38.8 ± 9.27[c]
Hydroxyphenylacetic acid sulphate	13.6 ± 3.83[a]	12.4 ± 6.10[a]	30.4 ± 9.06[ab]	40.9 ± 6.22[b]
Catechol	n.d.	2.73 ± 0.63[b]	0.68 ± 0.21[a]	4.32 ± 0.18[c]
Hippuric acid	161 ± 49.9[a]	186 ± 47.5[a]	223 ± 50.1[a]	367 ± 72.5[b]
Caecum content (nmols/g digesta)				
Dihydroxyphenylpropionic acid	89.0 ± 60.3[a]	195 ± 77.8[ab]	147 ± 25.9[ab]	403 ± 226[b]
Hydroxyphenylpropionic acid	n.d.	592 ± 143[b]	316 ± 70.4[a]	252.09 ± 87.8[a]
Dihydroxyphenylacetic acid	16.5 ± 5.26[a]	50.4 ± 13.9[b]	25.1 ± 7.40[a]	31.9 ± 12.5[ab]
Hydroxyphenylacetic acid*	65.1 ± 31.8[a]	707 ± 31.1[c]	493 ± 37.9[b]	309 ± 99.08[b]
Hydroxyphenylacetic acid sulpl	1.47 ± 0.97[a]	n.d.	6.14 ± 5.16[a]	34.9 ± 19.0[b]
Phenylacetic acid	n.d.	26.52 ± 9.69[a]	24.5 ± 2.18[a]	n.d
Catechol	0.36 ± 0.28[a]	1.62 ± 0.23[b]	1.13 ± 0.17[b]	1.67 ± 0.22[b]
Faeces (nmols/g)				
Hydroxybenzoic acid	0.63 ± 0.32[a]	0.46 ± 0.21[a]	2.97 ± 1.59[b]	5.82 ± 2.12[c]
Hippuric acid	8.67 ± 0.44[a]	6.65 ± 6.32[a]	16.1 ± 4.34[a]	48.84 ± 21.26[b]
Phenylacetic acid	52.7 ± 13.0[a]	70.2 ± 9.53[ab]	268 ± 43.1[b]	293 ± 50.0[b]

n.d.: not detected.
* ∑ *p*- and *o*-hydroxyphenylacetic acid.
[a–c] Indicate the levels which contain a significant difference at the 95.0% confidence level compared with the others in the same row.

esterification, oxidation and carboxylation (**Fig. 5**). This metabolic pathway was described in our previous work, in which OLE was fermented in vitro with human faeces (Mosele et al., 2014).

Once in the portal bloodstream, microbial metabolites can rapidly reach the liver, where they can be subjected to further phase II metabolism, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion. This could explain the detection of metabolites such as HPPAc-Sulf, HPAAc- Sulf and HPAAc-Glu in the small intestine and caecum contents, mainly after the OLE administration.

The biological activities of the products of microbial fermentation of olive phenols have not yet been systematically tested. However, a few reports have demonstrated that products of the colonic degradation of flavonoids, such as phenylacetic and phenylpropionic acids and their derivates, exhibit anti-inflammatory effects (Larrosa

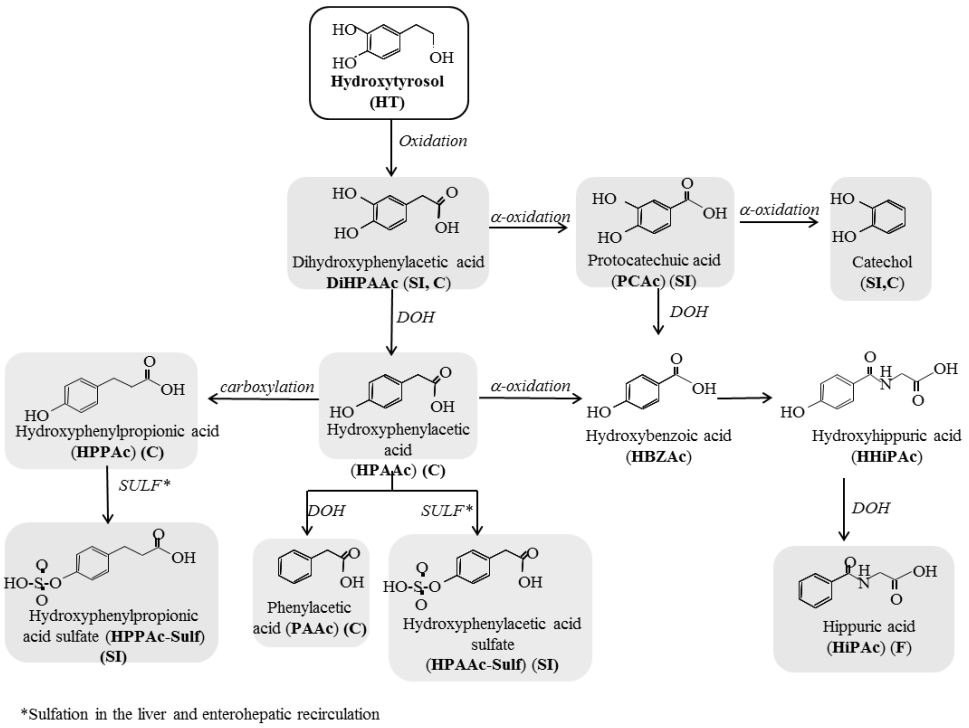


Fig. 3 – Proposed catabolism pathways of HT by the gut microbiota derived from the analysis of small intestine (SI) and caecum (C) contents and faeces (F). Compounds that were detected after HT treatment are highlighted in grey colour. DOH: dihydroxylation; SULF: sulphotransferase enzyme. *Phase II metabolism (sulphation) in liver of some compounds that were detected in the GIT after enterohepatic recirculation.

et al., 2009) as well as antioxidant powers (Jaganath, Mullen, Lean, Edwards, & Crozier, 2009). It has also been pointed out that the local concentration of intact polyphenols and its metabolites that reach the intestine may have a significant redox effect (Scalbert et al., 2000). This might be the case of olive phenols, which in the present study, no matter the precursor (HT, SEC or OLE), have all been demonstrated to undergo extensive transforma tions in the GIT, resulting in the release of a large number of putative bioactive compounds, varying depending on the precursor compound. Metabolites could also be reabsorbed and exert their action in the colon as well as in other target tissues after absorption. Wang, Williams, Ferruzzi, and D'Arcy (2013) demonstrated the transference of some phenolic fermentation products across the Caco-2 cell monolayer. The results from the microbial metabolism in the present study are in close agreement with our previous in vitro fermentation experiments performed with human faecal inoculums (Mosele et al., 2014), which

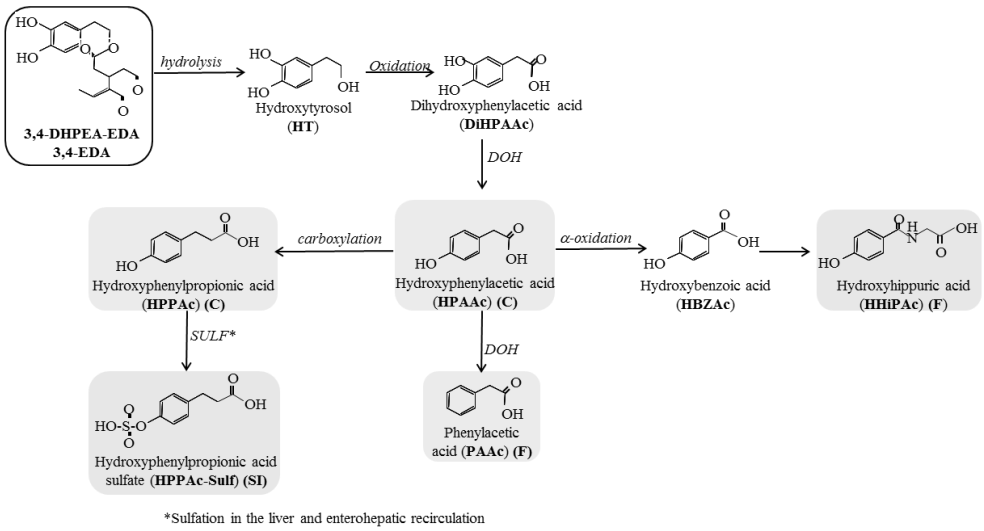


Fig. 4 – **Proposed catabolism pathway of 3,4-DHPEA-EDA (main compound in the SEC extract) by the gut microbiota derived from the analysis of small intestine (SI), caecum contents (C) and faeces (F). DOH: dihydroxylation; SULF: sulphotransferase enzyme. *Phase II metabolism (sulphation) in liver of some compounds that were detected in the GIT after enterohepatic recirculation.**

suggests that the rat model can be a good approach to performing a human approximation of the digestion and microbial metabolism of olive phenolic compounds. In fact, rodent animals are similar to humans at both the host geneticand the taxonomic levels of the microbiota, particularly at the phylum level. However, important differences may be appreciated at the lower taxonomic level (Kostic, Howitt, & Garrett, 2013). Moreover, it should remain clear that the amount and identity of the catabolites detected may significantly vary from individual to individual, as observed in the high deviation values in some determinations.

3.3. Plasma, urine excretion and tissue deposition

Table 3 shows the concentration of the phase I and II phenolic metabolites in plasma and urine after the sustained intake of free HT and its conjugated precursors OLE and SEC. The results in plasma revealed that very low concentrations of metabolites were detected (nanomolar range), probably due to the low daily dose administered in this study and the distribution of the dose over the day. No differences were observed between the three groups, except for EA-Glu, which was only detected in the plasma of OLE group. A previous study performed in our lab (Serra et al., 2012) showed that after an acute intake by rats of an olive phenolic

Table 3 – Concentration of the metabolites detected in plasma and urine obtained after the die supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as the mean ± SEM.

Phenolic compound	Control	HT	SEC	OLE
Plasma (µM)				
Hydroxytyrosol sulphate	n.d.	0.37 ± 0.03	0.36 ± 0.08	0.32 ± 0.07
Homovanillic alcohol sulphate	n.q	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
Elenolic acid glucuronide	n.q	–	0.09 ± 0.03[a]	0.22 ± 0.04[b]
Urine (µmol)				
Hydroxytyrosol	n.d.	0.03 ± 0.02[a]	5.38 ± 0.48[b]	4.01 ± 2.06[b]
Homovanillic acid	n.d.	4.26 ± 0.73[a]	9.11 ± 0.54[a]	169 ± 39.2[b]
Hydroxytyrosol sulphate	n.d.	57.4 ± 13.54[a]	72.6 ± 10.5[a]	169 ± 28.6[b]
Homovanillic acid sulphate	n.d.	85.6 ± 5.26[a]	92.6 ± 5.50[a]	557 ± 89.9[b]
Elenolic acid sulphate	n.d.	–	4.75 ± 0.90	6.95 ± 1.28
Hydroxytyrosol-4-*O*-glucuronide	n.d.	1.56 ± 0.25[b]	0.46 ± 0.06[a]	1.80 ± 0.38[b]
Homovanillic acid glucuronide	n.d.	n.d.	4.17 ± 0.30[a]	28.4 ± 5.35[b]
Elenolic acid glucuronide	0.38 ± 0.09[a]	–	18.5 ± 3.13[b]	61.0 ± 9.14[c]
Methyloleuropein aglycone sulphate	n.d.	–	n.d.	1.63 ± 0.29
Oleuropein	n.d.	–	n.d.	2.13 ± 0.66
Oleuropein aglycone glucuronide	n.d.	–	0.13 ± 0.02[a]	1.49 ± 0.22[b]
Oleuropein aglycone derivates	n.d.	–	n.d.	2.03 ± 0.13

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

[a–c] Indicate the levels which contain a significant difference at the 95.0% confidence level compared with the others in the same row.

extract rich in SEC, the sulphate conjugates of HT and the native forms of OLE and HT were detected in plasma at higher concentrations (between 21 and 89 µM) probably due to the higher administered dose (180 mg SEC/kg rat weight). We observed a similar metabolic fate after an acute intake of HT at different doses (1, 10, and 100 mg/kg rat weight), sulphation again being the most relevant conjugation pathway at the three administered doses (López de Las Hazas et al., 2015). The present study provides new data after a sustained intake of olive phenolic compounds, showing that when nutritionally relevant doses are administered in rats, a lower variety of metabolites is detected in the plasma (only HT-Sulf and HVAlc-Sulf) and at lower concentrations. In contrast to the plasma, urine recovery of metabolites showed significant differences among the three HT precursors administered. Urine excretion of phase I and II metabolites was significantly higher when OLE was admi nistered in comparison with HT or SEC (**Table 3**). In some cases, such as for HVAc, the metabolite was 50-fold higher in the OLE than the other groups. Thus, it can be concluded from the urine excretion results that, among the three groups, the bioavailability of HT was more effective

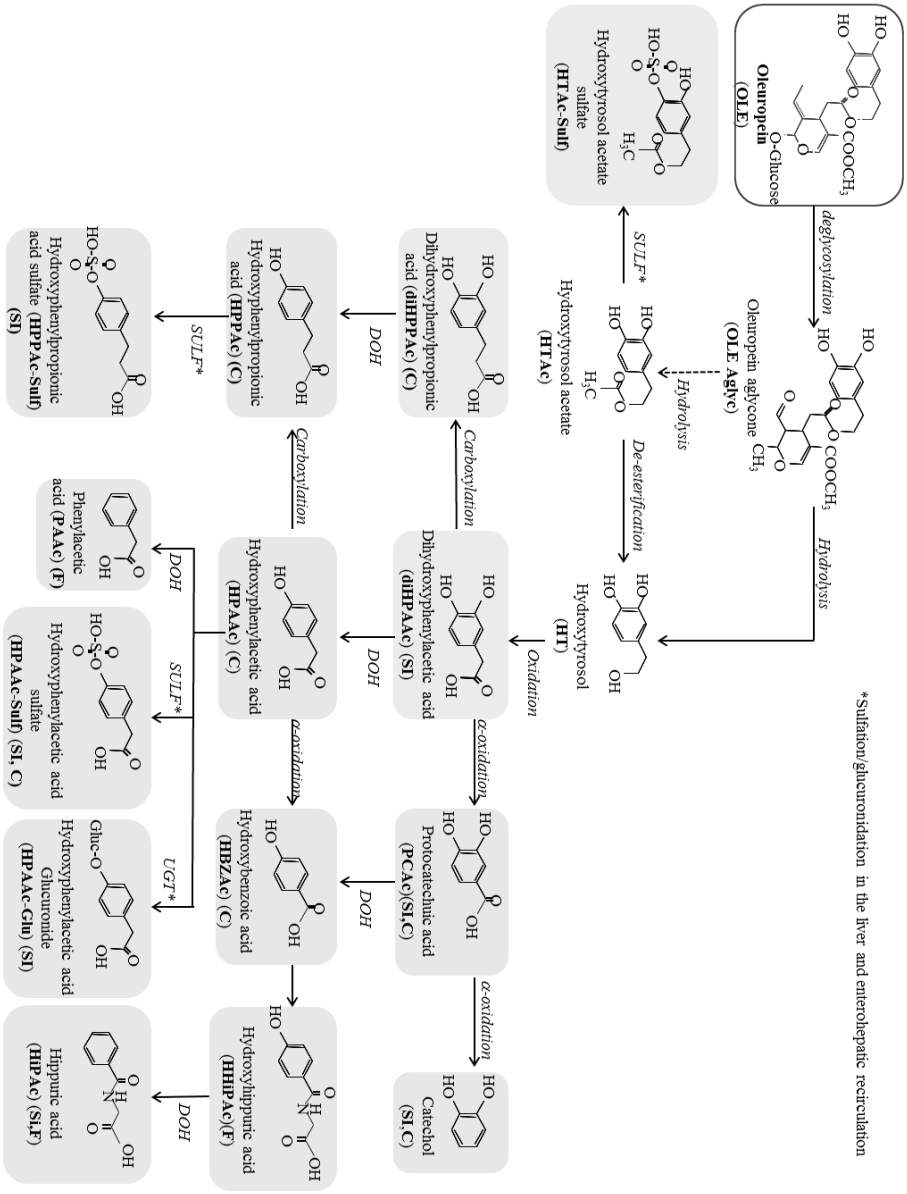


Fig. 5- Proposed catabolism pathway of OLE by the gut microbiota derived from the analysis of small intestine (SI), caecum contents (C) and faeces (F). DOH: dihydroxylation; SULF: sulphotransferase enzyme; UGT: glucuronosyl-transferase enzyme. *Phase II metabolism (sulphation and glucuronidation) in liver of some compounds that were detected in the GIT after enterohepatic recirculation.

after the diet supplementation with OLE. In fact, it has been established that 24 h-urine offers advantages over plasma measurements, mostly because they allow an accurate evaluation of the total polyphenols absorbed due to the very short plasma half-lives of the polyphenols (Medina - Remón, Tresserra - Rimbau, Arranz, Estruch, & Lamuela-Raventos, 2012). Regarding the colonic phenolic metabo-lites detected in the intestinal contents and faeces, a very low concentration of these metabolites was detected in the urine samples, and no significant differences were observed among the three groups or compared to the control (data not shown). These results could indicate rather that urine sampling of up to 24 h could be required to detect these HT microbial metabolites, or that they were not reabsorbed at colonic level.

So, results from plasma and urine showed that depending on the molecular structure of the HT precursors, the bioavailability could differ. In accordance, previous studies showed that the bioavailability of flavonoids also varied depending on their chemical structure. Results obtained from plasma and urinary levels of flavanol stereoisomers after their oral intake, showed that the ranking for the oral absorbability of flavanols was as follows: (−)-epicatechin > (+)-epicatechin = (+)-catechin > (−)-catechin, (−)-epicatechin (Ottaviani et al., 2011). Moreover, the sugar moiety of flavonoids has been suggested as an important determinant in their absorption in humans. Typically, absorption of flavonoid glycosides is associated with the release of the aglycone as a result of the action of LPH enzyme with β-glucosidase activity. However, a great part of the polyphenol conjugates with sugar moieties are resistant to the action of LPH enzymes and pass to the colon, where the colonic microbiota cleave conjugating moieties (Del Rio et al., 2013). This might be the case of the glycoside molecule of OLE, which could have been in part hydrolysed by LPH enzymes leading to HT in the upper part of the GIT, and another important amount of OLE could have passed to the colon.

Regarding the tissue distribution, in contrast to our previous study (Serra et al., 2012), in which a similar olive phenolic extract was administered to rats, in the present study, very few HT metabolites were detected in liver and kidney and at very low concentrations with no significant differences among the HT, SEC and OLE groups (**Table S4** of Supplementary Material). This fact could be due to the low daily dose administered (5 mg/kg rat weight/day) and the tissue perfusion with isotonic solution performed to remove the remaining blood irrigating tissues after the sacrifice of the rats. Accordinly with our previous study, HT-Sulf appeared again as the most deposited metabolite in both liver and kidney tissues.

4. CONCLUSIONS

The results from the present study show that, of the three HT precursors, the maximum efficiency in the bioavailability of HT and its metabolites was observed after diet supplementation with OLE. This could be due to the higher stability of OLE

under digestion conditions observed along the GIT, which could have led to a major exposure of OLE to phase II metabolism. The higher bioavailability of HT through OLE was mainly reflected in the urine excretion of phase I and II metabolites. Moreover, OLE as a glycoside molecule could have resisted in part the action of the β-glucosidase enzymes in the small intestine and passed unaltered to the colon with the resultant higher production of microbial fermentation catabolites with potential biological activity. The findings from this study revealed that the absorption and biotransformation of HT depends mainly on its precursor. Further studies are needed to find a possible correlation with biological efficacy, but in terms of bioavailability and microbial fermentation, the results indicate that OLE is the most suitable HT derivate for use in food or nutraceutical formulations.

Acknowledgments

This study was supported by the Spanish Ministry of Education and Science (AGL2012-40144-C03-03 and AGL2012-40144-C03-02 projects) and by the University of Lleida through the M.C. López de las Hazas grant.

REFERENCES

Beyoglu, D., & Idle, J. R. (2012). The glycine deportation system and its pharmacological consequences. Pharmacology & Therapeutics, 135, 151–167.

Briante, R., Patumi, M., Terenziani, S., Bismuto, E., Febbraio, F., & Nucci, R. (2002). Olea europaea L. leaf extract and derivatives: Antioxidant properties. Journal of Agricultural and Food Chemistry, 50, 4934–4940.

Corona, G., Tzounis, X., Assunta Dessì, M., Deiana, M., Debnam, E. S., Visioli, F., & Spencer, J. P. E. (2006). The fate of olive oil polyphenols in the gastrointestinal tract: Implications of gastric and colonic microflora-dependent biotransformation. Free Radical Research, 40, 647–658.

de Bock, M., Thorstensen, E. B., Derraik, J. G. B., Henderson, H., V, Hofman, P. L., & Cutfield, W. S. (2013). Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (Olea europaea L.) leaf extract. Molecular Nutrition & Food Research, 57, 2079–2085.

D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O., Morrica, P., Capasso, G., Pontoni, G., Galletti, P., & Zappia, V. (2001). Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. Drug Metabolism and Disposition: The Biological Fate of Chemicals, 29, 1492–1498.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxidants and Redox Signaling, 18, 1818–1892.

El Riachy, M., Priego-Capote, F., León, L., Rallo, L., & Luque de Castro, M. D.

(2011). Hydrophilic antioxidants of virgin olive oil. Part 2: Biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors. European Journal of Lipid Science and Technology, 113, 692–707.

Herrero, M., del Pilar Sánchez-Camargo, A., Cifuentes, A., & Ibáñez, E. (2015). Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. Trends in Analytical Chemistry: TRAC, 71, 26–38.

Jaganath, I. B., Mullen, W., Lean, M. E. J., Edwards, C. A., & Crozier, A. (2009). In vitro catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. Free Radical Biology and Medicine, 47, 1180–1189.

Khymenets, O., Joglar, J., Clapés, P., Parella, T., Covas, M. I., & de la Torre, R. (2006). Biocatalyzed synthesis and structural characterization of monoglucuronides of hydroxytyrosol, tyrosol, homovanillic alcohol, and 3-(4′- Hydroxyphenyl)propanol. Advanced Synthesis & Catalysis, 348, 2155–2162.

Kostic, A. D., Howitt, M. R., & Garrett, W. S. (2013). Exploring host- microbiota interactions in animal models and humans. Genes and Development, 27, 701–718.

Larrosa, M., Luceri, C., Vivoli, E., Pagliuca, C., Lodovici, M., Moneti, G., & Dolara, P. (2009). Polyphenol metabolites from colonic microbiota exert anti-inflammatory activity on different inflammation models. Molecular Nutrition & Food Research, 53, 1044–1054.

Lin, P., Qian, W., Wang, X., Cao, L., Li, S., & Qian, T. (2013). The biotransformation of oleuropein in rats. Biomedical Chromatography: BMC, 27, 1162–1167.

López de Las Hazas, M. C., Rubió, L., Kotronoulas, A., de la Torre, R., Solà, R., & Motilva, M. J. (2015). Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats. Molecular Nutrition & Food Research, 59, 10395–11399.

Manna, C., Galletti, P., Maisto, G., Cucciolla, V., D'Angelo, S., & Zappia, V. (2000). Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. FEBS Letters, 470, 341– 344.

Medina-Remón, A., Tresserra-Rimbau, A., Arranz, S., Estruch, R., & Lamuela-Raventos, R. M. (2012). Polyphenols excreted in urine as biomarkers of total polyphenol intake. Bioanalysis, 4(22), 2705–2713.

Mosele, J. I., Martín-Peláez, S., Macià, A., Farràs, M., Valls, R. M., Catalán, U., & Motilva, M. J. (2014). Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches. Molecular Nutrition & Food Research, 58, 1809– 1819.

Muthyala, R. S., Ju, Y. H., Sheng, S., Williams, L. D., Doerge, D. R., Katzenellenbogen, B. S., Helferich, W.

G., & Katzenellenbogen, J. A. (2004). Equol, a natural estrogenic metabolite from soy isoflavones. Bioorganic & Medicinal Chemistry, 12, 1559–1567.

Nimmagadda, D., Cherala, G., & Ghatta, S. (2006). Cytosolic sulfotransferases. Indian Journal of Experimental Biology, 44, 171– 182.

Ottaviani, J. I., Momma, T. Y., Heiss, C., Kwik-Uribe, C., Schroeter, H., & Keen, C. L. (2011). The stereochemical configuration of flavanols influences the level and metabolism of flavanols in humans and their biological activity in vivo. Free Radical Biology & Medicine, 50, 237–244.

Ouwehand, A., & Vesterlund, S. (2003). Health aspects of probiotics. IDrugs: The Investigational Drugs Journal, 6, 573–580.

Scalbert, A., Déprez, S., Mila, I., Albrecht, A. M., Huneau, J. F., & Rabot, S. (2000). Proanthocyanidins and human health: Systemic effects and local effects in the gut. Biofactors (Oxford, England), 13, 115–120.

Selma, M. V., Espin, J. C., & Tomas-Barberan, F. A. (2009). Interaction between phenolics and gut microbiota: Role in human health. Journal of Agricultural and Food Chemistry, 57, 6485–6501.

Serra, A., Rubió, L., Borràs, X., Macià, A., Romero, M.-P., & Motilva, M. J. (2012). Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. Molecular Nutrition & Food Research, 56, 486–496.

Serra, A., Rubió, L., Macià, A., Valls, R. M., Catalán, U., de la Torre, R., & Motilva, M. J. (2013). Application of dried spot cards as a rapid sample treatment method for determining hydroxytyrosol metabolites in human urine samples. Comparison with microelution solid-phase extraction. Analytical and Bioanalytical Chemistry, 405, 9179–9192.

Sikand, G., Kris-Etherton, P., & Boulos, N. M. (2015). Impact of functional foods on prevention of cardiovascular disease and diabetes. Current Cardiology Reports, 17, 593. doi:10.1007/ s11886-015-0593-9.

Suárez, M., Romero, M. P., Macià, A., Valls, R. M., Fernández, S., Solà, R., & Motilva, M. J. (2009). Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid- phase extraction plate and liquid chromatography-tandem mass spectrometry. Journal of Chromatography B, 877, 4097– 4106.

Suárez, M., Romero, M. P., Ramo, T., Macià, A., & Motilva, M. J. (2009). Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. Journal of Agricultural and Food Chemistry, 57, 1463–1472.

Tiihonen, K., Ouwehand, A. C., & Rautonen, N. (2010). Human intestinal microbiota and healthy ageing. Ageing Research Reviews, 9, 107–116.

Valdés, L., Cuervo, A., Salazar, N., Ruas-Madiedo, P., Gueimonde, M., & González, S. (2015). The relationship

between phenolic compounds from diet and microbiota: Impact on human health. Food & Function, 6, 2424–2439.

Vissers, M. N., Zock, P. L., Roodenburg, A. J. C., Leenen, R., & Katan, M. B. (2002). Olive oil phenols are absorbed in humans. The Journal of Nutrition, 132, 409–417.

Wang, D., Williams, B. A., Ferruzzi, M. G., & D'Arcy, B. R. (2013). Microbial metabolites, but not other phenolics derived from grape seed phenolic extract, are transported through differentiated Caco-2 cell monolayers. Food Chemistry, 138, 1564–1573.

Zhao, W., Wang, Y., Liu, S., Huang, J., Zhai, Z., He, C., & Meng, H. (2015). The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments. PLoS ONE, 10, e0117441. doi:10.1371/journal.pone.0117441.

Zhao, Z., Egashira, Y., & Sanada, H. (2004). Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in liver. The Journal of Nutrition, 134(11), 3083–3088.

SUPPLEMENTARY MATERIAL

Table 1 of Supplementary Material. Phenolic composition of the secoiridoid extract (SEC) and nutritional composition of the standard fed pellets.

Secoiridoid extract	
Compound	**Concentration (mg/kg extract)**
HT	4176.82 ± 185.40
p-HPEA-EDA	936.37 ± 60.66
3,4-DHPEA-EDA	40995.97 ± 1085.46
p-HPEA-EA	378.82 ± 21.63
3,4- DHPEA-EA	1628.98 ± 274.01
OLE	480.00 ± 70.33
Standard fed pellets	
Ingredients: Wheat, corn, corn gluten, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, aminoacids mix, minerals and vitamins.	
Component	**% of diet**
Proteins	14.3 %
Fat	4 %
Carbohydrates	48 %
Fibers (∑ crude & neutral)	21 %
∑ minerals	2.7 %
∑ aminoacids	10.5 %
∑ vitamins (A, D, E, K, B_1, B_2, Niacin, B_6, Pantothenic Acid, B_{12}, Biotin, Folate, Choline)	

Values of extracts are means ± SD (n= 4). HT: Hydroxytyrosol; *p*-HPEA- EDA: Dialdehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-EDA: Decarboxymethyl elenolic acid linked to hydroxytyrosol; *p*-HPEA-EA: Aldehydic form of elenolic acid linked to tyrosol; 3, 4- DHPEA-EA: Isomer of oleuropein aglycone. OLE: oleuropein.

Table 2 of Supplementary Material. SRM conditions for the analysis of phenolic metabolites by UPLC-MS/MS.

Phenolic compound	Quantification (SRM_1)			Identification (SRM_2)			Standard in which has been quantified
	SRM	Cone voltage (V)	Collision energy (eV)	SRM	Cone voltage (V)	Collision energy (eV)	
Hydroxytyrosol	153 > 123	35	10	153 > 95	35	25	Hydroxytyrosol
Hydroxytyrosol sulfate	233 > 153	40	15	233 > 123	40	25	Hydroxytyrosol-3-*O*-sulfate
Hydroxytyrosol disulfate	313 > 233	40	10	313 > 153	40	30	Hydroxytyrosol-3-*O*-sulfate
Hydroxytyrosol-3-*O*-glucuronide	329 > 153	40	20	329 > 123	40	25	Hydroxytyrosol
Hydroxytyrosol-4-*O*-glucuronide	329 > 153	40	20	329 > 123	40	25	Hydroxytyrosol
Homovanillic alcohol	167 > 152	35	15	167 > 122	35	25	Hydroxytyrosol
Homovanillic alcohol sulphate	247 > 167	40	15	247 > 152	40	25	Hydroxytyrosol
Homovanillic alcohol glucuronide	343 > 167	40	20	343 > 152	40	35	Hydroxytyrosol
Homovanillic acid	181 > 137	25	10	181 > 122	25	15	Hydroxytyrosol
Homovanillic acid sulphate	261 > 181	40	15	261 > 137	40	25	Hydroxytyrosol
Homovanillic acid glucuronide	357 > 181	40	20	357 > 137	40	30	Hydroxytyrosol
Hydroxytyrosol acetate sulphate	275 > 153	35	15	275 > 123	35	30	Hydroxytyrosol
Elenolic acid	241 > 139	30	15	241 > 127	30	20	Hydroxytyrosol
Elenolic acid sulphate	321 > 241	40	15	321 > 139	40	20	Oleuropein
Elenolic acid glucuronide	417 > 241	40	15	417 > 139	40	20	Oleuropein
Oleuropein	539 > 377	35	15	539 > 275	35	20	Oleuropein
Oleuropein glucuronide	715 > 539	55	15	715 > 377	55	25	Oleuropein
Oleuropein aglycone sulfate	457 > 377	40	15	457 > 275	40	20	Oleuropein
Oleuropein aglycone glucuronide	553 > 377	40	15	553 > 377	40	20	Oleuropein
Methyl oleuropein aglycone sulfate	471 > 391	40	15	391 > 275	40	20	Oleuropein
Oleuropein aglycone derivate 1	555 > 523	40	15	555 > 275	40	25	Oleuropein
Oleuropein aglycone derivate 2	571 > 539	40	15	571 > 377	40	25	Oleuropein
Catechol	108.9 > 90.9	40	15	-	-	-	Catechol
Phenylacetic acid	135 > 91	20	5	-	-	-	HPA
Hydroxyphenylacetic acid	151 > 107	20	10	-	-	-	HPA
Hydroxyphenylacetic acid sulphate	231 > 151	20	20	231 > 107	20	25	HPA
Hydroxyphenylacetic acid glucuronide	327 > 151	20	15	327 > 107	20	25	HPA
Dihydroxyphenylacetic acid	167 > 123	20	10	-	-	-	DHPA
Hydroxybenzoic acid	137 > 93	30	15	-	-	-	HPA
Protocatechuic acid	153 > 109	45	15	-	-	-	Hydroxytyrosol
Hydroxyphenylpropionic acid	165 > 121	20	10	-	-	-	HPP
Hydroxyphenylpropionic acid sulphate	245 > 165	35	15	245 > 121	35	20	HPP
Dihydroxyphenylpropionic acid	181 > 137	20	15	-	-	-	HPP
Hippuric acid	178 > 134	40	10	-	-	-	Hippuric acid
Hydroxyhippuric acid	194 > 100	40	10	-	-	-	Hippuric acid

HPA: *p*-Hydroxyphenylacetic acid; HPP: 3-(4-Hydroxyphenyl)propionic; DHPA: 3,4-Dihydroxyphenylacetic

Table 3 of Supplementary Material. Linearity, calibration curves, reproducibility, accuracy, LOD and LOQs for the analysis of the commercial studied phenolic compounds by UPLC-MS/MS in spiked biological samples.

a) Plasma samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (0.5 µM)	Accuracy (*n*=3) (0.5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.03-2.77	y = 9.425 x + 0.013	11.2	98	0.002	0.005
Hydroxytyrosol-3-O-sulfate	0.03-277	y = 3.486 x + 0.024	9.8	102	0.005	0.015

b) Urine samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (25 µM)	Accuracy (*n*=3) (25 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.03-94.98	y = 5.412 x + 0.042	14.2	103	0.01	0.03
Oleuropein	0.07-78.67	y = 63.158 x – 0.107	13.4	99	0.02	0.07

c) Liver samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (2.5 µM)	Accuracy (*n*=3) (2.5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.009-179	y = 266.2 x + 0.019	9.8	100	0.003	0.009
Oleuropein	0.05-54.03	y = 108.85 x – 0.085	7.5	97	0.02	0.05

d) Kidney samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (2.5 µM)	Accuracy (*n*=3) (2.5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.60-72.88	y = 7.811 x – 0.019	10.2	97	0.20	0.60
Oleuropein	0.08-21.95	y = 25.934 x – 0.019	9.5	96	0.03	0.08

e) Stomach content samples *

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (5 µM)	Accuracy (*n*=3) (5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.03-636.36	y = 59.92 x + 65.54	7.9	96	0.01	0.03
Oleuropein	0.40-191.67	y = 305.74 x – 389.78	8.6	102	0.15	0.40

f) Small intestine content samples

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (10 µM)	Accuracy (*n*=3) (10 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.3-636.36	y = 108.02 x – 99.243	10.1	104	0.15	0.3
Oleuropein	0.19-191.67	y = 133.37 x + 529.84	12.6	103	0.03	0.19
p-Hydroxyphenylacetic acid	0.01-112.83	y = 520.91 x + 469.32	10.1	99	0.003	0.01
3,4-Dihydroxyphenylacetic aci	0.55-544.64	y = 63.319 x – 569.53	8.6	102	0.18	0.55
3-(4-Hydroxyphenyl)propionic acid	0.84-1084.34	y = 24.08 x + 159.63	9.9	97	0.25	0.84
Hippuric acid	5.44-543.58	y = 80.624 x – 1640.4	12.8	95	2.44	5.44
Catechol	0.3-745.75	y = 97.53 x – 124.56	10.5	105	0.15	0.3

g) Caecum content samples

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (5 µM)	Accuracy (*n*=3) (5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.004-636.36	y = 110.12 x – 98.147	12.4	102	0.001	0.004
Oleuropein	0.004-592.67	y = 145.48x + 624.75	13.4	95	0.001	0.004
p-Hydroxyphenylacetic acid	0.002-728.3	y = 518.22 x + 396.24	12.4	96	0.001	0.003
3,4-Dihydroxyphenylacetic acid	0.45-544.64	y = 62.124 x – 475.04	11.5	99	0.15	0.45
3-(4-Hydroxyphenyl)propionic acid	0.84-1084.38	y = 20.084 x + 152.04	11.6	105	0.28	0.84
Catechol	0.75-745.75	y = 102.64 x – 113.29	12.2	102	0.001	0.004

h) Faeces

Phenolic compound	Linearity (µM)	Calibration curve	RSD (%) (*n*=3) (50 µM)	Accuracy (*n*=3) (5 µM)	LOD (µM)	LOQ (µM)
Hydroxytyrosol	0.01-636.36	y = 96.116 x – 60.08	12.4	97	0.003	0.01
Hydroxytyrosol-3-*O*-sulfate	0.004-428.21	y = 450.49x + 142.58	11.9	99	0.001	0.004
p-Hydroxyphenylacetic acid	0.004-567.70	y = 559.73 x + 1009.6	12.7	101	0.001	0.004
Hippuric acid	0.54-543.58	y = 52.505 x – 120.03	13.0	103	0.2	0.54
Catechol	0.75-745.75	y = 103.57 x + 139.85	12.2	102	0.001	0.004

* Calibration curves (based on peak abundance) were plotted using y=a+bx, where *y* is the peak abundance ratio (analyte/IS) and *x* is the concentration ratio (analyte/IS). This was applied when catechol (IS) was not detected in samples.

Table 4 of Supplementary Material. Concentration of the metabolites detected in metabolic tissues (liver and kidney) obtained after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol (HT), secoiridoids (SEC) and oleuropein (OLE). The results are expressed as the mean ± SEM.

Phenolic compound	Control	HT	SEC	OLE
Liver (nmol/g tissue)				
Hydroxytyrosol sulfate	0.03 ± 0.00[a]	2.74 ± 0.40[b]	3.23 ± 0.30[b]	2.05 ± 0.25[b]
Homovanillic alcohol sulfate	n.d.	0.34 ± 0.10[a]	0.60 ± 0.07[a]	0.25 ± 0.02[b]
Homovanillic acid glucuronide	0.01 ± 0.00[a]	8.65 ± 0.86[d]	2.70 ± 0.07[b]	3.30 ± 0.06[c]
Elenolic acid sulfate	n.d.	-	0.77 ± 0.49	0.55 ± 0.21
Elenolic acid glucuronide	n.d.	-	0.35 ± 0.02	0.38 ± 0.06
Methyloleuropein aglycone sulfate	n.d.	-	0.12 ± 0.01	0.12 ± 0.02
Oleuropein	n.d.	-	0.19 ± 0.02	0.22 ± 0.05
Kidney (nmol/g tissue)				
Hydroxytyrosol sulfate	1.82 ± 0.18[a]	8.69 ± 0.32[b]	6.65 ± 0.70[b]	5.62 ± 0.95[b]
Homovanillic alcohol sulfate	0.72 ± 0.12[a]	2.12 ± 0.22[b]	1.86 ± 0.19[b]	1.79 ± 0.28[b]
Homovanillic acid glucuronide	1.78 ± 0.08[c]	0.79 ± 0.05[a]	1.41 ± 0.11[b]	1.02 ± 0.11[a]
Elenolic acid sulfate	0.16 ± 0.03	-	0.16 ± 0.06	0.11 ± 0.03
Elenolic acid glucuronide	0.13 ± 0.04[a]	-	0.55 ± 0.02[b]	0.20 ± 0.02[a]

Publication 3

Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood

Talanta 159: 189-193 (2016)

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

APPLICATION OF DRIED BLOOD SPOT CARDS TO DETERMINE OLIVE OIL PHENOLS (HYDROXYTYROSOL METABOLITES) IN HUMAN BLOOD

María Carmen López de las Hazas [a], Maria José Motilva [a], Carme Piñol [b], Alba Macià [a,*]

[a] Food Technology Department, Agrotecnio Center, Escola Tècnica Superior d'Enginyeria Agrària, University of Lleida, Avda. Alcalde Rovira Roure 191, 25198 Lleida, Spain.
[b] Department of Medicine, Facultat de Medicina, University of Lleida, Avda/Alcalde Rovira Roure 80, 25198 Lleida, Spain

Abstract

In this study, a fast and simple blood sampling and sample pre-treatment method based on the use of the dried blood spot (DBS) cards and ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) for the quantification of olive oil phenolic metabolites in human blood was developed and validated. After validation, the method was applied to determine hydroxytyrosol metabolites in human blood samples after the acute intake of an olive oil phenolic extract. Using the FTA DMPK-A DBS card under optimum conditions, with 20 mL as the blood solution volume, 100 mL of methanol/Milli-Q water (50/50, v/v) as the extraction solvent and 7 disks punched out from the card, the main hydroxytyrosol metabolites (hydroxytyrosol-3-O-sulphate and hydroxytyrosol acetate sulphate) were identified and quantified. The developed methodology allowed detecting and quantifying the generated metabolites at low µM levels. The proposed method is a significant improvement over existing methods to determine phenolic metabolites circulating in blood and plasma samples, thus making blood sampling possible with the volunteer pricking their own finger, and the subsequent storage of the blood in the DBS cards prior to chromatographic analysis.

Keywords: Blood; Dried blood spot cards; Hydroxytyrosol; Olive oil extract; UPLC-MS/MS

1. INTRODUCTION

In recent years, the biological effects of food bioactive compounds (FBCs) have been related to their bioavailability and their temporal and spatial distribution in the body. The concept of bioavailability is complex and includes: (i) availability for absorption or "bioaccessibility"; (ii) absorption; (iii) tissue distribution and (iv) bioactivity [1]. Based on the intense biological metabolism of some FBCs, mainly phenolic compounds, it is very important to determine the metabolites circulating for a better understanding of the fate of the parent compounds in the food.

Only when the circulating forms and the pharmacokinetics of the FBCs are known, a more complete picture can be obtained about their bioavailability and possible correlation with bioefficacy [1]. That is why absorption studies of FBCs in general, and polyphenols in particular, are of great importance for establishing the dose-exposure relationship, the impact of the composition of the food matrix, metabolism after being consumed, and the kinetic of absorption, among other aspects. The knowledge of these factors that determine the polyphenol absorption is essential to all those involved in food production and nutritional assessment. Although human clinical trials are mandatory for testing a functional ingredient, these involve an extremely complex organization of volunteers, and are a huge technical and economical investment [1]. Ideally, as a first step, feasibility studies should be performed in a small group of humans as a proof-of-concept to explore the effect of the dose and food matrix composition on absorption based on the analysis of the FBCs or their metabolites circulating in blood or plasma samples. This first step is achieved through a so-called acute intake or post-prandial study. In these studies, the volunteers are cited on the day of the experiment after fasting overnight. After the acute ingestion of the test food, blood samples are obtained by venipuncture from volunteers and are collected under fasting conditions and at different times, usually between 6 and 8 h after the intake of the test food. After blood has been collected, plasma samples are obtained by centrifuging the Vacutainer™ tubes containing the EDTA-K2 anticoagulant. After that, the extraction of the FBC metabolites from the plasma samples is usually carried out by liquid-solid extraction prior to the chromatographic analysis.

Nevertheless, for these post-prandial studies, the collection of blood samples has serious limitations because, in general, is necessary to insert a cannula into the vein for multiple blood sampling, resulting in intense discomfort for the volunteers. Additionally, it requires staff qualified in blood extraction and special infrastructure where the volunteers must spend a long time. A strategy to overcome these limitations and simplify the blood sampling and sample pretreatment procedure could be the use of dried blood spot (DBS) cards. Filter paper has been used for the collection and analysis of human blood sampling to screen newborn babies for congenital metabolic diseases for over 50 years [2]. Over the last few years, DBS sampling has been used for clinical and pre-clinical pharmacokinetic studies, taking advantage of smaller sampling needs and simplified sample collection and handling [3]. Briefly, DBS sampling involves collecting and storing a small volume of blood obtained from a human or study animal, via a simple prick (on the heel, finger, toe, or tail) or other means, on a card made of cellulose or polymer materials. Due to the ease of collection, DBS cards can even be used at home by the volunteers themselves in the human clinical or epidemiological studies [4,5]. Besides the simple sample collection, this technique allows the

storage of blood samples on the card and recovery of the target compounds with an extraction solvent (sample pre-treatment for clean-up) prior to their chromatographic analysis. The future of this simple idea for collecting and transporting a valid biological sample seems to be unlimited.

In recent years, DBS with liquid chromatography coupled to mass spectrometry (LC-MS) has gained significant interest as a potentially powerful tool for analyzing small molecules in different areas, such as therapeutic drug monitoring, toxicology and pharmaceuticals [6–8]. Recently, we developed a method using DBS cards to determine hydroxytyrosol metabolites in urine samples after a sustained intake (21 days) of phenol-enriched olive oil [9]. Due to the satisfactory results obtained, and to expand the applicability of DBS cards to bioavailability studies, the aim of this study was to develop and validate a method for detecting and quantifying hydroxytyrosol metabolites in blood samples using DBS cards combined with ultra-performance liquid chromatography coupled to tandem MS (UPLC-MS/MS). The method developed was applied to the analysis of hydroxytyrosol metabolites in human blood samples collected from three volunteers after an acute intake of an olive oil phenolic extract (OOE) at different post-intake times, from 0 to 120 min. To our knowledge, this is the first study in which DBS cards have been applied for the extraction of hydroxytyrosol metabolites in blood samples.

2. EXPERIMENTATION

2.1. Chemicals and reagents

Catechol as the internal standard (IS) and hydroxytyrosol were from Sigma-Aldrich (St. Louis, MO, USA) and Seprox Biotech (Madrid, Spain), respectively. Hydroxytyrosol-3-O-sulphate and hydroxytyrosol acetate sulphate were kindly supplied by Dr. de la Torre (Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Institut de Recerca, Hospital del Mar, Barcelona, Spain), and synthesized according to the method reported by Khymenets et al. [10]. Stock solutions of individual phenolic standard compounds were prepared by dissolving each compound in methanol at a concentration of 2000 mg/L, and storing these in dark flasks at 4 °C. Methanol (HPLC grade), acetonitrile (HPLC grade), and acetic acid were purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Ortho-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). The water was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

2.2. Blood sample collection

The olive oil phenolic extract (OOE) was prepared in our laboratory and the contents of hydroxytyrosol and the oleuropein derivatives (secoiridoids) were quantified by UPLC-MS/MS [11]. The OOE extract (2,4 g) was dissolved in 50 mL of water equivalent to a dose of 120 mg of hydroxytyrosol and oleuropein derivatives. The protocol of the study was approved by the Ethical Committee of

Human Clinical Research at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEIC-164 1326). The volunteers (three healthy adults aged between 25 and 30) gave written informed consent before starting the experiment protocol. The volunteers were asked to follow a diet free of virgin olive oil for a week before the study day. On the day of the study, each volunteer ingested the OOE extract solution (50 mL) after fasting overnight. The whole blood was taken by pricking the volunteers' fingers with disposable lancets (Unistik®, Owen Mumford Ltd, Woodstock, UK). Then, the blood drops were directly collected with micro-capillary blood collection tubes that contained lithium-heparin as an anticoagulant (Microvette®CB 300 LH, sample volume 300 µL Sarstedt, Numbrecht, Germany) (**Fig. 1**). Blood samples were collected at the baseline (0 h) and different post-intake times (15, 30, 60 and 120 min). To compare the results of the quantification of phenolic metabolites between whole blood and plasma, plasma was obtained by centrifuging directly (8784g for 15 min) the whole blood (the obtained at 30 min).

Fifty microliters of blood (or plasma) were mixed with 15 µL of catechol as IS (3500 mg/L prepared in methanol), and then 20 µL of this solution was spotted onto a premarked circle on the FTA DMPK cards (DBS filter paper) (GB Healthcare, Buckinghamshire, UK) (**Fig. 1**). The cards were then dried in a desiccator in the dark at room temperature for 2 h. After this time, 2-mm diameter disks were punched out from the card and placed into an Eppendorf tube with 100 mL of methanol/Milli-Q water (50/50, v/v). The Harris Uni-Core punch and Cutting Mat were supplied by Whatman Inc. (Sanford, ME, USA). The Eppendorf was then vortexed for 5 min and centrifuged at 8784 x g for 5 min at 4 °C. Afterwards, the supernatant was placed in a chromatographic vial. Then, 2.5 µL of this solution was directly injected into the UPLC-MS/MS system and the hydroxytyrosol metabolites were analyzed.

2.3. Liquid chromatography (UPLC-MS/MS)

The UPLC-MS/MS method for the analysis of hydroxytyrosol and its metabolites was the reported in our previous study [12]. The selected reaction monitoring (SRM) conditions used to identify and quantify the generated metabolites were those reported by Serra et al. [9].

2.4. Method validation

The instrumental quality parameters of the developed method, such as the linearity, calibration curve, repeatability, reproducibility, accuracy, detection limits (LODs) and quantification limits (LOQs), extraction recovery (%R) and the matrix effect (%ME) were determined by spiking blood samples (obtained under fasting conditions) with known concentrations of the standard compounds studied. These quality parameters were evaluated and determined as previously reported [9] by using blood as the biological matrix instead

of urine. The repeatability values were obtained analysing the target compounds at the same day and by using the same card; and the reproducibility values were obtained at different days and by using different DBS cards.

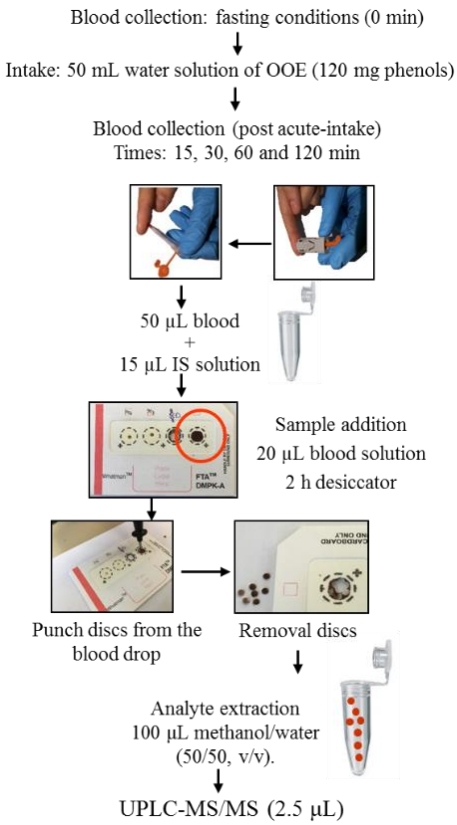


Fig. 1. Schematic procedure of the blood sampling and blood sample pre-treatment using DBS cards.

3. RESULTS AND DISCUSSION

3.1. Sample preparation and extraction: DBS cards

To obtain the maximum sensitivity to determine the hydroxytyrosol metabolites, the blood volume spotted onto the pre-marked circle on the card (from 5 to 20 µL) and the number of disks punched out from the card (from 1 to 7) were evaluated (Fig. 1). To carry out these experiments, hydroxytyrosol, hydroxytyrosol-3-O-sulphate, and hydroxytyrosol acetate-sulphate were spiked at known concentrations into blood samples obtained under fasting conditions (0 min). To optimize the blood solution volume applied to the card, four volumes: 5, 10, 15 and 20 µL were tested. The rest of the conditions were 100 µL of methanol/Milli-Q water (50/50, v/v) as the elution solvent, 2 h as the drying time, and one the number of the disks (extractions). As the blood volume applied to the card rose, the instrumental response of the standards also increased. Therefore, 20 µL of blood solution was chosen as the optimum blood volume. The extraction recovery (%R) and matrix effect (%ME) were similar for all four blood volumes tested (data not shown).

One potential of the DBS cards is its sample throughput. After the whole blood sample has been spotted onto a pre-marked circle, different extractions (disks) can be done by punching out from the entire card (both the centre and around it). Extractions from 1, 2, 4, 6 and 7 disks were studied in order to enhance the sensitivity of the method. The results showed that the instrumental response of hydroxytyrosol-3-O-sulphate increased linearly with the number of the disks punched from the card (**Fig. 2**). The other two standards, hydroxytyrosol and hydroxytyrosol acetate

sulphate followed the same behaviour (**Fig. S1 Supplementary data**). Therefore, 7 disks were selected.

3.2. Method validation

Under these optimum conditions (FTA® DMPK-A as the card, 20 µL as the blood solution volume, 100 µL methanol/Milli-Q water (50/50, v/v) as the solvent volume and nature, and 7 as the number of the disks), the %R and the %ME for standards of hydroxytyrosol and its metabolites were higher than 85% and lower than 10%, respectively (**Table 1**).

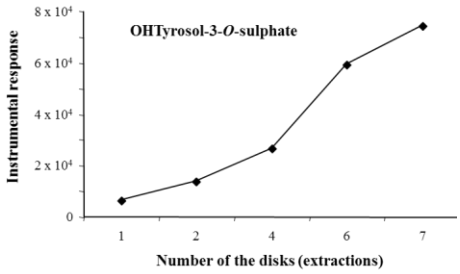


Fig. 2. Evaluation of the number of disks punched from the card for the analysis of hydroxytyrosol-3-O-sulphate spiked into whole blood sample at a concentration of 100 µM, and then analyzed by DBS and UPLC-MS/MS.

The instrumental quality parameters of the developed method, DBS with UPLC-MS/MS, were evaluated by spiking hydroxytyrosol, hydroxytyrosol-3-O-sulphate and hydroxytyrosol acetate sulphate into blank blood samples at different known concentrations (**Table 1**). The linearity range was from 0.5 to 100 µM. The calibration curves (obtained based on the integrated peak area) were calculated by using five points at different concentrations levels, and each concentration was injected three times. The determination coefficient (R2) of the calibration curves was higher than 0.994. The precision of the analytical method (repeatability and reproducibility) were determined by the relative standard deviation (% RSD) in terms of concentration, and these were calculated at three concentration levels of 10, 50 and 100 µM for hydroxytyrosol metabolites, and 20, 50 and 100 µM for hydroxytyrosol. The %RSDs for repeatability and reproducibility were lower than 7% and 11%, respectively. The accuracy was calculated from the ratio between the concentration found for the standard phenolic compounds studied compared

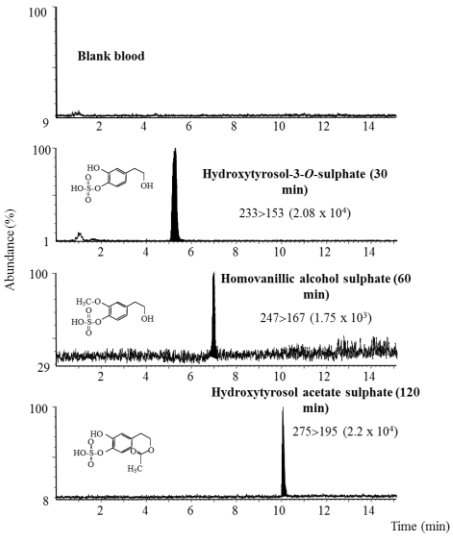


Fig. 3. Total ion chromatogram (TIC) of a blank blood sample (t¼ 0 min) and extracted ion chromatograms (EICs) of hydroxytyrosol metabolites obtained for the analysis of human blood samples after an acute intake of an OOE (120 mg hydroxytyrosol and oleuropein derivatives).

with the spiked concentration. This quotient was then multiplied by 100. This quality parameter was also studied at three concentration levels, the same as for the RSD%, and these ranged from 96% to 104%. The LODs and LOQs were calculated using the signal-to-noise ratio criterion of 3 and 10, respectively. The respective values were in the 0.15–3 to 0.5–10 µM range.

3.3. Determination of hydroxytyrosol metabolites in human blood samples

In order to show the applicability of the developed method, off- line DBS and UPLC-MS/MS was used to determine hydroxytyrosol and its metabolites generated in human blood samples obtained after an acute intake of OOE dissolved in water (equivalent to a dose of 120 mg of hydroxytyrosol derivatives). Three hydroxytyrosol metabolites were identified in the whole blood samples, these being hydroxytyrosol-3-O-sulphate, hydroxytyrosol acetate sulphate and homovanillic alcohol sulphate. **Fig. 3** shows the total ion chromatogram (TIC) of blank blood (t= 0 min) and the extracted ion chromatograms (EICs) of the hydroxytyrosol metabolites identified in the blood samples. These chromatograms correspond to hydroxytyrosol-3-O-sulphate at 30 min, homovanillic alcohol sulphate at 60 min, and hydroxytyrosol acetate sulphate at 120 min after the OOE intake, which were their maximum absorption times (tmax). As **Fig. 3** shows, no interference compounds were elutedat the same retention time as the identified phenolic metabolites. **Table 2** shows the concentration of the hydroxytyrosol metabolites detected in the whole blood at different times, and in the plasma at 30 min. The maximum concentrations of hydroxytyrosol-3-O-sulphate and hydroxytyrosol acetate sulphate were between 15 and 30 min after the OOE intake, and a second peak in the blood concentration was observed at 120 min.

This second adsorption peak could be explained to the resulting aglycones of the hydrolysis of oleuropein derivatives, which are complexes forms of hydroxytyrosol and the most abundant compounds in OOE [13]. Homovanillic alcohol sulphate was only detected at 60 min, coinciding with the decrease in the blood concentration of the main hydroxytyrosol metabolites. Due to the lack of a standard of this metabolite, this was tentatively quantified by using the calibration curve of hydroxytyrosol-3-O-sulphate.

Additionally, the quantification of hydroxytyrosol metabolites in the whole blood and plasma (obtained at 30 min) were compared (**Table 2**). The results of the present study are in agreement with a previous study where hydroxytyrosol-3-O-sulphate and hydroxytyrosol acetate sulphate were the main phenolic metabolites detected in the plasma after the acute intake (30 mL) of three phenol-enriched olive oils [13]. Additionally, the Cmax values in the plasma (1.35, 3.32, and 4.09 µmol/L) were similar to the plasma concentration detected in the present study at 30 min (**Table 2**), reinforcing the

applicability of the DBS cards for quantifying the circulating hydroxytyrosol metabolites. Comparing the results for the blood and plasma, the present study showed that the concentrations of hydroxytyrosol sulphate and hydroxytyrosol acetate sulphate determined in the plasma samples were between 6 and 2-fold lower than in the whole blood samples. Therefore, analysing blood samples allows the circulating phenolic metabolites to be determined at higher concentration levels compared with the plasma samples

4. CONCLUSIONS

The proposed method is a significant improvement over existing methods for determining the phenolic metabolites circulating in blood and plasma samples, thus making the blood sampling possible with the volunteers pricking their own fingers and subsequently storing the blood on the DBS cards. The method has also the advantage of simplifying the blood pre-treatment for the clean-up of sample prior to chromatographic analysis. For the first time, DBS cards combined with UPLC-MS/MS were successfully applied to the analysis of metabolites circulating as consumption biomarkers of virgin olive oil phenolics, such as hydroxytyrosol sulphate and hydroxytyrosol acetate, at concentration levels in the low µM range. In addition, the sensitivity was also enhanced by the fact of analysing whole blood rather than plasma. Based on the results of the present study, DBS sampling can be considered a good candidate to be

Table 1. Instrumental quality parameters for the analysis of hydroxytyrosol, hydroxytyrosol-3-O-sulphate and hydroxytyrosol acetate-sulphate in spiked blood samples by dried-spot-cards and UPLC-MS/MS.

Instrumental quality parameters	**Hydroxytyrosol**	**Hydroxytyrosol-3-*O*-sulphate**	**Hydroxytyrosol acetate-sulphate**
Extraction recovery (%R)	86	88	87
Matrix effect (%ME)	6	8	9
Linearity (µM)	10-100	0.5-100	0.8-100
Calibration curve	y = 158.38 x – 10.87	y = 2681.4 – 81.74	y = 1123.9 – 38.07
RSDs (n=3)			
C1 (10 µM)	10.5 *	10	9.5
C2 (50 µM)	8.5	7.5	7.0
C3 (100 µM)	7.5	6.5	6.5
Accuracy (n=3)			
C1 (10 µM)	95 *	98	100
C2 (50 µM)	99	100	102
C3 (100 µM)	101	102	104
LOQ (µM)	10	0.5	0.8
LOD (µM)	3	0.15	0.2

* The concentration analysed for hydroxytyrosol (C1) was 20 µM

applied, in the near future, for determining phenolic metabolites circulating in bioavailability (dose-exposure relationship, impact of food matrix composition or absorption kinetics) or epidemiological studies while reducing the limitations related to collecting blood sample from volunteers by venipuncture, handling these samples and the sample pre-treatment prior to the chromatographic analysis.

Table 2. Concentration of hydroxytyrosol metabolites in whole blood samples at different times (0 to 120 min) and in plasma (30 min) after the acute intake of OOE (n=3).

Metabolites (µM)	0 min	15 min	30 min		60 min	120 min
			blood	plasma		
Hydroxytyrosol-3-O-sulphat	n.d.	3.79 ± 0.76	10.8 ± 1.15	1.82 ± 0.37	1.96 ± 0.44	9.52 ± 1.22
Homovanillic alcohol sulphate	n.d.	n.d.	n.d.	n.d.	0.13 ± 0.06	n.d.
Hydroxytyrosol acetate sulphate	n.d.	5.11 ± 0.84	5.22 ± 0.89	3.07 ± 0.94	1.03 ± 0.21	10.2 ± 1.43

n.d.: not detected

Acknowledgements

This study was supported by the Spanish Ministry of Education and Science, the MEFOPC Project (AGL2012-40144-C03-03), and the University of Lleida through the M. C. López de las Hazas grant.

REFERENCES

[1] M.J. Motilva, A. Serra, L. Rubió, Nutrikinetic studies of food bioactive compounds: from in vitro to in vivo approaches, Int. J. Food Sci. Nutr. 66 (2015) S41–S52.

[2] J.V. Mei, J.R. Alexander, B.W. Adam, W.H. Hannon, Use of filter paper for the collection and analysis of human whole blood specimens, J. Nutr. 131 (2001) 1631S–1636S, http://dx.doi.org/10.1002/9781118890837.

[3] W. Li, M.S. Lee, Dried Blood Spots: Applications and Techniques, first edition., Wiley, Germany, 2014.

[4] M. González-Gross, C. Breidenassel, S. Gómez-Martínez, M. Ferrari, L. Béghin, A. Spinneker, L.E. Díaz, G. Maiani, A. Demailly, J. Al-Tahan, U. Albers, J. Wärnberg, B. Stoffel-Wagner, D. Jiménez-Pavón, C. Libersa, K. Pietrzik, A. Marcos, P. Stehle, Sampling and processing of fresh blood samples within a European multicenter nutritional study: evaluation of biomarker stability during transport and storage, Int. J. Obes. 32 (2008) S66–S75, http://dx.doi.org/10.1038/ijo.2008.185.

[5] U. Hoeller, M. Baur, F.F. Roos, L. Brennan, H. Daniel, R. Fallaize, H. Forster, E. R. Gibney, M. Gibney, M. Godlewska, K. Hartwig, S. Kolossa, C.P. Lambrinou, K. M. Livingstone, J.A. Lovegrove, A.L. Macready, Y. Manios, C.F.M. Marsaux, J. Martinez, C. Celis-Morales, G. Moschonis, S. Navas-Carretero, C. O'Donovan, R. San-Cristobal, W.H.M. Saris, A. Surwiłło, I. Traczyk, L. Tsirigoti, M.C.

Walsh, C. Woolhead, J.C. Mathers, P. Weber, Application of dried blood spots to determine vitamin D status in a large nutritional study with unsupervised sampling: The Food4Me project, Br. J. Nutr. 115 (2016) 202–211, http://dx.doi.org/10.1017/S0007114515004298.

[6] W. Li, F.L.S. Tse, Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules, Biomed. Chromatogr. 24 (2010) 49–65, http://dx.doi.org/10.1002/bmc.1367.

[7] A. Deep, P. Kumar, A. Kumar, A. Thakkar, Dry blood spot technique: a review, Int. J. Pharm. Sci. Rev. Res. 15 (2012) 90–94.

[8] J. Déglon, A. Thomas, P. Mangin, C. Staub, Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications, Anal. Bioanal. Chem. 402 (2012) 2485–2498, http://dx.doi.org/10.1007/s00216-011-5161-6.

[9] A. Serra, L. Rubió, A. Macià, R.-M. Valls, U. Catalán, R. de la Torre, M.J. Motilva, Application of dried spot cards as a rapid sample treatment method for determining hydroxy-tyrosol metabolites in human urine samples. Comparison with microelution solid-phase extraction, Anal. Bioanal. Chem. 405 (2013) 9179–9192, http://dx.doi.org/10.1007/s00216-013-7322-2.

[10] O. Khymenets, J. Joglar, P. Clapés, T. Parella, M.I. Covas, R. de la Torre, Biocatalyzed Synthesis and Structural Characterization of Monoglucuronides of Hydroxytyrosol, Tyrosol, Homovanillic Alcohol, and 3-(4′-Hydroxyphenyl) propanol, Adv. Synth. Catal. 348 (2006) 2155–2162, http://dx.doi.org/10.1002/adsc.200606221.

[11] M. Suárez, M.P. Romero, T. Ramo, A. Macià, M.J. Motilva, Methods for preparing phenolic extracts from olive cake for potential application as food anti-oxidants, J. Agric. Food Chem. 57 (2009) 1463–1472, http://dx.doi.org/10.1021/jf8032254.

[12] M.C. López de las Hazas, C. Piñol, A. Macià, M.P. Romero, A. Pedret, R. Solà, L. Rubió, M.J. Motilva, Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids, J. Funct. Foods 22 (2016) 52–63, http://dx.doi.org/10.1016/j.jff.2016.01.030.

[13] L. Rubió, R.M. Valls, A. Macià, A. Pedret, M. Giralt, M.P. Romero, R. de la Torre, M.I. Covas, R. Solà, M.J. Motilva, Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites, Food Chem. 135 (2012) 2922–2929, http://dx.doi.org/10.1016/j.foodchem.2012.07.085.

SUPPLEMENTARY MATERIAL

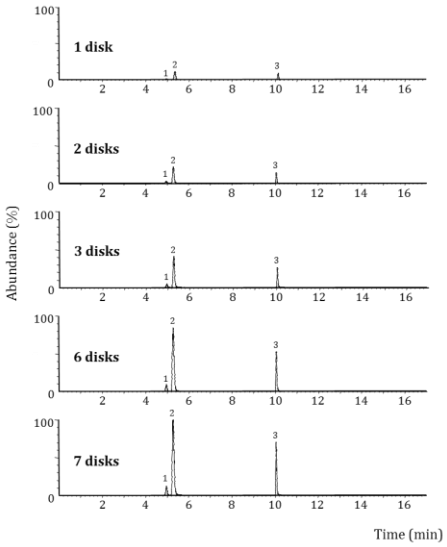


Figure S1-Supplementary data. Total ion chromatogram (TIC) obtained by using different number of disks for the analysis of spiked hydroxytyrosol and its metabolites in blood samples and then analysed by DBS and UPLC-MS/MS. Peak assignation was: 1) hydroxytyrosol, 2) hydroxytyrosol-3-O-sulphate, and 3) hydroxytyrosol acetate sulphate. Their concentrations were 100 µM. The intensity was 2.85 x 105.

Chapter 2

Biological effects of hydroxytyrosol and its biological plasma metabolites

Publication 4:

Molecular Nutrition Food Research 59: 2523-2536 (2015)

Publication 5:

Molecular Nutrition and Food Research 60:2114-2129 (2016).

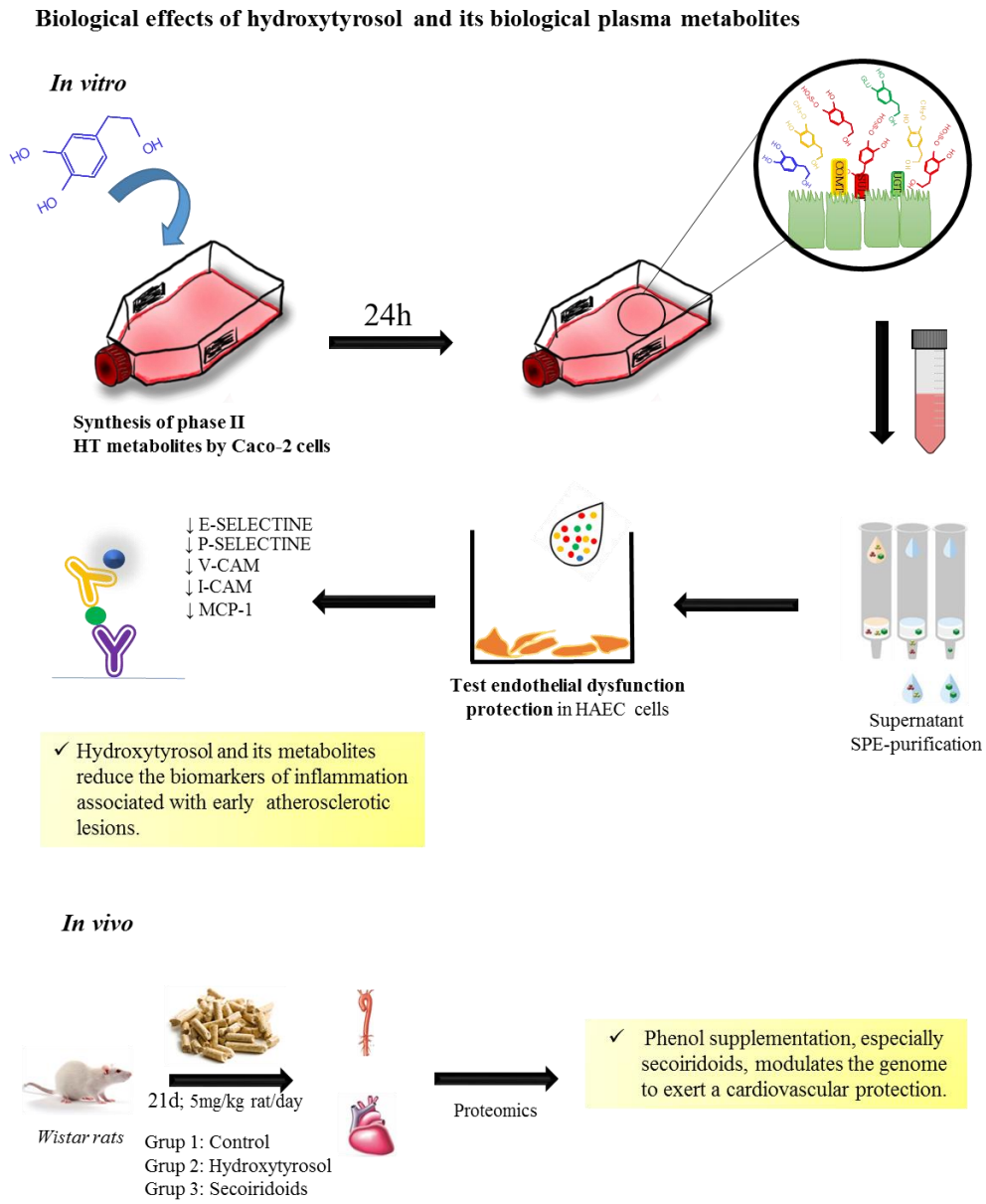


Figure 7: Experimental design and summatory of the main results of the chapter

Publication 4

Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells.

Molecular Nutrition Food Research 59: 2523-2536 (2015)

RESEARCH ARTICLE

PROTECTIVE EFFECT OF HYDROXYTYROSOL AND ITS PREDOMINANT PLASMATIC HUMAN METABOLITES AGAINST ENDOTHELIAL DYSFUNCTION IN HUMAN AORTIC ENDOTHELIAL CELLS

Úrsula Catalán[1*], Maria-Carmen López de las Hazas[2*], Laura Rubió[1,2], Sara Fernández-Castillejo[1], Anna Pedret[1], Rafael de la Torre[3], Maria-José Motilva[2] and Rosa Solà [1**]

[1] Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Reus, Spain
[2] Food Technology Department, Universitat de Lleida-AGROTECNIO Center, Lleida, Lleida, Spain
[3] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Facultat de Ciencies de la Salut i de la Vida, Universitat Pompeu Fabra (CEXS-UPF), Institut Hospital del Mar d'Investigacions Mediques (IMIM), Barcelona, Spain

Abstract

Scope: Hydroxytyrosol (HT) is the major phenolic compound in virgin olive oil (VOO) in free and conjugated forms that may exert health benefits against atherosclerosis. The native form of HT is undetectable in plasma due to an extensive first pass phase II metabolism. Therefore, it is necessary to find strategies to obtain HT metabolites and to demonstrate their protective role against the endothelial dysfunction.

Methods and results: Biosynthesis of the main plasmatic HT metabolites was performed through Caco-2 cells. The bioactivity of HT and the mixture of metabolites was tested at physiological concentrations (1, 2, 5, and 10 µM) in human aortic endothelial cells (HAEC) co-incubated with TNF-a (10 ng/mL) for 18 and 24 h. After the incubations, cells and media were analyzed to test possible deconjugation of metabolites or conjugation of HT. Both HT and metabolites significantly reduced the secretion of E-selectin, P-selectin, ICAM-1, and VCAM-1, but only HT metabolites further reduced MCP-1 at 24 h. HT underwent a conjugation process after incubation leading to its main metabolites in a dose-dependent manner.

Conclusion: Physiological HT metabolites, synthetized for the first time by using an intestinal cell model, might be responsible in part for the protection against endothelial dysfunction.

Keywords: Atherosclerosis; Caco-2; Human aortic endothelial cells; Hydroxytyrosol; Hydroxytyrosol metabolites.

1. INTRODUCTION

Virgin olive oil (VOO) a key component of the Mediterranean Diet (MD) has shown to contribute to cardiovascular disease (CVD) prevention. Part of this effect has been associated to VOO phenolic content [1, 2]. Results from the PREDIMED study, the first randomized clinical trial designed to assess the beneficial effects of the MD on the primary prevention of CVD, reported that the MD supplemented with VOO has a dual effect on the prevention of CVD, reducing classical cardiovascular risk factors and also having an intense anti-inflammatory effect through the downregulation of endothelial dysfunction markers related to atherosclerosis, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E- and P-selectin [3].

Hydroxytyrosol (HT) is the major phenolic compound in VOO in both free and conjugated form, mainly as oleuropein aglycone structures commonly named secoiridoids. HT has shown a wide range of biological functions, such as antioxidant, anticancer, anti-inflammatory, and neuroprotective activities, as well as having beneficial effects on the cardiovascular system [4, 5]. The potential health benefits of HT have stimulated intense research on the bioavailability and metabolism of VOO phenolic compounds, a requisite to support their potential benefits for human health. Previous studies have shown that VOO phenolic compounds undergo rapid hydrolysis under gastric and intestinal alkaline conditions resulting in significant increases in the amount of free HT entering the small intestine [6, 7]. Thus, HT becomes the major phenolic compound absorbed from the intestinal tract, which is further subjected to an extensive first pass metabolism, both in the intestinal epithelium and the liver, leading to the formation of phase II metabolites, mainly sulfate, methyl, and methyl–sulfate conjugates and glucuronides of HT [8–11]. Consequently, after the consumption of normal doses of VOO (less than 50 g/day) the native form of HT is not detected in plasma and the potential health benefits of VOO phenolic compounds could be either attributed to the biological activity of phase II metabolites or to free HT generated at cellular level by enzymatic deconjugation. So, it becomes essential to obtain the plasmatic HT metabolites, test them in cell-based experimental models at physiological concentrations and ex- amine a possible deconjugation process of these metabolites at the cellular level.

The physiological significance of most of the published articles on polyphenols bioactivity has been questioned as they still describe the effects of molecules that are only present in planta or in food. Nevertheless, detailed studies applying plasmatic and microbial metabolites of phenolic compounds are starting to establish reasonable mechanisms through which these physiological metabolites may exert beneficial effects [12]. In the case of VOO phenolics, a previous study tested the in vitro radical scavenging capacity of some of the HT metabolites observing that homovanillic alcohol (HVAlc) and the 3-

O-glucuronide conjugate appeared to be active in terms of antioxidant activity [13]. However, further observations suggested that none of the glucuronide conjugates of HT displayed antioxidant activity to protect LDL against oxidation [14].

Regarding the antiatherogenic effects, only HT in its native forms has been tested on preclinical experimental models describing its protective effects against atherosclerosis [15, 16] and no data have been published in relation to its biological metabolites. As reported by Valls et al., these metabolites could be involved in reducing the endothelial dysfunction in humans [17]. However, the mechanisms have not been demonstrated.

Endothelial dysfunction is involved in the early stages of atherosclerosis and is a key process in the development of CVD [18]. Briefly, the endothelial dysfunction in the early stages of atherosclerosis includes rolling (mediated by E- and P-selectin) and firm adhesion (mediated by VCAM-1 and ICAM-1) of blood leukocytes to the activated endothelial monolayer and directed migration (mediated by the monocyte chemotactic protein-1; MCP-1) of the bound leukocytes into the intima [18, 19]. So the endothelial dysfunction biomarkers E-selectin, P-selectin, VCAM-1, ICAM-1, and MCP-1 were selected in the present study as they have been considered as useful predictive biomarkers of atherosclerosis [20].The surface and soluble expression of these molecules is greatly increased in atherosclerotic lesions as a result of stimulation by pro-inflammatory cytokines, such as tumor necrosis factor-a (TNF-α). This cytokine was used in the present study as a stressor molecule for assessing the underlying mechanisms as it induces the release and surface expression of cell adhesion molecules [21, 22].

So, the main aim of the present study was to optimize the use of Caco-2 cell line as an alternative approach to obtain biological metabolites of HT previously detected in human plasma, and to evaluate their in vitro endothelial protective effect compared to HT alone at physiological concentrations [10].

2. MATERIALS AND METHODS

2.1. Solvents and reagents

HT (99.51% of purity, synthetic) was provided by Seprox Biotech (Madrid, Spain). Catechol, as the internal standard, (IS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxytyrosol-3-O-sulfate was custom-synthesized by Toronto Research Chemicals Inc. (Toronto, ON, Canada). Methanol (HPLC-grade), acetonitrile (HPLC-grade), ethanol, and dimethyl sulfoxide (DMSO) were purchased from Scharlab (Barcelona, Spain) and orthophosphoric acid (85%) from Panreac (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system(Millipore Corp., Medford, MA, USA).

2.2. Caco-2 cell culture treatments to obtain HT metabolites

Caco-2 cell line was purchased from ATCC (American Type Culture

Collection, US). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (HyClone, Thermo scientific, Logan, Utah) supplemented with 10% of fetal bovine serum (Sigma-Aldrich, Madrid, Spain), 1% L-glutamine (HyClone, Thermo Scientific, Logan, UT, USA), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; HyClone, Thermo Scientific), penicillin and streptomycin (100 mg/mL) (Gibco, Spain). Cells were cultured at 37°C, in a 5% CO_2 humidified incubator. Media culture was replaced every 2 days and phosphate-buffered saline (PBS; HyClone, Thermo Scientific) was used to wash the cells. In preliminary experiments, the stability of HT was evaluated over time (6, 12, 24, and 48 h) with DMEM or Hanks' Balanced Salt Solution (HBSS; HyClone, Thermo Scientific) media and finally, HBSS was chosen to perform all the

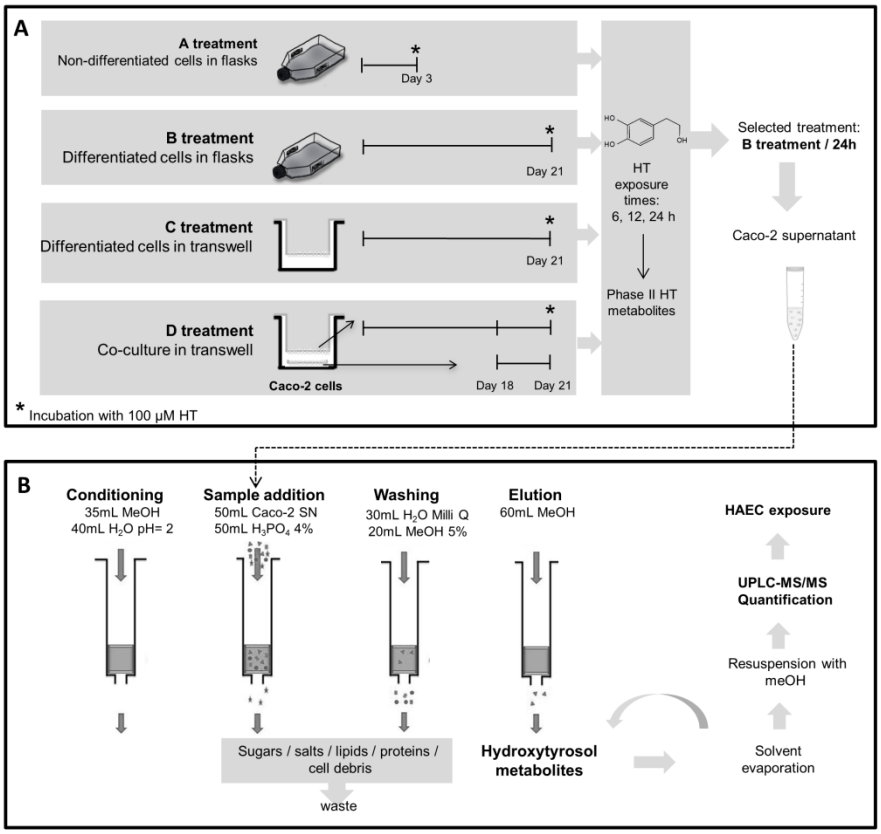


Figure 1. Caco-2 cell culture treatments for the production of HT metabolites and the methodology used for their extraction and purification. (A) Caco-2 cell culture treatments tested to obtain HT metabolites. T75 flask and transwell inserts were used as two different culture conditions to obtain HT metabolites with different differentiation states and at different times of HT exposure (6, 12, and 24 h). (B) Procedure for the extraction and purification of HT metabolites from Caco-2 cell supernatants.

experiments due to a better stability of HT over the time in these media. In order to select the optimal cell treatment to obtain HT metabolites, T75 flask and transwell inserts (both from Corning Costar, Netherlands) were tested as two different culture conditions (**Fig. 1**A). On the one hand, Caco-2 cells were seeded in T75 flasks at a density of 6.6×10^4 cells/mL and their HT conjugation rate was evaluated at two differentiation states: three days after seeding (A treatment) or after 21 days of culture (B treatment). On the other hand, Caco-2 cells were seeded in the apical side of transwell inserts at a density of 4.6×10^4 cells/mL and culturedfor 21 days (C treatment). D treatment consisted of 18 days of Caco-2 culture (4.6×10^4 cells/mL) in the apical side of transwell inserts and, after 18 days, an additional co-culture of Caco-2 cells was seeded in the basolateral side (5×10^4 cells/mL) for three more days, making a total of 21 days. Monolayer formation in transwell inserts was evaluated by transepithelial electrical resistance (TEER) measurement with a voltohmmeter (Millipore Corp.) selecting cells with TEER > 250 Ω/ cm^2. For all treatments (A, B, C, and D), the initial concentration of HT in the culture medium was 100 µM (HT stock solution 100 mM in methanol) and three exposure times were tested (6, 12, and 24 h). To preempt cytotoxicity, methanol concentrations never exceeded 0.1% (v/v) in culture media. Finally, supernatants of T75 flasks (A and B treatments) and the sum of the apical and basolateral supernatants from transwell inserts (C and D treatments) were collected. Prior to chromatographic analysis (see Section 2.4), HT and its metabolites were extracted from the cell media using micro-elution solid phase extraction (µSPE) plates with OASIS HLB µElution Plates 30 µm (Waters Corp., Milford, MA, USA) according to the previous study [23].

After the selection of 21-day cultured cells in T75 flasks with 24 h of HT incubation (B treatment) as the optimal treatment (see Section 3.2), different HT concentrations (10, 15, 20, 25, 50, and 100 µM) in the culture medium were assayed in order to test the effect of the HT concentration on the transformation yield of HT into its metabolites.

2.3 Purification of HT metabolites from Caco-2 culture medium

Once the B treatment (21-day cultured cells in T75 flasks) incubated with 100 µM HT for 24 h was selected as the optimal treatment for obtaining HT metabolites, the µSPE plates were replaced by SPE cartridges with a higher sorbent size (OASIS HLB Elution Plates 6 g, 35 cc; Waters, Milford, MA, USA) to obtain major amounts of HT metabolites. The conditioning of the SPE cartridges was done by adding sequentially 35 mL of methanol and 40 mL of milli-Q water acidified at pH 2 with acetic acid (**Fig. 1B**). Extractions were done by loading 50 mL of Caco-2 supernatant, which had been previously mixed with 50 mL of phosphoric acid at 4%. The loaded cartridges were washed with 30 mL of milli-Q water and 20 mL of methanol at 5%. Finally, the retained phenolic

compounds were eluted using 60 mL of methanol at 100%. This elution solvent was evaporated to dryness under a nitrogen stream in an evaporating unit at 30°C (PIERCE Model 18780, IL, USA). In order to obtain a more concentrated solution, after evaporation, eluates from several cartridges were collected and consecutively evaporated in the same tube. The residue was reconstituted with 1 mL of methanol. The extract was filtered through a 0.22 µm nylon syringe filter (Teknokroma, Barcelona, Spain) and transferred to the autosampler vial before the quantification of HT metabolites by liquid chromatography. This stock solution of HT metabolites was used to study their effectivity on endothelial dysfunction model in HAEC.

2.4 Chromatographic analysis of HT metabolites

HT metabolites were analyzed by AcQuity UPLCTM coupled to PDA detector and a triple quadrupole detector (TQD)TM mass spectrometer (Waters, Milford). The analytical column was a High Strength Silica (HSS) T3 column (100 × 2.1 mm, 1.8 µm). During the analysis, the column was kept at 30°C and the flow rate was 0.3 mL/min using 0.2% acetic acid as solvent A and methanol as solvent B. The elution started at 3% of eluent B and was linearly increased to 15% of eluent B in 6 min. Then, it was linearly increased to 70% in 8 min and further increased to 100% in 3 min. Then, it was returned to the initial conditions in 1 min, and the re-equilibration time was 2 min. The injection volume was 2.5 µL.

A TQD mass spectrometer was used as the detector system. Ionization was done by electrospray (ESI) in the negative mode and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters were capillary voltage of 3 KV, source temperature of 150°C, and desolvation temperature of 400°C with a flow rate of 800 L/h. Nitrogen (99% purity, N2LCMS nitrogen generator, Claind, Como, Italy) and argon (S99.99% purity, Alphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. Two transitions were acquired for HT and the metabolites generated, one for quantification and a second for confirmation purposes. The optimized SRM conditions are presented in Supporting Information Table 1. The software used was MassLynx 4.1. HT and HT-3-O-sulphate were quantified with their own calibration curves, and the other HT metabolites were tentatively quantified by using the calibration curve of HT. The calibration curves were constructed by using cellular medium free of HT metabolites.

2.5. HAEC cell culture for testing HT and HT metabolites activity

HAEC (Cascade BiologicsTM, Portland, OR, USA) at the fifth passage were seeded on Nunclon^{TM} Δ surface 12-well plates at a density of approximately 44×10^3 of viable cells/mL (1 mL/well). For the first 24 h, cells were maintained in complete cell culture medium (CM) composed of M-200

medium supplemented with 2% (v/v) low-serum growth supplement, 10 mg/mL gentamicin, 0.25 mg/ml amphotericin B (purchased from Gibco by Life Technologies, Madrid, Spain), 100 U/mL penicillin and 100 mg/mL of streptomycin (from Biowest-Labclinics, Barcelona, Spain). The cells were grown to confluence at 37°C in a humidified incubator (Heracell 150; Madrid, Spain) with an atmosphere containing 5% CO_2. The medium was replenished every 2 days with fresh CM. Viewed under an IMT2 microscope (Olympus, Barcelona, Spain), confluent monolayers displayed a typical monolayer phenotype of quiescent endothelial cells after 5 days in culture.

Experiments of time and dose-response were conducted in order to establish the final conditions of TNF-α as a stimulant. Stock solutions used for the experiments were: 5 mM of HT (commercial) dissolved in sterile H2O and 10 mM of the purified solution of HT metabolites dissolved in methanol (Panreac, Madrid, Spain). Appropriate dilutions were prepared until the final concentrations to be tested in the culture media (1, 2, 5, and 10 µM) were obtained. To preempt cytotoxicity, methanol concentrations never exceeded 0.1% (v/v) in culture media. Then, HAEC were co-incubated with HT or HT metabolites at 1, 2, 5, and 10 µM and TNF-α (10 ng/mL; Calbiochem, Darmstadt, Germany), the respective vehicle control alone (sterile H2O for HT or absolute methanol for HT metabolites) or TNF-α alone (10 ng/mL) for 18 or 24h. Additional experiments were performed to test HT at lower concentrations (<1 µM) equivalent to 9% of the residual HT remaining in the HT metabolites mixture at the concentrations tested (0.09, 0.18, 0.45, and 0.9 µM).

After co-incubation, supernatants were collected and stored at –20°C for batched measurements of the soluble forms of the selected cell adhesion molecules (VCAM-1/ CD106, E-selectin/CD62E, P-selectin/CD62P and ICAM-1/ CD54) or chemokine (MCP-1/CCL2) and for the activity of the lactate dehydrogenase (LDH) released. Cells were lysed with NaOH 0.5 M and stored at –80°C for measuring total cellular protein amount in order to adjust cell adhesion molecules and chemokine measurements.

In order to study the possible deconjugation of HT metabolites or conjugation of HT during the incubation with HAEC cells, the metabolite pattern in the cells and also in the culture media at the end of the incubation (24 h) was performed according to the same chromatographic method described in Section 2.4.

2.6. Cytotoxicity assays

The cytotoxicity effect of HT (25-200 µM) on Caco-2 cells was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay [24]. 200 000 Caco- 2 cells/mL were seeded in 24-multiwell plates and incubated with DMEM. After 24 h, DMEM media were replaced with the same media enriched with different concentrations of the HT for 24 h at 37°C and 5% CO2. Cells were then washed twice with PBS. DMEM enriched in 0.5 mg/mL of MTT (Sigma-Aldrich, Madrid,

Spain), was added to each well and incubated for 3 h. To determine the formazan production, once supernatants discarded, cells were lysed using an extraction solution composed of DMSO and ethanol at a concentration of 1:1. A colorimetric assay was performed at 570 nm optical density.

The cytotoxicity effect of HT or HT metabolites on HAEC was assessed with a Cytotoxicity Detection Kit LDH (Roche Applied Science, Mannheim, Germany) as previously described [25]. For these experiments, TNF-α treatment was considered the maximum stress condition for the cells. The activity of the LDH released from the cells was measured in cell-free supernatants collected at the end of the experiment. Results are expressed as the mean optical density (OD: 492 nm) and standard deviation (SD; error bars) of LDH produced by the cells under each treatment condition.

2.7. Total cellular protein quantification

In order to adjust the soluble cell adhesion molecules (E- selectin, P-selectin, VCAM-1, and ICAM-1) and chemokine (MCP-1) total cellular protein concentration from cell lysates was determined using the Bradford protein assay from Bio-Rad with bovine serum albumin (BSA). A range of concentrations of BSA were used as the calibration standards for the protein assay. The absorbance was measured at 595 nm. The results were expressed in milligrams of total cellular protein per milliliter compared to the maximum stimulation with TNF-α (10 ng/mL).

Measurement of soluble cell adhesion molecules and chemokine protein secretion by HAECs stimulated with TNF-α Luminex® Performance Assay was used to determine VCAM- 1, E-selectin, ICAM-1, and P-selectin protein secretion and DuoSet® enzyme-linked immunosorbent assays (ELISA) was used to detect MCP-1/CCL2 chemokine protein secretion. Both protein detection kits were from R&D Systems (Vitro, Madrid, Spain) and the tests were conducted according to the manufacturer's protocol.

All experimental data were compared to the outcomes in the TNF-α alone incubation, since this achieved the maximal secretion of soluble cell adhesion molecules or chemokine. A vehicle-alone control (sterile H2O for HT or absolute methanol for HT metabolites) was run in parallel and used as the control in the experiments. Results are expressed as the percentage of soluble cellular adhesion molecules or chemokine protein secretion adjusted by total cellular protein and standard error of the mean (SEM; error bars) compared to the maximum stimulation with TNF-α (10 ng/mL).

2.8. Statistical analyses

Unless otherwise stated, all experiments were performed at least twice and each incubation condition was set up in duplicate. One-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) post-hoc test was used for multiple comparisons. A

Table 1. HT and its metabolites concentration (µM) in culture media after HT exposure among different Caco-2 cell pretreatment models (T75 flask or transwell inserts) at different times of exposure (6, 12 and 24h). A: 3-day cultured cells in T75 flasks; B: 21-day cultured cells in T75 flasks; C: 21-day cultured cells in the apical side of transwell inserts and D: 18-day cultured cells in the apical side plus 3-day co-cultured cells in the basolateral side of transwell inserts (21 days in total). For treatments C and D values were the sum of apical and basolateral sides. The initial concentration of HT in culture media for all the experiments was 100µM. Based on the yield transformation of HT into phase II metabolites, the differentiated cells in flasks (B treatment) after 24 h of HT exposure were selected as the best approach.

Concentration (µM)	**6 h**				**12 h**				**24 h**			
	T75 FLASK		TRANSWELL		T75 FLASK		TRANSWELL		T75 FLASK		TRANSWELL	
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
HT *	76.10 ± 10,60^{a}	66.47 ± 10.47	41.87 ± 10.25^{c}	23.52 ± 9.81^{c}	45.15 ± 15.71^{a}	39.20 ± 5.24^{b}	9.29 ± 3.04^{c}	13.90 ± 1.53^{c}	39.91 ± 1.32^{a}	6.98 ± 0.20^{b}	5.18 ± 3.77^{b}	5.25 ± 0.33^{b}
sulfHT **	0.11 ± 0.00^{a}	10.77 ± 0.88^{b}	10.08 ± 3.02^{b}	10.37 ± 3.30^{b}	0.63 ± 0.77^{a}	27.73 ± 3.57^{b}	758 ± 2.62^{c}	7.27 ± 3.58^{c}	2.33 ± 0.83^{a}	63.42 ± 9.23^{b}	8.00 ± 2.44^{c}	7.99 ± 1.33^{c}
4-GlucHT*	n.d.	0.19 ± 0.04	1.03 ± 0.63	1.08 ± 0.72	n.d.	0.48 ± 0.19	0.62 ± 0.40	0.62 ± 0.35	0.13 ± 0.03^{a}	0.60 ± 0.13^{b}	0.64 ± 0.43^{b}	0.76 ± 0.39^{b}
3-GlucHT*	n.d.	0.06 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	n.d.	0.10 ± 0.11^{a}	0.01 ± 0.01^{b}	0.02 ± 0.01^{b}	0.02 ± 0.01^{a}	0.31 ± 0.06^{b}	0.02 ± 0.01^{a}	0.03 ± 0.00^{b}
sulfHVac*	n.q.	n.q.	0.08 ± 0.02	0.07 ± 0.04	n.q.	n.q.	0.06 ± 0.03	0.07 ± 0.06	n.q.	0.06 ± 0.04	0.03 ± 0.01	0.03 ± 0.01
glucHVac*	n.d.	n.d.	0.02 ± 0.02	0.03 ± 0.04	n.d.	n.d.	0.04 ± 0.02	0.03 ± 0.01	n.d.	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
sulfHVAlc	0.03 ± 0.00^{a}	0.28 ± 0.02^{b}	0.03 ± 0.00^{a}	0.05 ±0.02^{a}	0.02 ± 0.00	n.q.	0.03 ± 0.02	0.02 ±0.02	0.02 ± 0.02^{a}	2.27 ± 0.48^{b}	0.01 ± 0.00^{a}	0.02 ± 0.02^{a}
glucHVAlc	n.q.	0.11 ± 0.02*	n.q.	n.q.	n.q.	0.35 ± 0.08	n.q.	n.q.	n.q.	0.29 ± 0.16	n.q.	n.q.

HT: hydroxytyrosol; sulfHT: hydroxytyrosol sulfate; 4-GluHT: hydroxytyrosol 4' glucuronide; 3-GlucHT: hydroxytyrosol 3' glucuronide; sulfHVac: homovanillic acid sulfate; glucHVac: homovanillic acid glucuronide; sulfHVAlc: homovanillic alcohol sulfate; glucHVAlc: homovanillic alcohol glucuronide. n.d.: not detected; n.q.: not quantified. Different superscript letters (a,b,c) mean significant differences between treatments (A,B, C and D) in the same time tested for each metabolite. One-way Analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) post-hoc test was used for multiple comparisons ($p < 0.05$). * Quantified by using the calibration curve of HT.
** Quantified by using the calibration curve of HT-3-O-sulfate.

value of $p < 0.05$ was considered statistically significant. A requisite for the analytical quality of the model was the control of several aspects involved in the cellular process and analytical performance of measurements. Thus, we evaluated the precision of the model by calculating the SD, SEM, and the coefficients of variation (CV). All the results were analyzed with the Statistical Package for the Social Sciences (SPSS) software (version 22.0).

3. RESULTS

3.1. cytotoxicity

Results from the Caco-2 MTT cytotoxicity assay showed that HT was not cytotoxic at doses from 25 to 150 µM (data not shown). The cytotoxicity assay of the supernatants taken at the end of the study corresponding to the co-incubation of HT or HT metabolites at different concentrations (1, 2, 5, and 10 µM) and TNF-α (10 ng/mL) for different exposure times (18 and 24 h), measured as the activity of LDH release showed no difference compared to the TNF-α (10 ng/mL) treatment alone, confirming that HT and HT metabolites are not cytotoxic at the concentrations and times tested (**Supporting Information Fig. 1**). Lower concentrations of HT (<1 µM) did not show any toxicity (data not shown).

3.2. Effect of Caco-2 cell pretreatment on HT metabolite production

Table 1 shows the metabolism yield after exposing HT (100 µM) to different Caco-2 cell pretreatment models (A, B, C, and D) at different exposure times (6, 12, and 24 h). Comparing the T75 flask and transwell treatments (A and B versus C and D, respectively), the results showed that HT was much less stable in transwell inserts and also showed less metabolic activity compared to cells cultured in T75 flasks. Regarding the different exposure times, the highest metabolic transformation was obtained after 24 h. Longer exposure times were tested, but more than 24 h did not increase the metabolism yield and the total amount of metabolites obtained decreased with the time, probably due to a degradation process. Significant differences ($p < 0.05$) were observed on the biotransformation of HT into its main metabolites between the two T75 flask models (A and B) at all time points studied (6, 12, and 24 h), observing that Caco-2 cells under B treatment (21-day cultured cells) had a significantly higher metabolic activity compared to A treatment (3-day cultured cells). These results confirmed that differentiated Caco-2 cells can exert a higher conjugation enzymatic activity (catechol- O-methyltransferase, UDP-glucuronosyltransfe-rase, and sulfo-transferase) compared to non differentiated cells and thus, they appeared to be a more appropriate approach to obtaining HT phase II metabolites. In the case of the transwell models, no significant differ-rences were observed in the production of any HT metabolite when comparing C and D treatments. This could be attributed to the low enzymatic activity of the non differentiated co-cultured cells over the 3 days used in D treatment.

Based on the biotransformation yield of

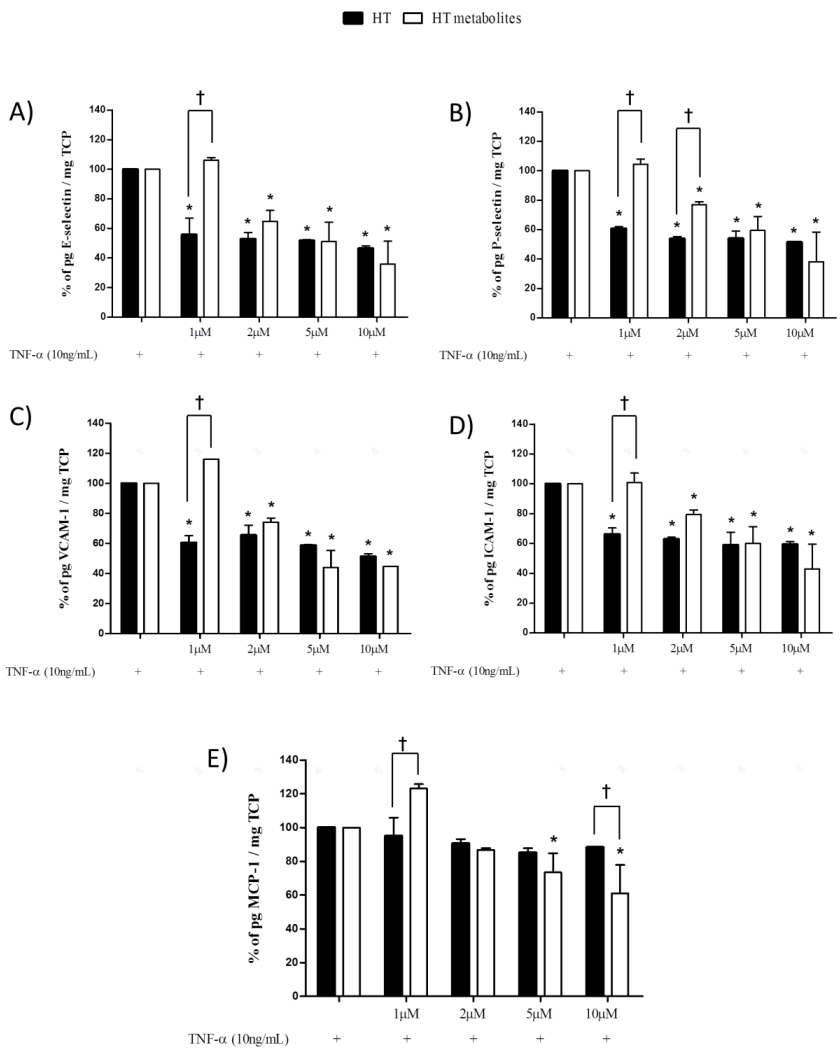


Figure 2. Effect of HT and HT metabolites on E-selectin, P-selectin, VCAM-1, ICAM-1, and MCP-1 protein secretion in HAEC stimulated by TNF-α after 24 h. HAEC were co-incubated with HT or HT metabolites at 1, 2, 5, and 10 µM and TNF-a (10 ng/mL) for 24 h. (A) Effect of HT or HT metabolites on E-selectin protein secretion. (B) Effect of HT or HT metabolites on P-selectin protein secretion. (C) Effect of HT or HT metabolites on VCAM-1 protein secretion. (D) Effect of HT or HT metabolites on ICAM-1 protein secretion. (E) Effect of HT or HT metabolites on MCP-1 protein secretion. Results are expressed as the percentage of soluble cellular adhesion molecules or chemokine protein secretion adjusted by total cellular protein and standard error of the mean (SEM; error bars). *$p < 0.05$ versus TNF-α alone. † $p < 0.05$ compared between HT and HT metabolites at the same concentration.

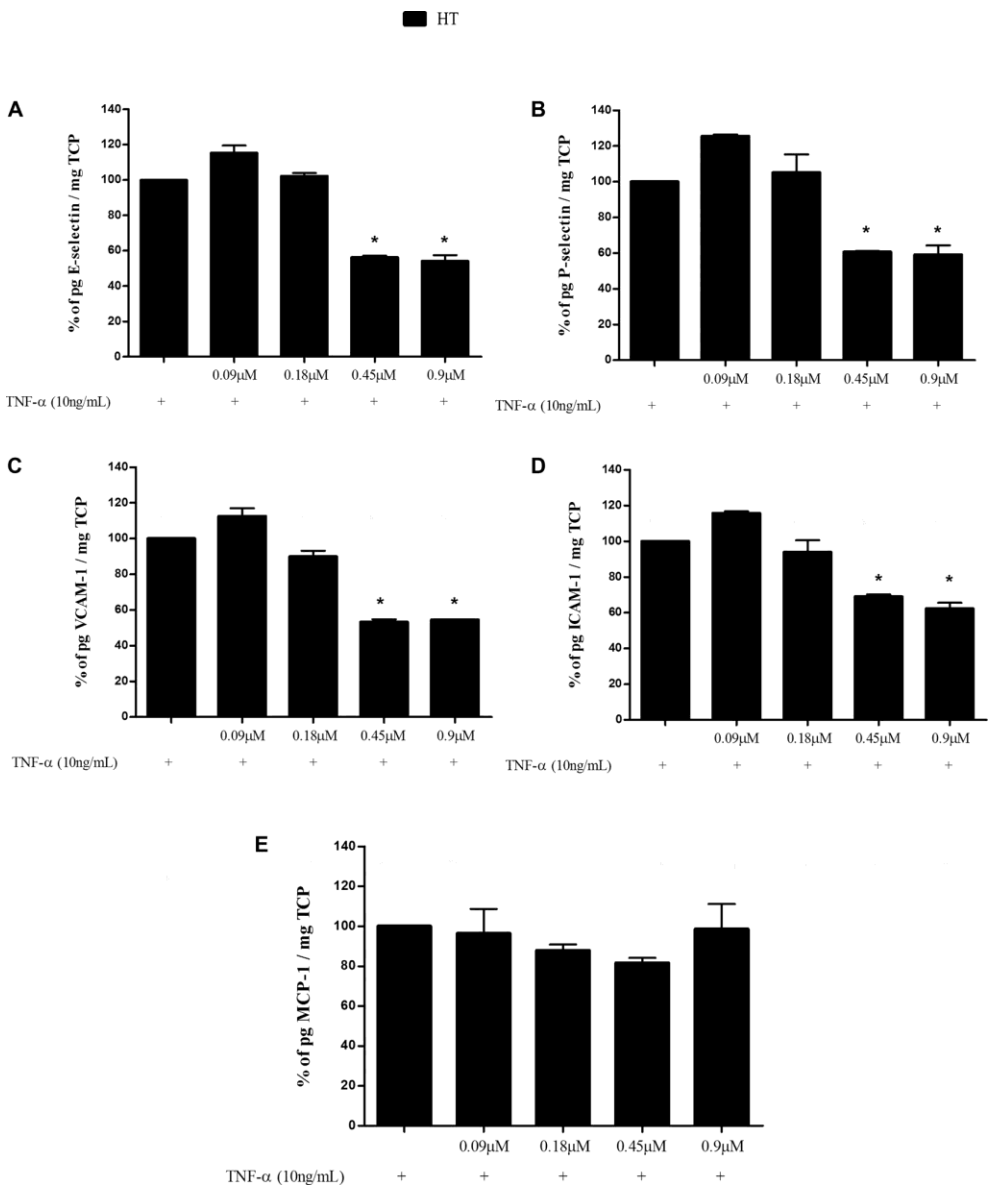


Figure 3. Effect of HT at lower concentrations (<1 µM) on E-selectin, P-selectin, VCAM-1, ICAM-1, and MCP-1 protein secretion in HAEC stimulated by TNF-α after 24 h. The tested concentrations (0.9, 0.45, 0.18, and 0.09 µM) were equivalent to the 9% of the tested doses of HT metabolites (10, 5, 2, and 1 µM).

HT into phase II metabolites, the differentiated cells in T75 flasks (B treatment) after 24 h of HT exposure were selected as the best approach to obtaining HT metabolites with the lowest concentration of residual free HT in the culture medium. Apart from being more efficient in terms of metabolic activity, T75 flasks are much cheaper, easier to handle, and have a bigger surface compared to transwell inserts, which allows higher amounts of metabolites to be obtained. Moreover, different HT concentrations in the culture medium were assayed in order to test the effect of the initial HT concentration on its transformation yield into phase II metabolites.

Results showed that the metabolic transformation was similar in all concentrations tested (10, 15, 20, 25, 50, 100 µM) (**Supporting Information Table 2**). Based on these results and considering that after the incubation of 100 µM HT a major amount of HT metabolites was obtained, 100 µM HT was selected to produce the stock solution of HT metabolites.

3.3. Effect of HT and HT metabolites on E-selectin, P-selectin, VCAM-1, ICAM-1, and MCP-1 protein secretion

After 18 h of co-incubation, significant reductions were observed with HT at all tested doses in E-selectin levels ($p<0.05$), with reductions ranging from 32.7 to 45.9% compared to TNF-α alone. HT metabolites significantly reduced E-selectin protein secretion by 36.6% ($p<0.05$) only at 10 µM (**Supporting Information Table 3 and Fig. 2**).

After 24 h of co-incubation, the reduction trend observed at 18 h was confirmed. HT reduced E-selectin, P-selectin, VCAM-1, and ICAM-1 at all tested doses (1, 2, 5, and 10 µM) compared to TNF-α alone ($p<0.05$) but the reductions did not follow a dose-dependent response (**Fig. 2**). HT metabolites reduced E-selectin, P-selectin, VCAM-1, and ICAM-1 at 2, 5, and 10 µM and additionally, they also reduced MCP-1 significantly at 5 and 10 µM compared to TNF-α alone. In thiscase, the molecules were reduced in a dose-dependent manner ($p < 0.05$; **Fig. 2**).

It is noteworthy that at 10 µM, HT metabolites were more effective than HT at reducing E-selectin, P-selectin, VCAM-1, ICAM-1, and MCP-1 with respective reductions of 62.4, 61.9, 55.4, 57.0, and 39.0% compared to HT (53.6, 48.6, 48.7, 40.6, and 11.7 %, respectively (**Supporting Information Fig. 2 and Table 3**). However, these reductions in HT metabolites compared to HT were only significant for the MCP-1 molecule.

To exclude the effects of the residual HT present in the HT metabolite mixture, lower concentrations of HT (0.9, 0.45, 0.18, and 0.09 µM) equivalent to the 9% of the tested doses of HT metabolites (10, 5, 2, and 1 µM, respectively) were tested (**Fig. 3**). Results showed that concentrations of 0.45 and 0.9 µM were effective in the reduction of VCAM-1, ICAM-1, P-selectin, and E-selectin indicating that the activity of the metabolite mixture could be due in part to the residual HT in the case of the highest doses (5 and 10 µM). However,

when testing 0.18 µM HT any significant effect was observed in the inhibition of the adhesion molecules, whereas the concentration of 2 µM of the HT metabolites mixture exerted a significant effect in the reduction of all adhesion molecules. In addition, the reduction percentages observed after testing 0.45 and 0.9 µM of HT were 10% lower compared to their respective HT metabolites mixtures (10 and 5 µM). These results indicate that HT metabolites are in part responsible for the attenuation of the inflammation-induced expression of adhesion molecules and could probably exert a synergistic effect together with HT. In the case of the chemokine MCP-1, lower concentration assays confirmed that HT has no effect on reducing MCP-1 at any concentration.

Vehicle control condition for HT (sterile H2O) or HT metabolites (absolute methanol) are not shown due to their low values of E-selectin, P-selectin, VCAM-1, ICAM-1, and MCP-1 protein secretion concentrations. They were not within the limits of detection of the techniques used for determining these molecules in this study, so they were assumed as practically zero.

3.4 Analysis of HT and metabolites in cells and culture media after incubation

The metabolite pattern in the cells and also in the medium at the end of the incubation was analyzed. Unexpectedly, results showed that after 24 h of incubation with HT, the native compound underwent a conjugation process leading to its main metabolites in a dose-dependent manner (**Fig. 4**), revealing that HAEC cells have conjugation enzymatic capabilities, mainly catechol-O-methyltransferase and sulfo-transferase activity (Supporting Information Table 4). On the other hand, after incubating the HT metabolite mixture with HAEC cells, small amounts of free HT were detected in cells and HT did not increase in parallel to the tested dose, indicating that the metabolites were not hydrolysed in cells during the assay and probably HT was degraded during incubation. Results from culture media presented a very similar profile compared to cells (**Fig. 4**).

4. DISCUSSION

The present study is among the first to obtain a mixture of physiological phase II metabolites of HT and demonstrating that they are able to exert an effect in the reduction of the endothelial dysfunction biomarkers in an in vitro endothelial cell model at plasmatic concentrations achievable following ingestion of olive oil [10]. The remaining amount of unaltered HT in urine is very low (<1%) [8, 11] and undetectable in plasma after the consumption of nutritional amounts of VOO phenols and, therefore, the role of HT phase II metabolites in preventing atherosclerosis must be considered.

However, in vitro studies with endothelial cell models carried out to date used HT [26, 27] rather than the majority forms that appear in human plasma. So, in order to perform mechanistic studies, first, it is necessary to obtain the HT metabolites,

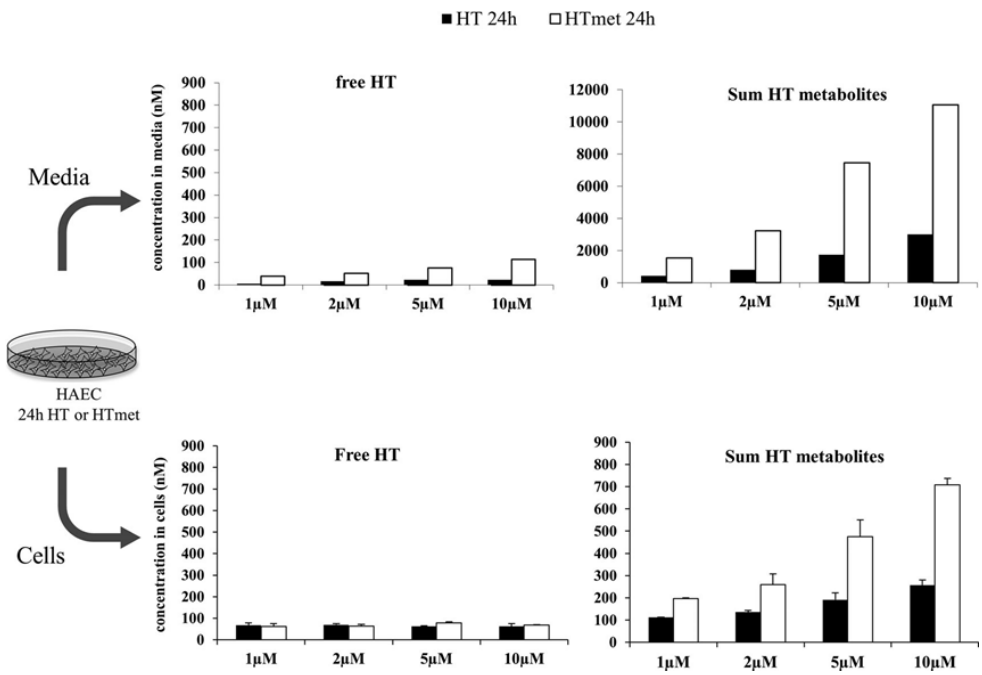


Figure 4. HT and metabolites detected in HAEC cells and culture media after co-incubation with HT or HT metabolites at 1, 2, 5, and 10 µM and TNF-a (10 ng/mL) for 24 h.

which are not commercially available. For this proposal, in the present study Caco-2 cells were incubated with HT in order to produce a mixture of HT metabolites resembling the human plasma after the intake of virgin olive oil. Previous studies have used different approaches to obtainning phase II metabolites of phenolic compounds [28–31]. The chemical synthesis approach could be considered the cheapest, but its main drawback is that most chemical procedures yield mixtures of α- and β– anomers along with side products [32]. Biocatalysis-assisted synthesis using tissue homogenates or recombinant conjugating enzymes (glucuronosyl-transferases and sulfotransferases) is an alternative strategy for preparing phenolic conjugates [33]. However, enzymatic synthesis requires the use of either expensive enzymes, co-factors (uridine 5'-diphosphoglucuronic acid and adenosine-5'-phosphosulfate), or liver microsomes and the degree of conversion is also very low [28]. Another alternative could be the use of biological samples to obtain phenolic phase II metabolites, which have been reported previously for the isolation of resveratrol glucuronide conjugates from human urine [30], thymol sulfate from rat urine [29], or catechin and quercetin from rat plasma [31]. The main drawback of this approach is that it requires the performance of an in

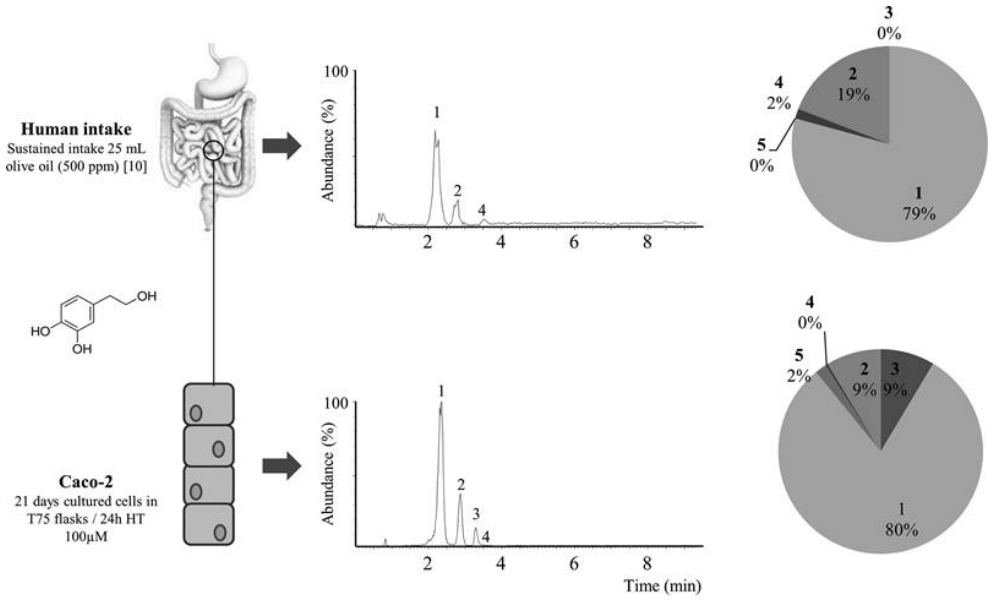


Figure 5. HT metabolites profile in human plasma versus Caco-2 cell production. Extracted ion chromatograms in human plasma after a sustained intake of virgin olive oil and in purified extract obtained from Caco-2 exposed to HT (differentiated cells in flasks/24-h exposure of HT 100 µM).

vivo study and the amounts obtained are very small. In relation to VOO phenolic metabolites, a previous study attempted to synthetize HVAlc and the glucuronic forms of HT using porcine liver microsomes [34].

In another study the potential hepatic metabolism of VOO phenolic compounds by HepG2 cell lines was tested, and the glucuronide, methyl- glucuronide, and methyl conjugates of HT were the main metabolites produced [35]. In both studies no sulfate conjugates were produced, which could be attributed to the low sulfotransferase activity of HepG2 cell line and its very low/partial expression of cytochrome P-450 [36]. Our previous studies in humans indicate that sulfation constitutes the major pathway for HT metabolism in humans [9, 10], and up todate, any effective and simple strategy to obtain a pool of the real phase II circulating HT metabolites has been developed. In the present study, we demonstrate that the use of Caco-2 cells as bioreactors for the generation of phase II metabolites of HT is a very appropriate tool given their availability, long life-span, stable phenotype, high metabolic efficiency, ease of handling, and the fact that they do not require the use of human volunteers or animals. Caco-2 cells, which are usually used as a model for studying the intestinal transport and metabolism of some dietary

compounds [37], are obtained from human colon adenocarcinoma and spontaneously differentiate in a confluence culture as intestinal absorptive cells due to changes in their morphology [38]. Once they are differentiated, Caco-2 cells exert phase II conjugate enzymes and cytochrome P-450 isoforms, and are useful for studying their metabolic activity and characterizing human circulating metabolites, as reported in previous studies in which the metabolic profile was determined after a polyphenol exposure [7, 37]. This approach also allowed to obtain a metabolic profile very similar to the human plasma after the consumption of nutritional amounts of VOO phenols described in our previous study [10]. In this study the main metabolites de- tected in plasma after a sustained intake of 25 mL of VOO with high phenolic content (500 mg total phenolic compounds/ kg oil) were the sulfated forms of HT (79%), homovanillic alcohol sulfate (sulfHVAlc) (19%), and in a minor proportion the homovanillic acid sulfate (sulfHVac). The Caco-2 intestinal cell model allowed us obtaining a mixture in which the main metabolites were HTsulf (80%), sulfHVAlc (10%) and in a minor proportion, HT glucuronides and free HT (**Fig. 5**). So the obtained mixture of metabolites is very close to the previously observed human plasma profile, with a 90% of resemblance.

Results from the TNF-α stimulated HAEC cells indicated that after 24 h of incubation both HT metabolites and free HT at physiological concentrations (1 to 10 µM) were effective at reducing E-selectin, P-selectin, VCAM-1, and ICAM-1 molecules involved at the first stages of atherosclerosis. In a previous study the inhibition of endothelial activation by free HT was described in a human umbilical vein endothelial cell (HUVEC) model, observing that HT was able to reduce lipopolysaccharide (LPS)-stimulated expression of VCAM-1 at low micromolar concentrations [15]. Another study observed reductions in E-selectin, VCAM-1, and ICAM-1 after HT exposure on HUVEC at doses of 5 and 25 µM of HT [39]. Regarding MCP-1, Richard N et al. also observed a significant reduction of MCP-1 after HT exposure (25 µM) on murine macrophages (RAW264.7 cells) [40]. In the two latter studies, the effect of HT metabolites was not tested, so the physiological significance of the results is questionable.

After the HT treatments we also observed that the remaining amounts of free HT in the cells and media did no differ significantly among the tested doses (**Fig. 4**), which was in accordance with the observed effects in the modulation of the cell adhesion molecules that were also maintained regardless of the dose.

However, when HT metabolites were incubated with HAEC cells, an increasing concentration of these metabolites was detected in the cells after the assay with a parallel dose-dependent inhibition. These data provide evidence of the potential effect of HT phase II metabolites in the reduction of the endothelial dysfunction biomarkers.

The residual amount of free HT in the

obtained metabolite mixture represents the major difference with the plasma data. Therefore, in order to test the possible activity of the residual amounts of HT present in the metabolite mixtures, lower concentrations were tested. Results indicated that in the case of adhesion molecules (VCAM-1, ICAM-1, E-selectin, and P-selectin) the attenuation effect of the metabolite mixtures could be due to a large extent to the residual HT. However, in all cases, reductions of the metabolite mixtures were 10% greater compared to their corresponding amounts of residual HT, indicating that metabolites also contribute in the inhibition. The effect of the metabolites was confirmed when the dose of 2 µM HT metabolites was tested, observing a significant inhibition of all adhesion molecules secretion, whereas its corresponding percentage of residual HT did not show any effect. From these results it can be concluded that metabolites might be in part responsible for the attenuation of the inflammation-induced expression of adhesion molecules.

In the case of the chemokine MCP-1, HT did not show any effect at any tested dose, whereas the doses of 5 and 10 µM of HT metabolites presented a significant effect, indicating in this case that metabolites clearly seem to be active on the inhibition of the secretion of this chemokine. The specific effect of HT metabolites on reducing MCP-1 could also indicate that free HT and HT metabolites could act reducing the endothelial dysfunction through different endpoints and having complementary activities. Unexpectedly, after the incubation assays with HT, an important amount of HT metabolites were detected in the cells and media, most of them sulfo and methylsulfo-conjugated (**Fig. 4**). Catechol-O-methyl-transferase have been previ-ously described in endothelial cells after incubating HUVEC with (–)-epicatechin, identifying the endothelial NADPH oxidase as a specific candidate target for the methylated forms [41]. SULT1A1, the main sulfotransferase enzyme responsible for the detoxification of xenobiotics, has been found in many different human tissues, with the highest abundance found in the liver, but it has never been detected in human aortic endothelial cells. Thus, the data provided in the present work add value to the study suggesting that aortic sulfotransferases may play an important role in reducing local cellular concentrations of native forms of phenolic compounds.

Although phase II phenolic metabolites have been generally considered to be pharmaco-logically inactive and targets for excretion [42], our results indicate that the inhibition of the endothelial dysfunction could be partly due to the HT metabolites. The effect of flavonoids (quercetin and (+)-catechin) and their different conjugates on cell adhesion has been recently reviewed [43] and it was established that conjugation can significantly affect the activity of aglycones both reducing and increasing the effects. So, given that the effects of conjugation can differ greatly on cell adhesion, research on the biological activity has to be done on a case-by-case basis.

Our results also demonstrate that effects

other than the reduction of ox-LDL and cholesterol, which have been demonstrated in humans [1, 44–46], may explain the anti-atherogenic action of VOO phenolic compounds. Two additional mechanisms involved in the vascular damage, platelet aggregation, and proliferation of smooth muscle cells, are also antagonized by the VOO phenolic compounds [16].

In conclusion, this is the first time that HT metabolites have been synthetized by using a differentiated Caco-2 cell line, obtaining a purified extract that resembles the human plasma HT metabolite profile after VOO intake. Results indicate that both free HT and HT metabolites are effective in the reduction of the endothelial dysfunction biomarkers but only HT metabolites reduce MCP-1 at physiological doses. Novel data are presented regarding the presence of xenobiotic detoxification enzymes in HAEC cells, which add value to the study suggesting that catechol-O-methyltransferase and sulfotransferases may play an important role in reducing local cellular concentrations of native forms of phenolic compounds in aortic endothelial cells. Our data suggest that HT metabolites might be responsible in part for the protection against endothelial dysfunction, and thus they are likely to contribute to the reduced risk in the early stages of atherosclerosis, which provide a clear future direction in olive oil phenols research.

Acknowledgements

This study was supported by the Spanish Ministry of Education and Science, The MEFOPC Project (AGL2012-40144-C03-03, AGL2012-40144-C03-02 and AGL2012-40144-C03-01) and by the University of Lleida through the M-C. López de las Hazas grant.

The authors have declared no conflict of interest.

REFERENCES

[1] Covas, M. I., Nyyssoönen, K., Poulsen, H. E., Kaikkonen, J. et al., The effect of polyphenols in olive oil on heart disease risk factors: A randomized trial. Ann. Intern. Med. 2006, 145, 333–341.

[2] Guasch-Ferré, M., Hu, F. B., Martínez-González, M. A., Fitó, M. et al., Olive oil intake and risk of cardiovascular disease and mortality in the PREDIMED Study. BMC Med. 2014, 12, 78.

[3] Casas, R., Sacanella, E., UrpıSarda, M., Chiva-Blanch, G. et al., The effects of the mediterranean diet on biomarkers of vascular wall inflammation and plaque vulnerability in subjects with high risk for cardiovascular disease. A randomized trial. PLoS One 2014, 9, e100084.

[4] Hu, T., He, X-W., Jiang, J-G., Xu, X-L., Hydroxytyrosol and its potential thera-peutic effects. J. Agric. Food Chem. 2014, 62, 1449–1455.

[5] Martín-Peláez, S., Covas, M. I., Fitó, M., Kusar, A. et al., Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Mol. Nutr. Food Res. 2013, 57, 760–771.

[6] Corona, G., Tzounis, X., Assunta Dessì, M., Deiana, M. et al., The fate of olive oil polyphenols in the gastrointestinal tract: implications of

gastric and colonic microflora-dependent biotransformation. Free Radic. Res. 2006, 40, 647–658.

[7] Rubió, L., Macià, A., Castell-Auví, A., Pinent, M. et al., Effect of the co-occurring olive oil and thyme extracts on the phenolic bioaccessibility and bioavailability assessed by in vitro digestion and cell models. Food Chem. 2014, 149, 277–284.

[8] Miro-Casas, E., Hydroxytyrosol disposition in humans. Clin. Chem. 2003, 49, 945–952.

[9] Suárez, M., Valls, R.M., Romero, M-P., Macià, A. et al., Bioavailability of phenols from a phenol-enriched olive oil. Br. J. Nutr. 2011, 106, 1691–701.

[10] Rubió, L., Farràs, M., de La Torre, R., Macià, A. et al., Metabolite profiling of olive oil and thyme phenols after a sustained intake of two phenol-enriched olive oils by humans: Identification of compliance markers. Food Res. Int. 2014, 65, 59– 68.

[11] Kotronoulas, A., Pizarro, N., Serra, A., Robledo, P. et al., Dose-dependent metabolic disposition of hydroxytyrosol and formation of mercapturates in rats. Pharmacol. Res. 2013, 77, 47–56.

[12] Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M. et al., Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid. Redox Signal. 2013, 18, 1818–1892.

[13] Tuck, K. L., Hayball, P. J., Stupans, I., Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. J. Agric. Food Chem. 2002, 50, 2404–2409.

[14] Khymenets, O, Fitó, M., Touriño, S, Muñoz-Aguayo, D. et al., Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. Drug Metab. Dispos. 2010, 38, 1417–1421.

[15] Carluccio, M.A., Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. Arterioscler. Thromb. Vasc. Biol. 2003, 23, 622–629.

[16] Petroni, A., Blasevich, M., Salami, M., Papini, N. et al., Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. Thromb. Res. 1995, 78, 151–160.

[17] Valls, R.-M., Farràs, M., Suárez, M., Fernández-Castillejo, S. et al., Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomised controlled trial. Food Chem. 2015, 167, 30–35.

[18] Libby, P., Ridker, P. M., Hansson, G. K., Progress and challenges in translating the biology of atherosclerosis. Nature 2011, 473, 317–325.

[19] Weber, C., Noels, H., Atherosclerosis: current pathogenesis and therapeutic options. Nat. Med. 2011, 17, 1410–1422.

[20] Barbaresko, J., Koch, M., Schulze, M. B., Nöthlings, U., Dietary pattern analysis and biomarkers of low-grade inflammation: a systematic literature review. Nutr. Rev. 2013, 71, 511–527.

[21] Stumpf, C., Fan, Q., Hintermann, C., Raaz, D. et al., Anti-inflammatory effects of danshen on human vascular endothelial cells in culture. Am. J. Chin. Med. 2013, 41, 1065– 1077.

[22] Hou, H., Zhou, R., Jia, Q., Li, Q. et al., Citreoviridin enhances tumor necrosis factor-a-induced adhesion of human umbilical vein endothelial cells. Toxicol. Ind. Health 2015, 31, 193–201.

[23] Suárez, M., Romero, M-P., Macià, A., Valls, R. M. et al., Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography-tandem mass spectro-metry. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2009, 877, 4097– 4106.

[24] Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 1983, 65, 55–63.

[25] Catalán, U., Fernández-Castillejo, S., Pons, L., Heras, M. et al., Alpha-tocopherol and BAY 11–7082 reduce vascular cell adhesion molecule in human aortic endothelial cells. J. Vasc. Res. 2012, 49, 319–328.

[26] Storniolo, C. E., Roselló -Catafau, J., Pintó, X., Mitjavila, M. T. et al., Polyphenol fraction of extra virgin olive oil protects against endothelial dysfunction induced by high glucose and free fatty acids through modulation of nitric oxide and endothelin-1. Redox Biol. 2014, 2C, 971–977.

[27] Zrelli, H., Matsuoka, M., Kitazaki, S., Zarrouk, M. et al., Hydroxytyrosol reduces intracellular reactive oxygen species levels in vascular endothelial cells by upregulating catalase expression through the AMPK-FOXO3a pathway. Eur. J. Pharmacol. 2011, 660, 275–282.

[28] Kittelmann, M., Rheinegger, U., Espigat, A., Oberer, L. et al., Preparative enzymatic synthesis of the acylglucuronide of mycophenolic acid. Adv. Synth. Catal. 2003, 345, 825–829.

[29] Rubió, L., Serra, A., Macià, A., Borrás, X. et al., Validation of determination of plasma metabolites derived from thyme bioactive compounds by improved liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2012, 905, 75–84.

[30] Radko, Y., Christensen, K. B., Christensen, L. P., Semi- preparative isolation of dihydroresveratrol-3-O-þ-d- glucuronide and four resveratrol conjugates from human urine after oral intake of a resveratrol-containing dietary supplement. J. Chromatogr. B 2013, 930, 54–61.

[31] Koga, T., Meydani, M., Effect of plasma metabolites of (+)- catechin and quercetin on monocyte adhesion to human aortic endothelial cells. Am. J. Clin. Nutr. 2001, 73, 941–948.

[32] Kaspersen, F. M., Van Boeckel, C. A. A., A review of the methods of chemical synthesis of sulphate and glucuronide conjugates. Xenobiotica 1987, 17, 1451–1471.

[33] Stachulski, A. V., Meng, X., Glucuronides from metabolites to medicines: a survey of the in vivo generation, chemical synthesis and properties of glucuronides. Nat. Prod. Rep. 2013, 30, 806.

[34] Khymenets, O., Joglar, J., Clapés, P., Parella, T. et al., Biocat- alyzed synthesis and structural characterization of monoglucuronides of hydroxy-tyrosol, tyrosol, homovanillic alcohol, and 3-(4-hydroxyphenyl)propanol. Adv. Synth. Catal. 2006, 348, 2155–

2162.

[35] Mateos, R., Goya, L., Bravo, L., Metabolism of the olive oil phenols hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate by human hepatoma HepG2 cells. J. Agric. Food Chem. 2005, 53, 9897–9905.

[36] Donato, M. T., Lahoz, A., Castell, J. V, Gómez-Lechón, M. J., Cell lines: a tool for in vitro drug metabolism studies. Curr. Drug Metab. 2008, 9, 1–11.

[37] Biasutto, L., Marotta, E., De Marchi, U., Beltramello, S. et al., Heterogeneity and Standardization of Phase II Metabolism in Cultured Cells. Cell. Physiol. Biochem. 2009, 23, 425–430.

[38] Smetanová, L., Stetinová, V., Svoboda, Z., Kvetina, J., Caco- 2 cells, biopharmaceutics classification system (BCS) and biowaiver. Acta Medica (Hradec Kralove) 2011, 54, 3–8.

[39] Dell'Agli, M., Fagnani, R., Mitro, N., Scurati, S. et al., Minor components of olive oil modulate proatherogenic adhesion molecules involved in endothelial activation. J. Agric. Food Chem. 2006, 54, 3259–3264.

[40] Richard, N., Arnold, S., Hoeller, U., Kilpert, C. et al., Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs cytokine and chemokine production in macrophages. Planta Med. 2011, 77, 1890–1897.

[41] Steffen, Y., Gruber, C., Schewe, T., Sies, H., Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. Arch. Biochem. Biophys. 2008, 469, 209–219.

[42] Perez-Vizcaino, F., Duarte, J., Santos-Buelga, C., The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids. J. Sci. Food Agric. 2012, 92, 1822–1825.

[43] Beekmann, K., Actis-Goretta, L., van Bladeren, P. J., Dionisi, F. et al., A state-of-the-art overview of the effect of metabolic conjugation on the biological activity of flavonoids. Food Funct. 2012, 3, 1008–1018.

[44] Castañer, O., Fitó, M., López-Sabater, M. C., Poulsen, H. E. et al., The effect of olive oil polyphenols on antibodies against oxidized LDL. A randomized clinical trial. Clin. Nutr. 2011, 30, 490–493.

[45] Cicero, A. F. G., Nascetti, S., López-Sabater, M. C., Elosua, R. et al., Changes in LDL fatty acid composition as a response to olive oil treatment are inversely related to lipid oxidative damage: the EUROLIVE study. J. Am. Coll. Nutr. 2008, 27, 314–320.

[46] De la Torre-Carbot, K., Chávez-Servın, J. L., Jaúregui, O., Castellote, A. I. et al., Elevated circulating LDL phenol levels in men who consumed virgin rather than refined olive oil are associated with less oxidation of plasma LDL. J. Nutr. 2010, 140, 501–508.

SUPPLEMENTARY MATERIAL

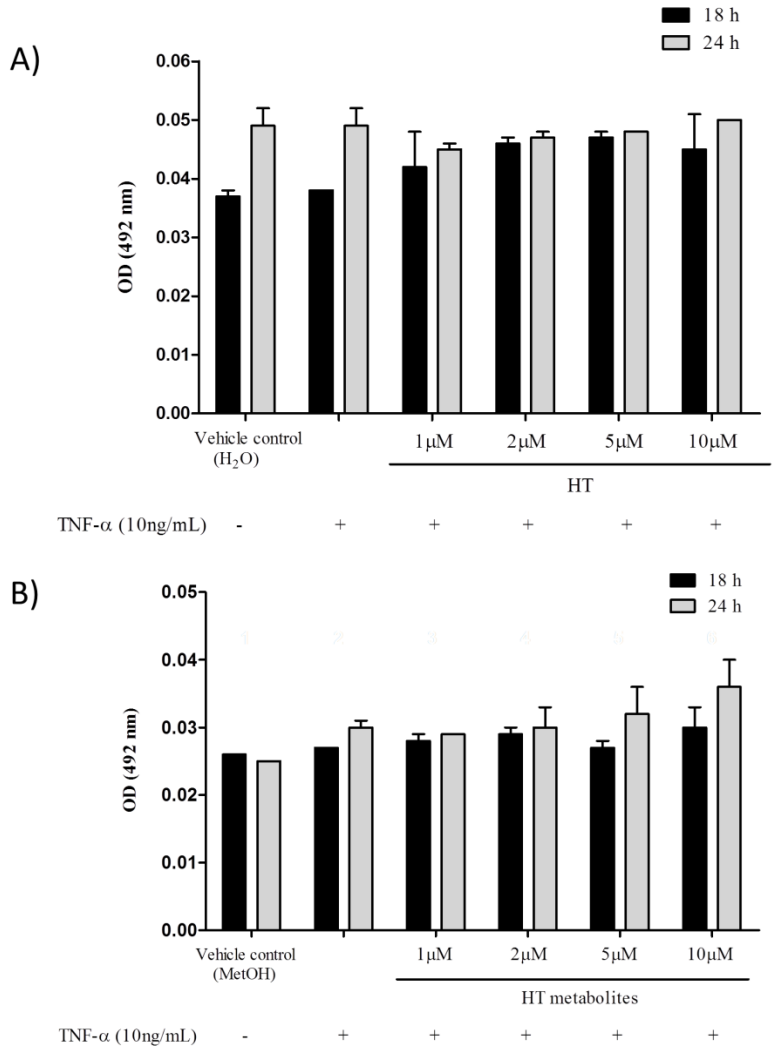


Figure S1. Cytotoxicity from HAEC supernatants after co-incubation with HT or HT metabolites and TNF-α. HAEC were co-incubated with HT (A) or HT metabolites (B) at 1, 2, 5 and 10 µM and TNF-α (10 ng/mL) for 18 and 24 h. Vehicle control refers to cells incubated with HT vehicle (H2O) or HT metabolites vehicle (Methanol). Results are expressed as the mean and the SD (error bars) of optical density (OD; 492 nm) from one representative experiment where each set of experimental conditions was run in duplicate. *P<0.05 compared to maximum stimulation TNF-α alone.

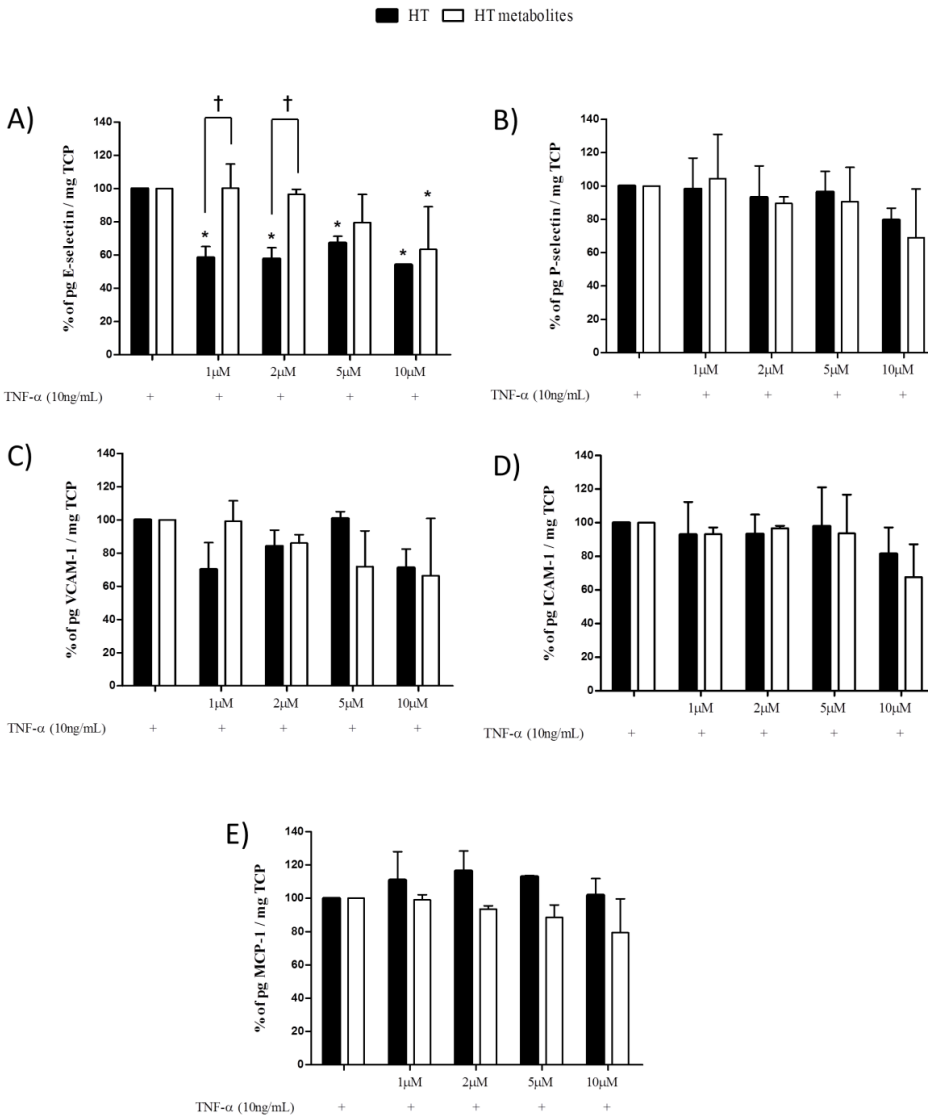


Figure S2. Effect of HT and HT metabolites on E-selectin, P-selectin, VCAM-1, ICAM-1 and MCP-1 protein secretion in HAEC stimulated by TNF-α after 18h. HAEC were co-incubated with HT or HT metabolites at 1, 2, 5 and 10 µM and TNF-α (10 ng/mL) for 18h. A) Effect of HT or HT metabolites on E-selectin protein secretion. B) Effect of HT or HT metabolites on P-selectin protein secretion. C) Effect of HT or HT metabolites on VCAM-1 protein secretion. D) Effect of HT or HT metabolites on ICAM-1 protein secretion. E) Effect of HT or HT metabolites on MCP-1 protein secretion. Results are expressed as the percentage of soluble cellular adhesion molecules or chemokine protein secretion adjusted by total cellular protein and standard error of the mean (SEM; error bars). * $p < 0.05$ versus TNF-α alone. † $p < 0.05$ compared between HT and HT metabolites at the same concentration.

Table S1 Supporting Information. Optimized SRM conditions for analysing the HT and their generated metabolites in Caco-2 cell line.

HT and their generated metabolites	MW (g/mol)	SRM quantification			SRM identification		
		Transition	Cone voltage (V)	Collision energy (eV)	Transition	Cone voltage (V)	Collision energy (eV)
HT	154	153 > 123	35	15	153 > 95	35	25
HT sulfate	234	233 > 153	40	15	233 > 123	40	25
Homovanillic alcohol sulfate	248	247 > 167	40	15	247 > 152	40	25
Homovanillic acid sulfate	262	261 > 181	40	15	261 > 137	40	25
HT glucuronide	330	329 > 153	40	20	329 > 123	40	25
Homovanillic alcohol glucuronide	344	343 > 167	40	20	343 > 152	40	35
Homovanillic acid glucuronide	358	357 > 181	40	20	357 > 137	40	30

SRM: selected reaction monitoring; MW: molecular weight; HT: hydroxytyrosol

Table S2 Supporting information. Relative percentage of HT and its metabolites, in the culture media after exposing HT to Caco-2 cells under B treatment (differentiated cells in T75 flasks / 24 h of HT exposure) with different initial doses of HT (10, 15, 20, 25, 50 and 100 µM) and the total HT metabolites obtained.

Initial HT concentration (µM)	**Relative percentage (%) in the cell media after Caco-2 cell exposure**					**Total HT metabolites obtained (µM)**
	HT	HT sulfate	HT glucuronides	HV alcohol metabolites	HV acid metabolites	
10	5.0	76.5	0.6	17.9	0.0	5.5
15	8.8	76.0	0.8	14.5	0.0	8.7
20	7.0	78.4	1.1	13.5	0.0	9.9
25	5.6	78.0	1.5	14.1	0.7	10.4
50	5.8	79.8	1.8	12.1	0.5	22.9
100	8.6	80.5	1.8	8.7	0.4	56.2

HT: hydroxytyrosol; HT glucuronides: hydroxytyrosol-3-*O*-glucuronide + hydroxytyrosol-4-*O*-glucuronide; HV alcohol metabolites: homovanillic alcohol sulfate + homovanillic alcohol glucuronide; HV acid metabolites: homovanillic acid sulfate + homovanillic acid glucuronide.

Table S3 Supporting Information. Percentages of reduction of HT or HT metabolites on E-selectin, P-selectin, VCAM-1, ICAM-1 and MCP-1 compared to TNF-α alone at 18 and 24h.

Concentra-tions	18h										24h									
	E-selectin (% of reduction)		P-selectin (% of reduction)		VCAM-1 (% of reduction)		ICAM-1 (% of reduction)		MCP-1 (% of reduction)		E-selectin (% of reduction)		P-selectin (% of reduction)		VCAM-1 (% of reduction)		ICAM-1 (% of reduction)		MCP-1 (% of reduction)	
	HT	HT met	HT	HT met	HT	HT met	HT	HT met	HT	HT met	HT	HT met	HT	HT met	HT	HT met	HT	HT met	HT	HT met
0.09 µM	-	-	-	-	-	-	-	-	-	-	n.r.	-	n.r.	-	n.r.	-	n.r.	-	3.6	-
0.18 µM	-	-	-	-	-	-	-	-	-	-	n.r.	-	n.r.	-	10.0	-	6.1	-	12.1	-
0.45 µM	-	-	-	-	-	-	-	-	-	-	43.7	-	39.3	-	46.8	-	31.0	-	18.4	-
0.9 µM	-	-	-	-	-	-	-	-	-	-	45.8	-	40.9	-	45.5	-	37.7	-	1.4	-
1 µM	41.6	n.r.	1.8	n.r.	29.9	0.8	7.2	6.9	n.r.	1.0	44.2	n.r.	39.3	n.r.	39.5	n.r.	33.8	n.r.	5.0	n.r.
2 µM	42.2	3.5	6.8	10.5	15.9	14.0	6.8	3.4	n.r.	6.4	47.1	35.3	46.2	23.2	34.5	25.8	37.1	20.6	9.5	13.2
5 µM	32.7	20.5	3.5	9.4	n.r.	28.1	2.1	6.4	n.r.	11.5	48.2	48.9	46.0	40.6	41.2	56.0	41.1	39.9	14.0	26.6
10 µM	45.9	36.6	20.5	31.0	28.8	33.6	18.6	32.4	n.r.	20.7	53.6	64.2	48.6	61.9	48.7	55.4	40.6	57.0	11.7	39.0

HT: hydroxytyrosol; HT met: hydroxytyrosol metabolites; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; n.r.: no reduction

Table S4 Supporting Information. HT and metabolites concentration (nM) in HAEC cells and culture media after co-incubation with HT or HT metabolites (HTmet) at 1, 2, 5 and 10 µM and TNF-α (10 ng/mL) for 24h.

		1µM		2µM		5µM		10µM	
		cells	media	cells	media	cells	media	cells	media
HT 24h	free HT	67	5	70	16	63	24	63	23
	Acid homovanillic	83	23	91	33	86	56	84	47
	Hydroxytyrosol sulfate	0	12	0	82	11	422	34	1211
	Alcohol homovanillic sulfate	30	374	45	675	94	1232	139	1697
	Acid homovanillic sulfate	0	34	0	23	0	32	0	31
	Hydroxytyrosol glucuronide	0	0	0	0	0	0	0	0
	Alcohol homovanillic glucuronide	0	0	0	0	0	0	0	0
	sum of metabolites	**113**	**442**	**136**	**813**	**191**	**1742**	**257**	**2986**
HTmet 24h	free HT	63	38	64	51	79	76	69	113
	Acid homovanillic	91	65	84	130	121	236	107	405
	Hydroxytyrosol sulfate	61	974	108	2085	222	4941	395	7276
	Alcohol homovanillic sulfate	39	418	55	805	115	1764	165	2503
	Acid homovanillic sulfate	4	60	8	99	10	229	18	358
	Hydroxytyrosol glucuronide	0	0	0	22	0	73	8	136
	Alcohol homovanillic glucuronide	3	33	4	92	7	222	16	392
	sum of metabolites	**197**	**1551**	**259**	**3233**	**475**	**7464**	**709**	**11070**

Publication 5

Hydroxytyrosol and its complex forms (secoiridoids) modulate aorta and heart proteome in healthy rats: potential cardio-protective effects

Molecular Nutrition and Food Research 60:2114-2129
(2016)

RESEARCH ARTICLE

HYDROXYTYROSOL AND ITS COMPLEX FORMS (SECOIRIDOIDS) MODULATE AORTA AND HEART PROTEOME IN HEALTHY RATS: POTENTIAL CARDIO-PROTECTIVE EFFECTS

Úrsula Catalán [1**], Laura Rubió [1,2**], Maria-Carmen López de las Hazas[2], Pol Herrero[3], Pedro Nadal[3], Núria Canela[3], Anna Pedret[1], Maria-José Motilva[2*] and Rosa Solà[1].

[1] Functional Nutrition, Oxidation and Cardiovascular Diseases Group (NFOC-Salut), Unit of Lipids and Atherosclerosis Research (URLA), Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari Sant Joan, IISPV, Technological Center of Nutrition and Health (CTNS), Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Sant Llorenç, Reus, Spain
[2] Food Technology Department, Universitat de Lleida-AGROTECNIO Center, Lleida, Spain
[3] Centre for Omic Sciences, Universitat Rovira i Virgili (COS-URV), Reus, Spain

Abstract

Scope: Hydroxytyrosol (HT) is the major phenolic compound in virgin olive oil (VOO) in both free and complex forms (secoiridoids; SEC). Proteomics of cardiovascular tissues such as aorta or heart represents a promising tool to uncover the mechanisms of action of phenolic compounds in healthy animals.

Methods and results: Twelve female Wistar rats were separated into three groups: a standard diet and two diets supplemented in phenolic compounds (HT and SEC) adjusted to 5 mg/kg/day during 21 days. Proteomic analyses of aorta and heart tissues were performed by nano-LC and MS. Ingenuity Pathway Analysis was used to generate interaction networks. HT or SEC modulated aorta and heart proteome compared to the standard diet. The top-scored networks were related to Cardiovascular System. HT and SEC downregulated proteins related to proliferation and migration of endothelial cells and occlusion of blood vessels in aorta and proteins related to heart failure in heart tissue. SEC showed higher fold change values compared to HT, attributed to higher concentration of HT detected in heart tissue.

Conclusion: Changes at proteomic level in cardiovascular tissues may partially account for the underlying mechanisms of VOO phenols cardiovascular protection being the SEC effects higher than free HT.

Keywords: Cardiovascular disease; Hydroxytyrosol; Healthy rats; Proteome; Secoiridoids

INTRODUCTION

Hydroxytyrosol (HT) is the major phenolic compound in virgin olive oil (VOO) in either free and complex forms, which are commonly named secoiridoids (SEC) or oleuropein aglycone derivates. HT has shown a wide range of biological functions, such as antioxidant, anticancer, and neuro-protective activities, as well as having beneficial effects on the cardiovascular system [1, 2]. With a more significant impact, VOO phenolic compounds have been shown to beneficially alter lipid composition, platelet, and cellular function as well as reduce the inflammation [3]. These effects have been related with the low rate of cardiovascular disease (CVD) mortality and certain types of cancer in populations residing in the Mediterranean countries [4].

The impact of the diet and dietary components on CVD has been widely recognized in recent years [4, 5]. Therefore, prevention through the introduction of lifestyle and proper nutrition habits is now considered a primary strategy for what we call healthy aging. Omics-based studies, including genomics, transcriptomics, proteomics, and metabolomics, have been recognized as powerful analytical tools in cardiovascular research [6–8]. Specifically, the proteomic approach offers an unbiased way to study changes in protein levels induced by different experimental conditions and a major challenge of proteome research is detecting clinically useful biomarkers of disease, treatment response, and aging [9, 10]. Moreover, proteomic approach instead of the analysis of gene expression, focuses on the products that perform the biological function and it is an important key to characterize and longitudinally monitor individuals, their disease courses and the therapeutic responses [11].

Despite that proteomics represents a novel and promising tool to uncover the mechanisms of action as a response to diet or nutrients, the actual use of this technique in dietary interventions is still rather limited [12]. Regarding proteomics focused on cardiovascular tissues, very few studies have been performed up to now investigating the possible protective effect on diseased animals of food bioactive compounds on the protein expression of aortic tissue testing vitamin E and omega-3 fatty acids [13, 14] and heart tissue testing resveratrol [15, 16]. Other studies analyzing proteome on diseased cardiovascular tissues [6], such as calcific aortic valve or infarcted myocardium have reflected important changes in protein induced by disease [17]. However, to the best of our knowledge, no study has reported so far the modulation of aorta and heart proteome by bioactive compounds, specifically phenolic compounds, in healthy animals to address future cardiovascular protection. Small animal models [18–20] have provided insight into the fundamental mechanisms driving early atherosclerosis, but it is increasingly clear that new strategies and research tools are needed to translate these discoveries into improved prevention and treatment of symptomatic atherosclerosis in humans.

The hypothesis of the present work is that VOO phenolic compounds could promote protective effects in cardiovascular system in healthy animals. In an effort to understand the underlying molecular mechanisms of VOO phenols and to identify their potential target protein molecules in cardiovascular tissues, in the present study we performed a proteomic comparative analysis of the aorta and heart tissues of healthy female rats in response to supplemented diet with the equivalent of 5 mg phenol/kg rat weight during 21 days of HT as a pure molecule or its complex occurring forms in VOO through an extract rich in SEC, respectively.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

HT was provided by Seprox Biotech (Madrid, Spain), homovanillic acid by Fluka Co. (Steinheim, Switzerland) and catechol from Sigma-Aldrich (St. Louis, MO, USA). HT- 3'-O-sulfate was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Methanol and acetonitrile (HPLC-grade) were purchased from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Medford, MA, USA). Tandem mass tag (TMT) 10-plex isobaric reagents were from Thermo Fisher Scientific (San Jose´, CA, USA). Organic solvents were LC-MS grade from Panreac (Barcelona, Spain). Unless otherwise noted, all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. *Secoiridoid extract*

Ethanolic phenolic extract rich in SEC was obtained from Arbequina olive cake by pressurized liquid extraction (ASE 100 Dionex, Sunnyvale, California, USA) based on the method of Suárez et al. [21]. Extraction conditions were: ethanol/water (80:20, v/v) at 80°C, 60% setting volume and two static cycles of 5 min in each extraction, then, sample was purged with nitrogen (S99.99% purity, Alphagaz, Madrid, Spain). After that, ethanol was rotary evaporated until its elimination (Buchi, New Castle, DE, USA). Aqueous extract was freeze-dried and stored at –80°C in N2 atmosphere until use. The extract was mainly composed by dialdehydic form of decarboxymethyl elenolic acid linked to HT or dialdehydic form of elenolic acid linked to HT (3,4-DHPEA-EDA) (85%). It also contained minor proportions of free HT and other secoiridoids providing HT such as the isomer of oleuropein aglycone or 3,4-DHPEA-EA (Supporting Information Table 1). The analytical method used to obtain the phenolic composition of olive cake extract is reported in our previous study [22]. In order to calculate the administered dose of 5 mg/kg rat weight of SEC, only 3,4-DHPEA-EDA was considered as it is the main secoiridoid derivative providing HT.

2.3. *Animals and experimental procedure*

Twelve female Wistar rats weighted among 300–350 g were obtained from Charles River Laboratories (Barcelona, Spain). They were separated into three groups with different diets (four rats in each group): control group (A), HT group (B), and SEC group (C). Animals were housed two per cage in a temperature (21 ± 1°C) and humidity (55 ± 10%) controlled room with a 12-h light/dark cycle. Food and water were avail- able ad libitum in cage and metabolic cages. The animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 10-06/14, 31st July 2014).

For the supplemented diets, commercial feed pellets (Harlan Laboratories, Madison, WI, USA) were crushed in an industrial mill and mixed with Milli-Q water containing the equivalent of 5 mg of SEC or HT/kg rat weight in 16 g of crushed pellet (average daily consumption per rat), respectively. New pellets were prepared and freeze-dried. Food and animals were weighed every 2 days to adjust the weekly dose of phenolic compound to 5 mg/kg rat weight/day. The dose administered results in a human equivalent dose of 0.81 mg/kg, which equates to 48.6 mg for a 60 kg person, a dose achievable with a phenol-rich olive oil.

After 21 days of diet supplementation, rats were sacrificed by intracardiac puncture after isoflurane anaesthesia (IsoFlo, Veterinarian Esteve, Bologna, Italy). After blood collection, rats were perfused by isotonic solution of NaCl 0.9% to remove the remaining blood irrigating tissues and then heart and aorta were excised from the rats. All samples were stored at –80°C until analysis.

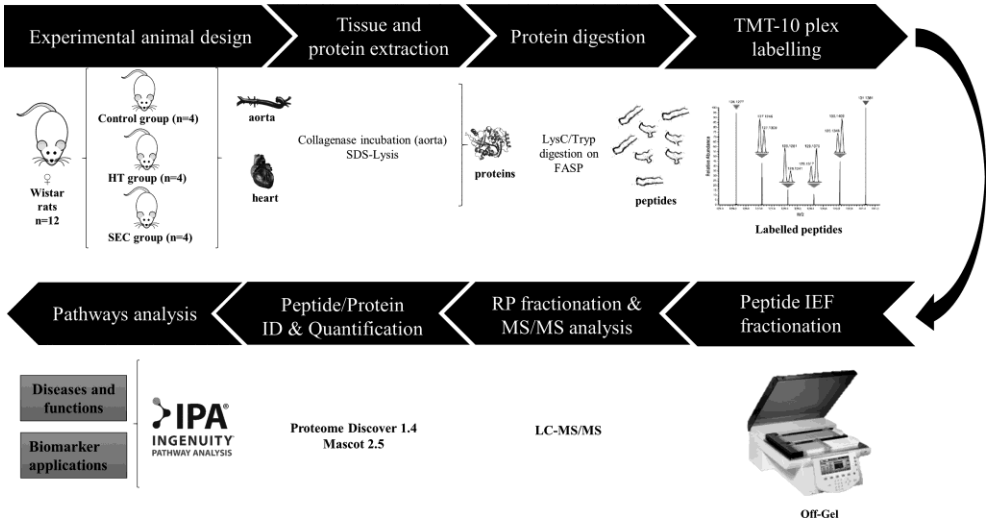


Figure 1. Experimental workflow chart.

2.4. *Heart tissue sample pretreatment and phenolic chromatographic analysis*

In order to study the disposition of phenolic compounds on heart tissue after HT or SEC supplemented diets, the free HT and its biological metabolites were determined. Aorta was not analyzed as it was entirely used for proteomic analysis. Prior to the chromatographic analysis, the heart tissue was freeze-dried and after homogenization 0.01g from each heart tissue sample were sequentially pretreated with a combination of liquid–solid extraction combined with micro solid phase extraction as previously described [23]. Phenolic compounds were then analyzed by Acquity Ultra-PerformanceTM liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) as the detector system from Waters (Milford, MA, USA), as reported in our previous study [23].

2.5. *Proteomic sample preparation and quantitative analysis of the aorta and heart tissues*

Protein extraction was performed from each individual rat sample of aorta and heart tissues, analyzing four samples per group and per tissue. A quantitative proteomic study was performed using tandem mass tag (TMT) isobaric tag labeling, off-gel fractionation, and MS based on nano-LC-Orbitrap technology was performed (Fig. 1). The different analytical steps are explained in detail in **Supporting Information: Materials and methods.** The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [24] partner repository with the dataset identifier PXD003699.

2.6. *Statistical analysis*

The statistical analysis to find the significant protein changes between conditions included in the present studies was done by Mass Profiler Professional software v. 13.0 from Agilent Technologies (Santa Clara, CA, USA). The statistical analysis was the same for aortic and heart tissue. The study comprises four samples from three different groups named as control, HT and SEC. A one-way analysis of variance (ANOVA) statistical test was applied with a p-value correction of Benjamin–Hochberg false discovery rate for multiple comparisons using the quantified proteins that appears in more than 66% of samples. Differentially expressed proteins have been selected based on their significant level. Moreover, multivariate statistical technique based on principal components analysis (PCA) was applied. There are three replicates for control condition (one is used for normalization and is not considered for statistical analysis) and four replicates for each treatment condition.

2.7. *Clustering and pathway analysis*

Various bioinformatics tools were employed for the biological interpretation of the results. Proteins were referred to by their gene encode symbol. Each protein

identifier (Swiss-Prot ID) was mapped to its corresponding protein object in the Wiki Pathways, KEGG, Reactome, and BioCyte data bases.

Ingenuity Pathway analysis (IPA; Ingenuity System Inc., Redwook, CA, USA, www.ingenuity.com) was used to analyze canonical pathways and protein networks involving the differentially expressed proteins for biological interpretation. Significance levels were assessed with Fisher's exact tests ($p < 0.01$). The differentially expressed proteins were overlaid with IPA-curated canonical pathways to explore possible metabolic and cell signaling pathways that were over- or underrepresented by the experimentally determined genes. Specifically, we conducted two analysis: (a) the common proteins up- or downregulated in the same direction after both treatments (HT and SEC) in aorta or heart tissue compared to control group, to study the potential common HT and SEC mechanisms of action, and (b) the whole dataset of proteins differentially expressed after HT or SEC supplemented diets in aorta or heart tissue compared to control group in order to acquire a global vision and focused on cardiovascular system(**Fig. 2**).

In addition, possible connections between mapped genes were evaluated and graphical networks were algorithmically generated. Nodes representing genes and gene products were linked by biological relationships. Networks were ranked by a score that defines the probability of a collection of nodes being equal to or greater than the number in a network achieved by chance alone.

3. RESULTS

3.1. *Disposition of HT and its metabolites in heart tissue*

As shown in Table 1, after diet supplementation with 5 mg of HT or SEC/kg rat weight/day during 21 days, HT was detected in heart tissue mainly in its free form and in a minor proportion as phase II metabolite HT-3-O-sulfate, which only appeared after HT diet. When comparing both diets, the free form of HT presented significant higher concentrations after SEC diet supplementation compared to HT. The free HT detected in the heart tissue of the control group could be related to the endogenous origin of this compound from dopamine metabolism previously described [25].

3.2. *Tissue proteome modulation by HT and SEC*

3.2.1. Aorta

After performing the proteome analysis of aorta tissue samples, we reached to identify and quantify 1247 proteins. The comparative analysis revealed that from the identified proteins, 115 significantly differed after HT and SEC treatments compared to control group ($p < 0.05$). All these up- or down- regulated proteins with their corresponding fold change (FC; the absolute value) values compared to the control group are listed in Table 2 and they

have been also classified in a Venn diagram in **Fig. 2A**. The corresponding information for all identified proteins in aorta is available in **Supporting Information Table 3**.

The comparative analysis between HT diet and control group in aorta tissue revealed an upregulation of 26 proteins ranging from 1.0 to 1.3 FC and a downregulation of 89 proteins from –1.0 to –1.7 of FC. When comparing SEC diet with control group, we observed a significant upregulation of 34 proteins ranging from 1.2 to 16.3 of FC and a downregulation of 81 proteins from –1.2 to –18.8 of FC. As shown in the Venn diagram (**Fig. 2**A), most of these proteins were significantly modulated in the same direction after the two supplemented diets (HT or SEC) compared to control group, and in most of the proteins FC values were strengthened after SEC diet supplementation compared to HT (**Table 2**). The PCA of aortic tissue samples after the statistical tests showed that only a

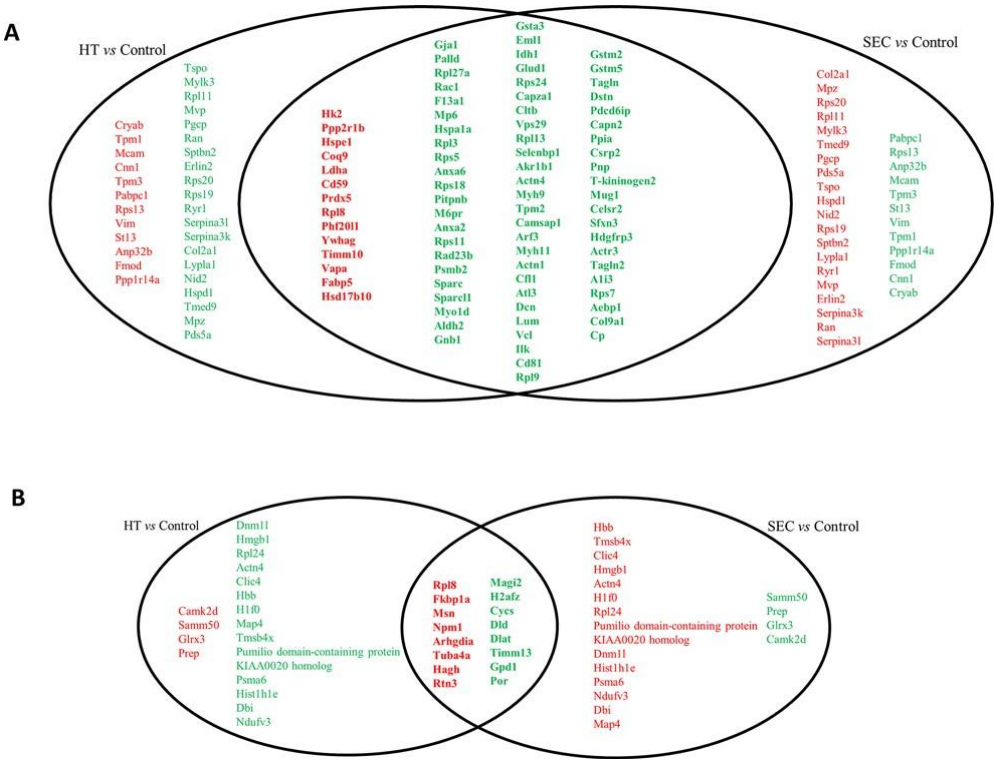


Figure 2. Venn diagram of aorta and heart tissue. Venn diagram showing intersections of proteins differentially expressed in aorta (A) or heart (B) tissue of healthy rats comparing HT versus con-trol and SEC versus control group. Red color indicates upregulated proteins and green color indicates downregulated proteins.

slightly differentiation between control group and HT group is observed but both are significantly different compared to SEC (**Supporting Information mation Fig. 1A**), which is in accordance with the higher FC values of SEC.

3.2.2. Heart

In heart tissue we identified and quantified 1124 proteins and 34 proteins that significantly differed after HT and SEC diets compared to control group ($p < 0.05$). All the up- or downregulated proteins with their corresponding FC values compared to the control group are listed in Table 3 and have been classified in a Venn diagram shown in Fig. 2B. The corresponding information for all the identified proteins in heart is available in **Supporting Information Table 4**.
The comparative analysis between HT supplemented diet and control group revealed an upregulation of 12 proteins ranging from 1.0 to 1.4 of FC and a downregulation of 22 proteins ranging from –1.0 to –1.4 of FC. Comparing SEC diet to control group, we observed an upregulation of 22 proteins ranging from 1.0 to 3.4 of FC and a downregulation of 12 proteins ranging from –1.1 to –1.5 of FC. Comparing SEC diet to HT diet revealed that 23 proteins were upregulated (from 1.0 to3.3of FC) and 11 were downregulated (from –1.0 to –2.0 of FC). As in aorta, some of the proteins were differentially expressed in the same direction after the two supplemented diets (HT or SEC) compared to control group (**Fig. 2B**), but in heart, most of the proteins were modulated in opposite directions depending on the treatment. The PCA analysis showed a clear differentiation between the three groups (control, SEC, and HT) in heart tissue (**Supporting Information Fig. 1B**). Among all the differentially expressed proteins, only two proteins, Actn4 and Rpl8, were found to be significantly modulated in both aorta and heart tissues.

3.3. Pathway analysis of common proteins modulated by HT and SEC

Following the pathway analysis strategy (a) described in Section 2.7, we analyzed in IPA the common proteins that were

Table 1. Concentration (nmols/g fresh tissue) of free hydroxytyrosol and the main metabolite, hydroxytyrosol-3-O-sulfate, detected in heart tissue after the diet supplementation (21 days) with 5 mg/kg rat weight/day of hydroytyrosol (HT) or secoiridois (SEC)

	Control (n = 4)	SEC (n = 4)	HT (n = 4)
Free hydroxytyrosol	5.78 (0.02)[a)]	19.6 (1.56)[c)]	8.75 (0.82)[b)]
Hydroxytyrosol-3-*O*-sulfate	n.d.	n.d.	0.52 (0.18)[a)]

n.d.: nondetectable.
a) b) c) Indicate significant differences among treatments in the same row at the 95.0% level of confidence. Results are expressed as mean (standard deviation; SD).

differentially up- or downregulated (14 up- and 69 downregulated proteins in aorta tissue and 8 up- and 8 downregulated proteins in heart tissue) in the same direction in response to both the HT and SEC consumption to provide common mechanisms after both treatments. IPA results regarding the top-scored canonical pathways, molecular and cellular functions, diseases and disorders and potential upstream regulators are presented in detail in Supporting Information: Results and discussion.

3.4. Global proteome changes after HT or SEC and its relation with cardiovascular system

Following the analysis strategy (b) the whole dataset of differentially expressed proteins after HT and SEC diets in aorta (115 proteins) or heart (34 proteins) tissue compared to control group was analyzed in IPA with special focus on cardiovascular system functions.

3.4.1. Aorta tissue

When the complete dataset of proteins differentially expressed in aorta was analyzed, the top network found by IPA was "Cardiovascular System Development" (score = 11). Eleven proteins of the 115 regulated proteins in aorta were part of this network. The graphical representation of the network is shown in **Fig. 3**, in which the modulated proteins (located in the cell compartments) have been highlighted in color and indicated when HT and SEC differently modulated a protein (up or downregulated).

We observed a significant increase of Hk2, Fabp5, Ldh proteins and a decrease of Akr1b1, Capn2, Gja1, Rac1, Ilk, Vcl, and Kng1/Kng1l1 proteins after both treatments. Vim protein was upregulated by HT and downregulated after SEC. Some of these proteins (Kng1, Hk2, Gja1, and Rac1) were found by IPA to have a strong relation with specific cardiovascular system functions, which are listed in **Table 4**. These proteins were mainly related to cardiac functions in this tissue such as occlusion of blood vessel and proliferation of endothelial cells.

Other proteins implicated in the network and connected with the proteins detected in our study were: NF-κB, Hspb1, Prkce, Pten, Mapk14, Prkca, Nos2, Erk1/2, Pka, Akt, Pdpk1, Pak2, Pxn, Rhoa, Nos3, Casp3, Pik3r1, Dock7, Cav3, Agtr2, Slc2a4, Igf1r, Cav1, and Ctgf. As shown NF-κB complex and Akt appeared to be key proteins in the network.

3.4.2. *Heart tissue*

In the case of heart tissue, the top network found by IPA was "Cardiovascular System Development" (score = 57). Twenty-one proteins of the 34 regulated proteins in heart were part of this network, which is represented in **Fig. 4**.

The upregulated proteins after HT and SEC supplemented diets implicated in this network were Npm1, Fkbp1a, Tuba4a, Arhgdia, Msn, and Alpha tubulin proteins and the downregulated were Por, Dlat, Dld, Cys, Cytochrome C, and Magi 2 proteins

Table 2. 115 significant proteins differentially expressed in aortic tissue of healthy rats after HT or SEC treatments compared to control group

Swiss-Prot code	Gene symbol	Protein name	MW (kDa)	*p*	FC (HT vs Control)	FC (SEC vs Control)
Q9QZA2	Pdcd6ip	Programmed cell death 6-interacting protein	96.6	1.4 × 10–5	–1.1	–2.0
P62850	Rps24	40S ribosomal protein S24	15.4	2.4 × 10–5	–1.1	–2.2
E9PT87	Mylk3	Myosin light chain kinase 3	85.5	3.1 × 10–5	–1.0	1.9
P62074	Timm10	Mitochondrial import inner membrane translocase subunit Tim10	10.3	3.3 × 10–5	1.0	3.1
P60868	Rps20	40S ribosomal protein S20	13.4	3.3 × 10–5	–1.1	3.3
P62282	Rps11	40S ribosomal protein S11	18.4	3.4 × 10–5	–1.1	–1.6
P10860	Glud1	Glutamate dehydrogenase 1. mitochondrial	61.4	3.9 × 10–5	–1.1	–1.5
P06907	Mpz	Myelin protein P0	27.6	3.9 × 10–5	–1.2	15.9
P62914	Rpl11	60S ribosomal protein L11	20.2	5.0 × 10–5	–1.0	1.9
Q99MC0	Ppp1r14a	Protein phosphatase 1 regulatory subunit 14A	16.7	7.7 × 10–5	1.0	–2.2
Q9EPF2	Mcam	Cell surface glycoprotein MUC18	71.3	8.3 × 10–5	1.1	–1.7
P17077	Rpl9	60S ribosomal protein L9	21.9	9.5 × 10–5	–1.1	–1.7
Q5I0E7	Tmed9	Transmembrane emp24 domain-containing protein 9	27.0	1.1 × 10–4	–1.1	1.8
P21531	Rpl3	60S ribosomal protein L3	46.1	1.2 × 10–4	–1.0	–1.7
P62083	Rps7	40S ribosomal protein S7	22.1	1.3 × 10–4	–1.2	–1.8
Q6AY20	M6pr	Cation-dependent mannose-6-phosphate receptor	31.1	1.4 × 10–4	–1.0	–1.4
P62919	Rpl8	60S ribosomal protein L8	28.0	1.5 × 10–4	1.1	1.7
P27274	Cd59	CD59 glycoprotein	13.8	1.5 × 10–4	1.1	1.5
Q9Z270	Vapa	Vesicle-associated membrane protein-associated protein A	27.8	1.6 × 10–4	1.0	1.6
P04904	Gsta3	Glutathione S-transferase alpha-3	25.3	2.0 × 10–4	–1.1	–1.7
Q4KMA2	Rad23b	UV excision repair protein RAD23 homolog B	43.5	2.1 × 10–4	–1.1	–1.4
P17074	Rps19	40S ribosomal protein S19	16.1	2.3 × 10–4	–1.1	1.4
Q9QWN8	Sptbn2	Spectrin beta chain. brain 2	270.9	2.3 × 10–4	–1.0	1.4
F1LMY4	Ryr1	Ryanodine receptor 1	565.1	2.4 × 10–4	–1.4	1.4
P05539	Col2a1	Collagen alpha-1(II) chain	134.5	2.8 × 10–4	–1.7	16.3
P18445	Rpl27a	60S ribosomal protein L27a	16.6	3.1 × 10–4	–1.0	–1.3
P08010	Gstm2	Glutathione S-transferase Mu 2	25.7	3.4 × 10–4	–1.1	–2.1
P50609	Fmod	Fibromodulin	43.2	3.5 × 10–4	1.0	–3.5
B5DFC9	Nid2	Nidogen-2	152.9	3.6 × 10–4	–1.1	1.5
Q9EPH8	Pabpc1	Polyadenylate-binding protein 1	70.7	3.8 × 10–4	1.1	–1.2
P05545	Serpina3k	Serine protease inhibitor A3K	46.5	4.1 × 10–4	–1.6	1.3
Q5XFX0	Tagln2	Transgelin-2	22.4	5.1 × 10–4	–1.2	–1.9
A2RUV9	Aebp1	Adipocyte enhancer-binding protein 1	128.0	5.5 × 10–4	–1.3	–2.3
B2RZ78	Vps29	Vacuolar protein sorting-associated protein 29	20.5	5.6 × 10–4	–1.1	–1.5
P23928	Cryab	Alpha-crystallin B chain	20.1	7.0 × 10–4	1.3	–5.6
P04692	Tpm1	Tropomyosin alpha-1 chain	32.7	7.4 × 10–4	1.1	–1.9
P53812	Pitpnb	Phosphatidylinositol transfer protein beta isoform	31.4	7.5 × 10–4	–1.0	–1.4
A4L9P7	Pds5a	Sister chromatid cohesion protein PDS5 homolog A	150.2	8.2 × 10–4	–1.3	1.7
O08619	F13a1	Coagulation factor XIII A chain	82.6	8.4 × 10–4	–1.0	–1.4
P61206	Arf3	ADP-ribosylation factor 3	20.6	8.9 × 10–4	–1.1	–1.5
Q63357	Myo1d	Unconventional myosin-Id	116.0	8.9 × 10–4	–1.1	–2.1

Table 2. Continuec

Swiss-Prot code	Gene symbc	Protein name	MW (kDa)	p	FC (HT versus control	FC (SEC versus control
P16257	Tspo	Translocator protein	18.9	9.9 × 10–4	–1.0	1.6
P70470	Lypla1	Acyl-protein thioesterase 1	24.7	1.1 × 10–4	–1.1	1.4
Q4V8C3	Eml1	Echinoderm microtubule-associated protein-like 1	89.7	1.1 × 10–3	–1.1	–1.7
P24050	Rps5	40S ribosomal protein S5	22.9	1.2 × 10–3	–1.0	–1.2
P0C5E3	Palld	Palladin (Fragment)	66.7	1.4 × 10–3	–1.0	–1.5
Q08290	Cnn1	Calponin-1	33.3	1.4 × 10–3	1.1	–3.8
Q07439	Hspa1a	Heat shock 70 kDa protein 1A/1B	70.1	1.4 × 10–3	–1.0	–2.3
P40307	Psmb2	Proteasome subunit beta type-2	22.9	1.5 × 10–3	–1.1	–1.4
P05544	Serpina3l	Serine protease inhibitor A3L	46.2	1.5 × 10–3	–1.6	1.2
P63039	Hspd1	60 kDa heat shock protein. mitochondrial	60.9	1.6 × 10–3	–1.1	1.5
P08932	N/A	T-kininogen 2	47.7	1.7 × 10–3	–1.2	–2.7
Q9QXQ0	Actn4	Alpha-actinin-4	104.8	1.8 × 10–3	–1.1	–1.7
P58775	Tpm2	Tropomyosin beta chain	32.8	1.8 × 10–3	–1.1	–2.1
D3Z9R8	Mp6	6.8 kDa mitochondrial proteolipid	6.9	1.8 × 10–3	–1.0	–4.1
P24054	Sparcl1	SPARC-like protein 1	70.6	1.8 × 10–3	–1.4	–2.8
P08082	Cltb	Clathrin light chain B	25.1	2.0 × 10–3	–1.1	–1.7
Q9Z1P2	Actn1	Alpha-actinin-1	102.9	2.1 × 10–3	–1.1	–2.5
Q01129	Dcn	Decorin	39.8	2.1 × 10–3	–1.1	–1.4
Q9R063	Prdx5	Peroxiredoxin-5. mitochondrial	22.2	2.2 × 10–3	1.1	1.6
P51886	Lum	Lumican	38.3	2.3 × 10–3	–1.1	–1.6
Q63862	Myh11	Myosin-11 (Fragments)	152.4	2.5 × 10–3	–1.1	–1.6
P61983	Ywhag	14-3-3 protein gamma	28.3	2.6 × 10–3	1.0	1.5
P14046	A1i3	Alpha-1-inhibitor 3	163.7	2.7 × 10–3	–1.2	–1.9
P31232	Tagln	Transgelin	22.6	2.8 × 10–3	–1.1	–2.0
Q62745	Cd81	CD81 antigen	25.9	2.9 × 10–3	–1.1	–2.0
Q68FT1	Coq9	Ubiquinone biosynthesis protein COQ9. mitochondrial	35.1	3.0 × 10–3	1.1	2.1
P16975	Sparc	SPARC	34.3	3.1 × 10–3	–1.1	–3.0
P41123	Rpl13	60S ribosomal protein L13	24.3	3.1 × 10–3	–1.1	–2.0
Q923W4	Hdgfrp3	Hepatoma-derived growth factor-related protein 3	22.4	3.1 × 10–3	–1.2	–1.5
Q9EST6	Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	31.0	3.2 × 10–3	1.0	–1.4
P08050	Gja1	Gap junction alpha-1 protein	43.0	3.5 × 10–3	–1.0	–1.7
P62278	Rps13	40S ribosomal protein S13	17.2	3.7 × 10–3	1.1	–1.3
Q4QQT4	Ppp2r1b	Serine/threonine-protein phosphatase 2A 65 kDan regulatory subunit A beta	66.0	3.7 × 10–3	1.2	1.6
Q7M0E3	Dstn	Destrin	18.5	3.8 × 10–3	–1.1	–2.1
P10111	Ppia	Peptidyl-prolyl cis-trans isomerase A		3.8 × 10–3	–1.1	–1.5
P13635	Cp	Ceruloplasmin	120.8	3.9 × 10–3	–1.4	–1.6
D3Z8E6	Camsap1	Calmodulin-regulated spectrin-associated protein 1	178.4	4.1 × 10–3	–1.1	–1.6
P85972	Vcl	Vinculin	116.5	4.3 × 10–3	–1.1	–1.6
Q4V9H5	Phf20l1	PHD finger protein 20-like protein 1	114.0	4.5 × 10–3	1.0	2.0
Q4V7C7	Actr3	Actin-related protein 3	47.3	4.6 × 10–3	–1.2	–1.5
Q8VIF7	Selenbp1	Selenium-binding protein 1	52.5	4.7 × 10–3	–1.1	–1.4

Table 2. Continued

Swiss-Prot code	Gene symbol	Protein name	MW (kDa)	p	FC (HT vs Control)	FC (SEC vs Control)
Q62812	Myh9	Myosin-9	226.2	4.9×10^{-2}	–1.1	–1.5
Q0ZHH6	Atl3	Atlastin-3	60.5	$5.0 \times 10^{-}$	–1.1	–1.4
Q99J82	Ilk	Integrin-linked protein kinase	51.3	$5.2 \times 10^{-}$	–1.1	–1.8
Q62908	Csrp2	Cysteine and glycine-rich protein 2	20.9	$5.2 \times 10^{-}$	–1.2	–2.2
P55053	Fabp5	Fatty acid-binding protein. epidermal	15.0	$5.3 \times 10^{-}$	1.0	2.5
Q63610	Tpm3	Tropomyosin alpha-3 chain	29.0	$5.3 \times 10^{-}$	1.1	–1.7
P11884	Aldh2	Aldehyde dehydrogenase. mitochondrial	56.5	$5.3 \times 10^{-}$	–1.1	–1.6
Q9QYP2	Celsr2	Cadherin EGF LAG seven-pass G-type receptor 2 (Fragment)	233.3	$5.4 \times 10^{-}$	–1.2	–3.0
Q9JHY2	Sfxn3	Sideroflexin-3	35.4	$5.6 \times 10^{-}$	–1.2	–1.9
B2GUZ5	Capza1	F-actin-capping protein subunit alpha-1	32.9	$5.8 \times 10^{-}$	–1.1	–1.6
B5DEH2	Erlin2	Erlin-2	37.7	$6.1 \times 10^{-}$	–1.1	1.4
P48037	Anxa6	Annexin A6	75.7	$6.4 \times 10^{-}$	–1.0	–1.3
Q6RUV5	Rac1	Ras-related C3 botulinum toxin substrate 1	21.4	$6.5 \times 10^{-}$	–1.0	–1.4
Q9Z1B2	Gstm5	Glutathione S-transferase Mu 5	26.6	$6.6 \times 10^{-}$	–1.1	–1.5
P54311	Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit	37.4	$6.6 \times 10^{-}$	–1.1	–1.5
P45592	Cfl1	Cofilin-1	18.5	$6.6 \times 10^{-}$	–1.1	-1.6
P62271	Rps18	40S ribosomal protein S18	17.7	$6.6 \times 10^{-}$	–1.0	–1.7
Q6IRK9	Pgcp	Plasma glutamate carboxypeptidase	52.0	$6.8 \times 10^{-}$	–1.0	1.8
P04642	Ldha	L-lactate dehydrogenase A chain	36.4	$7.0 \times 10^{-}$	1.1	1.9
P41562	Idh1	Isocitrate dehydrogenase [NADP] cytoplasmic	46.7	$7.0 \times 10^{-}$	–1.1	–1.3
P27881	Hk2	Hexokinase-2	102.5	$7.1 \times 10^{-}$	1.3	2.7
P50503	St13	Hsc70-interacting protein	41.3	$7.2 \times 10^{-}$	1.0	–1.8
P62828	Ran	GTP-binding nuclear protein Ran	24.4	$7.3 \times 10^{-}$	–1.0	1.3
P31000	Vim	Vimentin	53.7	$7.4 \times 10^{-}$	1.0	–1.9
Q62667	Mvp	Major vault protein	95.7	$7.4 \times 10^{-}$	–1.0	1.4
P85973	Pnp	Purine nucleoside phosphorylase	32.3	$7.6 \times 10^{-}$	–1.2	–1.5
Q03626	Mug1	Murinoglobulin-1	165.2	$7.6 \times 10^{-}$	–1.2	–2.0
P26772	Hspe1	10 kDa heat shock protein. mitochondrial	10.9	$7.8 \times 10^{-}$	1.1	2.5
P20850	Col9a1	Collagen alpha-1(IX) chain (Fragment)	31.2	$7.9 \times 10^{-}$	–1.3	–
Q07936	Anxa2	Annexin A2	38.7	$8.1 \times 10^{-}$	–1.1	–1.4
O70351	Hsd17b10	3-hydroxyacyl-CoA dehydrogenase type-2	27.2	$8.2 \times 10^{-}$	1.0	1.5
Q07009	Capn2	Calpain-2 catalytic subunit	79.9	$8.4 \times 10^{-}$	–1.1	–1.5
P07943	Akr1b1	Aldose reductase	35.8	$8.5 \times 10^{-}$	–1.1	–1.4

(**Fig. 4**). Some proteins, such as Hmgb1, Hbb, Actn4, Psma6, Dnm1l, Map4, and Clic4, were downregulated by HT and upregulated in response to SEC. On the other hand, Samm50, Glrx3, Prep, and Camk2d were upregulated by HT and downregulated after SEC. As in aorta, some of these proteins had a strong relation with specific cardiovascular system functions, which are listed in Table 4. Some of these proteins such as Camk2d, Fkbp1a, and Tuba4a, were related to cardiac functions and other proteins such as Dlat, Dld, and Por were related to lipid metabolism. Other proteins implicated in the network were Ppil4, Nf-kB, estrogen receptor, Hsp90, Calcineurin proteins, Caspase, Akt, P38 Mapk, Pka, Cd3, and N-methyl-R-salsolinol. NF-kB complex also resulted in a pivotal position as in aorta network.

4. DISCUSSION

Under the hypothesis that VOO phenolic compounds could promote benefits in cardiovascular system by inducing changes in the heart and aorta protein level, we performed a proteomic analysis of the aorta and heart tissues of healthy rats in response to a diet supplemented with HT, administrated either as a pure molecule (HT diet) or through its complex occurring forms in VOO named as secoiridoids (SEC diet).

Proteomic analysis revealed that the heart and aorta proteome significantly changed after the administration of VOO phenolics compared to the control group, observing a clearer differentiation when diet was supplemented with SEC. As expected, most of the proteins were similarly modulated after SEC and HT, and FC values did not exceed in most cases 2.5-fold. The relatively low FC after the supplemented diets could be attributed to the healthy status of the animals. Previous studies have focused on the proteomic profiling of aorta diseased tissues in order to gain knowledge of the molecular events underlying pathological processes such as the atherosclerotic lesions [26, 27]. However, based on the general consensus that VOO phenolic compounds have a protective role in the cardiovascular system [1], in this study we present the proteomic technique applied to healthy cardiovascular tissues as a novel approach to gain knowledge in the possible prevention mechanisms of these bioactive compounds.

There are scarce data in the bibliography regarding the effect of olive phenols on the tissue proteome modulation and so far no studies have been performed with heart and aorta tissues. Only some studies performed in injured liver of rat or mice and the hepatic proteome analysis revealed that VOO phenolic compounds could have benefits against chronic liver injury and steatosis, which was attributed to the modulation of proteins related to antioxidant mechanisms [28–30]. In other studies, the effect of VOO in the plasma proteome during aging in rats was analyzed [31] and also the impact on the HDL protein cargo of the intake of VOO and two other phenol-enriched VOO in hypercholesterolemic subjects [32]. In

Table 3. 34 significant proteins differentially expressed in heart tissue of healthy rats after HT or SEC treatments compared to control group

Swiss-Prot code	Gene symbol	Protein name	MW (kDa)	p	FC (HT vs control group)	FC (SEC vs control group)
Q9Z0W7	Clic4	Chloride intracellular channel protein 4	28.60	3.3 × 10–5	–1.1	1.4
P15865	Hist1h1e	Histone H1.2	22.00	3.9 × 10–5	–1.3	1.2
Q9QXQ0	Actn4	Alpha-actinin-4	104.80	4.0 × 10–5	–1.1	1.3
O35952	Hagh	Hydroxyacylglutathione hydrolase mitochondrial	34.10	1.6 × 10–4	1.1	3.4
P0C0S7	H2afz	Histone H2A.Z	13.50	2.7 × 10–4	–1.0	–1.4
P43278	H1f0	Histone H1.0	20.90	3.1 × 10–4	–1.2	1.3
Q5XIF6	Tuba4a	Tubulin alpha-4A chain	49.90	3.5 × 10–4	1.1	1.2
P13084	Npm1	Nucleophosmin	32.50	5.0 × 10–4	1.1	1.1
P15791	Camk2d	Calcium/calmodulin-dependent protein kinase type II subunit delta	60.00	5.6 × 10–4	1.4	–1.4
P62076	Timm13	Mitochondrial import inner membrane translocse subunit Tim13	10.50	6.6 × 10–4	–1.2	–1.5
Q9JLZ1	Glrx3	Glutaredoxin-3	37.80	6.8 × 10–4	1.1	–1.3
P00388	Por	NADPH–cytochrome P450 reductase	76.90	7.3 × 10–4	–1.3	–1.4
Q5XI73	Arhgdia	Rho GDP-dissociation inhibitor 1	23.40	7.7 × 10–4	1.1	1.2
P83732	Rpl24	60S ribosomal protein L24	17.80	7.8 × 10–4	–1.0	1.3
O70196	Prep	Prolyl endopeptidase	80.70	9.0 × 10–4	1.0	–1.2
P62919	Rpl8	60S ribosomal protein L8	28.00	1.0 × 10–3	1.2	1.3
Q6RJR6	Rtn3	Reticulon-3	101.50	1.1 × 10–3	1.0	1.2
P62329	Tmsb4x	Thymosin beta-4	5.00	1.2 × 10–3	–1.2	1.9
P60901	Psma6	Proteasome subunit alpha type-6	27.40	1.3 × 10–3	–1.2	1.1
P11030	Dbi	Acyl-CoA-binding protein	10.00	1.4 × 10–3	–1.3	1.1
Q562C7	N/A	Pumilio domain-containing protein KIAA0020 homolog	72.70	1.6 × 10–3	–1.2	1.2
P63159	Hmgb1	High mobility group protein B1	24.90	1.7 × 10–3	–1.0	1.4
Q6AXV4	Samm50	Sorting and assembly machinery component 50 homolog	51.90	1.8 × 10–3	1.1	–1.2
P62898	Cycs	Cytochrome c. somatic	11.60	1.8 × 10–3	–1.1	–1.3
O35763	Msn	Moesin	67.70	1.9 × 10–3	1.2	1.3
O88382	Magi2	Membrane-associated guanylate kinase. W W and PDZ domain containing protein 2	141.00	2.1 × 10–3	–1.0	–1.3
Q62658	Fkbp1a	Peptidyl-prolyl cis-trans isomerase FKBP1A	11.90	2.2 × 10–3	1.2	1.7
Q6P6R2	Dld	Dihydrolipoyl dehydrogenase mitochondrial.	54.00	2.2 × 10–3	–1.1	–1.1
Q6PCU8	Ndufv3	NADH dehydrogenase [ubiquinone] flavoprotein 3 mitochondrial	11.90	2.3 × 10–3	–1.4	1.1
O35077	Gpd1	Glycerol-3-phosphate dehydrogenase [NAD(+)],cytoplasmic	37.40	2.5 × 10–3	–1.2	–1.3
O35303	Dnm1l	Dynamin-1-like protein	83.90	2.5 × 10–3	–1.0	1.2
P08461	Dlat	Dihydrolipoyllysine-residue acetyltransferase component oof pyruvate dehydrogenase complex, mitochondrial	67.10	2.5 × 10–3	–1.1	–1.1
P02091	Hbb	Hemoglobin subunit beta-1	16.00	2.7 × 10–3	–1.1	2.2
Q5M7W5	Map4	Microtubule-associated protein 4	110.20	2.7 × 10–3	–1.2	1.0

HT, hydroxytyrosol; SEC, secoiridoids; MW, molecular weight; FC, fold change; N/A, not available. The fold change is positive if the treatments are upregulated and negative if they are downregulated.

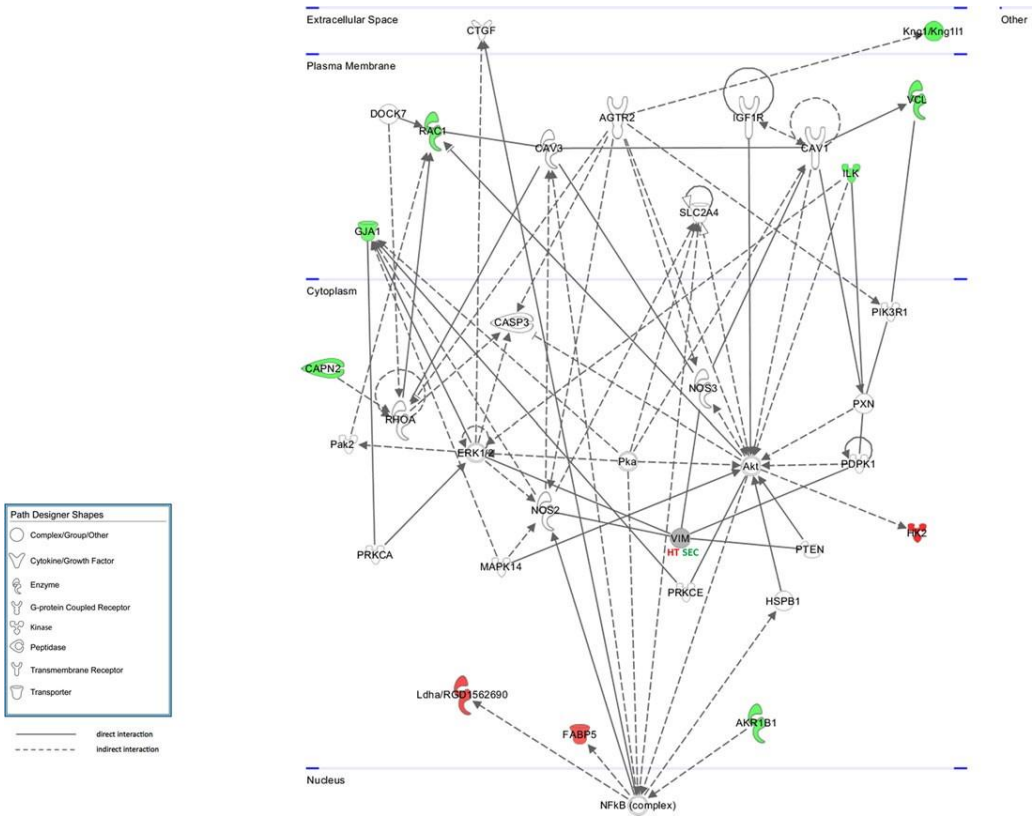


Figure 3. Aorta Cardiovascular System Network after HT or SEC supplemented diets. Interaction between the proteins differentially expressed in aorta after HT or SEC treatments and other important proteins related to the same network. HT or SEC are represented in red or green color if the protein is up- or downregulated, respectively.

both cases, a modulation of proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation was observed.

When comparing HT and SEC, diet supplementation with SEC leaded to higher FC values. These differences could be attributed to the higher concentration of free HT detected in heart tissue after SEC diet (**Table 1**). In our previous work, it was demonstrated that despite that HT and SEC were administered at the same dose (5 mg/kg rat weight), the urinary recovery of HT metabolites was higher with SEC, indicating that the bioavailability of HT was more effective with the intake of the more complex structure as SEC [33]. Consequently, higher amounts of HT and HT metabolites may be able to reach the target tissue (heart) and could have exerted a greater modulation of the proteome. These results highlight that the complementary information regarding the bioavailability and the tissue disposition of

the phenolic metabolites is critical to understand and determine the bioactivity exerted in a specific tissue. A previous study performed by our group demonstrated that free HT at physiological concentrations and in a dose-dependent manner, could exert significant effects reducing adhesion molecules (vascular adhesion molecule-1, intracellular adhesion molecule-1, E-selectin, and P-selectin) and in a chemokine (monocyte) chemoattractant protein-1) in human aortic endothelial cells stimulated by tumor necrosis factor alpha-1 [34], confirming the that depending on the HT dose reaching the target tissue the effect can differ.

The network analysis based on the whole differentially expressed proteins after HT and SEC diets demonstrated that the top-scored networks were related to Cardiovascular System Development in both tissues (**Figs. 3 and 4**), and specifically, some of the modulated proteins appeared to be involved in particular cardiac functions and lipid metabolism (Table 4). In the case of aorta (**Fig. 3**), the proteins involved in the top-scored network were mainly related to several cardiac functions. One of the downregulated proteins was T-kininogen 2 (–1.2-fold and –2.7-fold for HT and SEC, respectively), involved in the occlusion of blood vessel and proliferation of endothelial cells. Kininogens are multifunctional proteins that act as precursor of kinins, small vasoactive peptides that promote endothelial cell proliferation through kinin receptors and its plasma levels have been related to inflammatory and aging processes in rats and humans [35]. In accordance with our results, T-kininogen precursor was significantly decreased after the intake of VOO compared with sunflower oil in rats [31]. Therefore, the decrease of T-kininogen 2 after intake of HT and SEC suggests that HT could contribute to the decline of inflammatory processes. Together with T-kininogen 2, Gap junction alpha-1 protein (Gja1), which was also downregulated after HT (–1.0-fold) and SEC (–1.7-fold), is related to proliferation of endothelial cells, as well as with cardiogenesis and vasoconstriction of blood vessels.

Another relevant protein involved in the top-scored Cardiovascular System network in aorta was Rac1 (ras-related C3 botulinum toxin substrate 1), which was significantly downregulated after HT (–1.0-fold) and SEC (–1.4-fold) diets.

Rac1 is a small GTPase essential for the assembly and activation of NADPH oxidase. Several molecular and cellular studies have reported the involvement of Rac1 in different cardiovascular pathologies, such as vascular smooth muscle proliferation, atherosclerosis, and endothelial dysfunction [36]. An increased activation of NADPH oxidase by Rac1 has been reported in animals and humans after myocardial infarction and heart failure [36]. Due to all these findings, Rac1 has emerged as a new pharmacological target for the treatment of CVD [37].

Table 4. Functions and related proteins in aorta and heart tissue after HT or SEC treatments involved in the top-scored network: Cardiovascular system development

	Cardiovascular system functions	Aorta tissue proteins	Heart tissue proteins
Cardiac functions	Formation of thrombus	Anxa2	—
	Occlusion of blood vessel	Kng1/Kng1l1	—
	Cell viability of cardiomyocytes	Hk2, Hspd1, Hk2	—
	Survival of ventricular myocytes	Hk2,	—
	Proliferation of endothelial cells	Gja1, Kng1/Kng1l1	—
	Cardiogenesis	Gja1, Mylk3	—
	Vasoconstriction of blood vessel	Gja1, Hspa1a/Hspa1b	—
	Migration of endothelial cell lines	Rac1	—
	Heart rate	Rac1, Tpm1	—
	Cell death of cardiomyocytes	Hspd1, Hspe1	—
	Apoptosis of cardiomyocytes	Hspd1, Hspe1	—
	Hypertrophy of cardiomyocytes	Cryab	Camk2d, H2afz, Glrx3
	Pericarditis	—	Tuba4a
	Myocardial infarction	—	Tuba4a, Psma6
	Acute myocardial infarction	—	Tuba4a
	Hypertrophy of heart	—	Camk2d, Fkbp1a, H2afz, Glrx3
	Severe heart failure	—	Camk2d
	Failure of heart	—	Camk2d, Fkbp1a
	Hypertrophy of left ventricle	—	Fkbp1a
	Congestive heart failure	—	Fkbp1a
	Systolic dysfunction	—	Fkbp1a
Lipid metabolism	Synthesis of acetyl-coenzime A	—	Dlat, Dld,
	Fatty acid metabolism	—	Dlat, Dld, Dbi, Hbb, Msn, Por
	Acetylation of acetyl-coenzyme	—	Dlat
	A Acetylation of dihydrolipoic	—	Dlat
	acid Synthesis of lipid	—	Dlat, Dld, Dbi, Hbb, Por
	Synthesis of fatty acid	—	Dlat, Dld, Hbb
	Concentration of cholesterol	—	Dbi, Por, Arhgdia
	Quantity of very long chain fatty acid	—	Dbi
	Quantity of farnesyl pyrophosphate	—	Por
	Oxidation of testosterone	—	Por
	Conversion of androstenedione	—	Por
	Concentration of tretinoin	—	Por
	Concentration of retinol	—	Por
	Cytotoxicity of tacrolimus	—	Fkbp1a
	Oxidation of cardiolipin	—	Cycs
	Oxidation of linoleic acid	—	Cycs
	Binding of paclitaxel	—	Map4

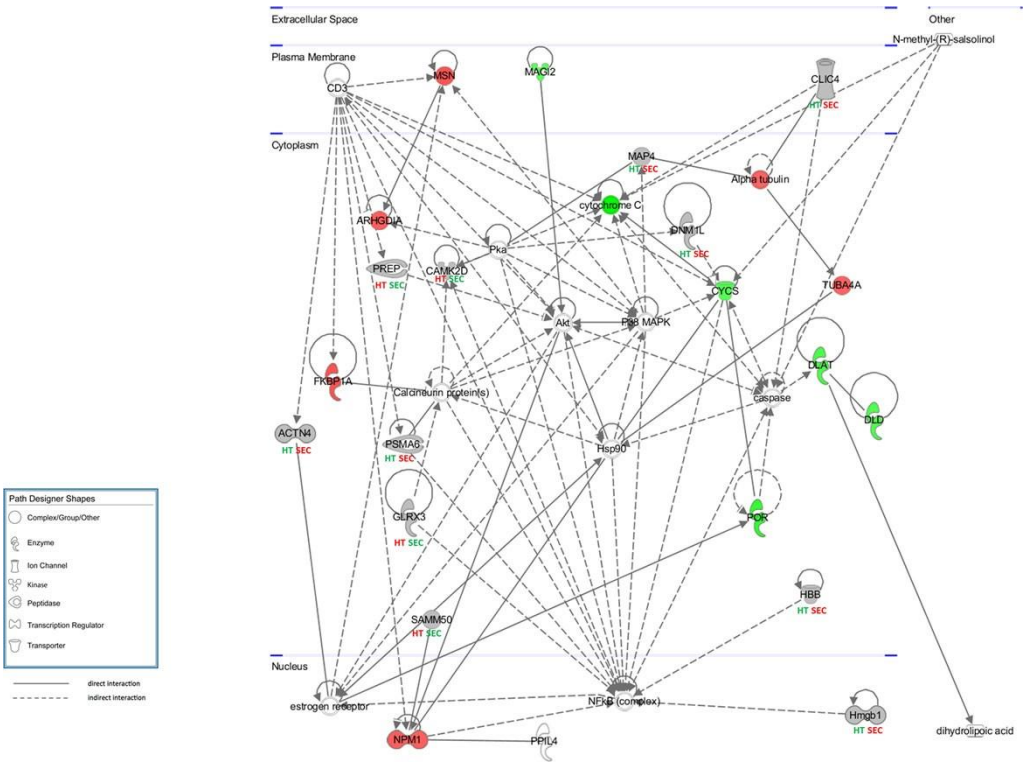


Figure 4. Heart Cardiovascular System Network after HT or SEC supplemented diets. Interaction between the proteins differentially expressed in heart after HT or SEC diets and other important proteins related to the same network. HT or SEC is represented in red or green color if the protein is up- or downregulated, respectively.

Thus, our results suggest that its downregulation after HT and SEC consumption could imply a cardio-protection effect.

Hexokinase-2 (HK2) also appeared to be one of the differentially expressed proteins (1.3-fold for HT and 2.7-fold for SEC) related to the cardiovascular network in aorta, playing an important role in cell viability of cardiomyocytes and survival of ventricular myocytes (Table 4). HKs are multifunctional proteins that orchestrate metabolic, antioxidant, and direct anti-cell death effects [38]. HK2 specifically has a role in shuttling glucose-6-phosphate to glycolysis and oxidative phosphorylation when bound to mitochondria [39]. Genetic reduction of HK2 levels in heterozygous HK2 knock-out mice increased susceptibility to ischemia/reperfusion injury [40] indicating its cardioprotective role. In this sense, HK2 also increased the cell viability of cultured ventricular myocytes [41]. The overexpression of HK2 after HT and SEC administration suggests that HK2 could be an important

protein target for HT. Heat shock protein family E member 1 (Hspe1; upregulated by HT 1.1-fold and SEC 2.5-fold) was also related to cardiac necrosis/cell death and previous studies showed that its upregulation decreased apoptosis of cardiac myocytes from newborn rat [42].

Heat shock 70 kDa protein (Hspa1a), which was downregulated after both supplemented diets (–1.0-fold for HT and –2.3-fold for SEC), acts as a cellular defense mechanism its expression being induced under stressful conditions.

In fact, it has been related to the development of atherosclerosis where Hspa1a is expressed within human atherosclerotic lesions [43], and also seems to be a clinically useful biomarker for prediction of mortality in heart failure patients [44]. Due to its protective role, high serum levels of Hsp1a1 have been associated with low risk of coronary artery disease [45]. The downregulation in Hspa1a found in our experiment could be related to the increased antioxidant capacity in response to diet supplementation with HT or SEC and therefore, a lower need for the expression of genes involved in stress and atherosclerotic lesions.

Apart from the implication of Hsp1a1 in the cardiovascular system, its overexpression has been linked to the development of some cancers, such as hepatocellular carcinoma, gastric cancers, colon cancers, breast cancers, and lung cancers, which led to its use as a prognostic marker for these cancers [46]. Circulating Hspa1a has been also recently stablished as a clinical marker of rheumatoid arthritis, used for its diagnosis and monitoring the disease activity [47]. Moreover, the overexpression of Annexin 2 (Anxa2), which was down-regulated after both diets (–1.0-fold for HT and –1.4-fold for SEC), has been defined as a prognostic marker in certain cancers such as cholangiocarcinoma [48]. These results together with all the presented evidence, suggest that olive phenols not only might be able to modulate proteins related to the prevention of CVD, but could also exert a protector effect against cancer modulating some cancer-relevant proteins [1, 49–51].

The network analysis in heart tissue revealed that the significantly expressed proteins were mainly implicated in cardiac functions. Peptidyl-prolyl cis-trans isomerase (Fkbp1a), which appeared to be related to heart failure, hypertrophy of left ventricle, congestive heart failure, and systolic dysfunction, was upregulated 1.2-fold and 1.7-fold by HT and SEC, respectively. Previous studies have shown that mutant mice deficient in Fkbp1a develop multiple abnormalities in cardiac structure, including lack of compaction and thin ventricular walls [52, 53], suggesting that its upregulation could imply a positive effect preventing all these anomalies. In the same line, calcium/calmodulin-dependent protein kinase type II subunit delta (Camk2d) was also modulated and it has been defined as a determinant of clinically important heart disease phenotypes, and it has been suggested that its inhibition can be a highly selective approach for targeting adverse myocardial hypertrophy, dilation, and dysfunction, in individuals with

myocardial infarction [54]. A downregulation of Camk2d was observed after SEC diet (–1.4-fold) indicating an ameliorant cardiac function [55]. In contrast, after HT diet an upregulation of this protein was observed (1.4-fold). Camk2d is regulated by different mechanisms, including intracellular Ca2+ levels [56], so the different modulation by HT and SEC could be explained by the different pathway regulation of Camk2d.

Another related protein that appeared to be downregulated was Cythocrome c (Cycs), a component of the electron transport chain in mitochondria involved in initiation of apoptosis. Cycs release in cardiomyocytes has been attributed to many mechanisms including ROS generation, cardiolipin peroxidation, and Ca2+ overload in the mitochondria and therefore, if the release of Cycs could be inhibited, apoptosis could be prevented, slowing the disease progression or limiting neurologic damage after trauma [57].

Only two proteins, alpha-actinin -4 (Actn4) and 60S ribosomal protein L8 (Rpl8), were modulated in both tissues (aorta and heart) after HT and SEC supplemented diets. Actn4 belong to a diverse group of cytoskeletal proteins and the Actn4 isoforms present in cardiac tissue and smooth muscle cells help to anchor the myofibrillar actin filaments. Its downregulation is associated with less inflammation in THP-1 monocytes [34] and also involved in vasorelaxation in human umbilical vein endothelial cells [35]. The downregulation of Actn4 after HT and SEC supplemented diets suggest they contribution to the decline of inflammatory processes. Rpl8, a ribosome that catalyze protein synthesis, has not been described, as far as we know, neither in heart nor in aorta and no literature was found linking Rpl8 and cardiovascular diseases. Its upregulation after both treatments and both tissues suggests that the novo protein synthesis could be slightly stimulated after HT and SEC supplementation.

One limitation of this study is the small sample size, thus, future studies increasing the number of treated rats are needed in order to confirm our results. Given that the present study has been performed in healthy animals, further studies are needed to assess the effect at the proteomic level of VOO phenols supplementation in animals with a cardiovascular disease such as ApoE-deficient mouse.

In conclusion, the findings of the present study revealed that HT and SEC may play a pivotal role in the Cardiovascular System through the modulation of several proteins. For the first time, a MS-based proteomic technique is used as a novel approach to gain knowledge in the root causes for the VOO phenol protective effects in the cardiovascular system. Results demonstrated that HT and SEC are able to positively regulate the expression of relevant proteins in aorta tissue related to atherosclerotic processes such as proliferation and migration of endothelial cells and occlusion of blood vessels. In heart, other relevant proteins related to cardiac functions such as heart failure were also positively modulated. Other prognostic markers for some cancers

appeared to be modulated after HT and SEC treatments, suggesting that olive phenols could also exert a protector effect against cancer.

Diet supplementation with SEC demonstrated higher FC values that was attributed to the higher concentration of HT detected in heart tissue as consequence of the more effective bioavailability of SEC. These results suggest that SEC, the main phenolics present in VOO, could have a higher cardio-protective effect than free HT.

Ú.C., L.R., M.-C.L., M.-J.M., and R.S. conceived and designed the experiments. M.-C.L. conducted the experiments. Ú.C., M.-C.L., and L.R. acquired the data. P.H., P.N., and N.C. processed the samples. Ú.C. and L.R. analyzed and interpreted the data. Ú.C., L.R., and P.H. drafted the article. Ú.C., L.R., M.-C.L., M.-J.M., and R.S. revising the article critically for important intellectual content.

This study was supported by the Spanish Ministry of Education and Science, The MEFOPC Project (AGL2012-40144-C03-03 and AGL2012-40144-C03-02) and by the University of Lleida through the M-C. López de las Hazas grant.

The authors have declared no conflict of interest.

REFERENCES

[1] Martín-Peláez, S., Covas, M. I., Fitó, M., Kusar, A., Pravst, I., Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Mol. Nutr. Food Res. 2013, 57, 760–771.

[2] Hu, T., He, X.-W., Jiang, J.-G., Xu, X.-L., Hydroxytyrosol and its potential therapeutic effects. J. Agric. Food Chem. 2014, 62, 1449–1455.

[3] Cicerale, S., Lucas, L., Keast, R., Biological activities of phenolic compounds present in virgin olive oil. Int. J. Mol. Sci. 2010, 11, 458–479.

[4] Sofi, F., Abbate, R., Gensini, G. F., Casini, A., Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. Am. J. Clin. Nutr. 2010, 92, 1189–1196.

[5] Meydani, M., Nutrition interventions in aging and age- associated disease. Ann. NY Acad. Sci. 2001, 928, 226–235.

[6] Cui, Z., Dewey, S., Gomes, A. V, Cardioproteomics: advancing the discovery of signaling mechanisms involved in cardiovascular diseases. Am. J. Cardiovasc. Dis. 2011, 1, 274–292.

[7] Karbassi, E., Vondriska, T. M., How the proteome packages the genome for cardiovascular development. Proteomics 2014, 14, 2115–2126.

[8] Shah, S. H., Newgard, C. B., Integrated metabolomics and genomics: systems approaches to biomarkers and mechanisms of cardiovascular disease. Circ. Cardiovasc. Genet. 2015, 8, 410–419.

[9] Anderson, L., Candidate-based proteomics in the search for biomarkers of cardiovascular disease. J. Physiol. 2005, 563, 23–60.

[10] Lindsey, M. L., Mayr, M., Gomes, A. V., Delles, C. et al., Transformative impact of proteomics on cardiovascular health and disease: a scientific statement from the American Heart Association.

Circulation 2015, 132, 852–872.

[11] Eckhard, U., Marino, G., Butler, G. S., Overall, C. M., Positional proteomics in the era of the human proteome project on the doorstep of precision medicine. Biochimie 2016, 122, 110–118.

[12] De Roos, B., McArdle, H. J., Proteomics as a tool for the modelling of biological processes and biomarker development in nutrition research. Br. J. Nutr. 2008, 99 (Suppl 3), S66– S71.

[13] Kaga, E., Karademir, B., Baykal, A. T., Ozer, N. K., Identification of differentially expressed proteins in atherosclerotic aorta and effect of vitamin E. J. Proteomics 2013, 92, 260– 273.

[14] Joumard-Cubizolles, L., Gladine, C., Ge´ rard, N., Chambon, C. et al., Proteomic analysis of aorta of LDLR −/− mice given omega-3 fatty acids reveals modulation of energy metabolism and oxidative stress pathway. Eur. J. Lipid Sci. Technol. 2013, 115, 1492–1498.

[15] Dekkers, D. H. W., Bezstarosti, K., Gurusamy, N., Luijk, K. et al., Identification by a differential proteomic approach of the induced stress and redox proteins by resveratrol in the normal and diabetic rat heart. J. Cell. Mol. Med. 2008, 12, 1677–1689.

[16] Sabe, A. A., Sadek, A. A., Elmadhun, N. Y., Dalal, R. S. et al., Investigating the effects of resveratrol on chronically ischemic myocardium in a swine model of metabolic syn- drome: a proteomics analysis. J. Med. Food 2015, 18, 60–66.

[17] Oda, T., Matsumoto, K.-I., Proteomic analysis in cardiovascular research. Surg. Today 2015, 46, 285–296.

[18] Almofti, M. R., Huang, Z., Yang, P., Rui, Y., Proteomic analysis of rat aorta during atherosclerosis induced by high cholesterol diet and injection of vitamin D3. Clin. Exp. Pharmacol. Physiol. 2006, 33, 305–309.

[19] Yao, W., Fan, W., Huang, C., Zhong, H. et al., Proteomic analysis for anti-atherosclerotic effect of tetrahydroxystilbene glucoside in rats. Biomed. Pharmacother. 2013, 67, 140–145.

[20] Ahmad, S., Kumar, K. A., Basak, T., Bhardwaj, G. et al., PPAR signaling pathway is a key modulator of liver proteome in pups born to vitamin B(12) deficient rats. J. Proteomics 2013, 91, 297–308.

[21] Suárez, M., Romero, M.-P., Ramo, T., Macià, A., Motilva, M.-J., Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. J. Agric. Food Chem. 2009, 57, 1463–1472.

[22] Rubió, L., Motilva, M.-J., Macià, A., Ramo, T., Romero, M.- P., Development of a phenol-enriched olive oil with both its own phenolic compounds and complementary phenols from thyme. J. Agric. Food Chem. 2012, 60, 3105–3112.

[23] Serra, A., Rubio´, L., Borràs, X., Macià A. et al., Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. Mol. Nutr. Food Res. 2012, 56, 486–496.

[24] Vizcaíno, J. A., Csordas, A., Del-Toro, N., Dianes, J. A. et al., 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 2016, 44, D447–D456.

[25] D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O. et al., Phar-

macokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. Drug Metab. Dispos. 2001, 29, 1492–1498.

[26] Xu, J., Jüllig, M., Middleditch, M. J., Cooper, G. J. S., Modelling atherosclerosis by proteomics: molecular changes in the ascending aortas of cholesterol-fed rabbits. Atherosclerosis 2015, 242, 268–276.

[27] De la Cuesta, F., Alvarez-Llamas, G., Gil-Dones, F., Martin-Rojas, T. et al., Tissue proteomics in atherosclerosis: elucidating the molecular mechanisms of cardiovascular diseases. Expert Rev. Proteomics 2009, 6, 395–409.

[28] Wang, H., Sit, W.-H., Tipoe, G. L., Wan, J. M.-F., Differential protective effects of extra virgin olive oil and corn oil in liver injury: a proteomic study. Food Chem. Toxicol. 2014, 74, 131– 138.

[29] Arbones-Mainar, J. M., Ross, K., Rucklidge, G. J., Reid, M. et al., Extra virgin olive oils increase hepatic fat accumulation and hepatic antioxidant protein levels in APOE-/- mice. J. Proteome Res. 2007, 6, 4041–4054.

[30] Rodríguez-Gutiérrez, G., Duthie, G. G., Wood, S., Morrice, P. et al., Alperujo extract, hydroxytyrosol, and 3,4- dihydroxyphenylglycol are bioavailable and have antioxi- dant properties in vitamin E-deficient rats—a proteomics and network analysis approach. Mol. Nutr. Food Res. 2012, 56, 1137–1147.

[31] Santos-González, M., López-Miranda, J., Pérez-Jiménez, F., Navas, P., Villalba, J. M., Dietary oil modifies the plasma proteome during aging in the rat. Age 2012, 34, 341– 358.

[32] Pedret, A., Catalán, Ú., Fernández-Castillejo, S., Farra` s, M. et al., Impact of virgin olive oil and phenol-enriched virgin olive oils on the HDL proteome in hypercholesterolemic subjects: a double blind, randomized, controlled, cross-over clinical trial (VOHF study). PLoS One 2015, 10, e0129160.

[33] López de las Hazas, M.-C., Piñol, C., Macià, A., Romero, M.-P. et al., Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids. J. Funct. Foods 2016, 22, 52–63.

[34] Catalán Ú., López de Las Hazas, M.-C., Rubio´, L., Ferna´ndez-Castillejo, S. et al., Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. Mol. Nutr. Food Res. 2015, 59, 2523–2536.

[35] Pérez, V., Leiva-Salcedo, E., Acuña-Castillo, C., Aravena, M. et al., T-kininogen induces endothelial cell proliferation. Mech. Ageing Dev. 2006, 127, 282–289.

[36] Carrizzo, A., Forte, M., Lembo, M., Formisano, L. et al., Rac-1 as a new therapeutic target in cerebro-and cardio-vascular diseases. Curr. Drug Targets 2014, 15, 1231–1246.

[37] Ferri, N., Contini, A., Bernini, S. K., Corsini, A., Role of small GTPase protein Rac1 in cardiovascular diseases: development of new selective pharmacological inhibitors. J. Cardiovasc. Pharmacol. 2013, 62, 425–435.

[38] Calmettes, G., Ribalet, B., John, S., Korge, P. et al., Hexoki- nases and cardioprotection. J. Mol. Cell. Cardiol. 2015, 78, 107–115.

[39] John, S., Weiss, J. N., Ribalet, B., Subcellular localization of hexokinases

I and II directs the metabolic fate of glucose. PLoS One 2011, 6, e17674.

[40] Smeele, K. M. A., Southworth, R., Wu, R., Xie, C. et al., Disrup- tion of hexokinase II-mitochondrial binding blocks ischemic preconditioning and causes rapid cardiac necrosis. Circ. Res. 2011, 108, 1165–1169.

[41] Roberts, D. J., Tan-Sah, V. P., Smith, J. M., Miyamoto, S., Akt phosphorylates HK-II at Thr-473 and increases mitochondrial HK-II association to protect cardiomyocytes. J. Biol. Chem. 2013, 288, 23798–23806.

[42] Lin, K. M., Lin, B., Lian, I. Y., Mestril, R. et al., Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and pre- vents apoptotic cell deaths induced by simulated ischemia-reoxygenation. Circulation 2001, 103, 1787–1792.

[43] Xu, Q., Metzler, B., Jahangiri, M., Mandal, K., Molecular chaperones and heat shock proteins in atherosclerosis. Am. J. Physiol. Heart Circ. Physiol. 2012, 302, H506–H514.

[44] Jenei, Z. M., Gombos, T., Förhécz, Z., Pozsonyi, Z. et al., Elevated extracellular HSP70 (HSPA1A) level as an independent prognostic marker of mortality in patients with heart failure. Cell Stress Chaperones 2013, 18, 809–813.

[45] Zhu, J., Quyyumi, A. A., Wu, H., Csako, G. et al., Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. Arterioscler. Thromb. Vasc. Biol. 2003, 23, 1055–1059.

[46] Wang, X., Wang, Q., Lin, H., Li, S. et al., HSP72 and gp96 in gastroenterological cancers. Clin. Chim. Acta. 2013, 417, 73–79.

[47] Najafizadeh, S. R., Ghazizadeh, Z., Nargesi, A. A., Mahdavi, M. et al., Analysis of serum heat shock protein 70 (HSPA1A) concentrations for diagnosis and disease activity monitoring in patients with rheumatoid arthritis. Cell Stress Chaperones 2015, 20, 537–543.

[48] Wang, A.-G., Yoon, S. Y., Oh, J.-H., Jeon, Y.-J. et al., Identification of intrahepatic cholangiocarcinoma related genes by comparison with normal liver tissues using expressed sequence tags. Biochem. Biophys. Res. Commun. 2006, 345, 1022–1032.

[49] Cárdeno, A., Sanchez-Hidalgo, M., Alarcón-de-la-Lastra, C., An up-date of olive oil phenols in inflammation and cancer: molecular mechanisms and clinical implications. Curr. Med. Chem. 2013, 20, 4758–4776.

[50] Casaburi, I., Puoci, F., Chimento, A., Sirianni, R. et al., Poten- tial of olive oil phenols as chemopreventive and therapeutic agents against cancer: a review of in vitro studies. Mol. Nutr. Food Res. 2013, 57, 71–83.

[51] Rafehi, H., Ververis, K., Karagiannis, T. C., Mechanisms of action of phenolic compounds in olive. J. Diet. Suppl. 2012, 9, 96–109.

[52] Towbin, J. A., Lorts, A., Jefferies, J. L., Left ventricular non- compaction cardiomyopathy. Lancet 2015, 386, 813–825.

[53] Shou, W., Aghdasi, B., Armstrong, D. L., Guo, Q. et al., Car- diac defects and altered ryanodine receptor function in mice lacking FKBP12. Nature 1998, 391, 489–492.

[54] Kreusser, M. M., Lehmann, L. H., Keranov, S., Hoting, M.-O. et al., Cardiac CaM kinase II genes δ and γ contribute to adverse remodeling but

redundantly inhibit calcineurin-induced myocardial hypertrophy. Circulation 2014, 130, 1262–1273.

[55] Toko, H., Takahashi, H., Kayama, Y., Oka, T. et al., Ca2+/calmodulin-dependent kinase IIdelta causes heart failure by accumulation of p53 in dilated cardiomyopathy. Circulation 2010, 122, 891–899.

[56] Quijada, P., Hariharan, N., Cubillo, J. D., Bala, K. M. et al., Nuclear calcium/calmodulin-dependent protein kinase II signaling enhances cardiac progenitor cell survival and cardiac lineage commitment. J. Biol. Chem. 2015, 290, 25411–25426.

[57] Hüttemann, M., Pecina, P., Rainbolt, M., Sanderson, T. H. et al., The multiple functions of cytochrome c and their reg- ulation in life and death decisions of the mammalian cell: from respiration to apoptosis. Mitochondrion 2011, 11, 369–381.

SUPPORTING INFORMATION

Table 1. Phenolic composition of the secoiridoid (SEC) extract.

Compound	Concentration (mg/kg extract)
HT	4176.82 ± 185.40
p-HPEA-EDA	936.37 ± 60.66
3,4-DHPEA-EDA	40995.97 ± 1085.46
p-HPEA-EA	378.82 ± 21.63
3,4- DHPEA-EA	1628.98 ± 274.01
OLE	480.00 ± 70.33

HT, Hydroxytyrosol; *p*-HPEA-EDA, Dialdehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; *p*-HPEA-EA, Aldehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-EA, Isomer of oleuropein aglycone and OLE, oleuropein.

Values are expressed as mean ± SD (n= 4).

Supporting information Table 2. Isotopic labelling mass tags for the three TMT kits used in the study.

Aorta				Heart			
Sample	Type	TMT-batch	TMT-label	Sample	Type	TMT-batch	TMT-label
PS2218_1	Control	Aorta-1	126	PS2218_9	Control	Heart-2	126
PS2218_2	Control	Aorta-1	127N	PS2218_10	Control	Heart-2	127N
PS2218_3	Control	Aorta-1	127C	PS2218_11	Control	Heart-2	127C
PS2218_4	Control	Aorta-1	128N	PS2218_12	Control	Heart-2	128N
PS2218_5	HT	Aorta-1	128C	PS2218_13	HT	Heart-2	128C
PS2218_6	HT	Aorta-1	129N	PS2218_14	HT	Heart-2	129N
PS2218_7	HT	Aorta-1	129C	PS2218_15	HT	Heart-2	129C
PS2218_8	HT	Aorta-1	130N	PS2218_17	HT	Heart-2	130N
PS2218_2	Control	Aorta-3	127N	PS2218_9	Control	Heart-3	126
PS2595_1	SEC	Aorta-3	128N	PS2595_5	SEC	Heart-3	127C
PS2595_2	SEC	Aorta-3	129N	PS2595_6	SEC	Heart-3	128C
PS2595_3	SEC	Aorta-3	130N	PS2595_7	SEC	Heart-3	129C
PS2595_4	SEC	Aorta-3	131	PS2595_8	SEC	Heart-3	130C

Supporting information: Table 3. Proteins identified and quantified in aorta tissue after proteome analysis of healthy rats. (*check at DOI: 10.1002/mnfr.201600052*)

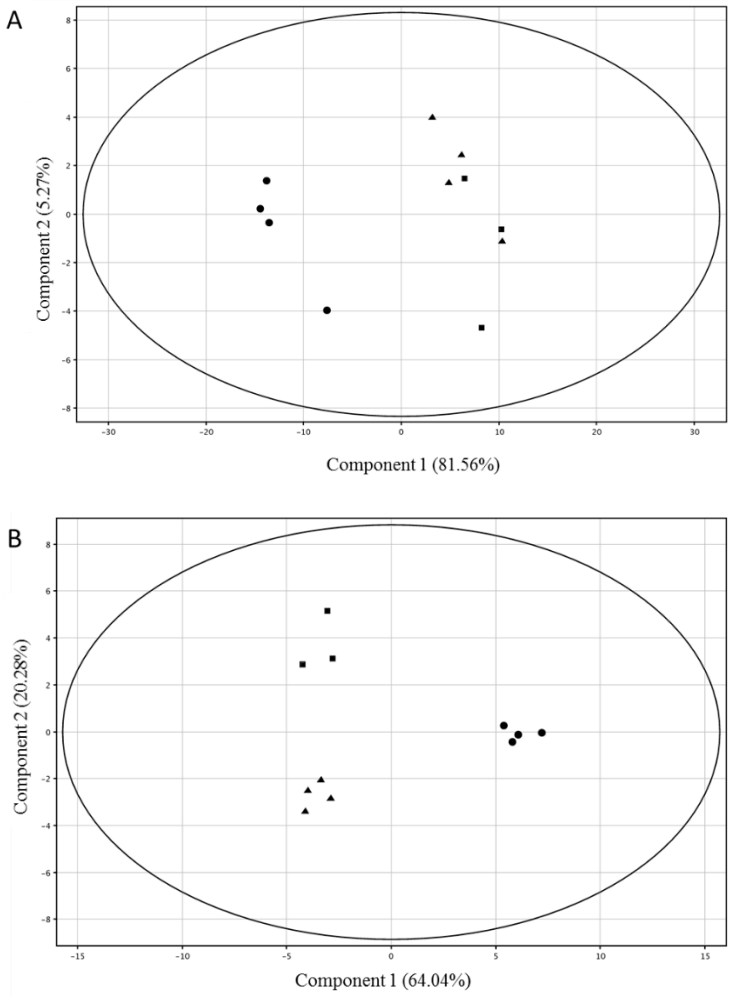
Supporting information: Table 4. Proteins identified and quantified in heart tissue after proteome analysis of healthy rats. (*check at DOI: 10.1002/mnfr.201600052*)

Supporting information: Table 5. Diseases and functions exerted within the top networks of the common differentially expressed proteins modulated by HT or SEC treatments after proteome analysis.

Tissue	Diseases and functions exerted within the top networks	Molecules in Network		Score*
		Differentially expressed proteins	Interaction with other proteins involved in the same Network	
Aorta	Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities	Actn1, Actn4, Anxa2, Glud1, Gnb1, Hspa1a/Hspa1b, Ldha, Mug1, Myh9, Myo1d, Pdcd6ip, Sfxn3, Vapa	Ap3d1, Ap3m2, Cacna1a, Cacna1c, Chmp2b, Dync1h1, Hspa8, Insr, Prkar2b, Rabl6, Secretase gamma	19
	Cardiac Hypertrophy, Cardiovascular Disease, Developmental Disorder	Akr1b1, Aldh2, Capn2, Fabp5, Gja1, Hk2, Ilk, Kng1/Kng1l1, Ldha, Rac1, Tpm1, Vcl	Agtr2, Akt, Cacna1a, Calpain, Cav1, Cav3, Ctnnb1, Dock7, Igf1r, Kcnj11, Mapk14, Nfkb (complex), Nos2, P38 Mapk, Pik3r1, Pka, Prkce, Pxn, Rhoa, Slca4, Tpm1, Tpm3	16
	Cellular Development, Skeletal and Muscular System Development and Function, Tissue development	Actr3, Anxa6, Arf3, Cd81, Cp, Csrp2, Hspe1, M6pr, Myh11, Sparc, Tagln	Adk, Akt, Cd63, E2f1, Itga3, Itgb1, Kcnj11, Lxn, mir-145, miR-145-5p, Myc, Myocd, Ncl, Nfkb (complex), Nos2 Pias1, Pka, Pkc, Ruvbl1, Slc2a4, Slpi, Srf, Tnf, Yap1	13
	Cancer, Cardiovascular Disease, Connective Tissue Disorders	Idh1	Insulin, Kcnb2	1
Heart	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	Cycs, Dlat, Dld, Rtn3	Kcnj11, Bace1	7
	Cancer, Connective Tissue Disorders, Developmental Disorder	Por	Cyp1a	2
	Protein Synthesis, Cellular Development, Cellular Growth and Proliferation	Magi2	Cnksr2, Ctnnb1	2
	Cardiovascular System Development and Function, Tissue Morphology, Cell Morphology	Arhgdia	Grm4, Itgb8, Pka	2

HT, hydroxytyrosol treatment; SEC, secoiridoids treatment

*Score is calculated by Ingenuity Pathway analysis software (IPA) on the resulting network and the network with the highest score is retained.



Supporting information Figure 1: PCA of aorta and heart tissue

SUPPORTING INFORMATION MATERIALS AND METHODS

2.5. Proteomic sample preparation and quantitative analysis of the aorta and heart tissues

2.5.1. Protein extraction

Prior to protein extraction [1], aortic tissues were pre-treated during 30 min at 37ºC with

collagenase (4 mg/mL) in a buffer containing 100 mM Tris, 100 mM $CaCl_2$ and pH 7.4. Afterwards, 50 mg of heart and pre-treated aortic tissues were homogenized using an Ultra-Turrax T 25 (IKA, Staufen, Germany) in 500 µL sodium dodecyl sulfate (SDS)-lysis buffer containing 4% SDS, 100 mM dithiothreitol (DTT), 150 mM Tris pH 7.6 and protease inhibitors. Following homogenization, samples were incubated at 95ºC for 5 minutes and placed in an ultrasonic cell crusher with probe (10 times, 80w, 10s each time with 15s interval). The crude extract was clarified by centrifugation at 14,000 x *g* for 20 min and quantified by reducing agent and detergent compatible protein assay (Bio-Rad, Madrid, Spain).

2.5.2. Protein digestion and purification

Aliquots containing 120 µg of protein were diluted with 200 µL of 8 M urea in 0.1 M Tris/HCl, pH 8.5 and placed in Microcon-30 kDa Centrifugal Filter Unit with Ultracel-30 membrane (Merk, Millipore, Billerica, MA, USA) to perform filter aided sample preparation. Briefly, proteins are reduced and alquilated using 10 mM DTT and 50 mM iodoacetamide, respectively, before perform a double digestion by endoproteinase LysC (1:66) for 4h and trypsin (1:100) overnight. Digested samples were dried in the centrifugal evaporator (Savant SPD 2010, Thermo Scientific, Waltham, MA, USA) and purified by C18 Sep-Pak SPE from Waters (Bedford, MA, USA).

2.5.3. TMT 10-plex labeling

TMT-10plex labelling reagents (Thermo Fisher Scientific, Waltham, MA, USA) were added to the peptide samples and incubated at room temperature for 60 min as is described in manufacturer's instructions. Afterwards, the reaction was quenched by the addition of 5% hydroxylamine and incubated for 15 min. After labelling, samples were subjected again to a C18 SPE (Sep-Pak, Waters, Bedford, MA, USA) desalting before Off-Gel fractionation [2].

2.5.4. Off-Gel fractionation

In order to increase the number of identified/quantified proteins in samples, peptides were fractionated by iso-electrofocusing before nanoLC-MS analysis. Pooled samples (heart and aortic samples were treated as a two separated pools) were fractionated in an Agilent 3100 OFFGEL Fractionator (Agilent Technologies, Santa Clara, CA, USA) through 24-well IPG strips (linear gradient from pH 3 to 10) according to the supplier's protocol. Initially, 24-cm-long IPG strips were hydrated with 40 µL of hydration buffer (12.5% Glycerol and 1.25% v/v Off-Gel buffer pH 3–10). Each pool was diluted in the focusing buffer to a final volume of 1.4 mL, and 150 µL was loaded in each well. The samples were focused at 50 µA, with voltages between 500 and 4500 V for a total of 50 kVh. After iso-electrofocusing fractionation, the 24 fractions for each TMT pool were reduced to 10 end fractions based on their different isoelectric point and combined fractions were analyzed by nanoLC-MS for quantitative proteomic analysis [3].

2.5.5. Nano Liquid Chromatography-Mass Spectrometry (nanoLC-MS)

The 10 Off-Gel fractions were directly separated onto a C18 reversed phase (RP) nano-column (75 µm I.D.; 15cm length; 3µm particle diameter, Nikkyo Technos Co. LTD, Japan) coupled to a trap nano-column (100 µm I.D.; 2cm length; 5 µm particle diameter, Thermo Fisher Scientific, San José, CA, USA). 1 µg of each sample were analyzed using a continuous acetonitrile gradient consisting of 0–5% B in 4 min, 5-15% B in 60 min, 15-35% B in 60 min and 35-95% B in 10 min where is maintained during 20 min (A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid).

A flow rate of 300 nL/min was used to elute peptides for real time ionization and peptide fragmentation on an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San José, CA, USA). An enhanced FT high resolution spectrum (R= 30,000 FHMW) followed by two data dependent MS/MS scan events were recorded. One consist on a higher-energy collisional dissociation fragmentation (40% normalized collision energy; NCE) and Fourier transform coupled to tandem mass spectrometry (FT-MS/MS) acquisition (R=30,000 FHMW) from most intense ten parent ions with a charge state rejection of one and dynamic exclusion of 0.5 min which is used for peptide quantification. The other event consist on a collision-induced dissociation fragmentation (35% NCE) and IT-MS/MS acquisition from the same most intense ten parent ions which is used for peptide identification.

2.5.6. Peptide/protein identification and quantification

MS and MS/MS spectra were analyzed on Proteome Discoverer software (v. 1.4.0.288) (Thermo Fisher Scientific, San José, CA, USA) using Mascot (v. 2.4.1.0, Matrix Science, Boston, MA, USA) as search engine node. Mascot was set up to search against SwissProt_2015_05. fasta database (548454 entries), restricting for *Rattus norvergicus* taxonomy (7935 sequences) and assuming trypsin digestion. Three missed cleavages were allowed and an error of 0.02 Da for FT-MS/MS fragment ion mass, 0.8 Da for IT-MS/MS fragment ion mass and 10.0 ppm for a FT-MS parent ion mass were allowed. Oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine and TMT-10plex on lysine were set as static modifications. The false discovery rate (FDR) and protein probabilities were calculated by Target Decoy PSM Validator working between 0.01 and 0.05 for Strict and relaxed respectively. For proteins identified only with one unique peptide, visual verification of fragmentation spectra was done.

For protein identification/quantification the LC-MS data were processed by multidimensional protein identification technology (MudPIT) analysis which combines the identification/quantification results from different fraction analyzed in one unique report. Quantification values were obtained averaging the TMT ratios for all unique peptides from a given protein and were normalized based on protein median. The normalization samples were labelled with TMT tags 126 and 127N for heart and aorta tissues respectively, as indicated

in **Supporting information Table 2.** The quantification is the average value of the ratio for the three most intense unique peptides for each protein and were normalized based on protein median. After MudPIT-nanoLC-MS analysis a total of 1303/1247 and 1172/1124 proteins were identified/quantified for aorta tissue and heart tissue, respectively.

References

[1] Yue, Q.-X., Xie, F.-B., Song, X.-Y., Wu, W.-Y., et al., Proteomic studies on protective effects of salvianolic acids, notoginsengnosides and combination of salvianolic acids and notoginsengnosides against cardiac ischemic-reperfusion injury. *J. Ethnopharmacol.* 2012, 141, 659–67.

[2] Paulo, J.A., McAllister, F.E., Everley, R.A., Beausoleil, S.A., et al., Effects of MEK inhibitors GSK1120212 and PD0325901 in vivo using 10-plex quantitative proteomics and phosphoproteomics. *Proteomics* 2015, 15, 462–73.

[3] Wiśniewski, J.R., Zougman, A., Nagaraj, N., Mann, M., Universal sample preparation method for proteome analysis. *Nat. Methods* 2009, 6, 359–62.

SUPPORTING INFORMATION: RESULTS AND DISCUSSION

Results

3.3. Pathway analysis of common proteins modulated by HT and SEC

3.3.1. Aorta

3.3.1.1. Canonical pathways

Results from IPA indicated that the top 5 canonical pathways modified by the common proteins (with their implicated proteins) were: Eukaryotic Initiation Factor 2 Signaling (Rpl3, Rpl9 and Rpl13 proteins located at nucleus and Rpl27a. Rps5, Rps7, Rps11, Rps18 and Rps24 proteins located at cytoplasm; p = 5.59E-08, ratio = 0.054); Integrin-linked Kinase Signaling (Actn1 and Actn4 transcription regulators located at cytoplasm, Cfl1 protein located at nucleus, Ilk kinase located at plasma membrane, Myh9, Myh11 both located at cytoplasm, Ppp2r1b phosphatase and Vcl enzyme both located at plasma membrane; p = 9.40E-07, ratio = 0.047); Integrin Signaling (Actn1 and Actn4 transcription regulators located at cytoplasm, Actr3 protein located at plasma membrane, Arf3 enzyme and Capn2 peptidase located at cytoplasm, Ilk kinase, Rac1 and Vcl enzymes located at plasma membrane; p = 2.07E-06, ratio = 0.042); Epithelial Adherens Junction Signaling (Actn1 and Actn4 transcription regulators located at cytoplasm, Actr3 protein located at plasma membrane, Myh9 enzyme and Myh11 protein located at cytoplasm, Rac1 and Vcl enzymes located at plasma membrane; p = 2.51E-06, ratio = 0.051) and Actin Cytoskeleton Signaling (Actn1 and Actn4 transcription regulators located at cytoplasm, Actr3 protein located at

plasma membrane, Cfl1 protein located at nucleus, Myh9 enzyme and Myh11 protein located at cytoplasm Rac1 and Vcl enzymes located at plasma membrane; p = 3.63E-06, ratio = 0.039).

3.3.1.2. Molecular and cellular functions

The top molecular and cellular functions and their related proteins in which HT and SEC could have an influence are Cell Cycle (Hspa1a/Hspa1b, Rac1, Tpm2 and Ilk), Cell-To-Cell Signaling and Interaction (Hspa1a/Hspa1b, Rac1, Tpm2, Gja1, Ywhag, Akr1b1 and Ilk), Cellular Growth and Proliferation (Hspa1a/Hspa1b, Rac1, Tpm2, Anxa2, Kng1/Kng1l1, Ldha, Lum, Akr1b1, CD59, CD81, Gja1, Ilk and Vapa), Cell Morphology (Actr3, Camsap1, Celsr2, Gja1, Ilk, Rac1, Vapa, Ywhag, Cfl1 and Hspa1a/Hspa1b) and Cellular Assembly and Organization (Actr3, Camsap1, Celsr2, Gja1, Ilk, Rac1, Vapa, Ywhag, Cfl1, Hk2 and Tpm2).

3.3.1.3. Diseases and disorders

The top 5 diseases and disorders significantly related to both HT and SEC were: CVD (Anxa2 and Kng1/Kng1l1), Cancer (Anxa2 and Gja1), Organismal Injury and Abnormalities (Anxa2, Gja1, Actn4, Actn1, Cp, Tagln and Rac1), Tumor Morphology (Anxa2) and Gastrointestinal Disease (Gja1).

3.3.1.4. Biological networks

IPA also identified a set of top biological networks when the common proteins for HT and SEC were analyzed (**Supporting information: Table 5**). The two top-scored networks in aorta were "Hereditary Disorder, Neurological Disease, Organismal injury and Abnormalities" (score = 19) with 13 proteins involved and other 11 proteins identified by IPA to interact with the quantified proteins and "Cardiac Hypertrophy, CVD, Developmental Disorder" (score = 16) with 12 proteins involved and 22 proteins identified by IPA interacting in the same network.

3.3.1.5. Upstream regulators

Moreover, an analysis of the potential upstream regulators was performed in order to identify the molecules upstream of the proteins that potentially explain the observed expression changes. In the case of aorta these proteins were Myc, Myocd, Pias1, Yap1 and Srf.

3.3.2. Heart tissue

3.3.2.1. Canonical pathways

The top 5 canonical pathways modified by the common differentially expressed proteins in heart tissue after the administration of HT or SEC included (with their implicated proteins): Acetyl-CoA Biosynthesis I Pyruvate Dehydrogenase Complex (modified by the enzymes Dlat and Dld located at cytoplasm; p = 1.51E-05, ratio= 0.286); Glycerol-3-phosphate Shuttle

(modified by the enzyme Gpd1 located at cytoplasm; p = 1.75E-03, ratio = 0.500); Methylglyoxal Degradation 1 (modified by the enzyme Hagh located at cytoplasm; p = 2.63E-03, ratio = 0.333); 1,25-dihydroxyvitamin D3 Biosynthesis (modified by the enzyme Por located at cytoplasm; p = 2.63E-03, ratio = 0.333) and 2-ketoglutarate Dehydrogenase Complex (modified by the enzyme Dld located at cytoplasm; p = 3.51E-03, ratio = 0.250).

3.3.2.2. Molecular and cellular functions

The top molecular and cellular functions that appeared to be significantly related were Lipid Metabolism (Dlat, Dld and Por), Nucleic Acid Metabolism (Dlat, Dld and Gpd1), Small Molecule Biochemistry (Dlat, Dld, Por and Gpd1), Cell Morphology (Arhgdia and Msn) and Cellular Assembly and Organization (Cycs and Msn).

3.3.2.3. Diseases and disorders

According to the IPA analysis, the top diseases and disorders in which the modulation of the common proteins could be involved in heart were: Renal and Urological disease (Arhgdia) and CVD, Developmental Disorder and Skeletal and Muscular Disorders (H2afz).

3.3.2.4. Biological networks

The analysis of top biological networks when the common proteins for HT and SEC were analyzed in heart tissue revealed that the top-scored biological network was "Cell morphology, cellular assembly and Organization, Cellular Function and Maintenance" (score = 7) with 4 proteins involved (Cycs, Dlat, Dld, Rtn3) and 2 more proteins identified by IPA involved in the same network (Kcnj11, Bace1) (**Supporting information: Table 5**).

3.3.2.5. Upstream regulators

The upstream regulators that appeared to be potentially implicated were E2f4 and PPAR-γ.

Discussion: Pathway analysis of common proteins modulated by HT and SEC

In a first step, we analyzed in IPA the common proteins that were differentially expressed in the same direction after HT and SEC supplementation. From these results we highlight the potential upstream regulators that appeared to be implicated, which allowed us to identify the molecules upstream of the proteins that potentially explain the observed expression changes. In the case of aorta, Myc, Myocd, Pias1, Yap1 and Srf are the main upstream regulators potentially implicated in the modulated pathways ($p<0.05$). All these proteins have been shown to play a role in proliferative vascular disorders in vascular smooth muscle cells (VSMC) [1]. Specifically, Yap1, Myc and Myocd have been related in the modulation of VSMC phenotype in response to the environmental stimuli through a process characterized by an increased proliferation and migration [2]. Phenotypic switch of VSMC is one of the

major cellular events underlying many VSMC-related pathological conditions, such as atherosclerosis.

In heart tissue, the top upstream regulators which are potentially implicated were E2f4 and PPAR-γ ($p<0.05$). The transcription factor E2f4 has been shown to act as an activator of cardiomyocyte proliferation, a required regulator for cardiac regeneration [3]. PPAR-γ has been well recognized to be a central player participating in various biological responses, including lipid metabolism, inflammation, and cell proliferation, the underlying pathological processes of metabolic diseases and cancer [4]. So, the potentially implication of these upstream regulators in aorta and heart tissues in response to olive phenolic compounds provides insight into the molecular mechanisms underlying their protective action on cardiovascular alterations.

[1] Ledee, D., Smith, L., Bruce, M., Kajimoto, M., et al., c-Myc Alters Substrate Utilization and O-GlcNAc Protein Posttranslational Modifications without Altering Cardiac Function during Early Aortic Constriction. *PLoS One* 2015, *10*, e0135262.

[2] Owens, G.K., Kumar, M.S., Wamhoff, B.R., Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* 2004, *84*, 767–801.

[3] van Amerongen, M.J., Diehl, F., Novoyatleva, T., Patra, C., et al., E2F4 is required for cardiomyocyte proliferation. *Cardiovasc. Res.* 2010, *86*, 92–102.

[4] Kim, J.-H., Song, J., Park, K.W., The multifaceted factor peroxisome proliferator-activated receptor γ (PPARγ) in metabolism, immunity, and cancer. *Arch. Pharm. Res.* 2015, *38*, 302–12.

Chapter 3

Biotransformations of phenols into its microbial metabolites and its health effect in colon cancer cells.

Publication 6:

Journal of Agricultural and Food Chemistry (2016 in press)

(*DOI: 10.1021/acs.jafc.6b04933)*

Publication 7:

Journal of Agricultural and Food Chemistry (2016 in press)

(*DOI: 10.1021/acs.jafc.6b04096*).

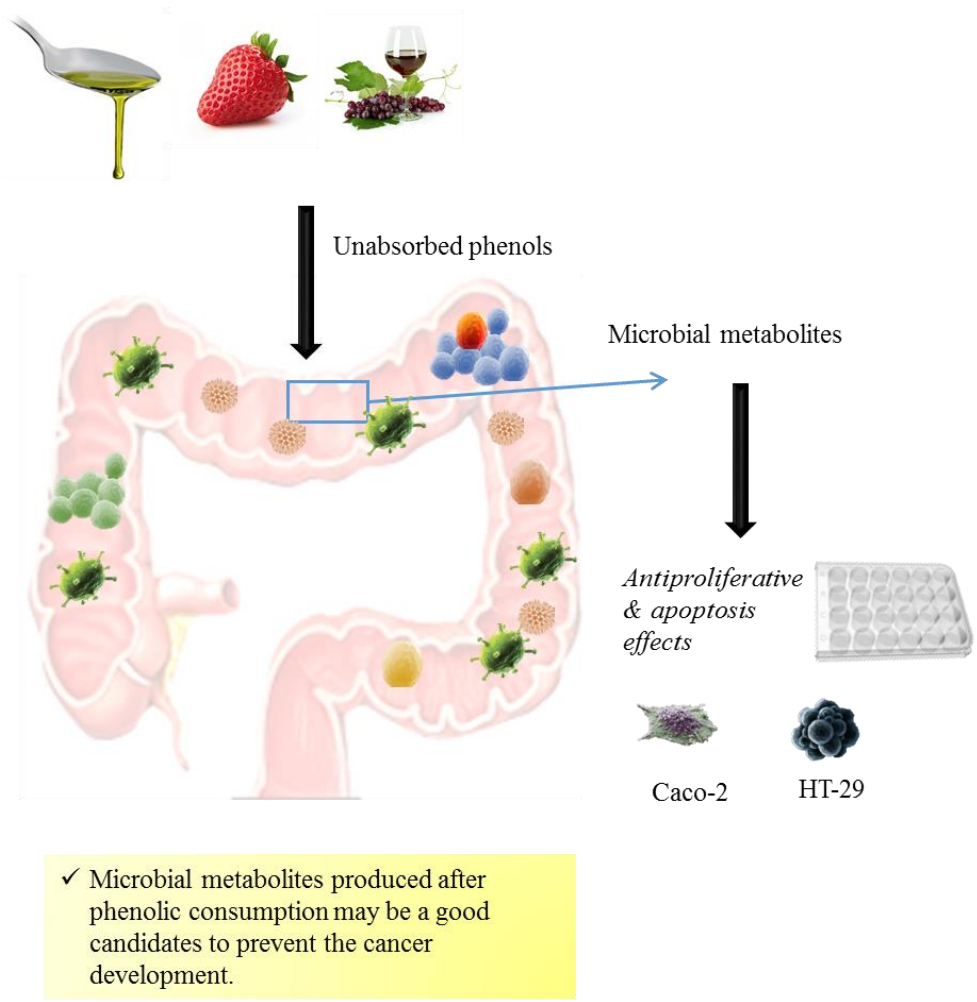


Figure 8: Experimental design and summatory of the main results of the chapter

Publication 6

Hydroxytyrosol and its colonic metabolites produce apoptosis and increase cell death in colon cancer cells

Journal of agricultural and food chemistry
DOI.10.1021/acs.jafc.6b04933

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

HYDROXYTYROSOL AND THE COLONIC METABOLITES DERIVED FROM VIRGIN OLIVE OIL INTAKE INDUCE CELL CYCLE ARREST AND APOPTOSIS IN COLON CANCER CELLS

Maria-Carmen López de las Hazas[a*], Carme Piñol[b], Alba Macià[a], Maria-José Motilva[a]

[a] Food Technology Department, Universitat de Lleida-Agrotecnio Center, Escuela Técnica Superior de Ingeniería Agraria, Lleida, Avda. Alcalde Rovira Roure 191, 25198 Lleida, Spain

[b] Department of Medicine, Universitat de Lleida-Institut de Recerca Biomèdica de Lleida (IRBLleida), Avda/Alcalde Rovira Roure 80, 25198 Lleida, Spain

Abstract

After the sustained consumption of virgin olive oil (VOO), the unabsorbed native phenols (mainly hydroxytyrosol (HT)) are transformed into its catabolites in the intestine by microbials. The role of these catabolites in preventing colon cancer has not been sufficiently investigated. This work aims to study the antiproliferative and apoptotic activities in colon (Caco-2; HT-29) cancer cell lines of the main catabolites detected in human feces (phenylacetic, phenylpropionic, hydroxyphenylpropionic, and dihydroxyphenylpropionic acids and catechol), after the sustained VOO intake. Additionally, an assessment of the ability of these colonic cells to metabolize the studied compounds was performed. The results showed that HT and phenylacetic and hydroxyphenylpropionic acids produce cell cycle arrest and promote apoptosis. HT-29 cells were more sensitive to phenol treatments than Caco-2. In synthesis, the results of the present study represent a good starting point for understanding the potential apoptotic and antiproliferative effects of VOO phenolic compounds and their colonic metabolites.

Keywords*:* apoptosis; caco-2; cell cycle; colorectal cancer; HT-29; hydroxytyrosol; metabolites

INTRODUCTION

The incidence and mortality of colon cancer is rising in developing countries, and it is the third most common cancer worldwide, after lung and breast cancers.[1] Epidemiological studies have highlighted that environmental factors are of more importance in cancer growth than genetic susceptibility. Exposure to carcinogenic agents produces an accumulation of genetic alterations that could trigger the variation of cell morphology and mechanisms of cell proliferation.[2,3] Apoptosis occurs to maintain the homeostasis replacement cells in tissues, mediated by the activation of a class of cysteine proteases called caspases.[4]

Failure to eliminate cells may contribute to the development of cancer and resistance to anticancer therapy,[5] so the enhancement of cancer cell apoptosis is one approach to cancer control by anticancer agents.

The gastrointestinal tract (GIT) is colonized by gut microbiota, composed of eukaryotic fungi, archaea, viruses, and predominantly bacterial communities,[6] which differ between individuals.[7] Gut microbiota contributes to maintain the healthy state of the colon, and it is involved in the control of obesity,[8] diabetes, steatohepatitis, psychiatric disorders, and multiple sclerosis,[9] among others. However, the presence of pathogenic microbiota promotes intestinal diseases and cancer and activates immune responses.[7,10,11] Some diet components are important regulators of the host microbial populations.[12] For diet phenolic compounds, the unabsorbed part arrives to the large intestine and suffers catabolic transformations by microbial metabolism, producing biological metabolites such as phenylacetic acids, benzaldehydes, hippuric acid derivatives, pyrogallol, and catechol among others.[13−15] These colonic catabolites may contribute to health by exerting beneficial effects on the colon as well as in the organs,[16,17] because they reach the bloodstream between 4 and 22 h postconsumption.[13] Also, the native phenols present in food could reach the colon by different paths: nonabsorbed food fraction, enterohepatic recirculation,18 or deconjugation of phase II metabolites,[19,20] which could exert different effects from their conjugate forms.[21] Traditionally, research into the biological activity of phenolic compounds has been focused on the native form present in foods. However, during gastrointestinal digestion, phenolic compounds undergo intense phase I and phase II metabolism as a result of digestive or hepatic activity. Transformations of dietary phenolics (as flavonols, flavan-3-ols, flavones, and anthocyanins) could lead to more potent microbial-inhibitory compounds, such as phenolic acids. However, few publications have focused on the bioactivity of dietary phenolic microbial metabolites. Microbial compounds seem to selectively influence the species of intestinal bacteria and therefore could affect the diversity and metabolic activity of the intestinal microbiota, including the transformation of phenolics in the gut.[22]

Virgin olive oil (VOO), the main source of dietary fat in the Mediterranean area, is associated with the reduction of the incidence and prevalence of cancer, including colorectal cancer.[23] Hydroxytyrosol (HT), the most important phenolic compound of VOO, is a potent antioxidant and has several biological activities,24 such as control of oxidative stress,[25,26] inhibition of proliferation in several tumor cell lines as a chemotherapeutic agent, and apoptosis promoter.[27,28] During gastric digestion, complex VOO phenols (oleuropein aglycone derivatives or secoiridoids) are transformed into HT,[29] which is thoroughly transformed into its

phase II metabolites (glucuronide and sulfate conjugates) and into hydroxytyrosol acetate (HT-AC) by the effect of the acetyl-CoA activity.[30,31] Once the unmetabolized native phenols arrive at the small and large intestines, the microbiota transforms into different catabolites mainly by oxidation and dehydroxylation reactions. In the intestine and cecum, HT is thoroughly transformed by microbials into phenylacetic (PA) and its derivatives; however, the catabolism of HT-AC produces phenyl-propionic (PP) derivatives. The catabolism of the oleuropein produces both PA and PP families of catabolites. Then, these colonic metabolites could be absorbed and subsequently transformed into phase II metabolites.[32] In a previous in vivo study, we investigated the presence of the HT colonic metabolites in human fecal samples obtained before and after the sustained intake of a daily dose of 25 mL of a phenol-enriched olive oil during 3 weeks.[32] The results revealed that a moderate daily intake of olive oil rich in its own phenols raised the concentration of phenylacetic and phenylpropionic acids in human feces, the increase in the concentration of free hydroxytyrosol being of special interest.

On the basis of the intense metabolism of VOO phenols during GIT digestion and the proximity with the colon we want to study the potential colon cancer preventive capacity of the catabolism products, this study was designed to assess the anti- proliferative effect, following the cell cycle progression, and the apoptotic activity of HT and the main microbial metabolites derived after olive oil consumption (PA, PP, 3-(4-hydroxyphenyl)propionic (HPP) acid, and 3-(2,4-dihydroxyphenyl)- propionic acid (diHPP)) previously detected in human feces after diet supplementation with phenol-enriched olive oil.[32] In addition, catechol (CAT), a common product of the phenol colonic metabolism, was tested as reference. Furthermore, with regard to the proportion of compounds found in feces, a mix of compounds (MIX) was prepared to see some possible synergic protective effects among the studied metabolites. For this proposal, two colonic cancer cell lines (Caco-2 and HT-29) were employed to study the cell cycle progression as well as the apoptosis. To measure the first stages (translocation of phosphatidylserine, PS) we employed annexin V (AnnV) staining, and to confirm the apoptosis we studied the caspase-3 activity as effector of apoptosis by flow cytometry. Flow cytometry is a common technique employed for the accurate quantification of apoptosis and distinguishes apoptotic from non-apoptotic cells by means of DNA staining. In addition, an assessment of the ability of these colonic cells to metabolize HT and the studied compounds was made.

MATERIALS AND METHODS

Chemical Reagents. HT (99.51% of purity, synthetic) was provided by Seprox Biotech (Madrid, Spain). CAT,

PA, PP, HPP acid, and diHPP were purchased from Sigma-Aldrich (St. Louis, MO, USA). Homovanillic acid (Fluka Co., Steinheim, Switzerland) and orthophosphoric acid (85%) were purchased from Panreac (Barcelona, Spain). Methanol (HPLC-grade), acetonitrile (HPLC-grade) and ethanol were purchased from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Medford, MA, USA).

Colon Cancer Cell Lines. The Caco-2 cell line was purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (HyClone, Thermo Scientific, Logan, UT, USA) supplemented with 10% of fetal bovine serum (Sigma-Aldrich, Madrid, Spain), 1% L-glutamine, and 1% (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) purchased from HyClone, penicillin, and streptomycin (100 mg/mL) (Gibco, Thermo Scientific). The cells were cultured at 37 °C in a 5% CO2 humidified incubator. The cell employed was from passage 15−25. The medium culture was replaced every 2 days; phosphate-buffered saline (PBS; HyClone) was used to wash the cells, and they were then cultured in flask bottles (Corning Costar, Corning, NY, USA) at an initial density of 0.6×10^6 until reaching a density of 3.5×10^6 cells.

The HT-29 cell line purchased from ATCC was cultured in McCoy's 5A medium (HyClone, Thermo Scientific) supplemented as previously mentioned as preview cells and cultured under the same conditions. The cell employed was from passage 15−25. To preempt cytotoxicity, methanol concentrations never exceeded 0.1% (v/v) in the culture media.

Cell Viability Study. The scheme of the experiments is shown in **Figure 1**. Dose selection of the phenolic compounds employed was performed by the cytotoxic effect of the individual phenolic standards (HT, PA, PP, HPP, diHPP, CAT) and the mixture of the phenol compounds (MIX). The Caco-2 and HT-29 cells were tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, Madrid, Spain)[33] with modifications. Briefly, the cells were seeded in 24-well plates (Corning Costar) at a density of 0.2×10^6 cells/mL. After the exposure time (8 and 48 h, respectively), the cells were washed with PBS, and then the MTT solution was added at 0.5 mg/mL. Two hours later, the supernatant was discarded and ethanol/DMSO (1:1, v/v) was used for cells lysis. Formazan formation was measured at 547 nm in a Multiskan GO microplate spectrophotometer (Thermo Scientific).

Cell Cycle Assay by Flow Cytometry. Cell cycle division, where the DNA content is duplicated for cell replication,

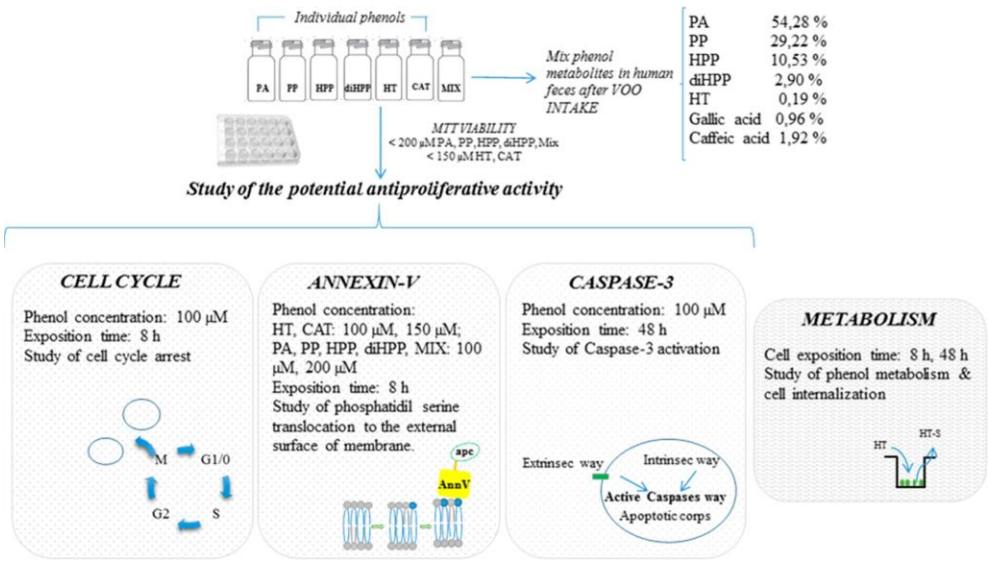


Figure 1. Experimental design of the study. PA, phenylacetic acid; PP, phenylpropionic acid; HPP, hydroxyphenylpropionic acid; diHPP, dihydroxyphenylpropionic acid; HT, hydroxytyrosol

is divided into four stages: G1, S, G2, and M. The distribution of HT-29 and Caco-2 cells in the phases of the cell cycle was quantified by flow cytometry. The HT-29 and Caco-2 cells were seeded in 24-well plates at a density of 0.2×10^6 cells/mL and treated with PA, PP, HPP, diHPP, CAT, and HT (100 µM) for 8 h (**Figure 1**). Then the cells were washed twice with PBS and harvested using trypsin/EDTA 1X (Hyclone, Thermo Scientific), collected with the supernatant, and centrifuged at 1300g for 5 min. The cellular pellet was fixed in 1 mL of cold ethanol (70%) and kept overnight at −20 °C. The pellet was washed twice with PBS and suspended for 30 min at room temperature and in darkness in RNase and propide iodurum (PI) solution (BD Biosciences, Santa Clara, CA, USA), and the cells were then analyzed by flow cytometry (BD Biosciences). The percentage of cells in the different cell cycle phases (G0/G1, S, and G2/M phases) was calculated using Coulter Epicx XL-MCL DNA analysis software (Beckman Coulter, Brea, CA, USA).

Annexin V−APC Assay. A PI/annexin V−APC apoptosis detection kit (Affymetrix eBioscience, San Diego, CA, USA) was used in the HT-29 and Caco-2 cells. This assay determines the translocation of PS (start cell death) that occurs under the effect of the compounds tested. Cells were incubated for 8 h at two different concentrations: HT and CAT at 100 and 150 µM, and their microbial fermentation products (PA, PP, HPP, diHPP, and MIX) at 100 and 200 µM. Differences in the

concentrations employed were due to the toxicity rate. After the incubation period, the cells were harvested and treated with AnnV and PI following the manufacturer's instructions (**Figure 1**). The degree of apoptosis was determined by flow cytometry (BD Biosciences). At least 10,000 events were recorded to assess the percentage of apoptotic cells. The results are expressed as early apoptosis (AnnV positive and PI negative, AnnV+/PI−); later apoptosis (AnnV positive and PI positive, AnnV+/PI+) and necrose/death cells (AnnV negative and PI positive, AnnV−/PI+).

Caspase-3 Assay. To determine the rate of later apoptosis and its activity after longer periods of exposure, an FITC active caspase-3 apoptosis kit (BD Biosciences) was used. The cells were incubated with HT and the microbial fermentation products (PA, PP, HPP, diHPP, CAT, and MIX) at 100 μM for 48 h (**Figure 1**). After this time, the cells were exposed to the caspase-3 antibody according to the instructions provided by the manufacturer. At least 10,000 events were recorded in the flow cytometer (BD Biosciences).

Analysis of HT Metabolites in Cell Culture Medium.

To study the capacity of the cell lines (Caco-2 and HT-29) to metabolize phenol, the individual phenolic compounds studied (100 μM) were incubated with the cells for 8 and 48 h. After the incubation time, the cell culture medium was collected. Aliquots were stored at −80 °C until the chromatographic analysis.

The cell culture medium samples were pretreated by microelution solid-phase extraction (μSPE), and OASIS HLB μElution plates 30 mm (2 mg) (Waters, Milford, MA, USA) were used to clean them up before the chromatographic analysis. These microcartridges were conditioned by adding sequentially 250 μL of methanol and 250 μL of methanol/Milli-Q water (50:50) acidified at pH 2.0 with acetic acid. Then, 350 μL of sample and 350 μL of orthophosphoric acid at 4% were loaded into the microcartridge. The loaded microcartridges were washed with 200 μL of Milli-Q water and 200 μL of methanol at 5%. Finally, the retained phenolic metabolites were eluted with 2 × 50 μL of methanol. Then, this solution was directly injected into the chromatographic system.

The phenolic compounds were analyzed by using Acquity ultraperformance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) from Waters (Milford, MA, USA), following the chromatographic method described by Catalan et al.[21] Briefly, the analytical column was an HSS T3 (100 × 2.1 mm, 1.8 μm) (also from Waters), and the mobile phase was 0.2% acetic acid (as eluent A) and methanol (as eluent B). The flow rate was 0.3 mL/min, and the injection volume was 2.5 μL. The detection system for the analysis of HT and the studied compounds was tandem MS. More detailed information

regarding the SRM conditions is shown in **Table 1S in the Supporting Information**.

Statistical Analysis. All of the experiments were conducted in duplicate from three biological replicates (n = 6). To perform the statistical analysis, the mean ± standard deviation of each independent experiment was used. Differences between treatments were calculated using a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. A value of $p < 0.05$ was considered statistically significant. All of the results were analyzed with the Statgraphics Centurion XVI Statistical Package (Statpoint Technologies Inc., Warranton, VA, USA).

RESULTS

The preliminary results of the group showed that a moderate daily intake of a phenol-enriched olive oil raised the concentrations of phenylacetic and phenylpropionic acids in human feces,[32] although this increase was only statistically significant for free HT. These results stimulate the interest in olive oil phenols, mainly HT and its products of catabolism, opening a promising line of research focused on their preventive action in the colon and bowel diseases.

Cell Viability Study. The cytotoxicityof HT and the studied compounds, as well as their mixture (MIX), was evaluated in the Caco-2 and HT-29 cancer cell lines using the MTT assay. Each compound was studied from 20 to 200 μM at two exposure times (8 and 48 h). According to the MTT assay, the results revealed similar behaviors in both cancer cell lines. HT and CAT showed dose-dependent cytotoxic effects, and the limit of 80% cell viability was observed at a concentration of 150 μM in both cell lines (data not shown). However, all of the studied compounds showed no cytotoxic effects up to 200 μM (80% viability).

Cell Cycle Arrest Capacity. Flow cytometry is a common technique for the accurate quantification of apoptosis and distinguishes apoptotic from non-apoptotic cells by means of DNA staining. Apoptotic cells are stained with fluorescent dye and passed through a beam of light at a single wavelength. Cell cycle division, where the DNA content is duplicated for cell replication, is divided into four stages: G1, S, G2, and M. **Figure 2** shows the antiproliferative activity of the compounds studied (100 μM) after 8 h of exposure of the cell lines. As can be seen HT promoted cell cycle arrest in phase G1 in both cancer cell lines (Caco-2 and HT-29), exhibiting a strong inhibitory effect on DNA duplication. HPP and the mix of HT metabolites (MIX) decreased G1, followed by an accumulation in the S phase, reaching arrest in the G2/M phase. In Caco-2, PA stimulates cell cycle arrest ($p < 0.05$) in the G2/M phase, whereas in HT-29, it did not produce any significant effect on cell cycle control. However, PP and diHPP did not show any antiproliferative effects. Catechol (CAT) in Caco-2

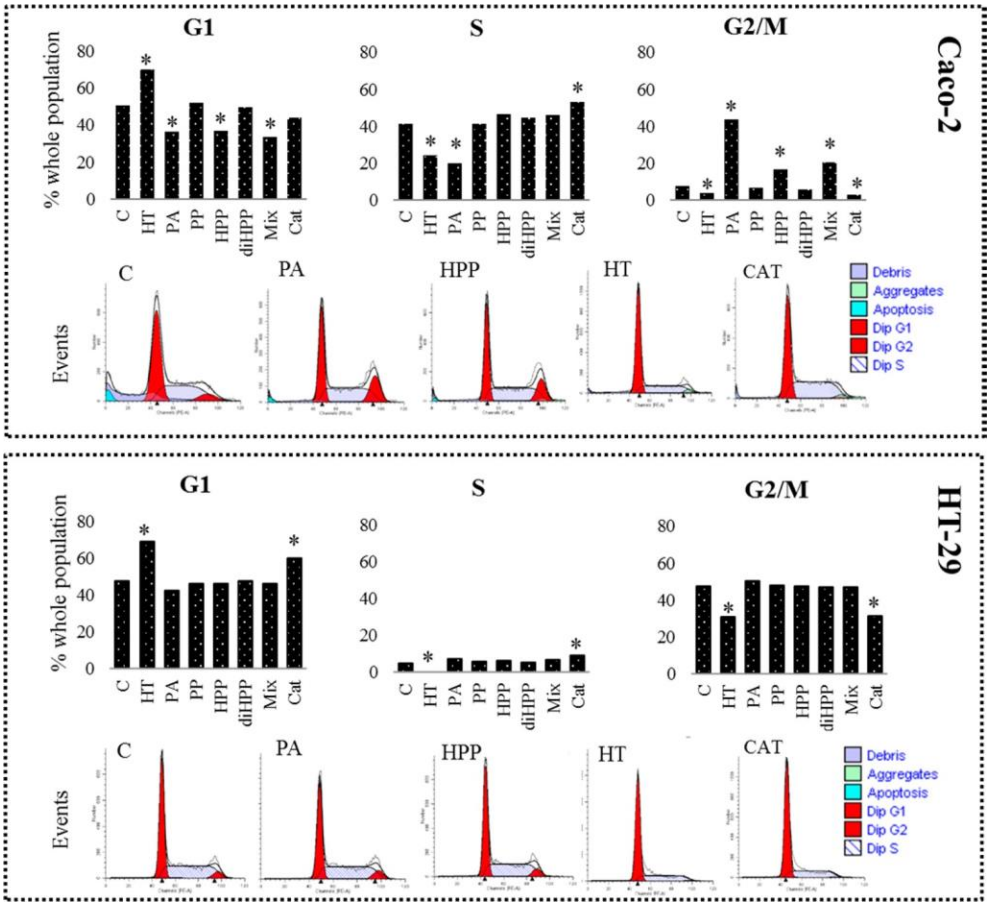


Figure 2. Cell cycle arrest capacity of the phenolic metabolites studied. Caco-2 and HT-29 cell lines were incubated with each compound (100 µM) for 8 h, and the number of cells in the cell cycle phases was determined by flow cytometry. Synthetic phase (S), generation of a single and faithful copy of cell genetic material; mitosis phase (M), partitioning of all cellular components between two identical daughter cells; G1 and G2, "gap" periods, during which cells prepare themselves for the successful completion of the S and M phases, respectively. The assays were conducted in duplicate from three biological replicates (n = 6). * indicates significant difference compared with control ($p < 0.05$). C, control; HT, hydroxytyrosol; PA, phenylacetic acid; PP, 2-hydroxyphenylacetic acid; HPP, hydroxyphenylpropionic acid; diHPP, dihydroxyphenylpropionic acid; MIX (**Figure 1**); CAT, catechol.

induced the cell cycle to stop in the S phase, whereas in HT-29, in phase G1 ($p < 0.05$).

Determination of Cell Apoptosis with the Annexin V−APC Assay.

Continuing the study of the apoptotic activity of the phenolic compounds, the annexin assay was carried out. The participation of the live cells and cells in early apoptosis or necrosis in the population of Caco-2 or HT-29 cell lines was evaluated. Cell apoptosis starts with the modulation of the gene expression and the activation of signaling cascades of caspases,34 which starts with the permeabilization of the outer mitochondrial membrane and release of cytochrome c into the cytosol to activate the caspase cascade.[35−39] Furthermore, liberation of cytochrome c induces the translocation of PS to the outer membrane that binds with annexin V. Non-apoptotic cell death is defined morphologically as a cell death process that is not mediated by caspases.[40] The most widely studied mechanism is necrosis, which is characterized by the loss of integrity of the membrane, releasing the cellular contents into the media in an uncontrolled way, resulting in an inflammatory response in the tissue and thus propagating cell death.[4] Under normal conditions, PS is located on the intracellular site of the membrane[41] and translocates into an external site when the cells undergo apoptosis, caspase-independent apoptosis, and autophagy.[42] The annexin V−APC assay (AnnV) was used in combination with PI, which binds to nucleic acids in later apoptosis. If the membrane disappears, the cell becomes necrotic or dead. In the experiments conducted (test annexin V + PI), the results of the present study showed that after 8 h of phenol treatment, most of the compound studied produced a significant ($p < 0.05$) decrease in the percentage of live cells (**Figure 3**).

It is noteworthy that the degree of induced apoptosis and necrosis depended on the compound and the cell line.

Comparing the induction of apoptosis under HT treatment (at 100 μM) with the other compounds studied, HT induced later apoptosis and increased the necrosis or cell death in Caco-2 and HT-29 (**Figure 3**). The apoptotic activity and mechanism of action of the studied compounds depended on the molecular structure and the cell line used. Whereas PA and HPP induced early apoptosis and death/necrotic cells in both cellular lines (Caco-2 and HT-29) to a significant degree ($p < 0.05$), PP and diHPP induced later apoptosis and cell death/necrosis only at 200 μM exclusively in HT-29 cells. The main difference between the two cell lines was observed with the MIX, which induced early apoptosis in Caco-2, whereas in HT-29 it induced late apoptosis and necrosis. Surprisingly, CAT produced procarcinogenic effects in the Caco-2 cells at 150 μM, decreasing the apoptosis rate (%) compared with the control (without phenol). The opposite effects occurred in HT-29, where CAT

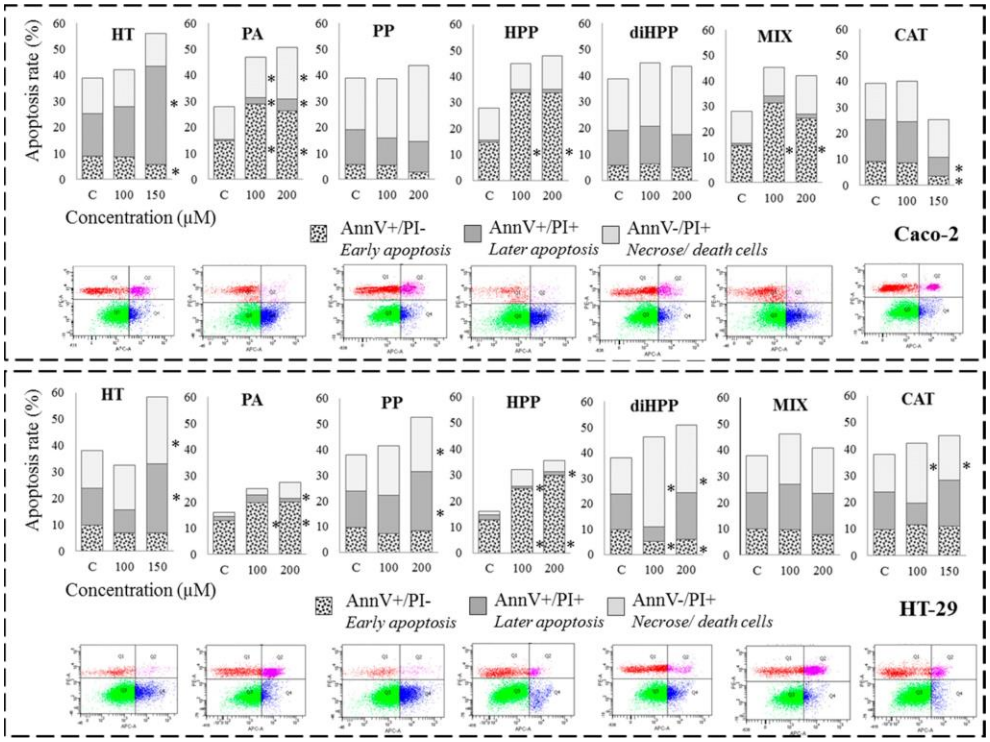


Figure 3. Results of the annexin V−PI test for Caco-2 and HT-29 cell lines treated for 8 h with HT and their metabolites at different doses (100−150 μM). Early apoptosis (AnnV positive and PI negative, AnnV+/PI−); later apoptosis (AnnV positive and PI positive, AnnV+/PI+); and necrose/death cells (AnnV negative and PI positive, AnnV−/PI+). The assays were conducted in duplicate from three biological replicates (n = 6). ∗ indicates a significant difference compared with control ($p < 0.05$). C, control; HT, hydroxytyrosol; PA, phenylacetic acid; PP, hydroxyphenylacetic acid; HPP, hydroxyphenylpropionic acid; diHPP, dihydroxyphenylpropionic acid; MIX, mimetics of the mixture of the fecal content after VOO intake (**Figure 1**); CAT, catechol.

produced a significant increase ($p < 0.05$) in necrosis/death cells. To sum up, HT and the PA and HPP microbial metabolites induced apoptosis and cell death in both cellular lines. CAT showed apoptotic activity only in the HT-29 cell line. The cytotoxic activity of PP and diHPP was based on cell death probably related with a quick apoptosis or direct necrosis.

Caspase-3 Activity. Caspase-3 is an effector key protease that is activated during the latest stages of apoptosis and, like other members of the caspase family, it is synthesized as an inactive pro-enzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. To complete the evaluation of apoptotic cell death, we investigated the potential of

the phenolic compounds studied to increase the caspase-3 activity, measured in the cell media (Caco-2 and HT-29) after 48 h of release. As shown in **Figure 4**, the caspase-3 activity in the untreated controls (C) was very low.

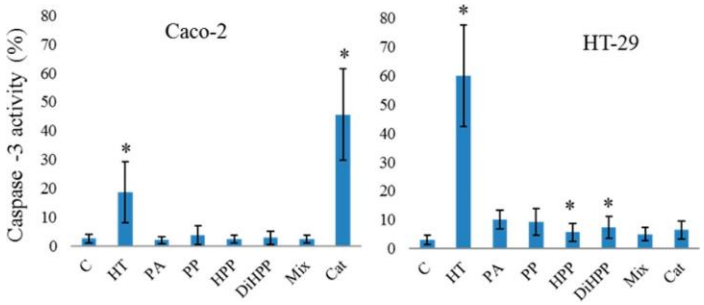


Figure 4. Representative flow cytometry histograms of caspase-3 activity, a biomarker of cell apoptosis for Caco-2 and HT-29 cells treated for 48 h with HT and their microbial metabolites at 100 μM. Values are mean ± SD (n = 6). C, control; PA, phenylacetic acid; PP, hydroxyphenylacetic acid; HPP, hydroxyphenylpropionic acid; diHPP, dihydroxyphenylpro- pionic acid; MIX, mimetics of the mixture of the fecal content after VOO intake (**Figure 1**); HT, hydroxytyrosol; Cat, catechol. ∗ indicates a significant difference compared with control ($p < 0.05$).

A significant increase ($p < 0.05$) in the caspase-3 activity was observed in Caco-2 treated with HT as well as CAT. In contrast, the levels of active caspase-3 in HT-29 increased with the cell treatment with HT and, to a lesser extent, with PA and PP ($p < 0.05$). **Metabolism of Phenolic Compounds in Cell Culture.** The stability of the tested compounds (HT, PA, PP, HPP, diHPP, CAT, and MIX) in the media (without cells) and the ability of colon cancer cells (Caco-2 and HT-29) to metabolize them, after 8 and 48 h of incubation, were studied. All of the compounds showed high stability under incubation conditions over both study times (8 and 48 h) (data not shown). Similarly, the exposure of the individual compounds to both cell lines (Caco-2and HT-29), during 8 and 48 h, revealed that only HT and CAT were extensively metabolized, as seen in the compounds detected in the media (**Table 1**). However, PA, PP, HPP, and diHPP were not detected as phase II metabolites. The HT-29 cell line showed higher metabolic capacity than Caco-2, generating a wide range of HT and CAT metabolites. After 8 h of exposure, HT remained mainly unmetabolized, but after 48 h, it was extensively metabolized, mainly in its sulfate form (HT-S) (**Table 1**). The presence of PA, HPA, and CAT conjugates at low concentration in the cell culture media could be related to the endogenous activity and cells. Despite its lower metabolic activity compared with HT-29, the Caco-2 cell line produced caffeic and ferulic acids after 48 h of HT exposure. As a consequence of the CAT exposure to cell lines, it was mainly metabolized in its glucuronide conjugate (CAT-G). **Figures 1S and 2S** in the

Supporting Information show the proposed metabolic pathways of HT and CAT after exposure to the Caco-2 and HT-29 cell lines.

DISCUSSION

Cells seem to possess multiple death mechanisms, including apoptosis, necrosis, caspase-independent apoptosis, and autophagy among others, with various efficiencies depending on the cell type. However, the contribution of each type of death mechanism remains unknown.[40,42] During the carcinogenic state, cells lose the death homeostasis and angiogenesis and survival increase. The present study demonstrates, for the first time, that some of the main colonic metabolites (especially PA and HPP) derived from the consumption of VOO may contribute to reducing the progression of cancer by inducing cell cycle arrest and stimulating cell death with similar activity as HT, which is a potential antioxidant native compound. Apart from the protective effects against cancer found in our study, PA, PP HPP, and diHPP showed anti-inflammatory activity through the inhibition of prostaglandin E-2 production in CCD-18 colon fibroblast cells stimulated by IL-1β. The compounds showed slightly anti-inflammatory activity, but diHPP showed a strong anti-inflammatory response.[43] HT, a strong antioxidant compound, inhibits cell proliferation and promotes apoptosis in several tumor cell lines by diverse mechanisms.[39,44−49] The present study finds that HT reduces the proliferation of the carcinogenic cells by inducing cell cycle arrest in the G1 stage (**Figure 2**). In line with our results, in human promyelocytic leukemia cells (HL60), Fabiani et al.[50] showed cell cycle detention in the G1 phase. This was through a decrease in the level of cyclin-dependent kinase 6 and an increase in cyclin D3. Furthermore, in MCF-7 breast cancer cells, HT arrested the cell cycle in G1 by decreasing cyclin B1.[28] In contrast, other authors showed the cell cycle arrest in the G2 phase in different cell lines, such as SKBR3 and T-47D breast adenocarcinoma,[49] in HCC human hepatocarcinoma cells,[51] and in TFK-1, KMBC cholangiocarcinoma, and GBS-SD gallbadder cancer cells.[36] In addition to cell cycle detention, our study showed that HT is able to induce apoptosis and cell death shortly after release (**Figure 3**), as well as to activate the caspase signaling in both the Caco-2 and HT-29 cell lines (**Figure 4**). In accordance with our study, Ragione et al.[52] showed in HL-60 (human promyelocytic leukemia cells) and peripheral blood mononuclear cells (PBMCs) that HT induced mitochondrial membrane permeability and cytochrome c release, as well as activation of caspase signaling. Additionally, the results of the present study are in accordance with a previous study conducted by our group.[53] In the earlier work, we studied the proteome of the heart and aorta of healthy adult Wistar rats treated during 21 days with a diet supplemented with 5 mg HT/kg/day. In that study, we observed that HT diet supplementation modulates the proteome and down-regulates the protein

Table 1. Concentration of the Generated Phenolic Metabolites (µM ± standard deviation). * Detected in Cell Line Media After the Exposition (8 and 48 h Respectively) of Hydroxytyrosol (HT) and Catechol (CAT) to Caco-2 and HT-29 Cell Lines. The Number of Replicates Was *n*=3.

Generated metabolites	Control				HT				CAT			
	Caco-2		HT-29		Caco-2		HT-29		Caco-2		HT-29	
	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h	8 h	48 h
HT	nd	nd	nd	nd	122.3±56.1[a]	3.78±0.96[b]	105.2±17.3[a]	12.91±7.35[b]	nd	nd	nd	nd
PA	nd	nd	nd	0.02±0.05	6.98±0.46[a]	14.95±0.83[b]	4.81±0.26[a]	7.49±1.21[b]	nd	nd	nd	nd
PP	nd	nd	nd	nd	nd	nd	nd	3.48±1.85	nd	nd	2.11±0.05	2.13±0.08
HPA	0.08±0.00	0.33±0.00	nd	nd	2.50±0.14[a]	6.86±0.44[b]	nd	nd	0.41±0.15	0.67±0.17	0.77±0.21[a]	1.81±0.26[b]
HVAlc	nd	nd	nd	nd	0.54±0.16[a]	2.75±0.68[b]	2.65±0.1[a]	4.45±0.54[b]	nd	nd	nd	nd
HT-S	nd	nd	nd	nd	5.59±1.16[a]	21.48±6.60[b]	14.69±1.31[a]	104.3±14.0[b]	nd	nd	nd	nd
HVAlc-S	nd	nd	nd	nd	nd	nd	1.00±0.1[a]	41.25±11.4[b]	nd	nd	nd	nd
HT Ac-S	nd	nd	nd	nd	nd	nd	3.16±0.22	3.75±0.29	nd	nd	nd	nd
HT-G**	nd	nd	nd	nd	1.49±0.32[a]	4.58±0.10[b]	7.45±0.23[a]	27.09±1.25[b]	nd	nd	nd	nd
HVAlc-G	nd	nd	nd	nd	nd	nd	0.82±0.06[a]	14.43±0.98[b]	nd	nd	nd	nd
HT Ac-G	nd	nd	nd	nd	nd	nd	1.35±0.06[a]	5.26±0.25[b]	nd	nd	nd	nd
CAT	nd	nd	nd	nd	nd	nd	nd	nd	123.3±16.7[a]	13.70±3.01[b]	104.5±19.6[a]	17.76±2.82[b]
CAT-S	1.70±0.48	2.19±0.27	2.60±0.35	3.43±0.61	1.77±0.41	2.23±0.27	1.86±0.20	1.91±0.31	64.35±12.2[a]	144.3±20.7[b]	65.28±24.1[a]	353.7±33.3[b]
Me-CAT-S	0.61±0.15	0.76±0.22	3.11±0.74	1.79±6.35	0.49±0.26[a]	1.69±0.59[b]	1.82±0.24	2.33±0.45	12.58±1.61[a]	139.3±24.7[b]	12.09±4.03[a]	200.9±13.7[b]
CAT-G	nd	nd	nd	nd	nd	nd	nd	0.11±0.02	92.69±21.3[a]	290.9±58.7[b]	192.5±64.9[a]	449.9±43.9[b]
Me-CAT-G	nd	nd	nd	nd	nd	0.50±0.79	nd	0.79±0.44	16.44±2.06[a]	156.5±14.4[b]	26.00±6.76[a]	110.3±5.85[b]
Caffeic acid-G	nd	nd	nd	nd	nd	12.79±1.97	nd	nd	nd	nd	nd	nd
Ferulic acid-G	nd	nd	nd	nd	nd	3.94±1.29	nd	nd	nd	nd	nd	nd

[a]The phase II metabolites (sulfate and glucuronide conjugates) were tentatively quantified with the respective standard (**Supporting Information Table 1S**). [b]The number of replicates was $n = 3$. Statistical differences ($p < 0.05$; a, b) in times of exposure on each cellular line calculated by ANOVA followed by LSD (least standard deviation) post hoc test. HT, hydroxytyrosol; PA, phenylacetic acid; PP, phenylpropionic acid; HPA, hydroxyphenylacetic acid; HVAlc, homovanillic alcohol; S, sulfate; G, glucuronide; CAT, catechol; Me, methyl; Acc acetate. [c]Sum of isomers.

expression of some biomarkers of cancers, such as annexin 2 and cytochrome C.

With regard to the HT catabolic metabolites, in a previous work[21] we studied the bioactivity of HT and its biological metabolites in human aortic endothelial cells. HT metabolites showed better effects than the native HT against endothelial dysfunction, inhibiting the MCP-1 and increasing the caspase-3 activity. The results of the present study showed that PA induced cell cycle arrest (**Figure 2**), which is correlated with the increase in injured cells in AnnV staining (**Figure 3**). The internalization of PA inside the cells (data not show) could promote the apoptotic cascade mediated by caspases as seen in the increased activity of caspase-3 in HT-29 cells (**Figure 4**). Our results contrast with the findings of Cohen et al.54 These authors observed that PA reduced the spontaneous apoptosis of the polymorphonuclear leucocytes and contributed to activating other inflammation markers. By contrast, in our study, PP did not show cell cycle arrest or cell death activity in the Caco-2 cell line (**Figures 2, 3, and 4**), but did show a moderate activity in HT-29, increasing apoptosis and cell death, although significantly only at 200 µmol in the annexin V experiments (annexin V–APC assay **Figure 3)** and caspase-3 activity (**Figure 4**). These results indicate that the apoptotic effect of PP could be efficacious at high concentrations and could be produced slowly over time. HPP promoted cell cycle arrest in Caco-2 at the G2 stage (**Figure 2**) and increased early apoptosis in both cell lines (**Figure 3**). However, HPP did not increase caspase-3 activity after 48 h (**Figure 4**), either because its progre ssion was quick or because HPP could induce cell death through other mechanisms such as caspase-independent apoptosis or autophagy.[42]By contrast, diHPP could exert its cytotoxic effects in the first hours after release. The early apoptosis (**Figure 3**) and low caspase-3 activity (**Figure 4**) could indicate the direct induction of cell necrosis. Yang et al.[55] identified diHPP as one of the most important products of the biotransformation of 4,5-O- dicaffeoyl-quinic acid methyl ester by human intestinal microbiota, showing an important antioxidant activity and the inhibition of NO production.

González de Llano et al.[14] studied the capacity of cranberry phenolic compounds and their microbial-derived metabolites, such as benzoic, phenylacetic, and phenylpropionic acids, to inhibit the adherence of uropathogenic *Escherichia coli* (UPEC) to T24 epithelial bladder cells. The results of this study proved that catechol and benzoic and phenylacetic acids showed anti-adhesive activity against UPEC in a concentration-dependent way, suggesting that the presence of microbial metabolites in the urine could reduce bacterial colonization and the progression of urinary tract infections. With regard to microbial-derived flavonoid metabolites, 3,4-dihydroxyphenylacetic and 3-

hydroxyphenylpropionic acids enhance glucose-stimulated insulin secretion in a beta cell line, INS-1E, and in rat pancreatic islets.[56] These findings strongly suggest that these flavonoid metabolites may have antidiabetic potential by promoting the survival and function of pancreatic beta cells.[56] With regard to CAT, the results of the present study showthat it is effective after long cell exposure times. CAT produces cell cycle arrest in the S phase, similar to that observed by Mateos et al.[57] with HT in a dose-dependent way. In the Caco- 2 cell line, CAT showed no positive effect in the first hours (8 h) in the annexin method (**Figure 3**). This fact could be related with the simple molecular structure of CAT, which facilitates its cellular uptake reaching high intracellular concentrations and thus producing pro-oxidant effects in the cells. This effect is normally associated with an increase of ROS content and could enhance the carcinogenesis in the first phases.[58] However, at 48 h, the activity of caspase-3 increased strongly in Caco-2 after CAT exposure (**Figure 4**). The biotransformation of CAT into phase II metabolites (sulfate and glucuronide conjugates) by the cells (**Table 1**) could reduce its pro-oxidant activity and consequently improve its chemopreventive effects.[58] In another study performed by Sirota et al.,[59] CAT provided scavenging ability and also contributed to increasing the phase II antioxidant enzymes by the activation of Nrf2/Keap1 via the production of hydrogen peroxide intracellularly or extracellularly. With regard to MIX, the results obtained showed no synergic effect among the compounds in any of the experiments.

The high content of CAT and HT phase II metabolites in the cell media in both cell lines (**Table 1**) could be involved in the high apoptosis mediated by caspases (**Figure 4**). Other authors observed that HT metabolites have the same or even higher bioactivity than the native compound. For instance, Atzeri et al.[60] evaluated the preventive effects of HT and HT-S against the oxidative stress induced by oxidized cholesterol in Caco-2 cells. HT and its metabolites decrease the malondialdehyde, ROS, and glutathione peroxidase cellular levels. Furthermore, a dose of 10 μM of HT, HT-AC, and HT-glucuronide was enough to protect red blood cells from hemolysis induced by H2O2.[61] Furthermore, these HT metabolites reduced the secretion of several adhesion molecules in human aortic endothelial cells stimulated by TNF-α.[21]

In conclusion, our results confirm the antiproliferative and pro-apoptotic activities of the native HT and the main microbial metabolites detected in feces after VOO diet supplementation in colonic cancer cells. Whereas PA and HPP inhibited proliferation and induced apoptosis in both cancer cell lines, Caco-2 and HT-29, PP and diHPP showed these activities exclusively in HT-29 cells. The MIX of metabolites has no synergic effects among the individual HT or HT microbial metabolites studied. In synthesis, the results of the present study represent a good starting point for understanding the potential apoptotic and antiproliferative effects of VOO phenolic compounds (HT)

and their colonic metabolites.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at **DOI: 10.1021/acs.jafc.6b04933**. SRM conditions for the analysis of the phenolic compounds by UPLC-MS/MS (**Table 1S**) and the proposal of the metabolic pathways produced by Caco-2 and HT-29 cell lines of HT (**Figure 1S**) and Catechol (**Figure 2S**) (PDF)

Corresponding Author

*(M.-C.L.H.) Phone: +34 973 702825. Fax: +34 973 702596.
E-mail: mclopez@tecal.udl.cat.

Funding

This study was supported by the Spanish Ministry of Education and Science (AGL2012-40144-C03-03 project) and the University of Lleida through the M.-C. López de las Hazas grant.

Notes

The authors declare no competing financial interest.

Abreviations used

AnnV, annexin V; CAT, catechol; diHPP, dihydroxyphenylpropionic acid; GIT, gastrointestinal tract; HPP, hydroxy-phenylpropionic acid; HT, hydroxytyrosol; HT-S, hydroxytyrosol sulfate; MIX, mixture of metabolites; PA, phenylacetic acid; PI, propide iodurum; PP, phenylpropionic acid; PS, phosphati-dylserine; ROS, radical oxygen species; VOO, virgin olive oil

REFERENCES

(1) Jin, Z.; Yan, W.; Jin, H.; Ge, C.; Xu, Y. Differential effect of psoralidin in enhancing apoptosis of colon cancer cells via nuclear factor-κB and B-cell lymphoma-2/B-cell lymphoma-2-associated X protein signaling pathways. *Oncol. Lett.* 2016, *11*, 267−272.

(2) Potter, J. D. Colorectal cancer: molecules and populations. *J. Natl. Cancer Inst.* 1999, *91*, 916−932.

(3) Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 2007, *35*, 495−516.

(4) Jin, Z.; El-Deiry, W. S. Overview of cell death signaling pathways. *Cancer Biol. Ther*. 2005, *4*, 147−171.

(5) Verheij, M. Clinical biomarkers and imaging for radiotherapy- induced cell death. *Cancer Metastasis Rev*. 2008, *27*, 471−480.

(6) Hollister, E. B.; Gao, C.; Versalovic, J. Compositional and functional features of the gastrointestinal microbiome and their effects on human health. *Gastroenterology* 2014, *146*, 1449−1458.

(7) Gagnière, J.; Raisch, J.; Veziant, J.; Barnich, N.; Bonnet, R.; Buc, E.; Bringer, M.-A.; Pezet, D.; Bonnet, M. Gut microbiota imbalance and colorectal cancer. *World J. Gastroenterol.* 2016, *22*, 501−518.

(8) Poutahidis, T.; Kleinewietfeld, M.; Smillie, C.; Levkovich, T.; Perrotta, A.; Bhela, S.; Varian, B. J.; Ibrahim, Y. M.; Lakritz, J. R.; Kearney, S. M.; et al. Microbial reprogramming inhibits Western diet-associated obesity. *PLoS One* 2013, *8*, e68596.Biedermann, L.; Rogler, G. The intestinal microbiota: its role in health and disease. *Eur. J. Pediatr*. 2015, *174*, 151−167.

(9) Belcheva, A.; Irrazabal, T.; Martin, A. Gut microbial metabolism and colon cancer:

can manipulations of the microbiota be useful in the management of gastrointestinal health? *BioEssays* 2015, *37*, 403−412.

(10) El Aidy, S.; Van den Abbeele, P.; Van de Wiele, T.; Louis, P.; Kleerebezem, M. Intestinal colonization: how key microbial players become established in this dynamic process: microbial metabolic activities and the interplay between the host and microbes. *BioEssays* 2013, *35*, 913−923.

(11) O'Keefe, S. J. D. Diet, microorganisms and their metabolites, and colon cancer. *Nat. Rev. Gastroenterol. Hepatol.* 2016, 13, 691−706.

(12) Feliciano, R. P.; Boeres, A.; Massacessi, L.; Istas, G.; Ventura, M. R.; Nunes dos Santos, C.; Heiss, C.; Rodriguez-Mateos, A. Identification and quantify-cation of novel cranberry-derived plasma and urinary (poly)phenols. *Arch. Biochem. Biophys.* 2016, *599*, 31−41.

(13) de Llano, D. G.; Esteban-Fernańdez, A.; Sańchez-Patań, F.; Martín Alvarez, P. J.; Moreno-Arribas, M. V.; Bartolomé, B. Anti-adhesive activity of cranberry phenolic compounds and their microbial-derived metabolites against uropathogenic *Escherichia coli* in bladder epithelial cell cultures. *Int. J. Mol. Sci.* 2015, *16*, 12119−12130.

(14) Dueñas, M.; Muñoz-Gonzaĺez, I.; Cueva, C.; Jimeńez-Giroń. Sańchez-Patań, F.; Santos-Buelga, C.; Moreno-Arribas, M. V.; Bartolomé, B. A survey of modulation of gut microbiota by dietary polyphenols. *BioMed Res. Int.* 2015, *2015*, 850902.

(15) Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J. P. E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signaling* 2013, 18, 1818−1892.

(16) Khanal, R.; Howard, L. R.; Prior, R. L. Urinary excretion of phenolic acids in rats fed cranberry, blueberry, or black raspberry powder. *J. Agric. Food Chem.* 2014, *62*, 3987−3996.

(17) D'Angelo, S.; Manna, C.; Migliardi, V.; Mazzoni, O.; Morrica, P.; Capasso, G.; Pontoni, G.; Galletti, P.; Zappia, V. Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metab. Dispos.* 2001, 29, 1492−1498.

(18) Selma, M. V.; Espín, J. C.; Tomaś-Barberań, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* 2009, *57*, 6485−6501.

(19) Valdeś, L.; Cuervo, A.; Salazar, N.; Ruas-Madiedo, P.; Gueimonde, M.; Gonzaĺez, S. The relationship between phenolic compounds from diet and microbiota: impact on human health. *Food Funct.* 2015, *6*, 2424−2439.

(20) Catalań, Ú.; Lopéz de Las Hazas, M.-C.; Rubió, L.; Fernańdez-Castillejo, S.; Pedret, A.; de la Torre, R.; Motilva, M.-J.; Solà, R. Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. *Mol. Nutr. Food Res.* 2015, *59*, 2523−2536.

(21) Cueva, C.; Moreno-Arribas, M. V.; Martín-Alvarez, P. J.; Bills, G.; Vicente, M. F.; Basilio, A.; Rivas, C. L.; Requena, T.; Rodríguez, J. M.; Bartolomé, B. Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Res. Microbiol.* 2010, *161*, 372−382.

(22) Hashim, Y. Z. H. Y.; Eng, M.; Gill, C. I. R.; McGlynn, H.; Rowland, I. R. Components of olive oil and chemoprevention of colorectal cancer. *Nutr. Rev.* 2005, *63*, 374−386.

(23) Rietjens, S. J.; Bast, A.; Haenen, G. R. M. M. New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. *J. Agric. Food Chem.* 2007, *55*, 7609−7614.

(24) Goya, L.; Mateos, R.; Bravo, L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Eur. J. Nutr.* 2007, 46, 70−78.

(25) Rodríguez-Ramiro, I.; Martín, M. Á.; Ramos, S.; Bravo, L.; Goya, L. Olive oil hydroxytyrosol reduces toxicity evoked by acrylamide in human Caco-2 cells by preventing oxidative stress. *Toxicology* 2011, *288*, 43−48.

(26) Anter, J.; Tasset, I.; Demyda-Peyrás, S.; Ranchal, I.; Moreno-Millán M.; Romero-Jimenez, M.; Muntané J.; Luque de Castro, M. D.; Muñoz-Serrano, A.; Alonso-Moraga, Á. Evaluation of potential antigenotoxic, cytotoxic and proapoptotic effects of the olive oil by- product "alperujo", hydroxytyrosol, tyrosol and verbascoside. *Mutat. Res., Genet. Toxicol. Environ. Mutagen.* 2014, *772*, 25−33.

(27) Bouallagui, Z.; Han, J.; Isoda, H.; Sayadi, S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food Chem. Toxicol.* 2011, *49*, 179−184.

(28) López de las Hazas, M.-C.; Piñol, C.; Macià, A.; Romero, M.-P.; Pedret, A.; Solà, R.; Rubió, L.; Motilva, M.-J. Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids. *J. Funct. Foods* 2016, *22*, 52−63.

(29) Rubió, L.; Macià, A.; Valls, R. M.; Pedret, A.; Romero, M.-P.; Solà R.; Motilva, M.-J. A new hydroxytyrosol metabolite identified in human plasma: hydroxytyrosol acetate sulphate. *Food Chem.* 2012, *134*, 1132−1136.

(30) López de las Hazas, M. C.; Motilva, M. J.; Piñol, C.; Macià, A. Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood. *Talanta* 2016, *159*, 189−193.

(31) Mosele, J. I.; Martín-Pelaéz, S.; Macià A.; Farràs, M.; Valls, R.-M.; Catalań, U.; Motilva, M.-J. Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches. *Mol. Nutr. Food Res.* 2014, *58*, 1809−1819.

(32) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 1983, *65*, 55−63.

(33) Seshacharyulu, P.; Pandey, P.; Datta, K.; Batra, S. K. Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Lett.* 2013, *335*, 9−18.

(34) Chimento, A.; Casaburi, I.; Rosano, C.; Avena, P.; De Luca, A.; Campana, C.; Martire, E.; Santolla, M. F.; Maggiolini, M.; Pezzi, V.; et al. Oleuropein and hydroxytyrosol activate GPER/ GPR30-dependent pathways leading to apoptosis of ER-negative SKBR3 breast cancer cells. *Mol. Nutr. Food Res.* 2014, *58*, 478−489.

(35) Li, S.; Han, Z.; Ma, Y.; Song, R.; Pei, T.; Zheng, T.; Wang, J.; Xu, D.; Fang, X.; Jiang, H.; et al. Hydroxytyrosol inhibits cholangiocarcinoma tumor growth: an in vivo and in vitro study. *Oncol. Rep.* 2014, *31*, 145−152.

(36) Guichard, C.; Pedruzzi, E.; Fay, M.; Marie, J.-C.; Braut-Boucher, F.; Daniel, F.; Grodet, A.; Gougerot-Pocidalo, M.-A.; Chastre, E.; Kotelevets, L.; et al. Dihydroxyphenylethanol induces apoptosis by activating serine/threonine protein phosphatase PP2A and promotes

the endoplasmic reticulum stress response in human colon carcinoma cells. *Carcinogenesis* 2006, *27*, 1812−1827.

(37) Han, J.; Talorete, T. P. N.; Yamada, P.; Isoda, H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. *Cytotechnology* 2009, *59*, 45−53.

(38) Ragione, F. Della; Cucciolla, V.; Borriello, A.; Pietra, V. Della; Pontoni, G.; Racioppi, L.; Manna, C.; Galletti, P.; Zappia, V. Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome c-dependent apoptosis. *Biochem. Biophys. Res. Commun.* 2000, *278*, 733−739.

(39) Tsujimoto, Y. Multiple ways to die: non-apoptotic forms of cell death. *Acta Oncol.* 2012, *51*, 293−300.

(40) Ogawa, K.; Aoki, M. Radiolabeled apoptosis imaging agents for early detection of response to therapy. *Sci. World J.* 2014, *2014*, 732603.

(41) Melino, G.; Knight, R. A.; Nicotera, P. How many ways to die? How many different models of cell death? *Cell Death Differ.* 2005, *12* (Suppl. 2), 1457−1462.

(42) Larrosa, M.; Luceri, C.; Vivoli, E.; Pagliuca, C.; Lodovici, M.; Moneti, G.; Dolara, P. Polyphenol metabolites from colonic microbiota exert anti-inflammatory activity on different inflammation models. *Mol. Nutr. Food Res.* 2009, *53*, 1044−1054.

(43) Sun, L.; Luo, C.; Liu, J. Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation. *Food Funct.* 2014, *5*, 1909−1914.

(44) Zou, X.; Feng, Z.; Li, Y.; Wang, Y.; Wertz, K.; Weber, P.; Fu, Y.; Liu, J. Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: activation of Nrf2 and JNK-p62/SQSTM1 pathways. *J. Nutr. Biochem.* 2012, *23*, 994−1006.

(45) Fabiani, R.; Sepporta, M. V.; Rosignoli, P.; De Bartolomeo, A.; Crescimanno, M.; Morozzi, G. Anti-proliferative and pro-apoptotic activities of hydroxytyrosol on different tumour cells: the role of extracellular production of hydrogen peroxide. *Eur. J. Nutr.* 2012, *51*, 455−464.

(46) Granados-Principal, S.; Quiles, J. L.; Ramirez-Tortosa, C.; Camacho-Corencia, P.; Sanchez-Rovira, P.; Vera-Ramirez, L.; Ramirez- Tortosa, M. C. Hydroxytyrosol inhibits growth and cell proliferation and promotes high expression of sfrp4 in rat mammary tumours. *Mol. Nutr. Food Res.* 2011, *55* (Suppl.1), S117−S126.

(47) Luo, C.; Li, Y.; Wang, H.; Cui, Y.; Feng, Z.; Li, H.; Li, Y.; Wang, Y.; Wurtz, K.; Weber, P.; et al. Hydroxytyrosol promotes superoxide production and defects in autophagy leading to anti-proliferation and apoptosis on human prostate cancer cells. *Curr. Cancer Drug Targets* 2013, *13*, 625−639.

(48) Elamin, M. H.; Hassan, Z. K.; Omer, S. A.; Daghestani, M. H.; Al-Olayan, E. M.; Virk, P.; Elobeid, M. A.; Mohammed, O. B. Apoptotic and antiproliferative activity of olive oil hydroxytyrosol on breast cancer cells. *J. Med. Plant Res.* 2013, *7*, 2420−2428.

(49) Fabiani, R.; Rosignoli, P.; De Bartolomeo, A.; Fuccelli, R.; Morozzi, G. Inhibition of cell cycle progression by hydroxytyrosol is associated with upregulation of cyclin-dependent protein kinase inhibitors p21(WAF1/Cip1) and p27(Kip1) and with induction of differentiation in HL60 cells. *J. Nutr.* 2008, *138*, 42−48.

(50) Zhao, B.; Ma, Y.; Xu, Z.; Wang, J.; Wang, F.; Wang, D.; Pan, S.; Wu, Y.; Pan, H.; Xu, D.; et al. Hydroxytyrosol, a natural molecule from olive oil, suppresses the growth of human hepatocellular carcinoma cells via

inactivating AKT and nuclear factor-kappa B pathways. *Cancer Lett.* 2014, *347*, 79−87.

(51) Della Ragione, F.; Cucciolla, V.; Criniti, V.; Indaco, S.; Borriello, A.; Zappia, V. Antioxidants induce different phenotypes by a distinct modulation of signal transduction. *FEBS Lett.* 2002, *532*, 289−294.

(52) Catalań, Ú.; Rubió, L.; López de las Hazas, M.-C.; Herrero, P.; Nadal, P.; Canela, N.; Pedret, A.; Motilva, M.-J.; Solà, R. Hydroxytyrosol and its complex forms (secoiridoids) modulate aorta and heart proteome in healthy rats: potential cardio-protective effects. *Mol. Nutr. Food Res.* 2016, *60*, 2114−2129.

(53) Cohen, G.; Raupachova, J.; Hörl, W. H. The uraemic toxin phenylacetic acid contributes to inflammation by priming poly-morphonuclear leucocytes. *Nephrol., Dial., Transplant.* 2013, 28, 421− 429.

(54) Yang, X.-W.; Wang, N.; Li, W.; Xu, W.; Wu, S. Biotransformation of 4,5-*O*-dicaffeoylquinic acid methyl ester by human intestinal flora and evaluation on their inhibition of NO production and antioxidant activity of the products. *Food Chem. Toxicol.* 2013, 55, 297−303.

(55) Fernańdez-Millań, E.; Ramos, S.; Alvarez, C.; Bravo, L.; Goya, L.; Martín, M. Á. Microbial phenolic metabolites improve glucose- stimulated insulin secretion and protect pancreatic beta cells against *tert*-butyl hydroperoxide-induced toxicity via ERKs and PKC pathways. *Food Chem. Toxicol.* 2014, *66*, 245−253.

(56) Mateos, R.; Pereira-Caro, G.; Bacon, J. R.; Bongaerts, R.; Sarriá, B.; Bravo, L.; Kroon, P. A. Anticancer activity of olive oil hydroxytyrosyl acetate in human adenocarcinoma Caco-2 cells. *J. Agric. Food Chem.* 2013, *61*, 3264−3269.

(57) Leoń-Gonzaĺez, A. J.; Auger, C.; Schini-Kerth, V. B. Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. *Biochem. Pharmacol.* 2015, *98*, 371−380.

(58) Sirota, R.; Gibson, D.; Kohen, R. The role of the catecholic and the electrophilic moieties of caffeic acid in Nrf2/Keap1 pathway activation in ovarian carcinoma cell lines. *Redox Biol.* 2015, *4*, 48−59.

(59) Atzeri, A.; Lucas, R.; Incani, A.; Peñalver, P.; Zafra-Gomez, A.; Melis, M. P.; Pizzala, R.; Morales, J. C.; Deiana, M.; Martin-Pelaez, S.; et al. Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells. *Food Funct.* 2016, *7*, 337−346.

(60) Paiva-Martins, F.; Silva, A.; Almeida, V.; Carvalheira, M.; Serra, C.; Rodrígues-Borges, J. E.; Fernandes, J.; Belo, L.; Santos-Silva, A. Protective activity of hydroxytyrosol metabolites on erythrocyte oxidative-induced hemolysis. *J. Agric. Food Chem.* 2013, *61*, 6636− 6642.

SUPPLEMENTAL INFORMATION

Table 1S. SRM Conditions (used for quantification) for the Analysis of the Phenolic Compounds by UPLC-MS/MS, as Well as in Which Standard Compound Has Been (Tentatively) Quantified Each Metabolite.

Phenolic metabolite	SRM quantification			Standard in which has been (tentatively) quantified
	Transition	Voltage cone (V)	Collision energy (eV)	
p-hydroxybenzoic acid	137 > 93	35	15	*p*-hydroxybenzoic acid
Phenylacetic acid	135 > 91	20	5	Phenylacetic acid
Hydroxyphenylacetic acid	151 > 107	20	10	Hydroxyphenylacetic acid
Phenylpropionic acid	149 > 105	20	5	Phenylpropionic acid
3-(4-Hydroxyphenyl)propionic acid	165 > 121	20	10	3-(4-Hydroxyphenyl)propionic acid
3-(2,4-Dihydroxyphenyl)propioni acid	181 > 137	20	10	Phenylpropionic acid
Hydroxytyrosol	153 > 123	35	10	HT
Homovanillic alcohol	167 > 152	35	15	HT
Hydroxytyrosol sulfate	233 > 153	40	15	HT
Hydroxytyrosol glucuronide	329 > 153	40	20	HT
Homovanillic alcohol sulfate	247 > 167	40	15	HT
Homovanillic alcohol glucuronid	343 > 167	40	20	HT
Hydroxytyrosol acetate sulfate	275 > 195	35	15	HT
Hydroxytyrosol acetate glucuronide	371 > 195	40	20	HT
Catechol	109.9 > 90.9	40	15	Catechol
Catechol sulfate	189 > 109	20	15	Catechol
Catechol glucuronide	285 > 109	40	15	Catechol
Methyl catechol sulfate	203 > 123	20	15	Catechol
Methyl catechol glucuronide	299 > 123	20	15	Catechol
Caffeic acid glucuronide	355 > 179	40	15	Caffeic acid
Ferulic acid glucuronide	369 > 193	35	15	Ferulic acid

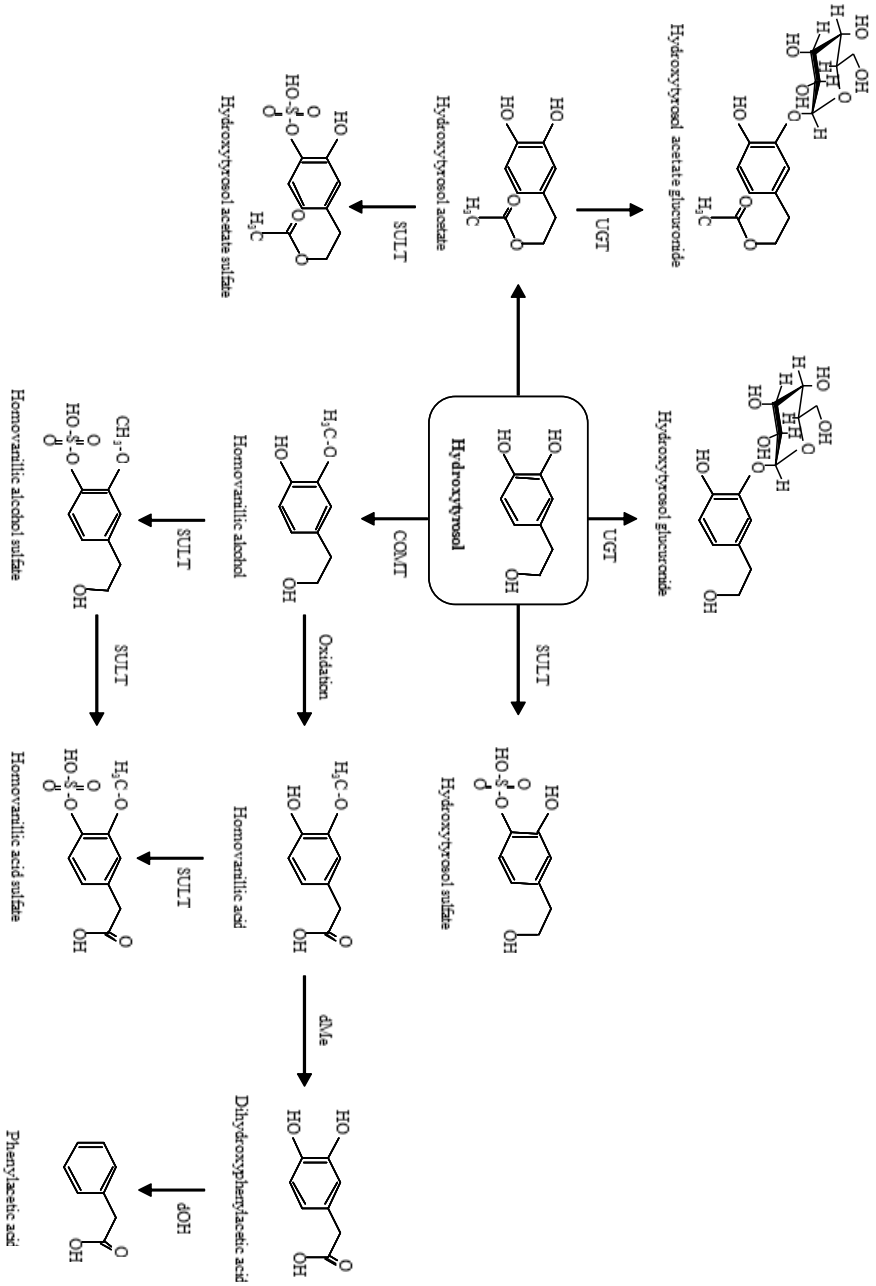


Figure 1S. Proposal of the Metabolic Pathways of Hydroxytyrosol Produced by Caco-2 or HT-29 Cell Lines. dOH: dehydroxylation; dMe: demethylation; SULT: Sulfotransferase enzyme; UGT: Glucuronosyl-transferase enzyme; COMT: Catechol-O-methyltransferase.

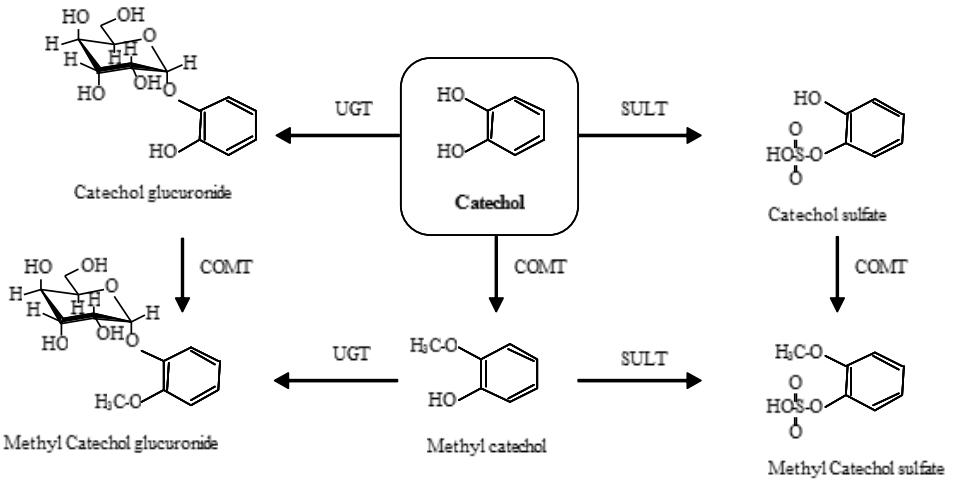


Figure 2S. Proposal of the Metabolic Pathway of Catechol Produced by Caco-2 or HT-29 Cell Lines. SULT: Sulfotransferase enzyme; UGT: Glucuronosyl-transferase enzyme; COMT: Catechol-O-methyltransferase.

Publication 7

Exploring the colonic metabolism of grape and strawberry anthocyanins fractions and their in vitro apoptotic effects in ht-29 colon cancer cells.

JAFC. DOI: 10.1021/acs.jafc.6b04096

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
pubs.acs.org/JAFC
Article

EXPLORING THE COLONIC METABOLISM OF GRAPE AND STRAWBERRY ANTHOCYANINS FRACTIONS AND THEIR *IN-VITRO* APOPTOTIC EFFECTS IN HT-29 COLON CANCER CELLS.

María-Carmen López de las Hazas, Juana I. Mosele, Alba Macià, Iziar A. Ludwig*, María-José Motilva

Food Technology Department, Agrotecnio Research Center, University of Lleida, Av/Alcalde Rovira Roure 191, 25198, Lleida, Spain.

ABSTRACT: Beneficial properties attributed to the intake of fruit and red wine have been associated with the presence of significant amounts of anthocyanins. However, their low absorption and consequent accumulation in the gut have generated the suspicion that colonic metabolites of anthocyanins are probably involved in their protective effects. Grape pomace and strawberries extracts, rich in malvidin- and pelargonidin-glucoside, respectively, were fermented *in-vitro* using human feces as microbial inoculum. After 8 h of anaerobic incubation, the anthocyanins were almost completely degraded while their microbial metabolites concentration was highest at 24 h. Syringic acid and tyrosol were the main metabolites of grape and strawberry fractions, respectively. Based on the metabolites detected, metabolic pathways of malvidin and pelargonidin glucosides were proposed. Anthocyanin-rich grape and strawberry fractions and their generated metabolites such as hydroxyphenylacetic acid showed apopototic effects in HT-29 colon cancer cells. This preliminary study may suggest their possible contribution as anti-carcinogenic agents.
KEYWORDS: anthocyanins; apoptosis; colon metabolism; malvidin-glucoside; pelargonidin-glucoside.

INTRODUCTION

Anthocyanins are a subgroup of flavonoids having the distinctive feature of conferring red, purple and blue colors to several fruits and vegetables, contributing to their attractiveness, one of the attributes most appreciated by consumers. Beyond this commercial perspective, anthocyanins have also been proposed as therapeutic agents for the prevention of cardiovascular diseases, cancer and certain metabolic disorders, such as type-2 diabetes and obesity [1–3]. Red fruit and products based on them (red wine, juices and extracts) are rich sources of several classes of anthocyanins, with one of them often being predominant [1–5]. For instance, malvidin and pelargonidin-glucosides are the

dominant anthocyanins in red grapes and strawberries, respectively (www.phenol-explorer.eu, access January 2016).

Diet can contribute to the maintenance of the harmonic symbiotic relationship between microbiota and host through the intake of phenolic compounds [6]. An important percentage of the anthocyanins ingested is not absorbed in the upper gastrointestinal tract and remains in the gut lumen. This apparently limited bioavailability of anthocyanins has been reported in different studies with ileostomy subjects [7,8] and animals [2]. The nonabsorbed anthocyanins reach the large intestine, where a numerous and varied class of local microorganisms, transform these natural food colorants into related microbial metabolites [2,4,7–9]. Despite the fact that the active structure in which anthocyanins exert their health benefits has still to be clarified, it is thought that during the digestive transit anthocyanins and/or their microbial derivatives could have considerable physiological implications due to their close proximity to the colon epithelium.

Digestive tract cancers, especially colorectal cancer, are among the most frequent in the Western population [10]. Although genetic predisposition (e.g. family history) is an important risk factor, others, such as diet and physical exercise, seem to have a relevant role in the appearance of colon malignancy [10,11]. Promising results have been obtained from in vitro and animal studies in terms of the use of phenolic-rich products in the prevention and treatment of colon cancer [4,12]. However, more evidence from human trials is needed to reach a more robust conclusion because there is no clear association between the intake of phenolic compounds and a reduction in the risk of colon cancer [12,13]. Programmed cell death (apoptosis) is an mechanism to maintain an equilibrium between growth and death and also to avoid the progression of damaged and malignant cells [14]. Cancer cells have deregulated several genes to avoid the apoptosis, such as p53, and these cells have high resistance to death compared with normal cells [15]. This not only ensures their viability but also increases the possibility of their spread (proliferation) and, therefore, the invasion of healthy tissues. For this reason, the different elements involved in triggering apoptosis are interesting targets for chemoprevention and/or chemotherapy. Apoptosis is mediated by the activation of caspases, cysteine-proteases that induce the cell death by the activation of cascade signals. The phenomenon starts with the permeation of mitochondrial membrane, which can be observed by the Annexin-V stain, and continues by the liberation of the caspases, such as caspase-3, an effector activated in the latest stages of apoptosis [14]. Several authors have studied the effect of anthocyanins or anthocyanin-rich products in the prevention of intestinal lesions. [3,5,16] Other studies have focused on the antiproliferative activity of individual microbial metabolites generated from the bacterial catabolism of anthocyanins. [1,3,16,17] However, these in vitro approaches do not reflect what really occurs in the intestinal tract, because anthocyanins

undergo progressive microbial transformations into different types of compounds. This phenolic cocktail, which is represented by a varied concentration of parent compounds (in this case anthocyanins) and microbial metabolites, could exert beneficial effects in a different way depending on the nature and amount of these compounds [4]. Successful prevention of colon diseases using phytochemicals may depend on the effect of the predominant substance or the mixture of metabolites generated during the digestion process.

The aims of the present study were first to define the gut metabolic fate of malvidin- and pelargonidin-glucosides, two of the most common anthocyanins, through the *in-vitro* fermentation of anthocyanin-rich extracts obtained from grape pomace and strawberries, respectively, and second, to study the potential apoptotic effects of malvidin- and pelargonidin-glucosides and their microbial metabolites on HT-29 colon cancer cells.

1. MATERIAL AND METHODS

1.1. Chemicals and reagents.

Different pure standards to identify and quantify the anthocyanins as well as their microbial metabolites were used. Cyanidin-, pelargonidin-, malvidin- and delphinidin-3-*O*-glucosides and tyrosol were purchased from Extrasynthese (Genay, France). Ferulic acid, 4-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, and 4-(3'-hydroxyphenyl)propionic acid were from Sigma Aldrich (St. Louis, MO, USA). Gallic acid was purchased from Panreac (Barcelona, Spain), and syringic and vanillic acids were from Fluka (Buchs, Switzerland).

Urea, KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ were obtained from Sigma Aldrich (St. Louis, MO, USA). $NaHCO_3$, KCl, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$ and $Mo_7(NH_4)_6O_{24} \cdot 4H_2O$ were purchased from Panreac Quimica S.A. (Barcelona, Spain) and $CuSO_4 \cdot 5H_2O$, NaCl, $Na_2SO_4 \cdot 10H_2O$ from Scharlau S.L. (Barcelona, Spain).

Acetonitrile (HPLC-grade) was from Romil (Tecknokroma, Barcelona, Spain). Methanol (HPLC-grade), hydrochloric acid (HCl) (37%), glacial acetic acid (99.8%), ethyl acetate (HPLC-grade), ethanol (HPLC-grade) and phosphoric acid (85%) were from Sharlau S.L. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Fisher BioReagents (Fisher Scientific; Pittsburgh, PA, USA) and Sigma Aldrich (St. Louis, MO, USA), respectively.

1.2. Grape and strawberry extract preparation.

Grape extract was prepared from grape pomace (byproduct of red wine making), kindly provided by a local winemaker, and strawberry extract was prepared from fresh strawberries purchased from a local market (Lleida, April 2015). Before their

lyophilization (Lyobeta 15 equipment, ImaTelstar, Terrassa, Barcelona, Spain), the grape pomace and sliced strawberries were frozen and then washed twice with ice water for sugar elimination. Extracts were obtained from the lyophilized product with an Accelerator Solvent Extraction (ASE 100 Dionex, Sunnyvale, CA) using acidified methanol/water solution (methanol/Milli-Q water/HCl, 79.9:20:0.1, v/v/v), at 80 ºC and two static cycles of extraction of 5 min each. The liquid extract was rotatory evaporated to remove the organic solvent and freeze dried. The resulting extract was purified by solid-phase extraction (SPE) following the method proposed by Taverniti et al. [18] to increase the anthocyanin concentration and to reduce the concentration of other phenolic compounds. Briefly, 0.1 g of lyophilized extract was treated with 10 mL of Milli-Q water, sonicated for 10 min and centrifuged at 8784g for 10 min at room temperature. This extraction procedure was repeated until the color had totally disappeared and then the supernatants were combined. The SPE cartridge (Oasis HLB 200 mg, from Waters) was preconditioned with 5 mL of methanol and 5 mL of Milli-Q water. Then, a mixture of 3 mL of the combined supernatants and 3 mL of acidified water (4% phosphoric acid) was loaded into the cartridge and cleaned up with 10 mL of HCl 0.01 N and 20 mL of ethyl acetate. The anthocyanin-rich fraction was eluted with 10 mL of methanol contained 0.1% of HCl. For the identification and quantification of anthocyanins, after filtration (Nylon filter 0.22 μm pore size), the extract was directly injected into the chromatographic system.

1.3. *In-vitro* colonic fermentation of malvidin- and pelargonidin-glucoside-rich extracts.

The in vitro procedure was performed as reported in our previous studies [19,20] with little modification. The colonic fermentation was designed to elucidate the colonic metabolism and the pathways of anthocyanins degradation, thus a reduced number of volunteers was considered appropiate. Fecal samples were obtained from three healthy volunteers after 4 days of an anthocyanin-deprived diet. Fresh fecal samples were transported to the laboratory and maintained in anaerobic conditions at 4 ºC and used within 2 hours of defecation. Fecal slurry was prepared by shaking 500 mL of prereduced carbonate-phosphate buffer with 5 g of fresh feces (1%, w/v) in a stomacher during 1 min. The fecal concentration of the slurry was lower than that reported in other fermentation experiments carried out by our group [19,20]. The reason was to slow down the microbial activity (decreasing the cell density) to observe the possible microbial intermediates of anthocyanins, especially aglycones, and other degradation products such as chalcones. [8,19]

Ten milliliters of filtered fecal slurry was distributed in disposable tubes (15 mL) containing the malvidin- and pelargonidin-glucoside-rich extracts (final concentrations of 25, 50 and 100 μmol/L malvidin- or pelargonidin-glucoside, respectively). Control tubes containing

fecal slurry but not the anthocyanin-rich extracts (control 1) and tubes containing carbonate-phosphate buffer and anthocyanin-rich extracts, but not feces (control 2), were also prepared. All the tubes were incubated in anaerobic chambers in an orbital shaker (60 rpm) at 37 ºC for 0, 1, 2, 4, 8 and 24 h. The anaerobic environment was achieved with anaerobic sacks and controlled with anaerobic strips (both from Becton Dickinson, MD, USA). The tubes collected at different time-points were acidified with 60 μL of HCl in order to stop the microbial metabolism, and aliquots of 5 mL were freeze-dried and stored at -80 ºC until analysis.

Anthocyanins and their microbial metabolites were extracted using methanol/water/acetic acid (79.9:20:0.1, v/v/v). Briefly, the lyophilized fermentation media (corresponding to 5 mL of fresh fermentation media) was reconstituted with 1 mL of the extraction solution, vortexed for 10 min, and centrifuged (8784*g*, 10 min at 4 ºC) The supernatant was filtered through 0.22 μm nylon filters and injected into the chromatographic system.

1.4. Chromatographic analysis.

The anthocyanin composition of the grape and strawberry extracts as well as the fermentation media, corresponding to different times of incubation, was qualitatively and quantitatively characterized using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA), equipped with a binary pump system (Waters, Milford, MA, USA) and applying the chromatographic method reported in our previous study[19]. The tandem mass spectrometry (MS/MS) analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. The analysis was done in the positive ion mode for the anthocyanins and in the negative ion mode for the other phenolic compounds. Data were acquired with the selected reaction monitoring mode (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 ºC; cone gas flow rate, 80 L/h and desolvation gas flow rate, 800 L/h; desolvation temperature, 400 ºC Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. Cone voltages and collision energies were optimized for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50:50, v/v) at a concentration of 10 mg/L. Two transitions were studied for each phenolic compound. The most abundant transition was used for quantification, and the second one for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software (Waters, Mildford, MA, USA).

The anthocyanin-glycosides and acetyl-glycosides were quantified using the calibration curves of their corresponding pure commercial standards, with the

exception of peonidin and petunidin derivatives, which were tentatively quantified with cyaniding- and malvidin-3-*O*-glucosides, respectively. Syringic acid, vanillic acid, tyrosol, gallic acid, hydroxyphenylpropionic acid, and hydroxyphenylacetic acid were quantified using the calibration curve of their respective commercial standards. 3-*O*-Methylgallic acid and dihydroferulic acid were tentatively quantified using the calibration curve of gallic and ferulic acids, respectively.

1.5. Cell-culture conditions.

The HT-29 cell line was obtained from the ATCC (American Type Culture Collection, VA, USA). The cells were cultured in a humidified incubator (5% CO_2, 37 ºC) in flask bottles (Corning Costar, NY, Netherlands) from an initial cell density of 0.6 x10^6 to a final cell density of 3.5 x10^6. The McCoy 5A cell medium (HyClone, Thermo Scientific, Logan, Utah, USA) supplemented with 10% of fetal bovine serum (Sigma-Aldrich, Madrid, Spain), 1% of L-glutamine, 1% of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (both from HyClone, Thermo Scientific, Logan, UT, USA), and 1% of penicillin and of streptomycin (Gibco, Spain) was replaced every 2 days after washing twice with phosphate buffered saline (PBS; HyClone, Thermo Scientific, Logan, Utah, USA).

1.6. Cell viability study.

The HT-29 cells were seeded in 24-well plates (Corning Costar, NY, Netherlands) at a density of 0.2 x 10^6 cells/mL. After 24 h of incubation and two washes with PBS, the wells were filled with cell medium enriched with different phenolic concentrations and incubated for 8, 24 and 48 h to observe variations in cell viability. The grape and strawberry extracts and the phenolic standards of syringic acid, vanillic acid, tyrosol, 3-(4´-hydroxyphenyl)propionic acid, 4-hydroxyphenylacetic acid and *p*-hydroxybenzoic acid were tested to study the cell viability. Freeze-dried grape and strawberry extracts were reconstituted with an extraction solution (methanol/water/acetic acid, 79.9:20:0.1, v/v/v) and added to the cell media so the final concentration of malvidin- and pelargonidin-glucosides was from 0 to 150 μmol/L. In the same way, individual and two different mixes of phenolic standards were also prepared in the same extraction solution and added to the cell media.

1.7. Preparation of standard mixes.

Mixes of standards were prepared to imitate the phenolic composition of the fermentation media after 24 h of the two anthocyanin-rich extracts fermentation, respectively. The use of standard mixtures instead of the media after fecal fermentation was justified mainly because the fermentation media contain high concentration of salts, which can interfere with the activity of phenols in the apoptotic assays.

As the extracts and phenolic standards were prepared in the extraction solution, the toxicity probably induced by this

solution was also tested. More detailed information regarding the composition of the cell media for the viability study is shown in Table 1S in the **Supporting Information**.

Cytotoxicity was measured by the MTT method at 8, 24 and 48 h following the instructions reported by Mosman et al.,[21] with some modifications. Briefly, after the incubation time, the cells were washed twice with PBS, and 0.5 mg/mL MTT culture media solution was added. Two hours later, the cell medium was discarded, and a mixture of ethanol/DMSO (1:1, v/v) was used to promote cells lysis. The intracellular ATP content was measured colorimetrically through formazan (Thermo Scientific, Logan, Utah, USA) at 547 nm in a microplate reader (Multiskan go, Thermoscientific, Madrid, Spain), using a 96-well plate. Calculation of the cell viability after incubation was based on the absorbance value of the control samples which were considered to contain 100% of live cells.

1.8. Apoptotic effect of malvidin- and pelargonidin glucoside-rich extracts and their microbial metabolites.

Annexin-V assay. A propidium iodide (PI)/annexin V-APC apoptosis detection kit (Affymetrix Bioscience, Santa Clara, California, USA) was used to distinguish between viable cells and different stages of cell death. for these experiments, the cells were seeded and incubated for 24 h in a 24-well plate (2 x 10^5 cells/well). After incubation and two washing steps with PBS, a cell medium enriched with 100 μmol/L of the same phenolic solution used for the cytotoxicity assay was added, and then the cells were incubated for 8 h. After this time, the cells were harvested and treated with annexin-V and PI following the manufacturer's instruction. The compounds and their concentrations used for the annexin-V assay are detailed in Table 2S in the **Supporting Information**. The degree of apoptosis was determined by flow cytometry (BD Biosciences, Santa Clara, CA, USA). At least 10000 events were recorded during acquisition.

Caspase-3 assay. The activation of caspase-3 was tested at 24 and 48 h after the incubation of the HT-29 cells with the anthocyanin-rich extracts, the individual phenolic compounds and the combination of phenolic compounds imitating the phenolic profile of the fermentations (mix of standards) (100 μmol/L). The compounds and concentrations used for the caspase-3 assay are detailed in **Table 2S** in the **Supporting Information**. Cellular content was processed following the FITC kit (BD Biosciences, Santa Clara, California, USA) manufacturer's instruction, and measured by flow cytometry (BD Biosciences, Santa Clara, California, USA). A minimum of 10000 events were recorded during acquisition.

1.9. Statistical analysis.

Each sample from the in vitro fermentation was analyzed in triplicate (three replicates from three volunteers; n = 9). Colonic fermentation data are presented as the mean values ± standard deviation (SD) of all the samples at each time (n = 9 per each

time) minus the concentration of the compound in the control 1. Experiments conducted in HT-29 cells were performed in duplicate in three biological replicates (n= 6) after the different phenolic treatments. ANOVA analysis was applied using the STAT-GRAPHICS Plus 5.1 software (Manugistics Inc., Rockville, MD, USA). A Least Significant Difference (LSD) multiple-range test was applied to observe statistical significance between groups when the one-way ANOVA analysis was $p < 0.05$.

2. RESULTS

2.1. Composition of anthocyanins-rich extracts.

The phenolic composition of the grape and strawberry extracts, which were purified by SPE, is shown in **Table 1**. Malvidin-glucoside (31.2 ± 0.33 μmol/L) was the main anthocyanin quantified in the purified extract obtained from grape pomace, and pelargonidin-glucoside (8.43 ± 0.03 µmol/L) from strawberries, respectively. **Figure 1** shows the extracted ion chromatograms (EICs) of the anthocyanins determined in A) grape extract, and B) strawberry extract. In both extracts, the total anthocyanin content was 85%, indicating the effectiveness of SPE at eliminating the other phenolic compounds in the crude extract. The total anthocyanins content in the two fractions was determined as the quotient between the anthocyanin concentration and the total phenolic concentration, and then the result was multiplied by 100. The concentration of all the phenolic compounds (anthocyanins and the rest of phenolic compounds) was determined by UPLC-DAD. Anthocyanins were quantified at 500 nm wavelength, flavonols at 339 nm and the rest of phenolic compounds at 278 nm.

As can be seen in **Table 1**, apart from anthocyanins, which were the most abundant phenolics, other phenolic compounds were also present in these two extracts, but at much lower concentration levels, around 15%. These phenolic compounds were phenolic acids (ferulic acid and gallic acid), flavan-3-ols (catechin and epicatechin) and flavonols in grape extract, and ellagic acid and its derivatives, flavan-3-ols (catechin, epicatechin and dimer) and flavonols in strawberry extract.

2.2. Microbial degradation of malvidin- and pelargonidin-glucoside

To consider only the metabolites associated with anthocyanin microbial metabolism, control 1 (fecal slurry) and control 2 (carbonate-phosphate buffer and anthocyanin-rich extract) were incubated in parallel with the experimental samples (fecal slurry plus anthocyanin-rich extract, grape and strawberry extracts). The control 1 samples were run to monitor the metabolites arising from the basal metabolism, and the generated compounds were subtracted from the metabolites produced in the presence of the anthocyanin-rich extract. On the other hand, control 2 was run to monitor the generation of the metabolites originated from chemical degradation. In short, **Figure 2** shows the kinetics of the

Table 1. Phenolic Composition (µmol/L) of Grape extract and Strawberry extract

Phenolic compounds	Grape Extract (Malvidin-glucoside-rich extract)	Strawberry extract (Pelargonidin-glucoside-rich extract)
Anthocyanins (85%)		
Pelargonidin derivatives (Total)	*0.13 ± 0.01*	*9.12 ± 0.04*
Pelargonidin-glucoside	0.13 ± 0.01	8.43 ± 0.03
Pelargonidin-rutinoside	n.d.	0.39 ± 0.03
Pelargonidin acetyl-glucoside	n.d.	0.21 ± 0.03
Pelargonidin malonyl-glucoside	n.d.	0.09 ± 0.02
Cyanidin derivatives (Total)	*0.29 ± 0.04*	*0.24 ± 0.01*
Cyanidin-glucoside	0.18 ± 0.03	0.24 ± 0.01
Cyanidin acetyl-glucoside	0.11 ± 0.02	n.d.
Delphinidin derivatives (Total)	*2.92 ± 0.05*	*n.d.*
Delphinidin-glucoside	2.08 ± 0.03	n.d.
Delphinidin acetyl-glucoside	0.84 ± 0.03	n.d.
Peonidin derivatives (Total)	*3.12 ± 0.05*	*0.05 ± 0.01*
Peonidin-glucoside	1.94 ± 0.07	0.02 ± 0.01
Peonidin acetyl-glucoside	1.18 ± 0.03	0.02 ± 0.01
Petunidin derivatives (Total)	*3.05 ± 0.06*	*n.d.*
Petunidin-glucoside	2.13 ± 0.04	n.d.
Petunidin-rutininoside	0.15 ± 0.01	n.d.
Petunidin acetyl-glucoside	0.78 ± 0.08	n.d.
Malvidin derivatives (Total)	*49.9 ± 0.39*	*0.16 ± 0.01*
Malvidin-arabinoside	0.05 ± 0.01	n.d.
Malvidin-glucoside	31.2 ± 0.33	0.11 ± 0.03
Malvidin-rutinoside	3.32 ± 0.05	0.01 ± 0.01
Malvidin acetyl-glucoside	15.3 ± 0.12	0.04 ± 0.01
Rest of phenolic compounds (15%)		
Phenolic acids and derivatives (Total)	*1.25 ± 0.77*	*0.94 ± 0.19*
Gallic acid	0.19 ± 0.02	n.d.
Ellagic acid	n.d.	0.08 ± 0.05
Ellagic acid pentoside	n.d.	0.18 ± 0.05
Ellagic acid deoxyhexoside	n.d.	0.67 ± 0.09
Ferulic acid glucoside	1.06 ± 0.75	0.01 ± 0.00
Flavan-3-ols (Total)	*6.14 ± 0.19*	*0.55 ± 0.19*
Catechin	4.07 ± 0.11	0.35 ± 0.09
Epicatechin	2.07 ± 0.08	0.06 ± 0.05
Dimer	n.d.	0.14 ± 0.05
Flavonols (Total)	*4.48 ± 0.76*	*0.48 ± 0.11*
Kaempferol glucoside	1.51 ± 0.13	0.47 ± 0.11
Quercetin	0.30 ± 0.09	n.d.
Quercetin glucoside	0.34 ± 0.05	0.01 ± 0.00
Quercetin glucuronide	0.31 ± 0.07	n.d.
Myricetin glucoside	0.48 ± 0.09	n.d.
Laricitrin glucoside	0.45 ± 0.08	n.d.
Syringetin glucoside	1.09 ± 0.25	n.d.

Values are expressed as mean ± SD; n.d.: not detected

degradation of anthocyanins and the generation of microbial metabolites by the microbiota of three volunteers as the mean value and standard deviation. The numerical data of this figure can be consulted in **Table 3S** in the **Supporting Information.***Fermentation of malvidin glucoside-rich extract: grape pomace*: **Figure 2**A shows the kinetics of malvidin-glucoside degradation and **Figure 2**B the parallel generation of microbial metabolites metabolites expressed as the mean of the three volunteers. In general, malvidin-glucoside was almost completely degraded after 8 h of incubation. With regard to the microbial metabolites, syringic acid was the main metabolite generated, and the highest recovery rate was observed at the end of the fermentation period (24 h). Taking into account the amounts recovered, hydroxyphenylpropionic acid and vanillic acid also increased during the in vitro fermentation of the grape extract. Hydroxyphenylpropionic acid and vanillic acid reached their maximum concentration in the incubation media between 8 and 24 h, depending of the microbiota of the volunteer. Although dihydroferulic acid, gallic acid and 4-methyl gallic acid were detected at low concentration levels, they were also considered malvidin microbial fermentation products because they were generated at higher amounts in the experimental samples than in controls 1 and 2 (**Tables 3S** A(1), A(2), and A(3) in the **Supporting Information**).

Fermentation of pelargonidin glucoside-rich extract: strawberries: **Figure 2**C shows the degradation rate of pelargonidin-glucoside and the generation of its microbial metabolites over the 24 h of the in vitro fermentation of the strawberry extracts. In a similar way to malvidin-glucoside, pelargonidin-glucoside was also almost completely degraded after 8 h of incubation, and the main microbial metabolite generated was tyrosol, with maximum recoveries after 24 h of fermentation. Other phenolic metabolites detected as microbial catabolism products of pelargonidin-glucoside were hydroxyphenylpropionic acid (maximum recovery at 24 h in the microbiota of volunteers 1 and 3 and at 4 h in the microbiota of the volunteer 2), hydroxyphenylacetic acid (maxima concentration at 24 h) and *p*-hydroxybenzoic acid (maxima concentration between 8 h and 24 h, depending on the microbiota of the volunteer).

2.3. Apoptotic effect of malvidin- and pelargonidin-glucoside-rich extracts and their main microbial metabolites.

Cell viability: HT-29 cells were incubated for 8, 24 and 48 h with different concentrations (from 0 to 150 μmol/L) of diverse types of phenolic compounds (Table 1S in the **Supporting Information**) to detect differences in cell viability. After 8, 24 and 48 h of incubation, the cell viability of all the experimental samples remained between 80 and 90% when compared with the control samples. Major toxicity was detected at a phenolic concentration of 100 μmol/L but no great differences were observed between

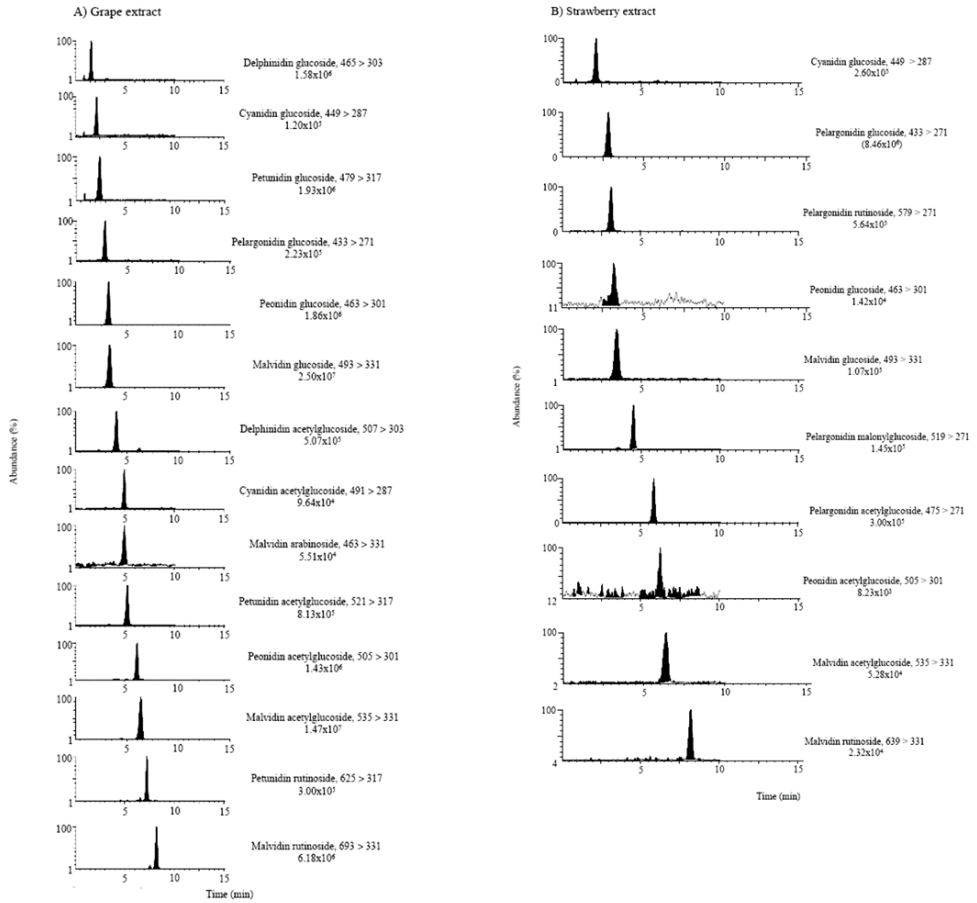


Figure 1: Extracted ion chromatograms (EICs) of the anthocyanins determined in the (A) grape extract and (B) strawberry extract.

concentrations of 100 and 150 μmol/L. The addition of the extraction solution (methanol/Milli-Q water/acetic acid, 79.9:20:0.1, v/v/v) to the cell media did not produce any alteration in cell viability (**Figure 1 Supporting Information**).

Annexin-V assay: The induction of apoptosis in HT-29 cells was assessed by PI/annexin-V APC assay. The annexin-V protein, which binds to phosphatidylserine (PS) in a highly selective manner, and the PI, which stains DNA and only enters dead cells, enables us to distinguish between viable cells (dot plot Q3, PI negative and annexin-V negative), early stage of apoptosis (dot plot Q4, PI negative and annexin-V positive), late stage of apoptosis (dot plot Q2, PI positive and annexin-V negative), and necrotic or dead cells (dot plot Q1, PI positive and annexin-V positive). **Figure 3** shows the percentages

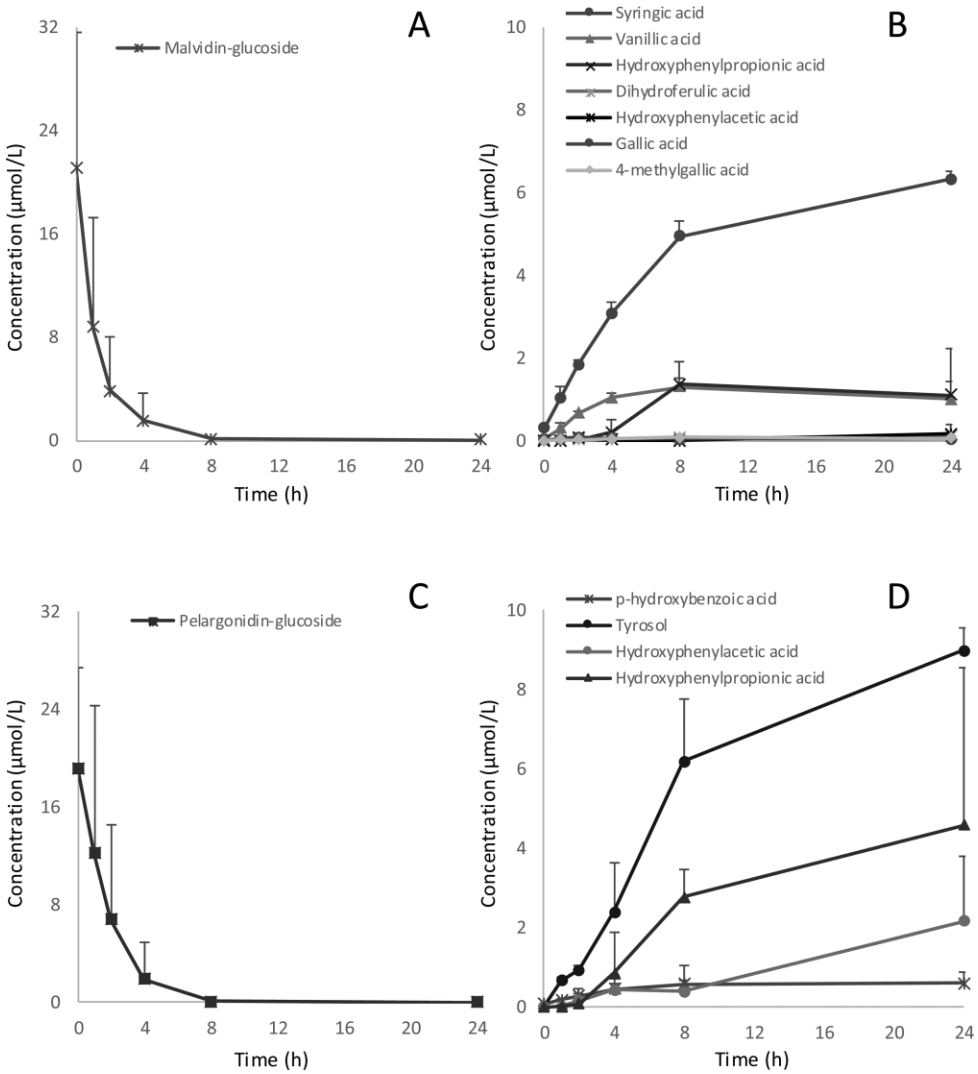


Figure 2: Kinetics of anthocyanin degradation and microbial metabolite generation during 24 h of in vitro fermentation of grape extract (A, B) and strawberry extract (C, D).

(considering the total cells) corresponding to those cells identified in dot plots Q1, Q2 and Q4.

Except for hydroxyphenylacetic acid, grape extract and strawberry extract, no significant differences were detected between the control and phenolic treatments. The differences were observed in the late stage of apoptosis between the control and hydroxyphenylacetic acid ($p<0.05$) and in the percentage of dead cells, which was higher after the treatment with hydroxyphenylacetic acid and

anthocyanin-rich extracts (grape and strawberry extracts) compared to the control ($p<0.05$).

Caspase-3 assay: The activation of the cysteine-proteases (caspases) cascade to confirm the induction of apoptosis cell death by the extracts and single microbial metabolites was measured in HT-29 cells. Caspase-3 activity was followed after 24 (**Figure 4**A) and 48 h (**Figure 4**B) of incubation with different phenolic treatments (Table 2S in the **Supporting Information**). After 24 h of phenolic treatment, no significant differences were observed between the treated and control cells (see **Figure 4**A). Following 48 h of incubation, only the mix that represented the phenolic profile of the grape extract fermentation (8-24 h) showed a significantly higher activation of caspase-3 in the HT-29 cells (see **Figure 4**B).

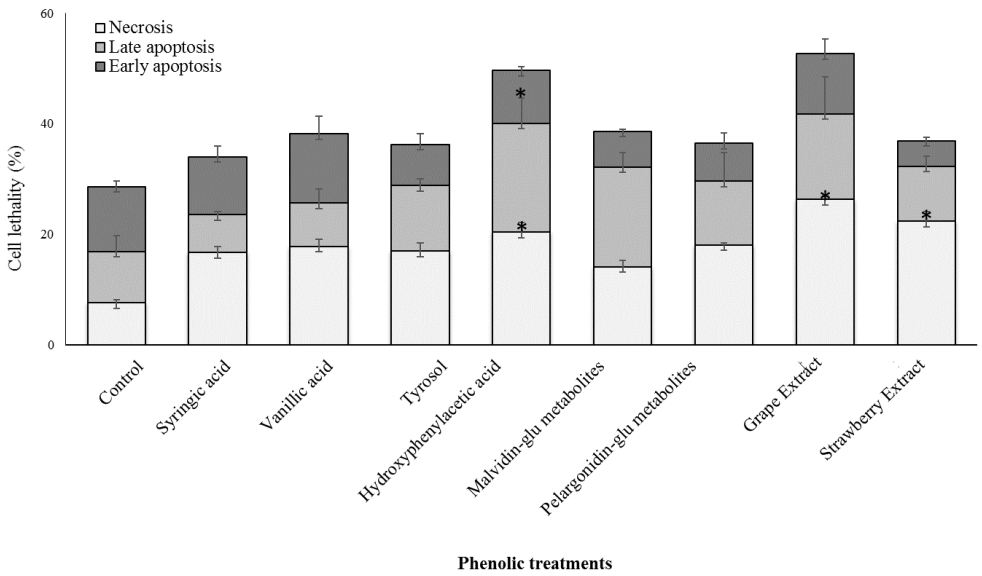


Figure 3. Annexin V－PI test (apoptosis) in HT-29 cells. Cells were treated for 8 h with main microbial catabolites detected after in vitro fermentation of grape and strawberry extracts comparing with the extracts as reference: early apoptosis (AnnV positive and PI negative, AnnV +/PI－); later apoptosis (AnnV positive and PI positive, AnnV+/PI+); and necrose/death cells (AnnV negative and PI positive, AnnV－/PI+). The assay was performed in three biological replicates n = 6. ∗ indicates significant difference ($p < 0.05$) compared with control cells.

1. DISCUSSION

The phenolic compounds present in the grape and strawberry extracts were mainly anthocyanins, representing 85% of the total phenolics. The remaining 15% were other phenolic compounds, such as phenolic acids, flavan-3-ols (catechin and epicatechin) and flavonols (kaempferol-, quercetin-, and syringetin-glucosides, among others).

Although the generated fermentation metabolites di- and hydroxyphenyl-propionic acids and di- and hydroxyphenylacetic acids are common from phenolic compounds, it was considered that these metabolites came mainly from anthocyanins, because these latter were present in the two fractions at 85% in comparison to 15% of the rest of phenolic compounds.The moderate consumption of anthocyanins through the intake of red wine and berries has been associated with an improvement of health biomarkers and a lower risk of colon cancer and other chronic diseases. [2,22,23] Nevertheless, the low rate of absorption of these flavonoids does not facilitate a consensus that definitely defines the form of the molecule responsible for the beneficial effects. Esposito et al. [2] observed areduction in weight gain and an improvement in glucose metabolism in mice fed a high-fat diet in combination with black currant extract (rich in delphinidin and cyanidin derivatives). Olsson et al. [24] showed that anthocyanins could be implicated in the antiproliferative effect in MCF-7 breast cancer cell line. Phenolic compounds may protect against the incidence of colon cancer indirectly by the interaction with microbiota. Beneficial effects observed after the intake of anthocyanins are produced in animals with intact microbiota but not in those with disrupted microbiota, highlighting the possible implication of microbial metabolites in the observed effects. [25,26] Furthermore, bioactive microbial metabolites are involved in the control of the microbial population, [27,28] which may regulate the antitumor response of immune cells. [29,30] In vitro fermentations using human feces as the microbial inoculum can be helpful for an approximation to the gut metabolism of phenolic compounds.

In the present work, we focused our attention on the transformation of malvidin- and pelargonidin-glucosides through the anaerobic incubation of anthocyanin-rich extracts (grape and strawberry extracts). Both anthocyanins were rapidly degraded by fecal bacteria, with only low amounts detected after 8 h of fermentation. A rapid disappearance seems to be a common characteristic of anthocyanin glucosides, [9,31,32] whereas other types of conjugates, such as acetyl-glycosides, offer more resistance to degradation. [4]

In our study, the metabolites generated as a product of the microbiota catabolism of malvidin- and pelargonidin-glucosides started to increase gradually until reaching maximum concentrations at the final fermentation times (8-24 h). In line with previous in vitro experiments, syringic acid was also detected as the main

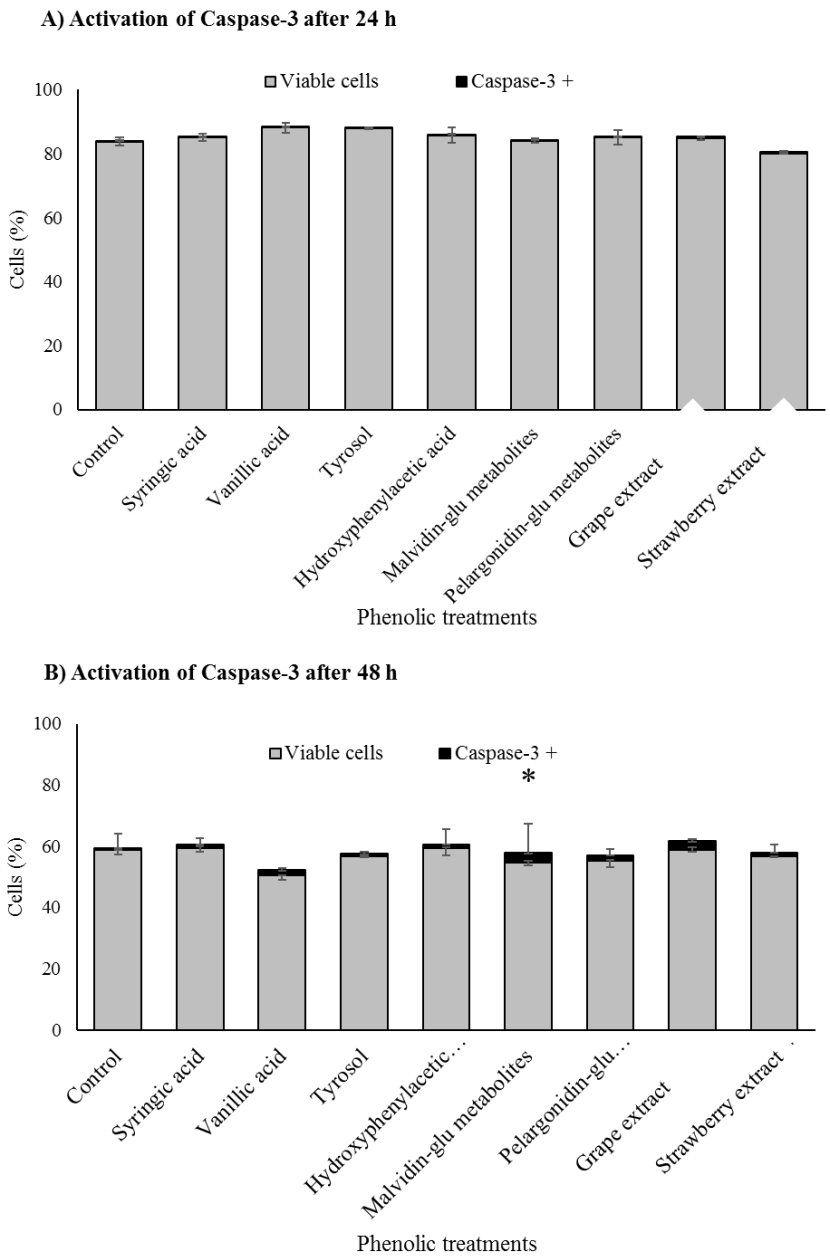


Figure 4. Apoptosis of HT-29 cells measured by caspase-3 activation staining. Cells were treated for (A) 24 h and (B) 48 h with main microbial catabolites detected after in vitro fermentation of grape and strawberry extracts comparing with the extracts as reference. The assay was performed in three biological replicates n = 6. ∗ indicates significant difference ($p < 0.05$) compared with control cells.

microbial metabolite of malvidin-glucoside. [4,31,32] However, we observed that the colonic fermentation of malvidin-glucoside also produced other simple phenolic compounds, such as vanillic acid, gallic acid, methylgallic acid, dihydroferulic acid, hydroxyphenylpropionic acid and hydroxyphenylacetic acid. Flores et al. [31] and Hidalgo et al. [32] have also described gallic acid as a microbial metabolite of malvidin-*O*-3-glucoside, but no other products of fermentation, except for pyrogallol, [32] were mentioned. On the basis of the different phenolic compounds detected in the fermentation media, we proposed the metabolic pathways of malvidin-glucoside (**Figure 5**A). The catabolic reactions involved in the metabolite generation were defined on the basis of the products detected throughout the 24 h of fermentation. The colonic metabolism of malvidin-glucoside produces a series of related metabolites but not all of these were detected (in grey) in the fermentation media, probably because of their low stability and/or their rapid transformation into other products.

Figure 5B shows the colonic metabolic fate of pelargonidin-glucoside based on the microbial metabolites detected during the 24 h of anaerobic incubation. The most notable aspect of the present work, observed for the first time, was the generation of tyrosol as the main microbial metabolite. Pelargonidin-glucoside, abundant in strawberries, has not been as widely studied as others such as cyaniding-, malvidin- and delphinidin-glucosides. [2,4,9,31] After a single oral dose of pelargonidin-glucoside, *p*-hydroxybenzoic acid was described as the main metabolite in rat biofluids.[33] In the present work, *p*-hydroxybenzoic acid was also detected, but at lower levels than hydroxyphenylpropionic and hydroxyphenylacetic acids, which were other compounds produced during the fecal incubation of the strawberry extract (**Figure 2**D and Table 3S in the **Supporting Information**). González-Barrio et al. [7] monitored the transformation of cyanidin-glucoside during the in vitro fermentation of raspberry extract. The authors observed a significant increase in tyrosol compared with the control samples. Cyanidin has the same chemical structure as pelargonidin except that it has an extra hydroxyl group in position 4 of the B ring. In two previous human intervention studies carried out by our group, we noted an increase in hydroxytyrosol in feces after the sustained intake of pomegranate juice (as a source of cyanidin-*O*-3-glucoside as the main anthocyanin) over 4 weeks [34] and in urine after an acute intake of a dealcoholized red wine (as a source of malvidin-glucoside). [35] These results suggest that the microbial transformation of anthocyanins is not limited to the generation of simple phenolic acids but also to the generation of such simple phenolic alcohols as tyrosol and hydroxytyrosol. Low absorption of anthocyanins during digestion increases the possibility of these compounds being accumulated in the gut lumen and coming into contact with the intestinal epithelium. However, in contact with gut

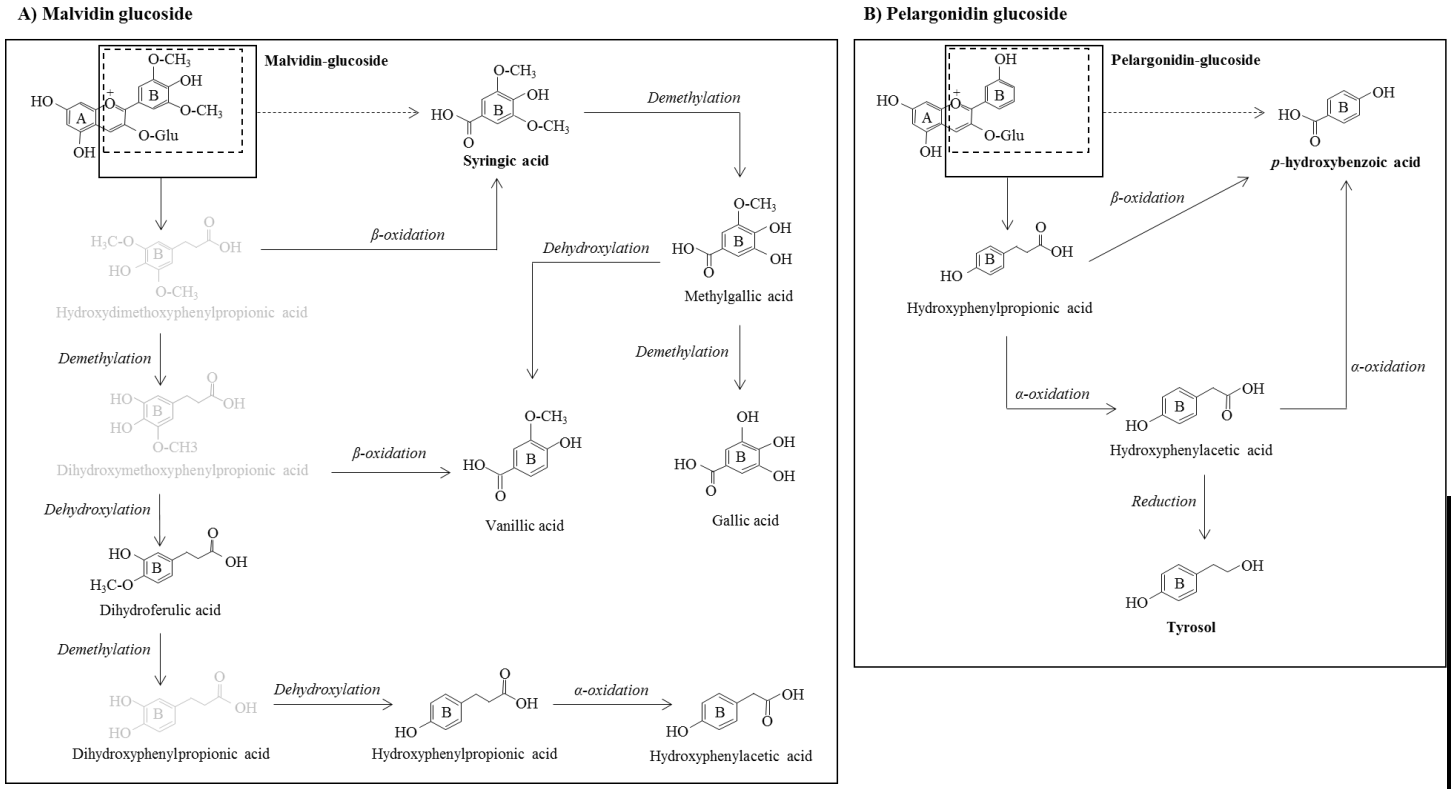


Figure 5. Colonic pathways proposed for (A) malvidin-glucoside and (B) pelargonidin-glucoside. Compounds in gray color are not detected intermediates

microbiota, anthocyanins seem to be rapidly converted into their microbial derivatives **(**see **Figure 2)**. This implies that the composition of the gut "digesta" probably changes during intestinal digestion toward a major presence of microbial metabolites as the bolus movestoward the colon. Few studies have focused on the study of the pro-apoptotic activities of anthocyanins [4,36,37] and their microbial metabolites [16,17] against colon cancer. In the present work, we tested a mix of phenolic compounds prepared from commercial standards to imitate the phenolic composition determined in the fermentation media at the final incubation times of the two anthocyanin-rich extracts (grape and strawberry extracts) studied (Table 1S and 2S in the **Supporting Information**). Furthermore, we used standard mixtures to avoid the harmful effects caused by salts from colonic digestion. The standard mixtures also gave a more realistic aspect and considered possible synergistic effects of microbial metabolites rather than individual compounds (Table 2S in the **Supporting Information**).

During gastrointestinal digestion, dietary compounds absorbed in the gut lumen are likely to come into contact with the intestinal cells and be able to exert their protective effect. To define if apoptosis is one of the mechanisms through which anthocyanins and their microbial metabolites can control the proliferation of colon cancer cells, the PI/annexin-V and caspase-3 assays were carried out. After 8 h of phenolic treatment of the HT 29 cell line, the PI/annexin-V assay revealed that the main cause of the loss of cell viability was the late apoptosis/necrosis with hydroxyphenyl-acetic acid, grape extract, and strawberry extract, being the most effective compounds (**Figure 3**). The activation of caspase-3 was only significant **after** 48 h of incubation with the phenolic mix representing the metabolic profile of grape extract (see **Figure 4**). Forester et al. [16] studied the apoptotic effect of other phenolic compounds derived of the catabolism of anthocyanins. Gallic acid, methylgallic acid and 2,4,6-trihydroxybenzaldehyde activate the caspases cascade response after 24h of release in Caco-2 cells.

In our study, hydroxyphenylacetic acid and the anthocyanin-rich extracts (grape and strawberry extracts) produced a significant effect on cell viability after 8 h of exposure, as the annexin-V indicated (see **Figure 3**). However, the lack of apoptotic evidence of an increase in caspase-3 activation could suggest that the HT-29 cells treated with the latter compounds do not reach the end stages of apoptosis (see **Figure 4**). The impossibility of confirming the cause of cell death through a programmed cell mechanism (apoptosis) could be explained by the activation of other forms of non-apoptotic cell death, as necrosis or autophagy. Probably, the caspase-dependent and caspase-independent mechanisms of colon cancer cell death may depend on the cell type and the compound that cells are exposed to.

Previous in vitro studies have postulated that a wide range of phenolic compounds, including anthocyanins and some

microbial metabolites, reduce the in vitro proliferation of colon cancer cells through the regulation of cellular mechanisms that resist cell death. Several pro-apoptotic mediators have been described as being activated by different phenolic compounds in many cancer cells lines, [16,36,37] but this mechanism was not altered in normal cells. [16] The results of the present study indicate that the mixture obtained from the in vitro fermentation of grape extract incubated with HT-29 cells is likely to act as antiproliferative agent. This effect was not observed when microbial metabolites were tested individually, suggesting a synergistic or additive effect of the microbial metabolites derived from grape anthocyanins. This fact contradicts the results of Correa-Betanzo et al., [4] who observed a more effective antiproliferative effect from the anthocyanin extract compared to the microbial degradation products in HT-29 cells after 24 and 48 h of exposure using the SRB (Sulforhodamine B) assay. Therefore, it is important to contrast the present results in in vivo studies because, on occasions, it was observed that the effects of phenolic compounds ingested as pure compounds or even through phenolic-rich food do not always imitate the chemopreventive effects observed under in vitro conditions [3].

The results obtained in the present study show that malvidin- and pelargonidin-glucosides are extensively metabolized by human gut microbiota in an in vitro model, respectively producing syringic acid and tyrosol as the main metabolites. The data obtained after the apoptotic assays suggested that malvidin- and pelargonidin-glucosides, hydroxyphenylacetic acid, and the combination of microbial metabolites generated during the in vitro fermentation of grape and strawberry extracts could induce cell death in HT-29 colon cancer cells. After the annexin-V assay, it was observed that malvidin- and pelargonidin-glucosides and hydroxyphenylacetic acid decreased cell viability, which could confirm the harmful effect against carcinogenic cells. On the other hand, the combination of microbial metabolites generated during the in vitro fermentation of grape extract could promote the activation of caspase-3, and induce cell apoptosis. On the basis of these findings, the present study suggests that both grape and strawberry anthocyanins as well as their microbial metabolites could be interesting candidates as preventive agents against colon cancer. However, the present study reports on results from a first screening of anthocyanins and their metabolites as potential apoptotic agents, and more studies are needed to verify their efficacy in other cancer cell lines and in colon cancer induced animal models.

Acknowledgments
We thank the volunteers who participated in the study.

Funding
This work was partly supported by the University of Lleida through a grant given to M. C. López de las Hazas and by the Generalitat de Catalunya through a grant given to J. I. Mosele. I. A. Ludwig was supported by a postdoctoral research

contract funded by the Spanish Ministry of Economy and Competitiveness (FJCI-2014-20689).

Notes
The authors declare no competing financial interest.

2. REFERENCES

(1) Coates, E. M.; Popa, G.; Gill, C. I. R.; McCann, M. J.; McDougall, G. J.; Stewart, D.; Rowland, I. Colon-available raspberry polyphenols exhibit anti-cancer effects on in vitro models of colon cancer. *J. Carcinog.* **2007**, *6*, 4.

(2) Esposito, D.; Damsud, T.; Wilson, M.; Grace, M. H.; Strauch, R.; Li, X.; Lila, M. A.; Komarnytsky, S. Black currant anthocyanins attenuate weight aain and improve glucose metabolism in diet-induced obese mice with intact, but not disrupted, gut microbiome. *J. Agric. Food Chem.* **2015**, *63*, 6172–6180.

(3) Kang, S.-Y.; Seeram, N. P.; Nair, M. G.; Bourquin, L. D. Tart cherry anthocyanins inhibit tumor development in ApcMin mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**; *194*, 13-19.

(4) Correa-Betanzo, J.; Allen-Vercoe, E.; McDonald, J.; Schroeter, K.; Corredig, M.; Paliyath, G. Stability and biological activity of wild blueberry (Vaccinium angustifolium) polyphenols during simulated in vitro gastrointestinal digestion. *Food Chem.* **2014**, *165*, 522–531.

(5) Jing, P.; Bomser, J. A.; Schwartz, S. J.; He, J.; Magnuson, B. A.; Giusti, M. M. Structure−function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. *J. Agric. Food Chem.* **2008**, *56*, 9391–9398.

(6) Dueñas, M.; Muñoz-González, I.; Cueva, C.; Jiménez-Girón, A.; Sánchez-Patán, F.; Santos-Buelga, C.; Moreno-Arribas, M. V.; Bartolomé, B. A survey of modulation of gut microbiota by dietary polyphenols. *Biomed Res. Int.* **2015**, *2015*, ID 850902.

(7) González-Barrio, R.; Edwards, C. A.; Crozier, A. Colonic catabolism of ellagitannins, ellagic acid, and raspberry anthocyanins: in vivo and in vitro studies. *Drug Metab. Dispos.* **2011**, *39*, 1680-1688.

(8) Kahle, K.; Kraus, M.; Scheppach, W.; Ackermann, M.; Ridder, F.; Richling, E. Studies on apple and blueberry fruit constituents: Do the polyphenols reach the colon after ingestion? *Mol. Nutr. Food Res.* **2006**, *50*, 418–423.

(9) Aura, A.-M.; Martin-Lopez, P.; O'Leary, K. A.; Williamson, G.; Oksman-Caldentey, K.-M.; Poutanen, K.; Santos-Buelga, C. In vitro metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* **2005**, *44*, 133–142.

(10) Johnson, I. . New approaches to the role of diet in the prevention of cancers

of the alimentary tract. *Mutat. Res. Mol. Mech. Mutagen.* **2004**, *551*, 9–28.

(11) Wei, E. K.; Giovannucci, E.; Wu, K.; Rosner, B.; Fuchs, C. S.; Willett, W. C.; Colditz, G. A. Comparison of risk factors for colon and rectal cancer. *Int. J. cancer* **2004**, *108*, 433–442.

(12) Rajamanickam, S.; Agarwal, R. Natural products and colon cancer: current status and future prospects. *Drug Dev. Res.* **2008**, *69*, 460–471.

(13) Il'yasova, D.; Martin, C.; Sandler, R. S. Tea intake and risk of colon cancer in African-Americans and whites: North Carolina colon cancer study. *Cancer Causes Control* **2003**, *14*, 767–772.

(14) Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **2007**, *35*, 495–516.

(15) Wong, R. S. Y. Apoptosis in cancer: from pathogenesis to treatment. *J. Exp. Clin. Cancer Res.* **2011**, *30*, 87.

(16) Forester, S. C.; Choy, Y. Y.; Waterhouse, A. L.; Oteiza, P. I. The anthocyanin metabolites gallic acid, 3-*O* -methylgallic acid, and 2,4,6-trihydroxybenzaldehyde decrease human colon cancer cell viability by regulating pro-oncogenic signals. *Mol. Carcinog.* **2014**, *53*, 432–439.

(17) Forester, S. C.; Waterhouse, A. L. Gut metabolites of anthocyanins, gallic acid, 3-O-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde, inhibit cell proliferation of Caco-2 cells. *J. Agric. Food Chem.* **2010**, *58*, 5320–5327.

(18) Taverniti, V.; Fracassetti, D.; Del Bo', C.; Lanti, C.; Minuzzo, M.; Klimis-Zacas, D.; Riso, P.; Guglielmetti, S. Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells. *J. Agric. Food Chem.* **2014**, *62*, 8346–8351.

(19) Mosele, J. I.; Macià, A.; Romero, M.-P.; Motilva, M.-J.; Rubió, L. Application of in vitro gastrointestinal digestion and colonic fermentation models to pomegranate products (juice, pulp and peel extract) to study the stability and catabolism of phenolic compounds. *J. Funct. Foods* **2015**, *14*, 529–540.

(20) Mosele, J. I.; Martín-Peláez, S.; Macià, A.; Farràs, M.; Valls, R.-M.; Catalán, U.; Motilva, M.-J. Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches. *Mol. Nutr. Food Res.* **2014**, *58*, 1809–1819.

(21) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.

(22) Wang, L.-S.; Stoner, G. D. Anthocyanins and their role in cancer prevention. *Cancer Lett.* **2008**, *269*, 281–290.

(23) Yousuf, B.; Gul, K.; Wani, A. A.; Singh, P. Health benefits of anthocyanins and their encapsulation for potential use in food systems: A review. *Crit. Rev. Food Sci. Nutr.* **2016**,

56, 2223–2230.

(24) Olsson, M. E.; Gustavsson, K. E.; Andersson, S.; Nilsson, A.; Duan, R. D. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. *J. Agric. Food Chem.* **2004**, *52*, 7264-7271.

(25) Morais, C. A.; de Rosso, V. V.; Estadella, D.; Pisani, L. P. Anthocyanins as inflammatory modulators and the role of the gut microbiota. *J. Nutr. Biochem.* **2016**, *33*, 1–7.

(26) Sears, C. L.; Garrett, W. S. Microbes, microbiota, and colon cancer. *Cell Host Microbe* **2014**, *15*, 317–328.

(27) Selma, M. V; Espín, J. C.; Tomás-Barberán, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485–6501.

(28) Faria, A.; Fernandes, I.; Norberto, S.; Mateus, N.; Calhau, C. Interplay between anthocyanins and gut microbiota. *J. Agric. Food Chem.* **2014**, *62*, 6898–6902.

(29) Viaud, S.; Saccheri, F.; Mignot, G.; Yamazaki, T.; Daillère, R.; Hannani, D.; Enot, D. P.; Pfirschke, C.; Engblom, C.; Pittet, M. J.; et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science.* **2013**, *342* (6161), 971-976.

(30) Nunes-Alves, C. Microbiome: Commensals promote anticancer immunotherapy. *Nat. Rev. Microbiol.* **2015**, *14* (1), 3.

(31) Flores, G.; Ruiz del Castillo, M. L.; Costabile, A.; Klee, A.; Bigetti Guergoletto, K.; Gibson, G. R. In vitro fermentation of anthocyanins encapsulated with cyclodextrins: Release, metabolism and influence on gut microbiota growth. *J. Funct. Foods* **2015**, *16*, 50–57.

(32) Hidalgo, M.; Oruna-Concha, M. J.; Kolida, S.; Walton, G. E.; Kallithraka, S.; Spencer, J. P. E.; de Pascual-Teresa, S. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J. Agric. Food Chem.* **2012**, *60*, 3882–3890.

(33) El Mohsen, M. A.; Marks, J.; Kuhnle, G.; Moore, K.; Debnam, E.; Kaila Srai, S.; Rice-Evans, C.; Spencer, J. P. E. Absorption, tissue distribution and excretion of pelargonidin and its metabolites following oral administration to rats. *Br. J. Nutr.* **2006**, *95*, 51–58.

(34) Mosele, J. I.; Gosalbes, M.-J.; Macià, A.; Rubió, L.; Vázquez-Castellanos, J. F.; Jiménez Hernández, N.; Moya, A.; Latorre, A.; Motilva, M.-J. Effect of daily intake of pomegranate juice on fecal microbiota and feces metabolites from healthy volunteers. *Mol. Nutr. Food Res.* **2015**, *59*, 1942–1953.

(35) Motilva, M.-J.; Macià, A.; Romero, M.-P.; Rubió, L.; Mercader, M.; González-Ferrero, C. Human bioavailability and metabolism of

phenolic compounds from red wine enriched with free or nano-encapsulated phenolic extract. *J. Funct. Foods* **2016**, *25*, 80–93.

(36) Wang, L.-S.; Kuo, C.-T.; Cho, S.-J.; Seguin, C.; Siddiqui, J.; Stoner, K.; Weng, Y.-I.; Huang, T. H.-M.; Tichelaar, J.; Yearsley, M.; et al. Black raspberry-Derived anthocyanins demethylate tumor suppressor genes through the inhibition of DNMT1 and DNMT3B in colon cancer cells. *Nutr. Cancer* **2013**, *65*, 118–125.

(37) Charepalli, V.; Reddivari, L.; Vadde, R.; Walia, S.; Radhakrishnan, S.; Vanamala, J. Eugenia jambolana (Java Plum) fruit extract exhibits anti-cancer activity against early stage human HCT-116 colon cancer cells and colon cancer stemcells. *Cancers.* **2016**, *8*, 29.

JAFC (2016) 10.1021/acs.jafc.6b04096

SUPPORTING INFORMATION

Supplementary information on composition of the media for the incubation of HT-29 cells to confirm toxicity (**Table 1S**) and apoptotic effects (annexin-V and caspase-3 assay) (T**able 2S**), the amounts of anthocyanins and metabolites detected during in vitro fermentation (**Table 3S**) and cytotoxicity results of the tested compounds after 48h of exposure to HT-29 cells (**Figure 1S**).

Table 1 of Supplementary Data. Composition of the Media for the Incubation of HT-29 Cells to confirm Toxicity

Compound	Mc Coy media (µL)	Final concentration (µM)	Solvent solution (µL)	Time of exposure (h)
-	1000	-	-	8 and 24
-	975, 950, 900	-	25, 50, 100	8 and 24
Grape extract	950, 975	100 and 50 µM malvidin glucoside	50, 25	8 and 24
Strawberry extract	962.25	75 µM pelargonidin glucoside	37.5	8 and 24
Syringic acid	997.5, 995, 992.5, 990, 987.5, 985	25, 50, 75, 100, 125, 150 µM	2.5, 5, 7.5, 10, 12.5, 15	8 and 24
Vanillic acid	997.5, 995, 992.5, 990, 987.5, 985	25, 50, 75, 100, 125, 150 µM	2.5, 5, 7.5, 10, 12.5, 15	8 and 24
4-Hydroxyphenylacetic acid	997.5, 995, 992.5, 990, 987.5, 985	25, 50, 75, 100, 125, 150 µM	2.5, 5, 7.5, 10, 12.5, 15	8 and 24
Tyrosol	997.5, 995, 992.5, 990, 987.5, 985	25, 50, 75, 100, 125, 150 µM	2.5, 5, 7.5, 10, 12.5, 15	8 and 24
Mix standards representing final time of grape extract fermentation (a)	997.5, 995, 992.5, 990, 987.5, 985	25, 50, 75, 100, 125, 150 µM (sum of compounds)	2.5, 5, 7.5, 10, 12.5, 15	8 and 24
Mix standards representing final time of strawberry extract fermentation (b)	997.5, 995, 992.5, 990, 987.5, 985	25, 50, 75, 100, 125, 150 µM (sum of compounds)	2.5, 5, 7.5, 10, 12.5, 15	8 and 24

Solvent solution: Methanol:Milli-Q water:acetic acid (79.9:20:0.1, v/v/v)

(a) Composition of the mix: 73.66 % of syringic acid, 14.01 % of vanillic acid and 13.27 % of 3-(4-hydroxyphenyl)propionic aicd

(b) Composition of the mix: 52 % of tyrosol, 38.5 % of hydroxyphenylpropionic acid, 5 % of 4-hydroxyphenylacetic acid and 4.5% of p-hydroxybenzoic acid

Table 2 of Supplementary Data. Composition of HT-29 Cells Media to study the Apoptotic Effects of Anthocyanins and their Microbial Products through Annexin-V (8 h of incubation) and Capsase-3 (24 and 48 h of incubation) probes.

Compound	Mc Coy media (µL)	Final concentration	Solvent solution (µL)	Time of exposure (h)
-	1000	-	-	8 , 24 and 48
-	975, 950	-	25, 50	8 , 24 and 48
Grape extract	975	100 µM malvidin glucoside	25	8 , 24 and 48
Strawberry extract	962.25	75 µM pelargonidin glucoside	37.5	8 , 24 and 48
Syringic acid	990	100 µM	10	8 , 24 and 48
Vanillic acid	990	100 µM	10	8 , 24 and 48
4-Hydroxyphenylacetic acid	990	100 µM	10	8 , 24 and 48
Tyrosol	990	100 µM	10	8 , 24 and 48
Mix standards representing final time of grape extract fermentation(a)	990	100 µM (sum of compounds)	10	8 , 24 and 48
Mix standards representing final time of strawberry extract fermentation(b)	990	100 µM (sum of compounds)	10	8 , 24 and 48

Solvent solution: Methanol:Milli-Q water:acetic acid (79.9:20:0.1, v/v/v)
(a) Composition of the mix: 73.66 % of syringic acid, 14.01 % of vanillic acid and 13.27 % of 3-(4-hydroxyphenyl)propionic aicd
(b) Composition of the mix: 52 % of tyrosol, 38.5 % of 3-(4-hydroxyphenyl)propionic acid, 5 % of 4- hydroxyphenylacetic acid and 4.5% of p-hydroxybenzoic acid

JAFC (2016) 10.1021/acs.jafc.6b04096

JAFC (2016) 10.1021/acs.jafc.6b04096

TABLE 3 OF SUPPLEMENTARY DATA.

Table 3A. Amounts of Malvidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Grape extract

Compound (µM)	Volunteer 1					
	0h	1 h	2 h	4 h	8 h	24 h
Hydroxyphenylacetic acid	0.00±0.01	n.d.	0.07±0.10	0.03±0.02	0.01±0.01	0.16±0.24
Hydroxyphenylpropionic acid	n.d.	0.01±0.02	0.02±0.02	0.21±0.32	1.37 ±0.56	1.10 ±1.12
Gallic acid	0.02±0.03	0.05±0.08	0.08±0.09	0.07±0.06	0.04±0.04	0.05 ±0.04
4-methylgallic acid	0.00±0.01	0.03±0.03	0.02±0.01	0.04±0.00	0.08±0.08	0.06±0.02
Vanillic acid	0.05±0.05	0.31±0.14	0.67±0.05	1.05±0.09	1.30±0.20	1.01±0.41
Dihydroferulic acid	n.d.	0.01±0.01	0.02±0.02	0.02±0.02	0.06±0.04	0.12±0.03
Syringic acid	0.30±0.00	1.04±0.29	1.82±0.13	3.09±0.25	4.94±0.39	6.32±0.21
Malvidin-glucoside	21.1±10.5	8.81±8.46	3.84±4.20	1.49±2.23	0.09±0.10	0.03±0.01

Table 3A(1). Amounts of Malvidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Grape extract using Feces from Volunteer 1

Compound (µM)	Volunteer 1					
	0h	1 h	2 h	4 h	8 h	24 h
Hydroxyphenylacetic acid	n.d.	n.d.	0.01±0.01	0.03±0.02	n.d.	0.04±0.01
Hydroxyphenylpropionic acid	n.d.	n.d.	n.d.	0.02±0.01	0.82 ±0.12	1.06 ±0.06
Gallic acid	n.d.	n.d.	0.01±0.02	n.d.	n.d.	n.d.
4-methylgallic acid	n.d.	0.02±0.00	0.02±0.02	0.04±0.01	0.03±0.00	0.04±0.00
Vanillic acid	n.d.	0.47±0.11	0.63±0.03	0.98±0.03	1.07±0.16	0.81±0.01
Dihydroferulic acid	n.d.	n.d.	n.d.	0.01±0.01	0.05±0.01	0.11±0.02
Syringic acid	0.26±0.30	1.30±0.10	1.96±0.28	3.04±0.17	4.50±0.62	6.44±0.62
Malvidin-glucoside	9.00±5.42	6.43±0.67	1.53±0.34	0.38±0.25	0.03±0.02	0.03±0.02

Table 3A(2). Amounts of Malvidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Grape Extract using Feces from Volunteer 2

Compound (µM)	Volunteer 2					
	0h	1 h	2 h	4 h	8 h	24 h
Hydroxyphenylacetic acid	n.d.	n.d.	0.01±0.00	0.05±0.01	0.02±0.04	0.43±0.35
Hydroxyphenylpropionic acid	n.d.	0.03±0.01	0.02±0.02	0.58±0.60	1.94±0.27	n.d.
Gallic acid	0.06±0.05	0.15±0.14	0.04±0.01	0.08±0.02	0.07±0.06	0.08±0.02
4-methylgallic acid	n.d.	0.01±0.01	0.02±0.01	0.04±0.01	0.04±0.01	0.07±0.02
Vanillic acid	0.05±0.07	0,20±0,21	0.65±0.42	1.02±0.26	1.42±0.67	0.75±0.27
Dihydroferulic acid	n.d.	0.02±0.01	0.02±0.02	0.04±0.02	0.02±0.03	0.09±0.03
Syringic acid	0.33±0.29	1.08±0.16	1.80±0.63	2.87±0.55	5.07±0.61	6.08±2.05
Malvidin-glucoside	27.9±8.29	18.2±2.92	8.69±3.94	4.06±1.41	0.20±0.17	0.02±0.01

Table 3A(3). Amounts of Malvidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Grape Extract using Feces from Volunteer 3

Compound (µM)	Volunteer 3					
	0h	1 h	2 h	4 h	8 h	24 h
Hydroxyphenylacetic acid	0.01±0.01	n.d.	0.18±0.02	0.01±0.00	n.d.	n.d.
Hydroxyphenylpropionic acid	n.d.	n.d.	0.04±0.01	0.04±0.02	1.36±0.14	2.24±0.43
Gallic acid	0.01±0.00	0.01±0.01	0.18±0.01	0.12±0.03	0.06±0.03	0.06±0.11
4-methylgallic acid	0.01±0.00	0.07±0.01	0.03±0.01	0.04±0.00	0.18±0.16	0.06±0.02
Vanillic acid	0.09±0.13	0.27±0.37	0.73±0.07	1.16±0.02	1.41±0.15	1.48±0.08
Dihydroferulic acid	n.d.	n.d.	0.03±0.00	0.02±0.01	0.10±0.02	0.15±0.01
Syringic acid	0.32±0.22	0.73±0.34	1.71±0.02	3.37±0.18	5.24±0.13	6.43±0.10
Malvidin-glucoside	26.3±8.16	1.80±0.82	1.31±0.31	0.04±0.00	0.03±0.01	0.03±0.03

JAFC (2016) 10.1021/acs.jafc.6b04096

JAFC (2016) 10.1021/acs.jafc.6b04096

Table 3B. Amounts of Pelargonidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Strawberry Extract

Compound (µM)	Volunteer 1					
	0 h	**1 h**	**2 h**	**4 h**	**8 h**	**24 h**
p-hydroxybenzoic acid	0.08±0.01	0.18±0.04	0.29±0.04	0.45±0.15	0.58±0.10	0.60±0.27
Tyrosol	0.02±0.02	0.65±0.12	0.94±0.12	2.39±1.24	6.18±1.60	8.99±0.57
Hydroxyphenylacetic acid	n.d.	0.01±0.01	0.17±0.29	0.44±0.36	0.39±0.66	2.17±1.64
Hydroxyphenylpropionic acid	n.d.	0.02±0.03	0.08±0.07	0.84±1.06	2.76±0.69	4.59±3.98
Pelargonidin-glucoside	19.2±8.22	12.2±12.1	6.76±7.75	1.90±3.03	0.05±0.02	0.02±0.03

Table 3B(1). Amounts of Pelargonidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Strawberry Extract using Feces from Volunteer 1

Compound (µM)	Volunteer 1					
	0 h	**1 h**	**2 h**	**4 h**	**8 h**	**24 h**
p-hydroxybenzoic acid	0.08±0.01	0.22±0.00	0.33±0.05	0.49±0.42	0.63±0.00	0.30±0.01
Tyrosol	n.d.	0.79±0.11	0.99±0.11	1.69±0.72	7.79±0.66	9.39±1.09
Hydroxyphenylacetic acid	n.d.	n.d.	0.01±0.01	0.03±0.00	0.02±0.02	1.59±0.17
Hydroxyphenylpropionic acid	n.d.	n.d.	n.d.	0.26±0.00	3.39±0.51	6.92±0.84
Pelargonidin-glucoside	10.7±1.36	6.38±0.51	2.50±0.78	0.22±0.01	0.04±0.03	n.d.

Table 3B(2). Amounts of Pelargonidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Strawberry Extract using Feces from Volunteer 2

Compound (μM)	**Volunteer 2**					
	0 h	**1 h**	**2 h**	**4 h**	**8 h**	**24 h**
p-hydroxybenzoic acid	0.08±0.01	0.18±0.00	0.27±0.01	0.58±0.27	0.65±0.00	0.69±0.06
Tyrosol	0.02±0.01	0.60±0.15	1.02±0.20	3.82±0.19	6.16±0.13	8.34±0.76
Hydroxyphenylacetic acid	n.d.	0.02±0.03	n.d.	0.60±0.07	1.16±0.08	4.03±0.66
Hydroxyphenylpropionic acid	n.d.	n.d.	0.10±0.03	2.06±0.33	2.03±0.26	n.d.
Pelargonidin-glucoside	27.1±1.58	26.1±0.03	15.7±1.50	5.40±0.18	0.05±0.03	0.01±0.00

Table 3B(3). Amounts of Pelargonidin-glucoside and its Metabolites detected during *In-vitro* Fermentation of Strawberry Extract using Feces from Volunteer 3

Compound (μM)	**Volunteer 3**					
	0 h	1 h	2 h	4 h	8 h	24 h
p-hydroxybenzoic acid	0.07±0.00	0.15±0.02	0.26±0.03	0.28±0.04	0.46±0.01	0.81±0.01
Tyrosol	0.03±0.01	0.56±0.04	0.80±0.09	1.66±0.28	4.59±0.73	9.23±1.09
Hydroxyphenylacetic acid	n.d.	n.d.	0.50±0.00	0.70±0.00	n.d.	0.90±0.00
Hydroxyphenylpropionic acid	n.d.	0.06±0.00	0.13±0.00	0.20±0.00	2.87±0.00	6.85±0.00
Pelargonidin-glucoside	19.8±2.68	4.03±0.46	2.08±0.46	0.09±0.02	0.07±0.01	0.06±0.01

n.d. no detected

JAFC (2016) 10.1021/acs.jafc.6b04096

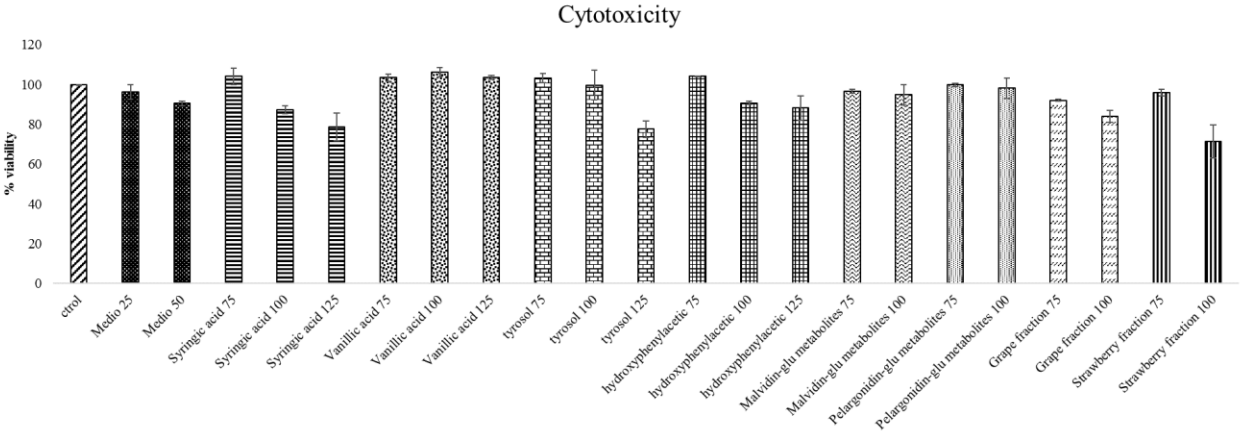
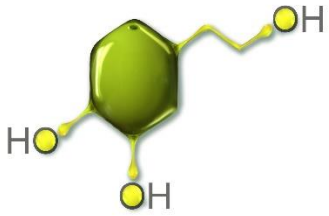


Figure 1S of Supplementary Data. Cytotoxicity results of the tested compounds after 48h of exposure to HT-29 cells

General discussion



Olive oil (OO) contains an exclusive phenolic family characterized for having hydroxytyrosol (HT) esterified with elenolic acid enclosed within its complex structure. The health benefits associated with OO phenol intake have been thoroughly studied (Cárdeno et al., 2013; Granados-Principal et al., 2010; Rigacci et al., 2016; Rodríguez-Morató et al., 2015). Despite the fact that there are several secoiridoid structures in olive and OO products, during gastro-intestinal digestion the complex forms are transformed, releasing into the digesta the unique bioavailable and active molecule: hydroxytyrosol (López de las Hazas et al., 2016a; Serra et al., 2012).

Many in-vitro studies support the huge antioxidant activity of HT and other biological effects for controlling different diseases. In addition, epidemiological and interventional studies (Covas et al., 2006; Konstantinidou et al., 2010; Owen et al., 2000; Weinbrenner et al., 2004) support the recent EFSA health claim (EFSA, 2012) related to the cardioprotective effects of VOO phenols. Due to the beneficial effects attributed to this phenolic family, a variety of products based on HT or its precursors (oleuropein and derivatives) have recently appeared on the market as useful supplements aimed at improving health status and preventing the development of certain diseases, especially, cardiovascular diseases (CVD).

It has also been found that unabsorbed HT continues to be metabolized in the last part of the intestine and caecum by microbiata, and these form catabolic products that may further contribute to the beneficial effects of OO. At the time of writing this Doctoral Thesis, the lack of information about the bioavailability and fate of phenols in tissues was a problem in terms of supporting the health effects attributed to phenolic compounds in general and OO phenols in particular.

For these reasons, the results of the Doctoral Thesis represent an advance in the knowledge of the biotransformation of HT and related compounds. By means of animal and human studies, I evaluate the impact of the dose administered and the impact of the chemical structure of HT and related compounds on metabolic fates.

A knowledge of phenol biotransformation and tissue deposition helps to establish the chemopreventive site of action and elucidate the relationship among the native and metabolic forms at cellular level. Also, the results of the Doctoral Thesis are an advance in the understanding of the molecular mechanisms of action of HT and its biological metabolites at cellular level. For this proposal, two target tissues for CVD (aorta and heart) and colon cancer cells were selected.

In this section, an overview of the main results obtained from the studies conducted as part of this Doctoral Thesis work is presented, based on the proposed objectives and discussed in relation to other recently published works.

1. METABOLIC FATE OF HYDROXYTYROSOL AND RELATED COMPOUNDS

The transformation of dietary phenolic compounds during digestion and absorption has been thoroughly studied for many years (Liu, 2007; Medina-Remón, 2012; Scalbert, 2000; Teng et al., 2012). In **Figure 9** a brief diagram of the biotransformation of phenols in the body is presented and exemplified with the fate of olive phenolic compounds (HT derivatives).

Under gastric conditions, most complex phenolic compounds like oleuropein (López de las Hazas et al., 2016a) and anthocyanins (Feliciano et al., 2016) are hydrolyzed to form simple phenolic structures (phenolic acids and alcohols). These simple phenolic compounds are highly available to be widely conjugated into its phase I and especially phase II metabolites, with glucuronidation or sulfation being the most habitual biotransformations (Liu & Hu, 2007). The enzymes involved are UDP-glucuronosyltransferases (UGTs) (Shelby et al., 2003) and sulfotransferases (SULTs) (Dunn et al., 1998) respectively, which are located mainly in the liver,

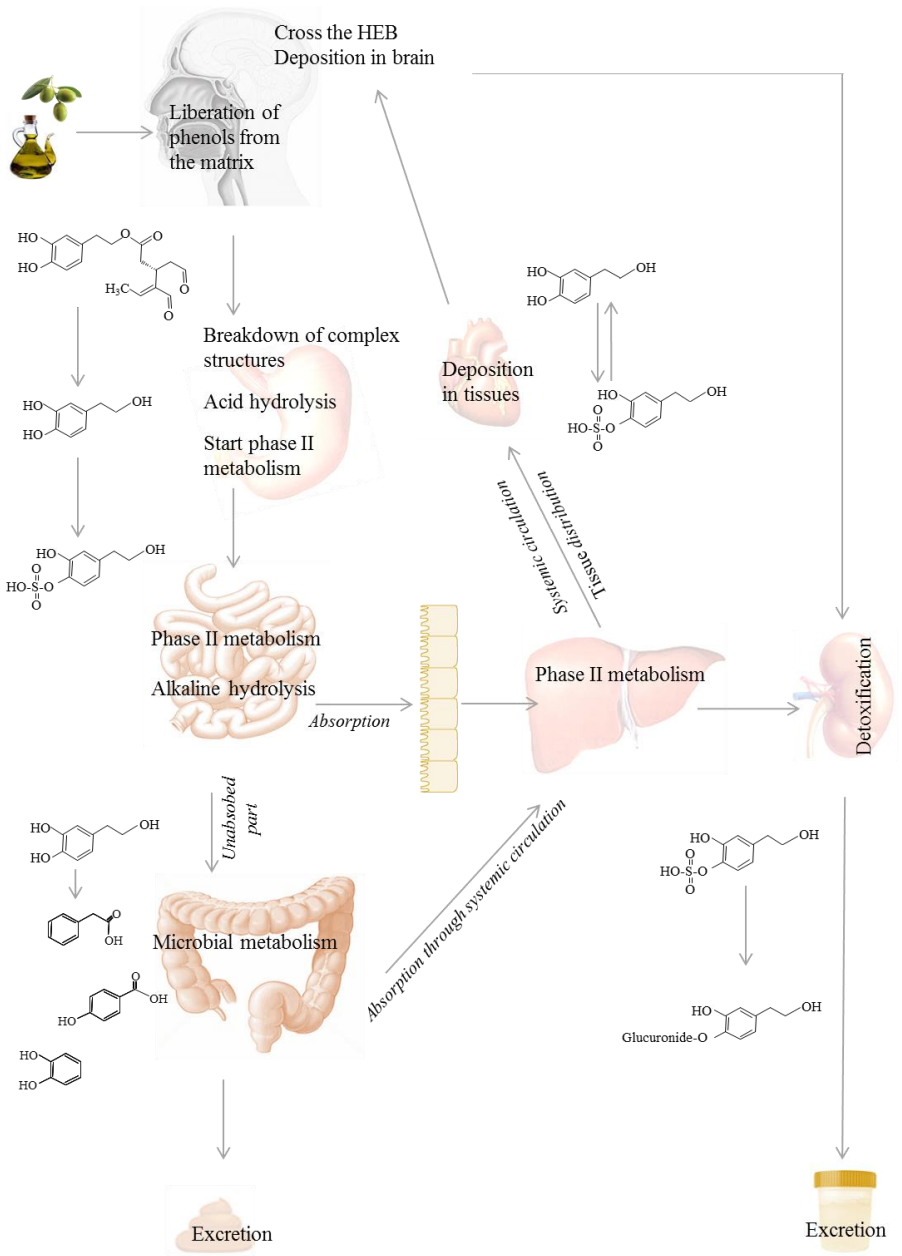


Figure 9. Diagram of the biotransformation of phenols in their pass through the tissues

kidney and intestine (Ying et al., 2015). The affinity of food native phenols to transform preferentially into one conjugate depends on the affinity of each phenolic structure. For instance, HT is preferentially transformed into its sulfate form (Catalán et al., 2015; López de Las Hazas et al., 2015; López de las Hazas et al., 2016a; Lopez de las Hazas et al., 2016b; Rubió et al., 2012). By contrast, other phenols such as quercetin are transformed preferentially into glucuronide forms (Yang et al., 2016).

Traditionally, the most suitable methods for predicting the transformation of phenolic compounds in the body had been in-vitro digestions combined with intestinal cell lines.

Nevertheless, animal models are a valuable tool for studying the metabolic fate of phenols. For this reason, the first study of the Doctoral Thesis was designed to assess the dose-effect metabolization and tissue uptake of HT. My results confirm that the metabolization of phenolic compounds is dose-dependent (**Figure 10**) (López de las Hazas et al., 2015), as previously noted by (Kotronoulas et al., 2013; Pinto et al., 2011; Visioli et al., 2000). However, in an earlier group study (Rubió et al., 2012), we observed that dietary supplementation with a daily dose of 30 mL of phenol-enriched olive oils (250 to 750 mg phenols/kg oil) promoted a dose-dependent response of phenol conjugate-metabolites in the human systemic circulation. However, the plasma pharmacokinetics of HT metabolites did not show a complete linear response after the intake of the high phenol-enriched oil (750 mg/kg) compared to the medium phenol-enriched oil (500 mg/kg). This fact could be related with a saturation of the transport systems involved in the intestinal efflux and conjugating enzymes.

Additionally, we observed that gender affects the bioavailability of HT and its metabolites, detecting a higher accumulation of compounds in female rat tissues than in male rat tissues, especially in the liver. Recently, Soukup et al. (2016) founded that levels of testosterone influenced the availability of soy isoflavones,

because androgens such as testosterone and other sex hormones regulate the expression of several UGTs and SULTs (Alnouti et al., 2011; Buckley et al., 2009; Dunn et al., 1998; Shelby et al., 2003).

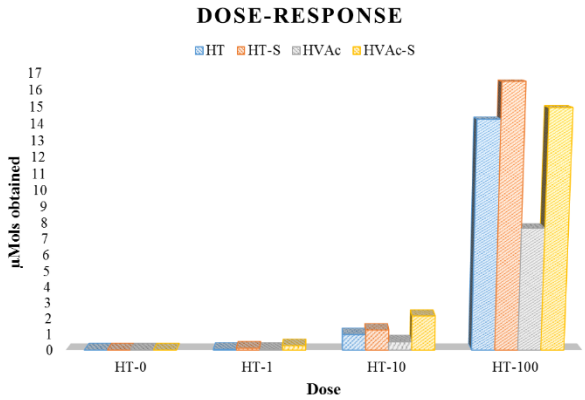


Figure 10. Chart of the plasmatic dose-response of the main phenols presents in rats

In addition, we found different plasma pharmacokinetic responses and tissue depositions of HT metabolites depending on the dose administered. **Figure 11** shows the relative percentage for each phenol of total phenolic compounds quantified in each dose in order to compare the biodistribution of the compounds.

The information obtained from the liver (**Figure 11**) help to understand the dose-dependent metabolism of compounds. At the lowest dose (HT-1), the disposition of phase I metabolites (as homovanillic acid) seems to be preferential, and they are subsequently transformed into their sulfo-conjugates. By contrast, at a higher dose (HT-10), HT is conjugated to homovanillic alcohol (HVAlc), which is also quickly transformed into homovanillic alcohol sulfate. Differences in HT metabolism could be related to the saturation of the enzymatic pathways. At the

highest dose studied, HVAc remained unmetabolized in the tissue, maybe because SULT enzymes have more affinity for HT than HVAc. It seems that, at higher doses, COMT enzymes are saturated and HT is transformed preferentially into its phase II metabolites (sulfate and glucuronide form).

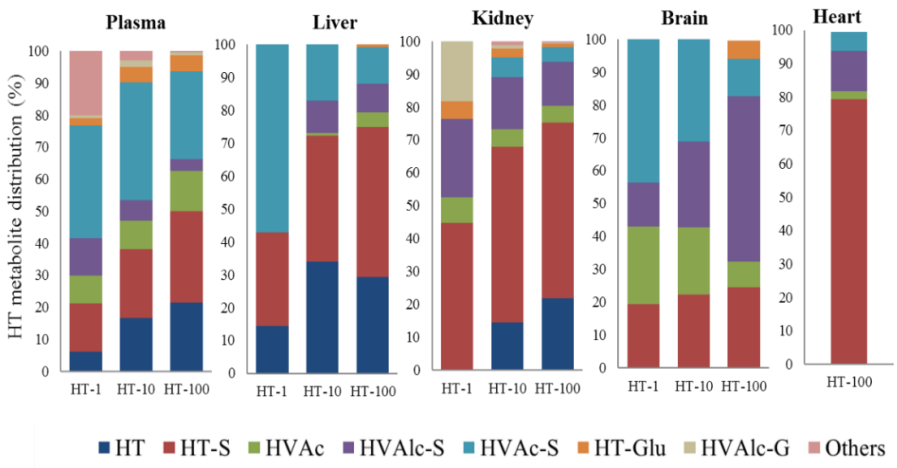


Figure 11. Uptake of metabolites in rat tissues after 5 hours of acute intake of different doses of HT

It appears that phase-II enzyme behaviour depends on the administered HT dose that determines the activation/inactivation of different metabolic enzymatic pathways (**Figure 12**).

The results of this Doctoral Thesis are consistent with the work previously published by our research group, in which we observed that after an acute intake of three different olive oils enriched with their own phenols (250 to 750 mg total phenols/kg oil), the biotransformation of HT into its metabolites did not follow a linear response of the C_{max} (Rubió et al., 2012). Of particular interest in **Figure 11** is the presence of free HT circulating in plasma after an acute intake, even at the lowest dose administered; this could be a consequence of the metabolic enzymatic system saturation by substrate. Also of note are the differences in HT metabolite

deposition depending of the tissue (D'Angelo et al., 2001; López de Las Hazas et al., 2015; López de las Hazas et al., 2016a; Serra et al., 2012).

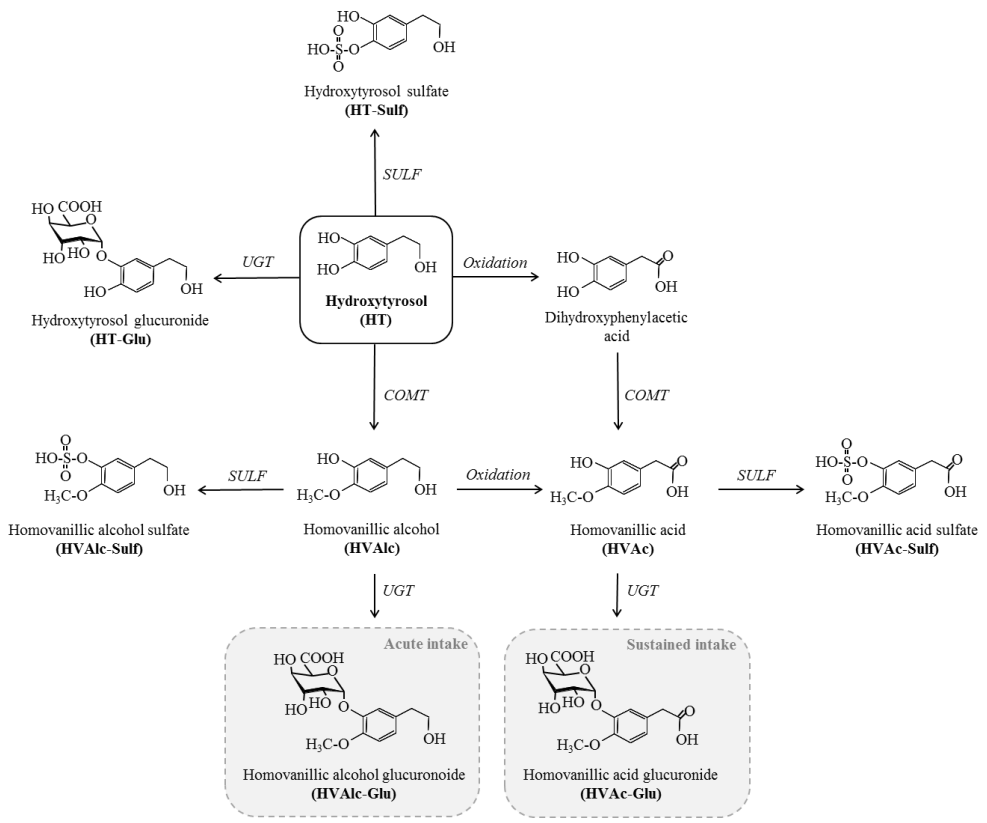


Figure 12. Proposed metabolic fate of HT in rats.

Based on the results observed we hypothesized that the differences between plasmatic and liver HT metabolites could be produced by (i) the different affinity of cell-blood interactions, which determines the plasma profile, or by (ii) the conjugative/deconjugative metabolic capacity of tissue cells. As parallel results to this Doctoral Thesis, Catalán et al. (2015) observed that the free HT was extensively metabolized into its sulfate and homovanillic derivates after HT incubation with HAEC cells. Also, we have observed that HT is extensively

metabolized to its phase II metabolites by HT-29 and Caco-2 colon cancer cells (López de las Hazas, 2016d). Similarly in a previous study, we observed that red blood cells are able to deconjugate HT plasmatic metabolites under oxidative conditions (Rubió et al., 2014). In line with these results, other authors found the hydrolysis of flavonoid glucuronides in human neutrophils (Shimoi et al., 2001) and carcinoma cell lines (Lee-Hilz et al., 2008), suggesting that the biological activity of flavonoid metabolites could be related to their deconjugation in situ, releasing the active free form. For these reasons, other authors have previously established that bioconversion of native phenols into their metabolites and vice versa depends on the kind of cells (Patel et al., 2013; Terao et al., 2011). Cell conjugation is easier than deconjugation because the free form of phenolic compounds can cross the cell membrane by passive diffusion or active process (Lançon et al., 2004), while, the internalization of the phenolic conjugates into the cells necessarily requires a transporter, and each kind of cells possesses a different gene expression and number of active transporters (Patel et al., 2013).

Coming back to the in-vivo metabolism of OO phenols, other studies in the references were designed with doses higher than the daily recommended dose and conducted using an acute intake by oral gavage or by venous injection (D'Angelo et al., 2001; Serra et al., 2012), or focused on the study of in-situ metabolism by intestinal perfusion (Edgecombe et al., 2000; Pinto et al., 2011). The lack of studies focusing on the evaluation of the effect of sustained consumption of OO phenols motivated me to conduct a second study. For this proposal, I conducted a rat diet supplementation study for 21 days at a dose of 5 mg/day of HT or its precursors oleuropein and secoiridoids based on oleuropein aglycone structures. This daily dose is similar to the equivalent human dose recommended by the EFSA (EFSA, 2011). The results of this study revealed that the chemical structure of the ingested phenol notably influenced HT bioavailability. The complex structures (oleuropein

and secoiridoids) are poorly absorbed in this native form (Edgecombe et al., 2000; López de las Hazas et al., 2016a). During digestion, these complex structures could protect HT, preventing its degradation by the effect of the gastric and intestinal conditions, resulting in a more effective absorption of HT compared to the effective bioavailability detected when HT was administered in its free form (López de las Hazas et al., 2016a). Also, it has been founded that changes in the lipophilicity of HT by adding an alkyl chain increases bioavailability across the Caco-2/TC7 cell monolayers (Pereira-Caro et al., 2010), maintaining the same bioactivity as HT or even improving it (Pereira-Caro et al., 2009, 2011). In addition, the sterochemical configuration has an influence on polyphenol bioavailability. Ottaviani et al. (2011) supplemented the diet of rats with 1.5 mg flavanols ((−)epicatechin, (−)catechin, (+)catechin) and (+)epicatechin)/kg body weight). The results of this study revealed that both epimers and enantiomers influence uptake and the flavanol metabolism, with the bioavailability being: (−)-epicatechin > (+)epicatechin = (+)catechin > (−)catechin.

Comparing the metabolism of HT after the acute or sustained dietary intake, some differences were observed (**Figure 13**). After the acute intake (one dose), HT is extensively metabolized into its phase II conjugates, but their accumulation in tissues is very low. However, the sustained consumption of HT may induce modest changes in the metabolizing capacity of phase II enzymes, resulting in the production of fewer metabolites but at higher concentration. Surprisingly, HVAlc-G was exclusively detected after the acute intake of HT while HVAc-G appeared only after the sustained intake of HT.

These observed differences in HT metabolism could be associated with differences in phase II enzyme metabolism or with differences in the feeding method; because, in the acute intake study, HT was administered by gavage dissolved in water, while in the sustained intake study, HT was administered

through food. Translating this into human terms, these differences in metabolism could be comparable to a phenol intake as nutraceutical pills or as a fortified or enriched food. It is important to highlight that the sustained intake of dietary supplements at high doses could lead to important changes in the body and might induce toxicity in certain organs. So, the uncontrolled intake of phenols without a knowledge of the side effects could represent a public health risk.

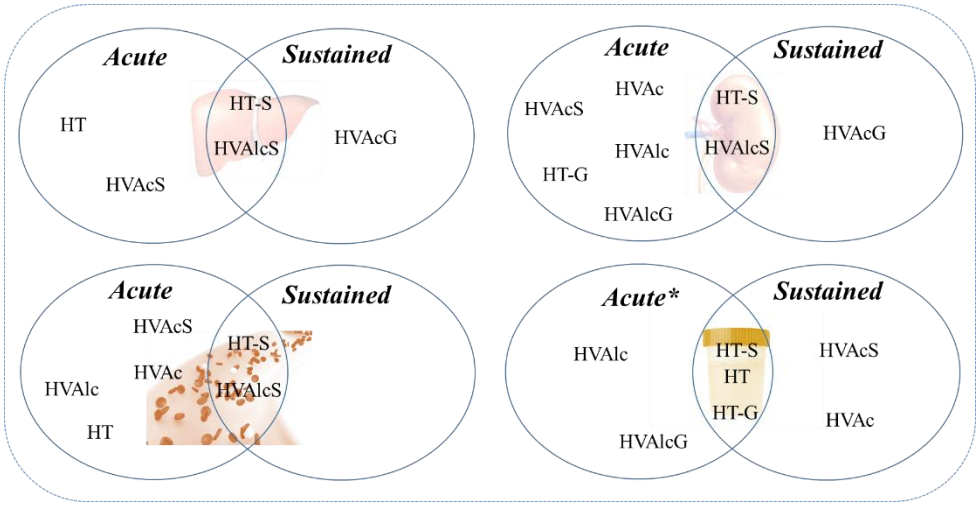


Figure 13. Qualitative comparison of the results obtained from the accumulation of HT and its metabolites in the liver through an acute intake of HT (1 mg/kg rat) (López de las Hazas, 2015) and after the sustained intake of HT (5 mg/kg rat/day) (López de las Hazas, 2016a) in the liver, kidney, plasma and urine. *These results come from Kotronoulas et al., 2013.

Although the bioavailability of phenols has been studied in animal models, it is necessary to verify the results in humans. Despite the fact that bioavailability studies are really important as predictor of phenol behaviour in the body, its use is limited because blood sampling, mainly in pharmacokinetic studies, raised important limitations: (i) it requires intensive blood sampling (numerous samples), (ii) demands special supervision of the volunteers by qualified staff and (iii) has to

be performed in specialist centres. An important contribution of this Doctoral Thesis has been the optimization of a simple blood sampling procedure for subsequent application to studies on polyphenol bioavailability in particular and on bioactive compounds in foods in general. This procedure is based on the use of dried blood spot (DSB) cards for direct blood sampling of volunteers, also simplifying the subsequent sample pre-treatment necessary for the chromatographic analysis in order to determine the circulating OO phenolic metabolites by UPLC-MS/MS (López de las Hazas, 2016b). After optimization, the procedure was validated by performing an acute intake of 120 mg of OOPCs. The main HT metabolites identified in the blood were hydroxytyrosol-3-O-sulfate, hydroxytyrosol acetate-sulfate and homovanillic alcohol-sulfate. It is important to highlight that these HT metabolites have previously been detected in plasma obtained by venipuncture (Rubió et al., 2012). Additionally, the maximum concentrations of these HT metabolites detected in complete blood were twice that detected in plasma.

2. BIOLOGICAL EFFECTS OF HYDROXYTYROSOL AND ITS BIOLOGICAL PLASMA METABOLITES

Mechanistic studies are an important tool for evaluating the effects of a pure compound or a mixture of bioactive compounds, and if they possess synergistic effects. After intake, food phenols are extensively metabolized into their phase II conjugates, which are traditionally considered pharmacologically inactive because they are less antioxidant than parent compounds. The low bioavailability of native forms of food polyphenols and their low plasma concentration leads to the consideration that conjugates could be active and produce beneficial effects (Del Rio et al., 2013). Recently, different protective mechanisms of action of native phenolic compounds have been proposed, but the effectiveness and molecular

mechanism of action of the conjugates remain unknown. The difficulty in obtaining these metabolites as standard delays the inclusion of these compounds. On this topic, another important contribution of the Doctoral Thesis has been the focus on the synthesis of HT metabolites for their subsequent use in mechanistic studies of cardiovascular protection. My first approach was to study the bioactivity of HT metabolites and compare it to that of native HT in order to understand the potential role of these compounds in cardiovascular protection. In order to achieve this goal, we designed a strategy to obtain HT conjugate-metabolites (phase II) by using of a Caco-2 cell line as a biological approximation to HT metabolism. After exposing HT to Caco-2 cells, the metabolic profile detected in cell media was similar to the human plasma profile observed in previous studies after the intake of VOO phenols. After the identification and quantification of the HT metabolite mixture, its bioactivity was tested against endothelial dysfunction in human aortic endothelial cells (HAEC) stimulated with TNF-α, and its activity was compared to that of native HT (Catalán et al., 2015). Our findings revealed that HT and its metabolites contributed to restoring the endothelial function, with decreases in V-CAM, I-CAM, E and P-Selectin. In addition, the metabolites led to a decrease in MCP-1. Actually, there are some documents in the references that support the conjugate forms as bioactive molecules. HT-S protects the intestinal cells against oxidative damage with an efficiency comparable to the native compound or even better (Atzeri et al., 2016), and the glucuronide form of HT reduces endoplasmic reticulum stress more than native HT does (Giordano et al., 2015). Also, resveratrol metabolites induce autophagy and possess antiproliferative activity in colon cancer cell lines (Patel et al., 2013).

Considering that, the concentration of the HT metabolites in plasma differs after the intake of HT or its complex form SEC, reaching the administration of SEC higher than after the intake of an equivalent dose of HT (López de las Hazas,

2016a). This diet supplementation also affects also to the modulation of the heart and aorta proteome observed in healthy rats. From this exploratory approach, we showed for the first time that HT and, especially, SEC consumption prevents events like those that induce cardiovascular disorders (preventing the migration of endothelial cells in the aorta and reducing the risk of heart failure). Besides the cardiac functions, it modulates proteins involved in lipid metabolism and cancer. Supplementation with both treatments, HT and SEC, significantly modulates the rat proteome in the same way, but SEC modulates the proteins more strongly than HT and also regulates more proteins in comparison to HT. To sum up, the in-vivo study in healthy rats indicates that a regular consumption of olive phenols modifies protein expression to improve health status. Complex phenol structures, such as SEC, may be more cardioprotective because of its higher bioavailability in relation to HT.

3. BIOTRANSFORMATION OF PHENOLS INTO THEIR MICROBIAL METABOLITES AND THEIR HEALTH EFFECT IN COLON CANCER CELLS

The unabsorbed part of dietary phenols reaches the caecum, where it is extensively biotransformed into phase I derivatives (microbial metabolites) by colonic microbiota. Additionally, food native phenols and their microbial metabolites could modulate gut microbiota composition. However, while the effects of the phenol colonic metabolites in the body have been studied very little it is known if there is a close relationship between the individual microbiota composition and the function of different organs like brain (Smith et al., 2015). Evaluating the biological activity of microbial phenol derivatives in cellular models as well as their plasma pharmacokinetic and tissue deposition are important tasks to

undertake in future mechanistic experiments. The pharmacokinetic response of these microbial metabolites starts 4 hours after phenol consumption, and they are present in the bloodstream up to 22 hours after consumption (Feliciano et al., 2016).

During the digestive transit, the close proximity of phenols and their microbial derivatives to the colon epithelium may have considerable physiological implications. These microbial compounds may contribute to a reduction in the incidence of bowel diseases, thus improving health status.

The analysis of different studies in the literature reveals that most of the microbial metabolites from phenolic compounds are in common. For this reason, the last part of the Doctoral Thesis focused on the study of the colonic metabolism of both OO phenols (López de las Hazas, 2016c) and anthocyanins (López de las Hazas, 2016d), and their potential in the prevention of colon cancer.

A previous study conducted by our research group looked into the microbial metabolites formed from OO phenols by in-vitro colonic fermentation (Mosele et al., 2014). It was found that the native phenols present in VOO are extensively metabolized, with phenylacetic acid being the main metabolite formed. Additionally, hydroxyphenylpropionic and phenylpropionic acids are formed by the colonic metabolism of HT and HT-acetate. It is important to emphasize the presence of free HT in the fermentation medium.

As an approach in the Doctoral Thesis, we followed the biotransformation of OO phenols analysing the gastric, duodenal and caecum digesta obtained from Wistar rats fed with OLE, SEC extract and free HT, respectively (López de las Hazas et al., 2016a). We found that the biotransformation of OOPCs into their microbial metabolites starts in the small intestine and increases exponentially in caecum. It was found that free HT and its sulfate derivative remained

unmetabolized in the intestinal tract and reached the colon, being excreted in faeces from the three rat groups studied (OLE, SEC and HT). However, the diversity of HT metabolites detected in rat faeces was strongly influenced by the form in which HT was ingested. Dietary supplementation with OLE resulted in a large diversity of metabolites in faeces and in a higher concentration than in supplementation with SEC or HT (López de las Hazas et al., 2016a).

In an approach to the human metabolism, Mosele et al. (2014) analysed the phenolic content of human faeces after dietary supplementation for 3 weeks with 25 mL of a phenol-enriched olive oil (500 mg/kg oil). After the supplementation period, the results showed an increase in the concentration of the 2-(4'-hydroxyphenyl)acetic, 2-(3'-hydroxyphenyl)acetic, 3-(4'-hydroxyphenyl)propionic acids in faeces, as well as in the free HT concentration. Based on the faeces metabolite profile found by Mosele et al. (2014), in this Doctoral Thesis we have evaluated the antiproliferative activity of HT colonic metabolites, individually and as a mixture, using Caco-2 and HT-29 carcinogenic colon cell lines. The main results of this study revealed that phenylacetic and hydroxyphenylpropionic acids produce cell cycle arrest and promote apoptosis.

2.1. Anthocyanins metabolism

In order to compare the antiproliferative activity of HT colonic metabolites to metabolites from other phenolic structures, we studied the antiproliferative activity of anthocyanins, one of the most common phenolic groups present in the diet, which is characterized by its complex molecular structure. Despite the fact that anthocyanins remained stable during in-vitro gastrointestinal digestion (Wang et al., 2016), some human interventional studies have shown that their bioavailability is limited (Feliciano et al., 2016; Xie et al., 2016), with the most bioavailable compounds being the phenolic acids resulting from the colonic fermentation of

undigested anthocyanins (Feliciano et al., 2016). The unmetabolized anthocyanins reach the colon where they are biotransformed into their microbial derivatives. In consequence, these colonic metabolites can reach the bloodstream and be distributed to tissues, thus producing effects that are beneficial to health.

In the last part of the Doctoral Thesis, I studied the colonic biotransformation of anthocyanins from two typical sources, grape pomace (source of malvidin-glucoside) and strawberry (source of pelargonidin-glucoside). For this proposal, the respective anthocyanin-rich extracts were submitted to an in-vitro colonic fermentation model. The results showed that during colonic fermentation, anthocyanins were completely degraded and produced syringic acid and, surprisingly, tyrosol as the main metabolites, among others. The subsequent study of the antiproliferative activity of the mixture of anthocyanin catabolites obtained after colonic fermentation of the grape pomace and strawberry extracts, respectively, using HT-29 colon cancer cells, suggests their possible contribution to the prevention of the development of colon cancer (López de las Hazas et al., 2016c)

In short, the results of this study represent a good starting point for understanding the potential apoptotic and antiproliferative effects of the colonic metabolites of two important phenolic compounds from the Mediterranean Diet: HT and derivates from VOO and anthocyanins from red grape.

4. REFERENCES

Alnouti, Y., & Klaassen, C. D. (2011). Mechanisms of gender-specific regulation of mouse sulfotransferases (Sults). *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *41*(3), 187–97.

Atzeri, A., Lucas, R., Incani, A., Peñalver, P., Zafra-Gómez, A., Melis, M. P., … Torre, R. de la. (2016). Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells. *Food Funct.*, *7*(1), 337–346.

Buckley, D. B., & Klaassen, C. D. (2009). Mechanism of gender-divergent UDP-glucuronosyltransferase mRNA expression in mouse liver and kidney. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *37*(4), 834–40.

Cárdeno, A., Sánchez-Hidalgo, M., & Alarcón-de-la-Lastra, C. (2013). An up-date of olive oil phenols in inflammation and cancer: molecular mechanisms and clinical implications. *Current Medicinal Chemistry*, *20*(37), 4758–76.

Catalán, Ú., López de Las Hazas, M.-C., Rubió, L., Fernández-Castillejo, S., Pedret, A., de la Torre, R., … Solà, R. (2015). Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. *Molecular Nutrition & Food Research*, *59*(12), 2523–36.

Covas, M.-I., Nyyssönen, K., Poulsen, H. E., Kaikkonen, J., Zunft, H.-J. F., Kiesewetter, H., … Marrugat, J. (2006). The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Annals of Internal Medicine*, *145*(5), 333–41.

D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O., Morrica, P., Capasso, G., … Zappia, V. (2001). Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *29*(11), 1492–8.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling*, *18*(14), 1818–92.

Dunn, R. T., & Klaassen, C. D. (1998). Tissue-Specific Expression of Rat Sulfotransferase Messenger RNAs. *Drug Metabolism and Disposition*, *26*(6).

Edgecombe, S. C., Stretch, G. L., & Hayball, P. J. (2000). Oleuropein, an antioxidant polyphenol from olive oil, is poorly absorbed from isolated perfused rat intestine. *The Journal of Nutrition*, *130*(12), 2996–3002.

Feliciano, R., Istas, G., Heiss, C., & Rodriguez-Mateos, A. (2016). Plasma and Urinary Phenolic Profiles after Acute and Repetitive Intake of Wild Blueberry. *Molecules*, *21*(9), 1120.

Feliciano, R. P., Boeres, A., Massacessi, L., Istas, G., Ventura, M. R., Nunes dos Santos, C., … Rodriguez-Mateos, A. (2016). Identification and quantification of novel cranberry-derived plasma and urinary (poly)phenols. *Archives of Biochemistry and Biophysics*, *599*, 31–41.

Giordano, E., Dangles, O., Rakotomanomana, N., Baracchini, S., Visioli, F., Visioli, F., … Visioli, F. (2015). 3-O-Hydroxytyrosol glucuronide and 4-O-hydroxytyrosol glucuronide reduce endoplasmic reticulum stress in vitro. *Food Funct.*, *6*(10), 3275–3281.

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Sanchez-Rovira, P., & Ramirez-Tortosa, M. C. (2010). Hydroxytyrosol: from laboratory investigations to future clinical trials. *Nutrition Reviews*, *68*(4), 191–206.

Konstantinidou, V., Covas, M.-I., Muñoz-Aguayo, D., Khymenets, O., de la Torre, R., Saez, G., … Fito, M. (2010). In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *24*(7), 2546–57.

Kotronoulas, A., Pizarro, N., Serra, A., Robledo, P., Joglar, J., Rubió, L., … de la Torre, R. (2013). Dose-dependent metabolic disposition of hydroxytyrosol and formation of mercapturates in rats. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society*, *77*, 47–56.

Lançon, A., Delma, D., Osman, H., Thénot, J.-P., & Latruffe, B. J. N. (2004). Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochemical and Biophysical Research Communications*, *316*(4), 1132–1137.

Lee-Hilz, Y. Y., Stolaki, M., van Berkel, W. J. H., Aarts, J. M. M. J. G., & Rietjens, I. M. C. M. (2008). Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation. *Food and Chemical Toxicology*, *46*(6), 2128–2134.

Liu, Z., & Hu, M. (2007). Natural polyphenol disposition via coupled metabolic pathways. *Expert Opinion on Drug Metabolism & Toxicology*, *3*(3), 389–406.

López de las Hazas, M.-C., Piñol, C., Macià, A., Romero, M.-P., Pedret, A., Solà, R., … Motilva, M.-J. (2016a). Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids. *Journal of*

Functional Foods, *22*, 52–63.

López de las Hazas, M. C., Motilva, M. J., Piñol, C., & Macià, A. (2016b). *Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood*. *Talanta* (Vol. 159).

López de las Hazas, M.-C., Piñol, C., Macià, A., Motilva, M.-J. (2016c). *Hydroxytyrosol and its colonic metabolites produce apoptosis and increase cell death in colon cancer cells*. *Journal of Agricultural and Food Chemistry* Under corrections

López de las Hazas, M.-C., Mosele, J. I., Macià, A., Ludwig, I. A., & Motilva, M.-J. (2016d). Exploring the Colonic Metabolism of Grape and Strawberry Anthocyanins and Their in Vitro Apoptotic Effects in HT-29 Colon Cancer Cells. *Journal of Agricultural and Food Chemistry*, acs.jafc.6b04096.

López de Las Hazas, M.-C., Rubió, L., Kotronoulas, A., de la Torre, R., Solà, R., & Motilva, M.-J. (2015). Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats. *Molecular Nutrition & Food Research*.

Medina-Remón, A., Tresserra-Rimbau, A., Arranz, S., Estruch, R., & Lamuela-Raventos, R. M. (2012). Polyphenols excreted in urine as biomarkers of total polyphenol intake. *Bioanalysis*, *4*(22), 2705–13.

Mosele, J. I., Martín-Peláez, S., Macià, A., Farràs, M., Valls, R.-M., Catalán, U., & Motilva, M.-J. (2014). Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches. *Molecular Nutrition & Food Research*, *58*(9), 1809–19.

Ottaviani, J. I., Momma, T. Y., Heiss, C., Kwik-Uribe, C., Schroeter, H., & Keen,

C. L. (2011). The stereochemical configuration of flavanols influences the level and metabolism of flavanols in humans and their biological activity in vivo. *Free Radical Biology & Medicine, 50*(2), 237–44.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Würtele, G., Spiegelhalder, B., & Bartsch, H. (2000). Olive-oil consumption and health: the possible role of antioxidants. *The Lancet Oncology, 1*(2), 107–112.

Patel, K. R., Andreadi, C., Britton, R. G., Horner-Glister, E., Karmokar, A., Sale, S., … Brown, K. (2013). Sulfate Metabolites Provide an Intracellular Pool for Resveratrol Generation and Induce Autophagy with Senescence. *Science Translational Medicine, 5*(205), 205ra133-205ra133.

Pereira-Caro, G., Madrona, A., Bravo, L., Espartero, J. L., Alcudia, F., Cert, A., & Mateos, R. (2009). Antioxidant activity evaluation of alkyl hydroxytyrosyl ethers, a new class of hydroxytyrosol derivatives. *Food Chemistry, 115*(1), 86–91.

Pereira-Caro, G., Mateos, R., Saha, S., Madrona, A., Espartero, J. L., Bravo, L., & Kroon, P. A. (2010). Transepithelial Transport and Metabolism of New Lipophilic Ether Derivatives of Hydroxytyrosol by Enterocyte-like Caco-2/TC7 Cells. *Journal of Agricultural and Food Chemistry, 58*(21), 11501–11509.

Pereira-Caro, G., Sarriá, B., Madrona, A., Espartero, J. L., Goya, L., Bravo, L., & Mateos, R. (2011). Alkyl hydroxytyrosyl ethers show protective effects against oxidative stress in HepG2 cells. *Journal of Agricultural and Food Chemistry, 59*(11), 5964–76.

Pinto, J., Paiva-Martins, F., Corona, G., Debnam, E. S., Jose Oruna-Concha, M., Vauzour, D., … Spencer, J. P. E. (2011). Absorption and metabolism of olive

oil secoiridoids in the small intestine. *The British Journal of Nutrition*, *105*(11), 1607–18.

Rigacci, S., & Stefani, M. (2016). Nutraceutical Properties of Olive Oil Polyphenols. An Itinerary from Cultured Cells through Animal Models to Humans. *International Journal of Molecular Sciences*, *17*(6), 843.

Rodríguez-Morató, J., Xicota, L., Fitó, M., Farré, M., Dierssen, M., & de la Torre, R. (2015). Potential Role of Olive Oil Phenolic Compounds in the Prevention of Neurodegenerative Diseases. *Molecules*, *20*(3), 4655–4680. http://doi.org/10.3390/molecules20034655

Rubió, L., Macià, A., Valls, R. M., Pedret, A., Romero, M.-P., Solà, R., & Motilva, M.-J. (2012). A new hydroxytyrosol metabolite identified in human plasma: hydroxytyrosol acetate sulphate. *Food Chemistry*, *134*(2), 1132–6.

Rubió, L., Serra, A., Macià, A., Piñol, C., Romero, M.-P., & Motilva, M.-J. (2014). *In vivo distribution and deconjugation of hydroxytyrosol phase II metabolites in red blood cells: A potential new target for hydroxytyrosol*. *Journal of Functional Foods* (Vol. 10).

Rubió, L., Valls, R.-M., Macià, A., Pedret, A., Giralt, M., Romero, M.-P., … Motilva, M.-J. (2012). Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites. *Food Chemistry*, *135*(4), 2922–9.

Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition*, *130*(8S Suppl), 2073S–85S.

Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol

concentrations (ID 1639), mainte. (2011). *EFSA Journal*, *9*(4), 2033.

Serra, A., Rubió, L., Borràs, X., Macià, A., Romero, M.-P., & Motilva, M.-J. (2012). Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. *Molecular Nutrition & Food Research*, *56*(3), 486–96.

Shelby, M. K., Cherrington, N. J., Vansell, N. R., & Klaassen, C. D. (2003). Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *31*(3), 326–33.

Shimoi, K., Saka, N., Nozawa, R., Sato, M., Amano, I., Nakayama, T., & Kinae, N. (2001). Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *29*(12), 1521–4.

Smith, P. A. (2015). The tantalizing links between gut microbes and the brain. *Nature*, *526*(7573), 312–314.

Soukup, S. T., Müller, D. R., Kurrat, A., Diel, P., & Kulling, S. E. (2016). Influence of testosterone on phase II metabolism and availability of soy isoflavones in male Wistar rats. *Archives of Toxicology*, 1–13.

Teng, Z., Yuan, C., Zhang, F., Huan, M., Cao, W., Li, K., … Mei, Q. (2012). Intestinal absorption and first-pass metabolism of polyphenol compounds in rat and their transport dynamics in Caco-2 cells. *PloS One*, *7*(1), e29647.

Terao, J., Murota, K., & Kawai, Y. (2011). Conjugated quercetin glucuronides as bioactive metabolites and precursors of aglyconein vivo. *Food Funct.*, *2*(1), 11–17.

Visioli, F., Galli, C., Bornet, F., Mattei, A., Patelli, R., Galli, G., & Caruso, D. (2000). Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Letters*, *468*(2), 159–160.

Wang, S., Amigo-Benavent, M., Mateos, R., Bravo, L., & Sarriá, B. (2016). Effects of *in vitro* digestion and storage on the phenolic content and antioxidant capacity of a red grape pomace. *International Journal of Food Sciences and Nutrition*, 1–13.

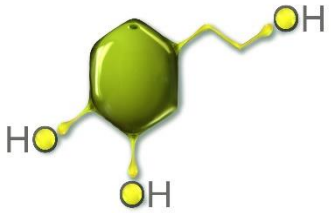
Weinbrenner, T., Fitó, M., de la Torre, R., Saez, G. T., Rijken, P., Tormos, C., … Covas, M.-I. (2004). Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. *The Journal of Nutrition*, *134*(9), 2314–21.

Xie, L., Lee, S. G., Vance, T. M., Wang, Y., Kim, B., Lee, J.-Y., … Bolling, B. W. (2016). Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract. *Food Chemistry*, *211*, 860–868.

Yang, L.-L., Xiao, N., Li, X.-W., Fan, Y., Alolga, R. N., Sun, X.-Y., … Ohara, K. (2016). Pharmacokinetic comparison between quercetin and quercetin 3-O-β-glucuronide in rats by UHPLC-MS/MS. *Scientific Reports*, *6*, 35460.

Ying L. H., Shi Ho Q., Ping W. K. Interplay of metabolizing enzymes and transporter of xenobiotics. *Xenobiotica*, 46, 1, 201

Conclusions



CONCLUSIONS

The structure of the Conclusions section is organized in accordance with the different chapters of the Doctoral Thesis, and the main achievements of the research are summarized under each one:

Metabolic fate of hydroxytyrosol and related compounds

I. The acute administration of different doses of hydroxytyrosol in rats follows a dose-response effect on bioavailability affecting the proportion of the different circulating plasma hydroxytyrosol metabolites and their tissue disposition.

II. The administration of hydroxytyrosol or hydroxytyrosol complex structures (oleuropein and secoiridoids) critically affects the concentration of the plasma hydroxytyrosol metabolites. Oleuropein was the precursor yielding the highest number and concentration of plasma hydroxytyrosol metabolites.

III. A fast and simple blood sampling and sample pre-treatment method based on the use of the dried blood spot (DBS) cards combined with ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) has been applied for the quantification of olive oil phenolic metabolites in human blood after the acute intake of a phenolic extract rich in secoiridoids. DBS sampling can be considered a good candidate to be applied, in the near future, for determining phenolic metabolites circulating in dose-response or epidemiological studies.

Biological effects of hydroxytyrosol and its biological plasma metabolites

IV. Biological hydroxytyrosol metabolites have been synthetized for the first time by the exposition of hydroxytyrosol to differentiated Caco-2 cell line, obtaining a purified extract that resembles the human plasma hydroxytyrosol metabolic profile after virgin olive oil intake.

V. The exposition of the purified extract of hydroxytyrosol metabolites, at physiological doses, to endothelial cells (HAEC cell line) has demonstrated the efficacy in the reduction of the endothelial dysfunction biomarkers (E-Selectin, P-selectin, V-CAM and I-CAM) similarly as the effect of native hydroxytyrosol; also, the mix of hydroxytyrosol metabolites reduced the expression of the MCP-1. These data suggesting the contribution of the hydroxytyrosol metabolites in the protection against endothelial dysfunction and reducing the risk in the early stages of atherosclerosis development.

VI. The sustained administration of hydroxytyrosol and secoiridoids in healthy rats induces positive changes in the expression of relevant proteins of aorta tissue, related to atherosclerotic processes such as proliferation and migration of endothelial cells and occlusion of blood vessels. In heart, other relevant proteins related to cardiac functions such as heart failure are also positively modulated. Other prognostic markers for some cancers appeared to be modulated after HT and SEC treatments, suggesting that olive oil phenols could also exert a protector effect against cancer.

VII. Diet supplementation with SEC demonstrated higher fold changes values in the protein expression that could be attributed to the higher concentration of HT detected in heart tissue as consequence of the more effective bioavailability of SEC. These results suggest that SEC, the main phenolics present in VOO, could have a higher cardio-protective effect than free HT.

Biotransformations of phenols into their microbial metabolites and their health effect in colon cancer cells

VIII. The unabsorbed phenols derived from the sustained consumption of virgin olive oil are transformed in the intestine by microbials into its catabolites (phenylpropionic, hydroxyphenylpropionic, dihydroxyphenylpropionic phenylacetic acids and catechol). These catabolites may display a role in the prevention of cancer development showing phenylacetic and hydroxyphenylpropionic acids antiproliferative and pro-apoptotic activities similarly to hydroxytyrosol in colonic cancer cells suggesting their contribution on maintaining the healthy state of colon.

IX. Red wine and strawberries are a rich diet source of anthocyanins (malvidin and pelargonidin-glucosides respectively) which are poorly absorbed after its consumption and reach the gut at high concentrations. In an invitro colonic fermentation model, human gut microbiota extensively metabolize the digested extracts rich in malvidin and pelargonidin-glucosides producing syringic acid and tyrosol as the main metabolites. The malvidin- and pelargonidin-

glucosides and the formed metabolites may contribute to prevent the colon cancer by its ability to induce cell apoptosis in colonic cancer cells.