POMPEU FABRA UNIVERSITY
Department of Experimental and Health Science

HEALTH BENEFITS OF OLIVE OIL: CONTRIBUTION OF PHENOLIC COMPOUNDS AND TRANSCRIPTOMIC RESPONSE IN HUMANS

DOCTORAL THESIS

Olha Khymenets

Neurophsycopharmacology Programme
Hospital del Mar Research Institute
(IMIM)

Barcelona, April 2010

POMPEU FABRA UNIVERSITY
Department of Experimental and Health Science

Doctoral Programme: Health and Life Science

HEALTH BENEFITS OF OLIVE OIL: CONTRIBUTION OF PHENOLIC COMPOUNDS AND TRANSCRIPTOMIC RESPONSE IN HUMANS

Dissertation presented by Olha Khymenets to obtain the PhD degree from the Pompeu Fabra University. This work was carried out under the supervision of Rafael de la Torre in the Neurophsycopharmacology Programme, Hospital del Mar Research Institute (IMIM).

Rafael de la Torre **Olha Khymenets**

Barcelona, April 2010

Присвячується моїм батькам,
Вірі та Миронові Хименець.

Dedicated to my parents,
Vira and Myron Khymenets.

ACKNOWLEDGEMENTS

After all those years, I have got quite a list of people who one way or another contributed to this thesis, for which I would like to express thanks.

Foremost, I would like to thank Dr. Rafael de la Torre, the director of this PhD. thesis, for his continual support, for his advice and expertise throughout this study and for the trust he always placed on me. His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a good basis for the present thesis.

During this work I have collaborated with many colleagues for whom I have great regard, and I wish to express my sincere thanks to all those who have helped me with my work in IMIM, CSIC, UPF, and beyond.

I am deeply grateful to Dr. Maria-Isabel Covas for her detailed and constructive comments, and for her important support throughout this work. My warm thanks are due to Dr. Montserrat Fitó and other people form ULEC who collaborate in the work on this thesis. Their kind support and guidance have been of great value in this study.

I would like to express my sincere gratitude to Dr Joglar J. for all his help over the years; especially his knowledge and expertise, critical reading and always having time for me. Dr José Lluis Torres and Dr Pere Clapés, and fellow research staff are gratefully acknowledged for their collaborative work and use of laboratory facilities at CSIC. All my ex-labmates and friends from the CSIC for their hospitality, friendship, readiness to help and for all the funny things we have enjoyed together.

I wish I would have place for personal thanks to all my mates and ex-mates from our department (IMIM): for their help, their companionship,

and for all nice moments we shared within the department walls and outside, and which I will never forget.

I wish to thank everybody with whom I have shared experiences in life over all those years. To people, who accompanied me in my work, who have made Barcelona a very special place to live, and who made my days more enjoyable. Special thanks to all my friends, for their permanent support, and for all the great moments and the laughs we have enjoyed together during these years.

Financial support from the Fundació IMIM (FIMIM) in printing of the thesis is appreciatively recognised.

I cannot finish without saying how grateful I am with my family. I wish to thank my parents for their faith in me, for all love and support they have always given me that I will never be able to acknowledge enough. To them I dedicate this work. A special mention to my sister and her family: for their loving support.

// Неможливо завершити без висловлення моєї вдячності усій моїй родині. Моя найбільша та найглибша подяка - моїм батькам. За їхню віру в мене, за безмежну батьківську любов і постійну підтримку, які завжди дарували мені і котрі я особливо ціную зараз, так далеко від тепла батьківських обійм. Вам, дорогі мої, присвячую цю працю. Особлива подяка моїй сестричці та її родині за їхню теплу і люблячу підтримку. //

And finally last but not least to Jesús: for loving me, for helping me always in everything he can, for making everyday special, for all great moments we have enjoyed together, for making me feel *at home.*

FINANTIAL SUPPORT

- PREDIMED network (ISCIII G03/140)

- QLK1-CT-2001-00287 from the European Commission (EUROLIVE)

- *MIPPAO* MCYT (SAF2004-08173-C03-00)

- CIBER de Fisiología de Obesidad y Nutrición (CIBEROBN) is sponsored by ISCIII

- Generalitat de Catalunya (AGAUR 2009 SGR 718)

- D'ajut per a la finalització de la tesi doctoral, FIMIM - Fundació IMIM

ABBREVIATIONS

3,4-DHPEA-EA - EA linked to HOTYR (DHPEA), OLE-aglycon;

3,4-DHPEA-EDA - dialdehydic form of EA linked to HOTYR (DHPEA);

8-epi-PGF$_{2\alpha}$ - 8-epi prostaglandin-F$_{2\alpha}$, F$_{2\alpha}$-isoprostanes;

8-OH-dG - 8-hydroxy-7,8-dihydro-2´-deoxyguanosine;

ABST - 2,2-azinobis-(3-ethyl-benzo- thiazoline-6-sulfonic acid)**;**

ADH - alcohol dehydrogenase;

ALDH - aldehyde dehydrogenase;

ALR - aldehyde/aldose reductase;

AO - antioxidant;

AP-1 - activator protein 1;

ApoB - apolipoprotein B;

ArOH - phenolic antioxidant;

AT - atherosclerosis;

BDE - Bond Dissociation Enthalpy;

BSTFA - bis-trimetylsilyl-trifluoro-acetamide;

CAE - caffeic acid equivalent;

CAT - catalase;

CD - conjugated dienes;

cDNA - complementary DNA;

CF - concentration factor;

Cl-Tyr - 3-chlorotyrosine;

CO - corn oil;

COMT - catechol-*O*-methyl transferase;

COO - common olive oil;

COX - cyclooxygenase;

CRP - C-reactive protein;

CVD - cardio-vascular disease;

CYP - cytochrome P;

DHPEA-EA - OLE-aglycon;

DF - dilution factor;

Di-Tyr - *o*,*o*-dityrosine;

DMPD - N, N'-Dimonomethylphenyl-p-phenylenediamine;

DOPAC - 3,4-dihydroxy-phenylacetic acid;

DOPAL - 3,4-dihydroxy-phenylacetaldehyde;

DOPET - 3,4-dihydroxy-phenylethanol (DHPEA), hydroxytyrosol;

DPPH - 1,1-diphenyl-2-picrylhydrazyl radical**;**

EA - elenolic acid;

ESI - electro spray ionization;

ET - electron transfer;

EVOO - extra virgin olive oil;

FP - fluorescence detection;

FRAP - ferric reducing ability of plasma;

FVIIa - activated factor VII;
GAE - gallic acid equivalent;
GAPDH - glyceraldehyde 3-phosphate dehydrogenase;
GC - gass chromatography;
GE - gene expression;
GI - gastro-intestinal**;**
GO - gene ontology;
GSH - glutathione reduced;
GSH-Px - glutathione peroxidase;
GS-R - glutathione reductase;
GSSG - glutathione oxidized;
HAT - H-atom transfer;
HDL - high density lipoproteins;
HDL-C - HDL cholesterol;
HMG-CoA - 3-hydroxy-3-methyl-glutaryl-CoA reductase;
HNE - 4-hydroxy-2-nonenal;
HOTYR - hydroxytyrosol; 3,4-dihydroxyphenylethanol (DHPEA);
HOTYRAc - hydroxytyrosol acetate;
HPC - high phenolic content;
HPLC - high performance liquid chromatography;
HVA - homovanillic acid;
HVAlc - homovanillyl alcohol;
ICAM - intercellular adhesion molecule;
IL - interleukin;
INF-γ - interferon gamma;
iNOS - inducible nitric oxide synthase;
IP - Ionization Potencial;
I.S. - internal standard;
IVI - intra venous injection;
LC - liquid chromatography;
LDL - low density lipoprotein;
LDL-C - LDL cholesterol;
LGS - ligstroside; TYR (*p*-HPEA) ester of EA-glucoside;
LLE - liquid-liquid extraction;
LOD - limit of detection;
LOQ - limit of quantification;
LPC - low phenolic content;
LPO - lipoperoxidase;
LTB(4) - leukotriene B_4;
MAO - monoaminoxidase;
MAPK - mitogen-activated protein kinase;
MDA - malondialdehyde;
mitDNA - mitochondrial DNA;
MNC - mononuclear cells;
MOPET - 3-hydroxy-4-methoxyphenylethanol, HVAlc;
MPC - medium phenolic content;
mRNA - messenger RNA;
MS - mass spectrometry;
MS-MS - tandem mass spectrometry;
MSTFA - N-methyl-N-trifluoro-acetamide;
MUFA - monounsaturated fatty acid;

NADPH - nicotinamide adenine dinucleotide phosphate;

NF-κB - nuclear factor-light-chain-rnhancer of activated B cells;

NMR - nuclear magnetic resonance;

$NO_{(x)}$ - nitrates/nitrites;

NO-Tyr - 3-nitrotyrosine;

NR - not reported;

OA - orally administrated;

OD - oxidative damage;

OLE - oleuropein; HOTYR (DHPEA) ester of EA-glucoside;

OO - olive oil;

OOPhEx - olive oil phenolic extracts;

ORAC - oxygen radical absorbance capacity;

OS - oxidative stress;

oxLDL - oxidized LDL;

P-(I-VI) - publication (I-VI);

PAI-1 - plasminogen activator inhibitor-1;

PAP - 3´-phosphoadenosine-5´-phosphate;

PAPS - 3´-phosphoadenosine-5´-phosphosulfate;

***p*-HPEA-EA -** EA linked to TYR (*p*-HPEA), LGS-aglycon;

***p*-HPEA-EDA -** dialdehydic form of EA linked to TYR (*p*-HPEA);

PKC - kinase protein kinase C;

PTP - protein tyrosine phosphatase;

qPCR - quantitative PCR;

Ref - reference;

RIN - RNA integrity number;

ROO - refined olive oil;

RP - reverse phase;

rRNA - ribosomal RNA;

RS - reactive species;

RT - reverse transcription;

SAH - S-adenosyl homocystein;

SAM - S-adenosyl methionine;

sICAM - soluble ICAM;

SIM - selective ion monitoring;

SO - sunflower oil;

SOD - superoxide dismutase;

SPE - solid phase extraction;

SRM - selective reaction monitoring;

SULT - sulphotransferase;

sVCAM - soluble VCAM;

TC - total cholesterol;

TG - triglyceride;

TGL - TG rich lipopritein;

TNF-α - tumor necrosis factor alfa;

TRL - triglyceride rich lipoproteins;

TXB(2) - thtomboxane B_2;

TYR - tyrosol; 4-hydroxy-phenylethanol (*p*-HPEA);

UDPGA - uridine diphosphate glucuronic acid;

UDPGT - UDP-glucuronosyl transferase;

UDP - uridinediphosphate;

UPLC - ultra performance liquid chromaptography;

UV - ultra violet;

VCAM - vascular adhesion molecule;

VOO - virgin olive oil;

WB - whole blood;

WHO - World Health Organization;

ABSTRACT

The evaluation of olive oil antioxidants, hydroxytyrosol and tyrosol, *in vivo* biological activities is challenged due to scarce data on their metabolic disposition and activities of their glucuronides, main metabolites found in humans in different biological matrices after olive oil consumption. In addition, the *in vivo* gene expression activity of virgin olive oil (VOO) as a dietary component has been never investigated in humans. Therefore, this thesis was focused on three main aspects: (i) analysis of bioavailability of hydroxytyrosol and tyrosol glucuronides in humans; (ii) evaluation of the impact of glucuronidation on antioxidant activities of olive oil phenolics; and (iii) identification mechanisms underlying beneficial action of VOO analysing induced *in vivo* transcriptome response in humans. To complete with the objectives, the glucuroconjugated standards, required for bioavailability and antioxidant activities studies were synthesized, and the preparative methodological studies for VOO-transcriptomic experiment were carried out. As a result of experimental work performed within this dissertation, the glucuronidation was shown to account for 75% of recuperated in urine olive oil phenols, and to have negative impact on their antioxidants activities, diminishing their antiradical and inhibitory against LDL oxidation activities. The transcriptome studies revealed 10 genes as potential targets of VOO action against atherosclerosis.

ABSTRACT (Català)

La avaluació *in vivo* de les activitats biològiques dels polifenols del oli d'oliva (OVV) hidroxitirosol i tirosol es un repte degut a les dades molt limitades que tenim de la seva depuració metabòlica i de les activitats biològiques dels seus principals metabòlits en matrius biològiques: els seus glucuronoconjugats. A més a més s'ha avaluat l'expressió gènica induïda en humans per la ingesta de OOV. Així la present tesi doctoral s'ha focalitzat en els següents aspectes: l'avaluació de la biodisponibilitat del hidroxitirosol i tirosol en humans; l'impacte de la glucuronoconjugació sobre les activitats antioxidants dels polifenols del OOV; i la identificació dels mecanismes subjacents a las accions benèfiques per la salut humana, analitzant la resposta transcriptòmica in vivo resultant de la ingesta OOV. Per complir amb els objectius de la tesi, ha estat necessari, sintetitzar patrons dels glucurònids i realitzar diversos estudis metodològics per tal d'estandarditzar l'avaluació de l'expressió gènica. S'ha demostrat que la glucuronoconjugació es un 75% dels polifenols recuperats en orina i que aquesta comporta la pèrdua de la capacitat bescanviadora de radicals i de la seva capacitat antioxidant (test *ex-vivo* d'oxidació de la LDL i DPPH). Els estudis transcriptòmics han detectat 10 gens rellevants pels efectes antiateroscleròtics induïts per OVV.

CONTENTS

PREFACE

HEALTH BENEFITS OF OLIVE OIL: CONTRIBUTION OF PHENOLIC COMPOUNDS AND TRANSCRIPTOMIC RESPONSE IN HUMANS.

Oxidative damage of tissue and cellular components is a primary or secondary causative factor in many different human diseases (e.g. cardiovascular, cancer, diabetes…) and aging processes (Cutler, 2005a). The oxidative stress status is under tight regulatory control for most individuals over a wide range of lifestyle variables including diet and exercise.

It has been shown that the elevated oxidative stress in individuals could be lowered to a normal level by antioxidant supplements (Cutler, 2005a). This fact has some clinical implications but also brings another important message about how the daily food intake, simply being rich in natural antioxidants, could prevent and defend our organism against incidence of specific age-dependent diseases.

There are growing scientific evidences supporting the beneficial effects of the Mediterranean diet on human fitness. It has been observed that this type of diet lowers incidence of coronary heart diseases (Katan, 1995) and of some types of tumours (Willett, 1995) and prevents from development of cardiovascular diseases (De Logeril, 1999). The health properties of the Mediterranean diet were attributed to a large amount of plant foods consumption and to a regular use of olive oil, as the main source of fat.

Olive oil composition includes a large proportion of unsaturated fatty acids (oleic, linoleic and linolenic acids), micronutrients, represented mainly by vitamins (A, E and β-carotene), and microconstituents (e.g. phenolic compounds or chemicals present in the unsaponificable fraction). Although the main health beneficial effects of olive oil have been primarily attributed to well-known chemicals with antioxidant properties, such as tocopherols and β-carotene, and to its unsaturated fatty acids composition, the phenolic micronutrients may also play a significant role (Covas, 2006b). The total phenol content in virgin olive oil has been reported to vary from 100 mg/kg to 1 g/kg (Tsimidou, 1998).

A set of intervention experiments on human volunteers has provided preliminary results showing a significant contribution of phenols to beneficial effects of olive oil (Fitó, 2002, 2005; Marrugat, 2004; Weinbrenner; 2004a). These studies indicate that they are actively involved in the modulation of the oxidative/antioxidative status in humans and that they are able to produce changes in oxidative stress biomarkers at postprandial state in a dose-dependent manner in a dose range compatible with their dietary intake. Therefore, they may account for the protection of the endogenous antioxidant defences. These findings support the hypothesis that olive oil consumption could provide benefits in the prevention of oxidative processes in humans.

The main phenolic compounds in olives are the glycosilated forms of oleuropein and ligstroside (Bleas, 2002; Brenes, 1999). The glucose residue is removed by enzymatic hydrolysis giving rise to

the aglycone forms of both compounds. In olive oil under acidic conditions, both oleuropein and ligstroside give rise to the polar phenolic compounds hydroxytyrosol (HOTYR) and tyrosol (TYR) (Brenes, 2001). HOTYR may also be the product of the enzymatic hydrolysis of its own corresponding glycoside (Rometo, 2002). Free forms of TYR and HOTYR and their secoroid derivatives have been described as representing around 30%, and other conjugated forms such as oleuropein and ligstroside aglycones represent almost half of the total phenolic content of a virgin olive oil (Owen, 2000).

All olive oil phenolic compounds are expected to have strong antioxidant activities due to their chemical structures. Their antioxidant capability is defined by the potent redox properties of phenolic hydroxyl groups and the structural relationships in the chemical configuration of molecules (Cheng, 2002). Phenolic compounds can scavenge free radicals derived from molecular oxygen and attenuate the oxidative stress (Visioli, 2002). Therefore, it was believed that the additive and synergistic effects of these minor antioxidant compounds could significantly contribute to the human health benefits of the olive oil. Following this hypothesis the most important acting compounds should be HOTYR and TYR as the most abundant ones. However, that is not exactly a case because they are extensively metabolised, and are detected in blood and urine mainly in the form of HOTYR and TYR glucuronide, sulfate and methylated conjugates (Caruso, 2001; Tuck, 2002). More than 95% of the recovery of HOTYR and TYR in urine is in the form of conjugated metabolites resulting from the activity of Phase II metabolic enzymes (Tuck, 2002). Although

concentrations of HOTYR and TYR metabolites in biological fluids are relatively low (Miró-Casas, 2001a, 2003a), there is a factual reason to suggest their participation to beneficial effects of olive oil. However, this still remain questioned, since the metabolism of HOTYR and TYR has not yet been well characterized.

All previously done *in vivo* and *in vitro* investigation was based only on the intrinsic biological activities of HOTYR and TYR as key phenolic compounds of olive oil. They are well known as *in vitro* scavengers of various free radicals, reactive nitrogen species, superoxide anions and hypochlorous acid, breaking peroxidative chain reactions, and preventing metal ion catalyzed production of reactive oxygen species (Visioli 1998a, 2004). HOTYR and TYR as well express a set of biochemical and cellular actions, which are also apparent *in vivo*, exerting cardioprotective effects such as inhibition of LDL oxidation and endothelial cells activation (Turner, 2005). The role of their conjugated metabolites, which could influence either in the same or different way on biological systems in human body, has not been yet seriously considered (Tuck, 2002). Little is known on their conjugated metabolites, mainly because there were no studies conducted due to the lack of a good characterization of their disposition and due to the lack of adequate reference compounds. At present it can be only guessed about phenolic compounds behaviour in human body and the role played by HOTYR and TYR metabolic derivatives. Even taking into account the extremely poor bioavailability of natural phenols, the contribution of metabolites to health benefits in humans is a hypothesis worth being tested. Some preliminary studies support

information that the conjugated forms of olive oil phenols should also exert certain antioxidant activities (Tuck, 2002).

This hypothesis should promote further investigation directed to the qualitative identification and quantification HOTYR and TYR metabolites in biological fluids. This will require the development of very sensitive analytical methods, based on a direct identification of conjugated forms using appropriate standards. Unfortunately, due to the lack of commercially available reference standards, these developments are quite challenging. The evaluation of the biological activities of HOTYR and TYR conjugated forms also requires the availability of pure reference material. Therefore, a synthetic procedure for the production of metabolite conjugates of HOTYR and TYR could be of great practical use to follow up with the research in these areas.

The availability of HOTYR and TYR metabolites should allow characterizing qualitatively their metabolic disposition and estimating quantitatively the contribution of each metabolic pathway. These results should be combined with those obtained in studies designed at the evaluation of their biological activity. The confirmation of their biological activity should allow to review past clinical studies or to design new ones where the contribution of phenol compounds to biological effects should be revised. At this stage it is proposed that this evaluation should be performed applying alternative experimental approaches to those applied until now.

Recent development of “omics” technologies (genomics, transcriptomics, proteomics and metabolomics) has brought new approaches in biomedical investigation conducted on humans. So far they were based on exploration of physiological (such as cardiovascular activity) or biochemical (enzyme activities, markers of bioactivities) levels. Post-genome technologies have revealed more profound and fundamental levels of biological system responses to pharmacologic treatments, nutritional interventions and the development of pathological conditions. An access to the transcriptome level is expected to give a simultaneous and global analysis for all functional components in biological system: oxidative stress, metabolism and specific pathologic processes markers, which were too difficult to be estimated using only physiological and biochemical methods. This approach should contribute to our understanding of mechanisms underlying beneficial effects of olive oil and to verify its impact on human health.

The subject matter of this thesis is structured into 2 introduction chapters according to the objectives defined:

CHAPTER 1: ANTIOXIDANT PROPERTIES OF OLIVE OIL PHENOLS HOTYR AND TYR AND TRANSCRIPTOME ACTIVITIES OF OLIVE OIL AS A COMPLEX FOOD COMPONENT

CHAPTER 2: METABOLISM AND DISPOSSITION OF OLIVE OIL PHENOLIC COMPOUNDS HOTYR AND TYR

Each chapter includes a detailed review of literature, existing hypothesis and main achievements within the area of investigation, motivating, therefore, a formation of the goals for given thesis, which are numerically structured and formulated within the “Objectives” part. Following it the “Methodological Approaches” section describes and justifies the technological approaches applied in this work for accomplishment with the determined tasks. The “Results and Discussions” part reports on the achieved outcomes of the investigation within defined objectives. It is presented in the form of six original publications each one comprising corresponding parts: materials and methods, results and discussion on a meaning of the findings in the scope of each specific research area. The “Concluding Remarks" overviews the main achievements of the present dissertation and defines their impact on the state of the investigation in the area of olive oil in cardiovascular diseases (CVDs) prevention, which at the end are briefly annotated within the separate section ”Conclusions”. Finally, additional information collected for supporting introductory part and protocols of clinical studies applied within this thesis are presented in “Supplementary Material” and “Appendices”, respectively.

INTRODUCTION

CHAPTER I

ANTIOXIDANT PROPERTIES OF OLIVE OIL PHENOLS HOTYR AND TYR AND TRANSCRIPTOME ACTIVITIES OF OLIVE OIL AS A COMPLEX FOOD COMPONENT

1. Oxidative stress and CVD

Oxidative damage of tissue and cellular components is a primary or secondary causative factor in many pathological conditions and aging processes (Cutler, 2005a, b; Kregel, 2007). Many human diseases are strongly associated with the steady-state level of oxidative damage in tissues. On an individual level this damage is defined as the oxidative stress (OS) status.

OS targets principal organs and systems of human organism and is associated with many of the major age-related diseases: cardio-vascular diseases (CVDs), different type of cancer, impaired function of organs and tissues, etc (Kregel, 2007; Valko, 2007). In general, the greater the OS status of individual, the higher the risk for disease development (Cutler, 2005a, b).

1.1. Oxidative stress, oxidative damage in aging related diseases

Oxidative stress

Oxidation[1] reaction is crucial for life and the generation of reactive species (RS). These by-products of oxidation reactions are essential to maintain homeostasis of human organism (Seis, 1997) (***Supplemental Table I***). However being in excess, RS can start chain reactions leading to cell damage and death. Antioxidants terminate these chain reactions by removing reactive species intermediates, and inhibit other oxidation reactions. In this way they interact to oxidant and keep the redox system (the interplaying activities of oxidant and antioxidant system) in balance (***Fig. 1A***).

The biological oxidative effects of both endogenously and exogenously derived RS within organism/cell are controlled by a wide spectrum of antioxidants that altogether compose the cell/organism antioxidant defence system (Cutler, 2005a; Sies, 1997). Endogenous antioxidant compounds in cells can be classified as (i) enzymatic and (ii) non-enzymatic antioxidants (***Fig. 1A***). The major antioxidant enzymes directly involved in the neutralization of reactive species are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GS-R).

The non-enzymatic antioxidants, usually low molecular weight molecules, are divided into (i) metabolic antioxidants and (ii) nutrient antioxidants (***Fig. 1A***). Metabolic antioxidants belonging

[1] Oxidation is a chemical reaction that transfers electrons/protons from a substance to an oxidizing agent, where an oxidizing agent (oxidant, oxidizer) is a chemical compound that readily transfers oxygen atoms, or a substance that gains electrons in a redox chemical reaction, and a reducing agent (reductant, reducer, antioxidants) readily donates its electrons/protons to another substance, and is, thus, oxidized itself.

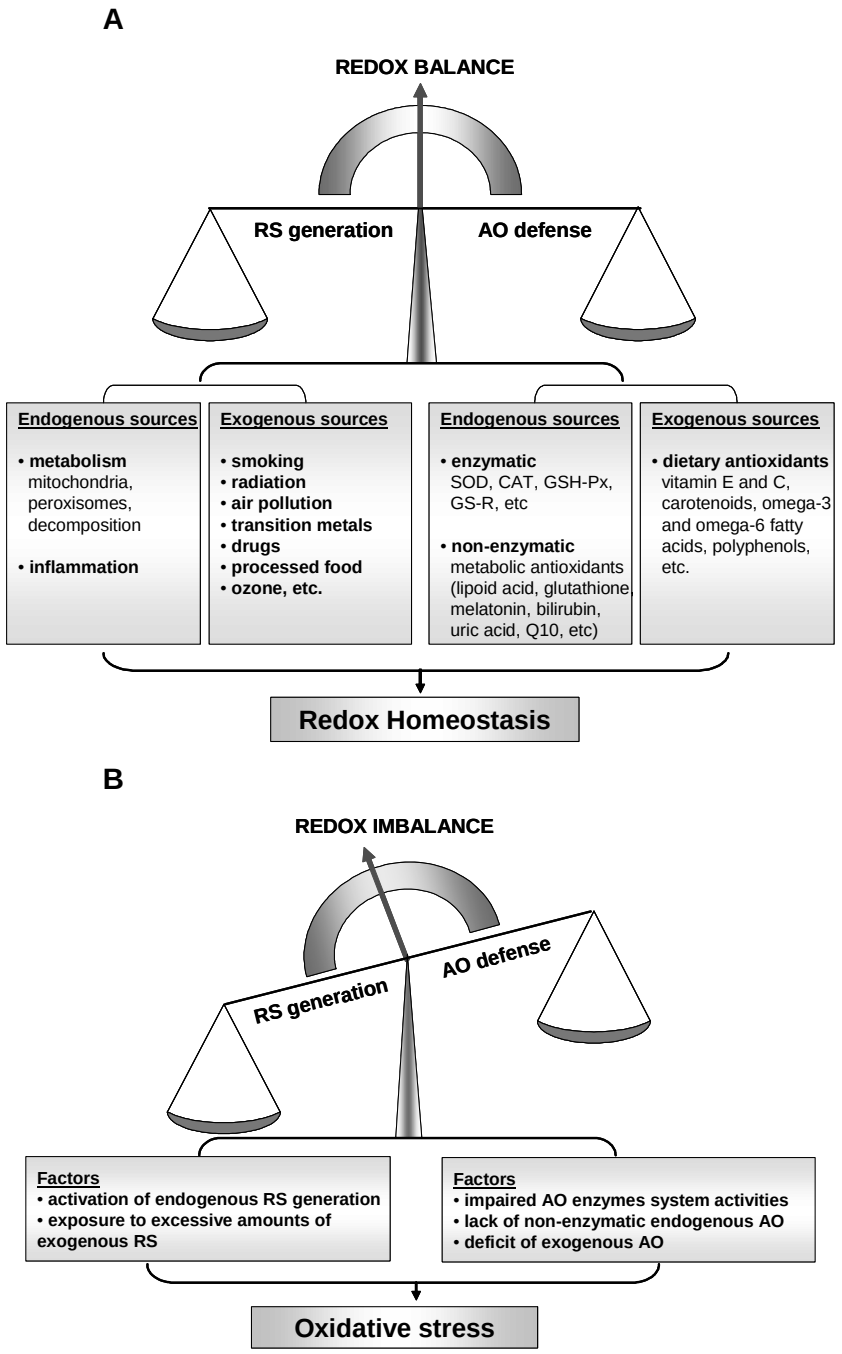


Figure 1 *Homeostasis (**A**) and unbalancing in redox system (**B**).*

to endogenous antioxidants are produced by metabolism in the body, such as lipoic acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc. While nutrient antioxidants belonging to exogenous antioxidants, are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, flavonoids, omega-3 and omega-6 fatty acids, polyphenols, etc (***Fig. 1A***).

"Oxidative stress" (OS) refers to a serious imbalance between RS production and antioxidant defences (***Fig. 1B***). The balance between RS production and antioxidant defences determines the degree of oxidative stress.

Regardless of how or where RS are generated, a rise in intracellular oxidant concentrations has two potentially important effects:

(i) damage to various cell components (Finkel, 2000; Valko, 2007);

(ii) triggering of the activation of specific signalling pathways (Owuor, 2002; Finkel, 2000; Valko, 2007).

Both effects can influence numerous cellular processes linked to aging and the development of age-related diseases (***Fig. 2***).

Aging is an inherently complex process that is manifested within an organism at genetic, molecular, cellular, organ, and system levels. Although the fundamental mechanisms are still poorly understood, a growing body of evidence points toward reactive species (RS) as one of the primary determinants of aging. The "oxidative stress

theory" holds that a progressive and irreversible accumulation of oxidative damage caused by RS impacts on critical aspects of the aging process and contributes to impaired physiological function, increased incidence of disease, and a reduction in life span. While compelling correlative data have been generated to support the oxidative stress theory, a direct cause-and-effect relationship between the accumulation of oxidative mediated damage and aging has not been strongly established (Kregel, 2007).

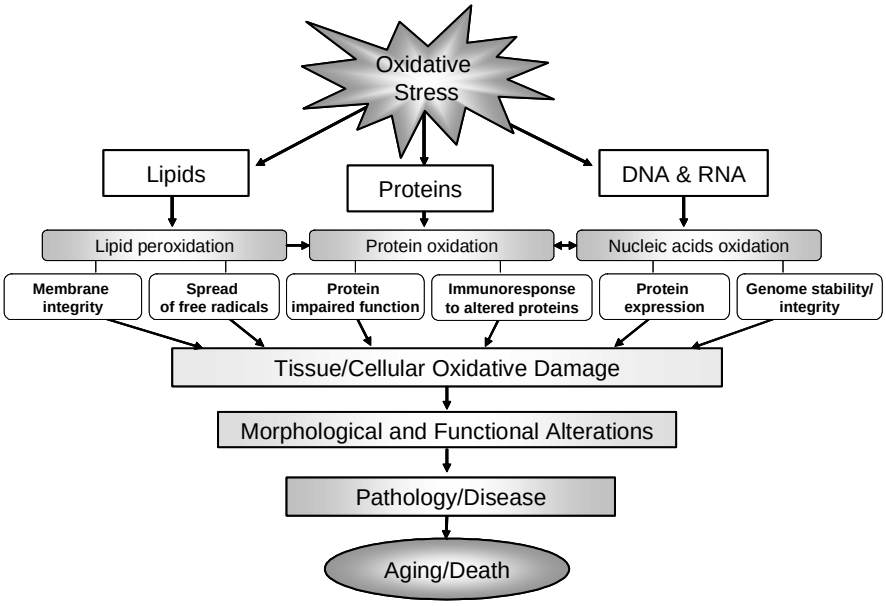


Figure 2 *Levels of oxidative damage and their consequences to living organism.*

OS is an important part of many human diseases and dysfunctions. However, it is unclear whether OS is the cause or the consequence of disease. In most cases the association between OS and pathology is secondary or beyond rather than primary. The most common OS-linked diseases are the following:

(i) heart and cardiovascular disease (CVD);

(ii) cancer disease of all tissues;

(iii) nervous and muscle system dysfunctions;

(iv) eye degenerative processes;

and many other tissue and organ dependent pathological conditions (Cutler, 2005a).

Oxidative damage

OS is defined (Sies, 1997) as a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage. Such damage includes modification of molecules and other cellular components, and is called "oxidative damage" (OD). Main targets for OD are proteins, lipids and nucleic acids of living organism (***Fig. 2***) and some of their oxidation products are often used as biomarkers[2] of OS and/or OD related processes (Dalle-Donne, 2006b; Blumberg, 2004).

Proteins modification caused by oxidative damage

Proteins are major targets for RS due to their high overall abundance in biological systems and because they are primary responsible for most functional processes within cell. It has been estimated that proteins can scavenge the majority (50%-75%) of RS generated (Davies, 1999). Exposure of proteins to RS may alter every level of protein structure from primary to quaternary (if multimeric proteins), causing major physical changes in protein structure. OD to proteins is induced either directly by RS or indirectly by reaction of secondary by-products of OS and can

[2] Biomarkers are defined as characteristics that can be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Dalle-Donne, 2006b).

occur via different mechanisms, leading to peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid (Davies, 1999, 2005; Stadtman, 1997) (***Fig. 3***). These oxidative modifications have a wide range of downstream functional consequences, such as inhibition of enzymatic and binding activities, increase susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity (Dean, 1997) (***Fig. 2***). In addition, accumulation of the modified proteins disrupts cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathways.

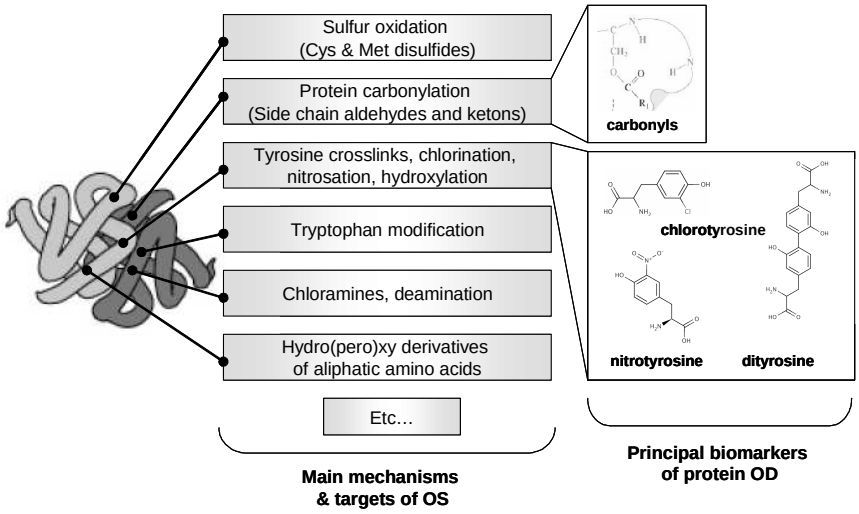


Figure 3 *Main sites of oxidative damage and relative modifications caused by RS in proteins. Main biomarkers of protein oxidative damage.*

The most widely studied OS-induced modification to protein is the formation of carbonyl derivatives (Dalle-Donne, 2006a). Another broadly acknowledged modifications are formation of *O,O*-dityrosine (Di-Tyr), 3-nitrotyrosine (NO_2-Tyr), and 3-chlorotyrosine

(Cl-Tyr), which provoke protein inactivation (Dalle-Donne, 2006b). Both types of biomarkers (***Fig. 3***) are potentially useful indicators of redox status and have been shown to accumulate during aging and age-related disease in variety of organisms (Levine, 2001; Dalle-Donne, 2006b).

DNA oxidative damage

Several studies have shown that aging cells and organisms accumulate increased levels of oxidant-damage nuclear DNA (Wei, 1998). DNA damage can be caused by RS generated under

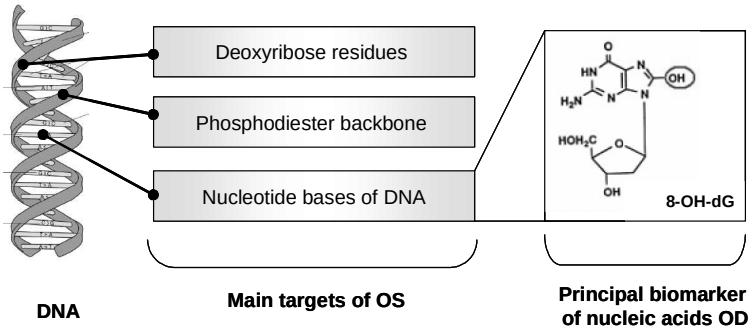


Figure 4 *Main sites of oxidative damage caused by RS in DNA. Main biomarker of DNA oxidative damage.*

different conditions and can result from reactions with nucleic acid bases, deoxyribose residues, or the phosphodiester backbone, but the majority of collected information is related to damage on base or degradation of deoxyribose (Marnett, 2001; Poulsen, 2005) (***Fig. 4***). Accumulation of mutations from oxidative DNA damage represents a crucial step in human carcinogenesis (Poulsen, 1998; Evans, 2004) (***Fig. 2***). The most extensively studied DNA lesion is the formation of 8-hydroxy-2´-deoxyguanosine (8-OH-dG), which is widely used as an index of oxidative DNA damage (Evans, 2004).

Lipids oxidation and modification caused by OS

Lipids are also important targets for oxidation by RS. OS induced peroxidation of membrane lipids can be very damaging because it leads to alterations in the biological properties of the membrane, such as degree of fluidity, and can lead to inactivation of membrane bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability.

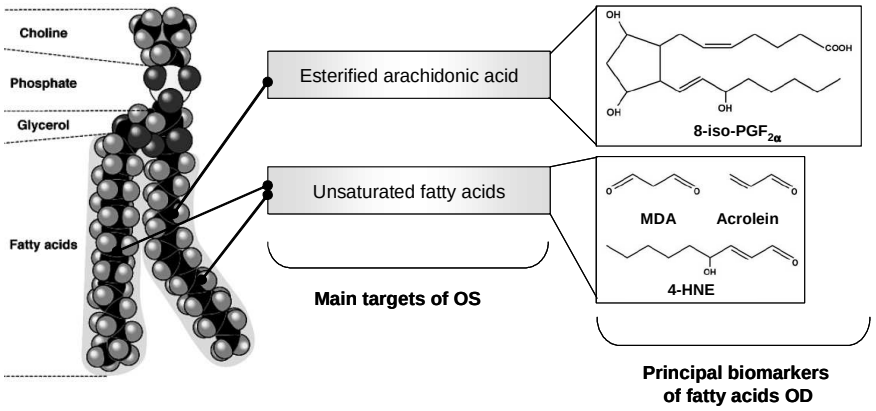


Figure 5 *Main sites of oxidative damage caused by RS in phospholipids as an example. Main biomarkers of lipids oxidative damage.*

Moreover, lipid peroxidation may contribute to and amplify cellular damage resulting from generation of oxidized products (***Fig. 2***), some of which are chemically reactive and covalently modify critical macromolecules. Lipid peroxidation generates a variety of relatively stable decomposition end products, mainly reactive aldehydes, as malonaldehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein), and isoprostanes (Niki, 2009) (***Fig. 5***). These compounds could be used as an indirect index of lipid oxidative stress (Dalle-Donne, 2006b).

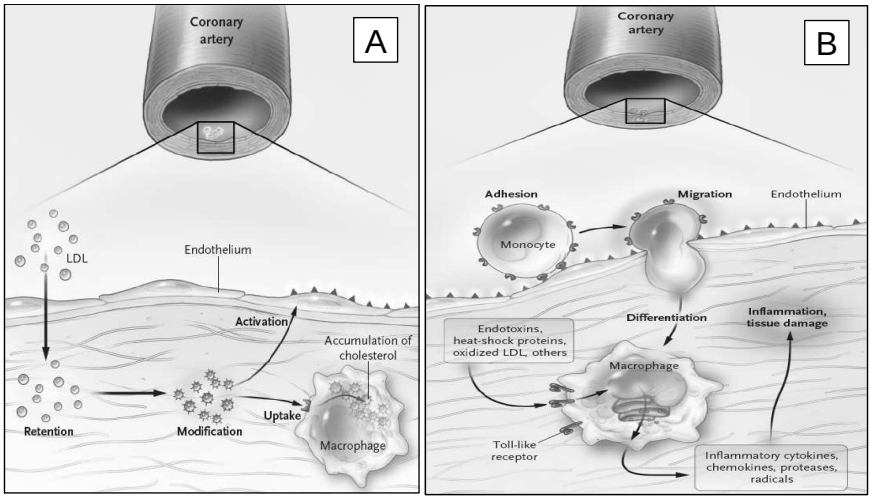
1.2. Role of oxidative stress in CVD

1.2.1. The atherogenic origin of CVD. Oxidative theory of atherosclerosis

Atherosclerosis (AT) is the condition in which an artery wall thickens as the result of a build-up of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophage white blood cells and promoted by low-density lipoproteins (LDL) without adequate removal of fats and cholesterol from the macrophages by functional high-density lipoproteins (HDL) (***Fig. 6***).

Cardiovascular disease (CDV) is the class of diseases that involve the heart or blood vessels (arteries and veins) and includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. While the term technically refers to any of the diseases that affect the cardiovascular system, it is usually used to refer to those related to atherosclerosis (arterial disease).

The "oxidative theory" of atherosclerosis proposes that intimal oxidation of lipid/lipoproteins generates biologically active products that are causal in atherosclerosis (Jessup, 2004). The oxidative modification of LDL and formation of oxidized LDL (oxLDL) in the sub-endothelial space of the arterial wall is a key initiating step in AT because it contributes to foam cell generation, endothelial dysfunction, and inflammatory processes (***Fig. 6***).



LDL oxidative modification activates inflammation in the artery

Recruitment of macrophages in inflammation of the artery

Figure 6 *Oxidative events leading to development and progression of AT (Hansson, 2005):* ***A*** *– LDL infiltrates the artery and its oxidative and enzymatic modifications lead to the release of inflammatory lipids that induce endothelial cells to express leukocyte adhesion molecules. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells.* ***B*** *– Monocytes recruited through the activated endothelium differentiate into macrophages. Macrophages are activated by variety of molecules (e.g. oxLDL) and lead to the release of inflammatory cytokines, chemokines, oxygen and nitrogen radicals, and other inflammatory molecules and, ultimately, to inflammation and tissues damage.*

Lipoproteins are susceptible to structural modifications by oxidation, particularly the small dense LDL particles. Little is known about the molecular mechanisms underlying LDL oxidation *in vivo*, but reactions involving transition metals, such as cooper and iron, free radicals, hypochlorous acid, peroxynitrite, and activity of selected enzymes, such as myeloperoxidase, lipoxygenase, xanthine oxidase and NADPH oxidase, released by endothelial

cells from the arterial wall have been claimed to play a role (Burkit, 2009; Steinberg, 1999). It has been shown that the oxidation targets several sites of LDL (ApoB, cholesterol, cholesterol esters, triglycerides, fatty acids etc.) among which lipid peroxidation is a key process of oxidation (Jessup, 2004; Parthasarathy, 2010) (***Fig. 7***). The oxidation of LDL in physiological fluids is prevented by proportional concentration of water-soluble antioxidants and by incorporated within LDL lipid bilayer liposoluble antioxidants (Burkit, 2009).

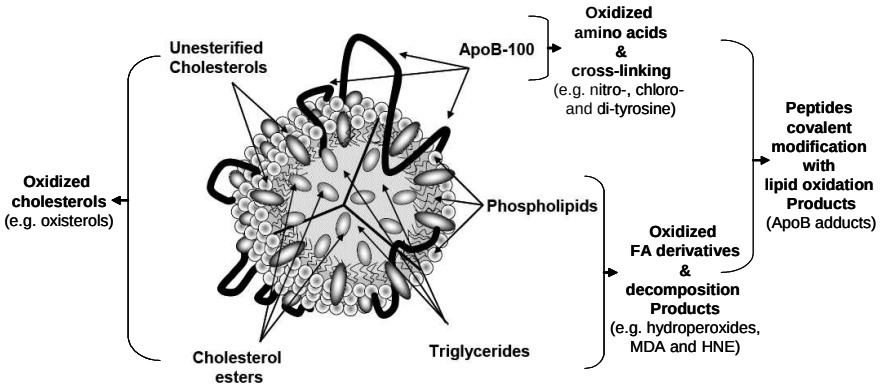


Figure 7 *LDL particle structure and its main targets for oxidation.*

Oxidation of the lipid part (Steinberg, 1989) or directly of the apoB of the LDL particle (Hazen, 1997), leads to a change in the lipoprotein conformation by which the LDL is better able to enter into the monocyte/macrophage system of the arterial wall, and develop the atherosclerotic process (Witzum, 1994). The modified apoB has immunogenic properties prompting the generation of auto-antibodies against oxidized LDL (Steinberg, 1989). In addition, chloro- and nitro-tyrosine generation, via myeloperoxidase activity,

in high density lipoproteins (HDL) converts the lipoprotein in a pro-inflammatory HDL, and reduces its capacity to remove cholesterol from cells (Fogelman, 2004).

AT, typically asymptomatic for decades, eventually produces two main health disorders: (i) atheromatous plaques (***Fig. 8***), an

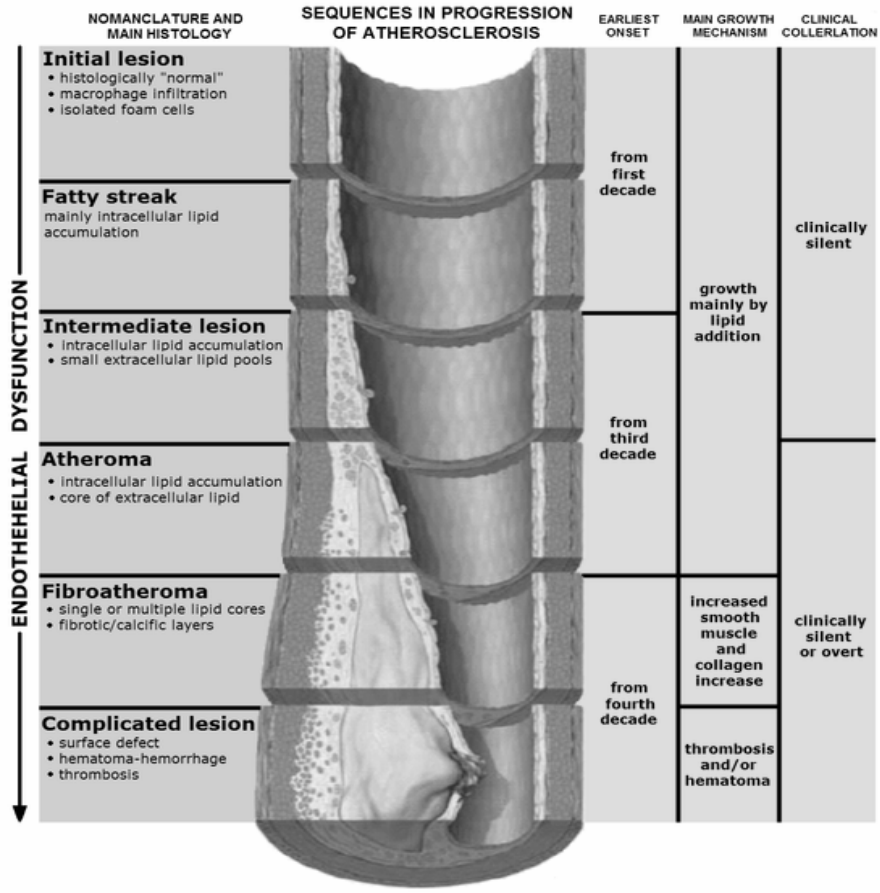


Figure 8 *Stages of endothelial dysfunction in atherosclerosis. (released under the GNU Free Documentation License: http://commons.wikimedia.org/wiki/File:Endo_dysfunction_Athero.PNG,)*

accumulation and swelling in artery walls that is made up mostly by macrophage cells or cell debris, that contain lipids (cholesterol and fatty acids), calcium and a variable amount of fibrous connective tissue; and (ii) aneurysm, a localized, blood-filled dilation of a blood vessel. AT typically begins in early adolescence, and is asymptomatic up till is causing serious health threatening cardiovascular problems (***Fig. 8***), as hard as a heart attack or sudden cardiac death. Unlike many other chronic medical conditions, CVD of atherogenic origin is treatable and to some extent reversible. The treatment, as in case with disease prevention, is primarily focused on diet (Hu, 2009). Therefore, much effort is put on preventing atherosclerosis by modifying risk factors, such as healthy eating, exercise and avoidance of smoking (Levenson, 2002).

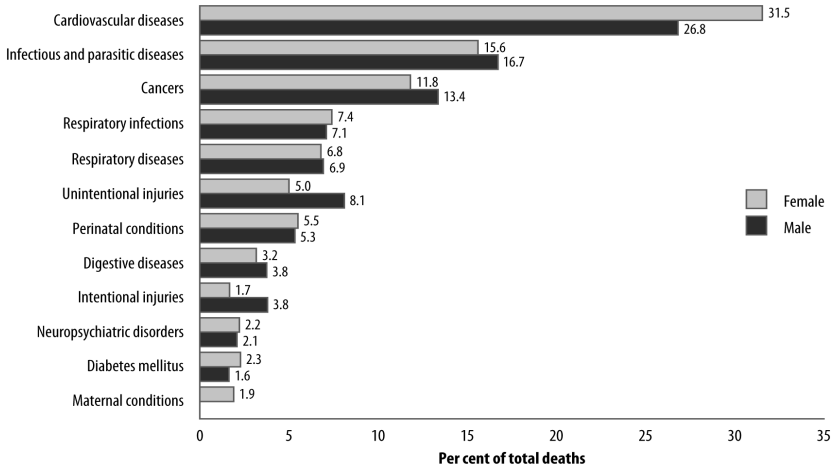


Figure 9 *Distribution of deaths by leading cause groups, in males and females in the world, by 2004 year (according to WHO report, 2004)*

The prevalence of cardiovascular diseases rises with aging and is one of the main causes of mortality in western countries. In

general, most countries face high and increasing rates of cardiovascular disease (WHO report, 2004) (***Fig. 9***). In view of the progressively aging population, there is an urge for a better understanding of age-associated CVDs and their underlying molecular mechanisms. The major causes of the CVDs are tobacco use, physical inactivity, and an unhealthy diet (WHO report, 2004).

1.2.2. CVD and oxidative stress risk factors: role of antioxidants

CVD is a life course disease that begins with the evolution of risk factors that in turn contribute to the development of subclinical atherosclerosis (Levenson, 2002; Hu, 2009). There are a variety of risk factors that contribute to CVD morbidity and mortality:

(i) overweight and obesity;
(ii) unhealthy eating;
(iii) physical inactivity;
(iv) high blood pressure and high blood cholesterol;
(v) diabetes;
(vi) cigarette smoking, etc.

When risk factors are combined, risk for CVD can increase. The majority of CVD risk factors are preventable or treatable by applying adequate diet: restriction in caloric intake, substitution saturated fatty acids by unsaturated, vitamins and antioxidants intake (Hu, 2002).

Increased production of RS has been directly and indirectly implicated in the initiation and progression of CVD and, therefore, OS accounts for an increased risk of developing this disease

(Singh, 2006). OS affects the availability and/or balance of key-regulators of vascular homeostasis and favours the development of cardiovascular pathology (De Rosa, 2010). Pharmacological therapies are continuously being investigated for counteracting harmful or damaging effects of oxidation in cells or tissues. Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing CVD. Although some studies have suggested antioxidant supplements have health benefits, other large clinical trials did not detect any benefit for the formulations tested, and excessive supplementation may be harmful (Victor, 2009; Singh, 2006). This controversy in results still remains unexplained. Therefore, hypothesis-driven and rigorous carefully designed studies in well-defined patient populations are warranted to provide a definitive answer (Sachidanandam, 2005; Willcox, 2008).

1.2.3. CVD associated biomarkers of oxidative stress

In clinical practice, pathology specific parameters, biomarkers, are employed to demonstrate that a treatment has a beneficial, unfavourable, or null effect on health promotion and CVD disease prevention or treatment. In addition, biomarkers can help to identify high-risk individuals, to diagnose disease conditions and in the prognosis of treated patients with CVD disease (Dalle-Donne, 2006b). Some of the biomarkers most frequently associated with CVD are listed in ***Table 1.***

In clinical research studies, some of the biomarkers of oxidative damage could be employed to reflect environmental pro-oxidant

exposures, status of the endogenous redox system, dietary antioxidant intake or to serve as a surrogate measure of a process in oxidative stress related disease. DNA, lipid, and protein oxidation products provide an extensive and growing array of potential

Table 1 *Oxidative status and other biomarkers associated with CVD.*

Status	Process and Mechanism	Biomarkers
Oxidative status	Lipid oxidative damage	HNE Acrolein F_2-IsoP (F_2-isoprostance) MDA Lipoperoxidase activity (LPO)
	Antioxidant system	Serum/plasma antioxidant capacity GSH/GSSG ratio GSH-Px and GS-R activities
	RS production	$NO_{(x)}$ production;
	Protein oxidative damage	Protein carbonyls; NO_2-Tyr Cl-Tyr Di-Tyr
	DNA oxidative damage	8-HO-dG total (urinary) and cellular (mitDNA origin)
Plasma lipid status:	hyperlipidemia hypercholesteremia	concentrations of triglycerides (TG) total cholesterol (TC) HDL/LDL cholesterol ratio
	LDL status	oxLDL concentration; LDL fatty acids and antioxidants composition; LDL resistance to oxidation
Endothelial dysfunction:	Thrombogenic state	PAI-1 FVIIa
	Epithelia/immune cell adhesion	E-selectin sICAM-1 and sVCAM-1,
Inflammation:	Cytokines synthesis	C-reactive protein (CRP) TXB(2),LTB(4) and IL-6,

oxidative stress biomarkers for CVD and AT. However, the relation between their status in cells and tissues, and biological matrices (plasma and urine), and the development of pathology still remain to be elucidated. Biomarkers of oxidative stress in CVD and AT are general markers of oxidative damage and correspond poorly to

CVD disease specific processes and its outcome (Valko, 2007; Dalle-Donne, 2006b), however they are widely used in the CVD research especially in those areas where oxidative stress theory is concerned (Stephens, 2009).

2. Olive oil antioxidants and CVD health

2.1. Mediterranean diet and health prevention

The traditional dietary habits of the Mediterranean area have been consistently associated with lower incidence of cardiovascular disease (CVD) and cancer (de Lorgeril, 2006) (Trichopoulou, 1997, 2000 & 2003) and perhaps other chronic conditions (de Lorgeril, 2008). On one hand, the involvement of excessive free radical production involved in development and progression of above-mentioned diseases points out that dietary antioxidants likely play a protective role (Seifried, 2007). On the other hand, low content of saturated vs. high content of monounsaturated fatty acids (mainly coming from olive oil as a main source of fat) in this diet was been shown to be associated with the lower risk of certain diseases, where this food element could be implicated: colon cancer, CVD and AT, hypertension, etc. (de Lorgeril, 2006). Investigating the health benefits promoted by this dietary pattern the concept of the Mediterranean diet was originated (Keys, 1980).

On the whole, the traditional Mediterranean diet is characterized by eight principal components (Trichopoulou, 2000):

1. high monounsaturated/saturated fat ratio;
2. moderated ethanol consumption;

3. high consumption of legumes;
4. high consumption of cereals (particularly bread);
5. high consumption of fruits;
6. high consumption of vegetables;
7. low consumption of meat and meat products;
8. moderate consumption of milk and dairy products.

Because of abundant plant foods plenty of vitamins, antioxidants and high content of monounsaturated fatty acids (olive oil as a principal source of fat), all of them being potentially active in protection against the age-related diseases, the diet from the Mediterranean basin was recognized as “functional diet” with respect to human health protection (Ortega, 2006).

2.2. Cardiovascular health and olive oil

A substantial body of knowledge demonstrates that the Mediterranean diet conveys a markedly lower risk of coronary disease (Trichopoulou, 1997, 2003). In addition, the adherence to the Mediterranean diet has been shown to be effective in the secondary prevention of coronary heart disease in intervention studies (de Lorgeril, 2006).

Olive oil, the primary source of fat in the Mediterranean diet, was associated with a low mortality for cardiovascular disease (Trichopoulou, 2001). The data from clinical studies show that consumption of olive oil can provide heart health benefits such as favourable effects on cholesterol regulation and LDL oxidation, and that it exerts anti-inflammatory, antithrombotic, antihypertensive as

well as vasodilator effects both in animals and in humans (Covas, 2007). Additional clinical evidence suggests that the olive oil phenolic content, may contribute to its cardioprotective benefits. Which were reasons and which facts supporting this statement will be reviewed in detail in the following sections after revealing the complexity of olive oil as a food component.

2.3. Olive oil phenols and CVD

2.3.1. Evidence from intervention studies in humans

Acute/postprandial studies

Postprandial lipemia[3] has been recognized as a risk factor for CVD and especially for AT development on its own, together with postprandial hyperglycemia[4], and associated with oxidative changes (Roche, 2000; Hyson, 2003) and inflammatory response (Alipour, 2008). After a high-fat meal an oxidative stress occurs triggering inflammation, endothelial dysfunction, hypercoagulability, and a cascade of other atherogenic changes (O'Keefe, 2007). However, the consumption of fatty meals with suitable sources of antioxidants can minimize this postprandial oxidative stress (Sies, 2005).

Some randomized and crossover clinical studies, summarized in ***Supplementary Table II***, have examined the postprandial effect of

[3] a physiological effect leading to an excess of lipids in the blood that occurs between 2 and 12 hours after the ingestion of food.
[4] a physiological effect leading high concentration of glucose in the blood. that occurs between first hours after the ingestion of food.

olive oil phenolic compounds on biomarkers of oxidative stress (see first part of the ***Table 1***). Although they were well planed, results of postprandial studies are difficult to evaluate and compare because some of them do not mention whether postprandial lipemia and/or hyperglycemia occurred (Bogani, 2007; Visioli, 2000a). In addition, the dosages of polyphenols chosen in some studies were quite dissimilar for representative dietetic levels of high and low phenolic content olive oil antioxidants (Visioli, 2000a; Ruano, 2005); populations were usually small and mainly consisted in healthy male individuals, only one study was performed in mixed population (women and man) of hypercholesterolemic patients (Ruano, 2005).

In clinical trials previously performed in our institution it was shown that the ingestion of a 25-mL olive oil does not promote postprandial oxidative stress with independence of the phenolic content of the olive oil (Weinbrenner, 2004a, b), whereas single doses of 40 mL (Covas, 2006a) and 50 mL (Fitó, 2002) did.

With olive oil doses at which oxidative stress occurs, data from randomized, crossover, controlled postprandial studies in human show that:

(i) virgin olive oil polyphenols increase serum antioxidant capacity;
(ii) virgin olive oil polyphenols modulate the degree of lipid and LDL oxidation, in a dose dependent manner.

Sustained doses intervention studies

Most intervention studies with olive oil were made on the basis of single-dose administration. The argument has been made that single doses are not representative of the actual dietary situation with olive oil consumption. There are two main drawbacks:

(i) in most cases consumption of olive oil, as a natural dietary component, is of sustained character;
(ii) the repeated administrations of it could be necessary to reach to see some of the effects of its actions.

Lipid oxidative damage was investigated in the majority of the studies with sustained doses intervention. Two studies with a similar approach in experimental design of study, a short term intervention study (Weinbrenner, 2004a) with a strict very low-antioxidant diet in both wash-out and intervention periods and a 3-weeks intervention study (Marrugat, 2004) with a strictly controlled low antioxidant consumption diet, reported on the protective effects of olive oil phenols *in vivo* on the basis of two lipid oxidative damage biomarkers: plasma oxLDL and urinary MDA concentrations.

The acute and short term intervention studies performed in our group were able to demonstrate that olive oil phenolic content modulates the oxidative/antioxidative status of healthy subjects (Weinbrenner, 2004a). These preliminary results were further supported by results obtained in a controlled, crossover international study (EUROLIVE) where participants (n=200) were randomly assigned to 3 sequences of daily administration of 25 mL of 3 olive oils for 3 weeks. Olive oils had a low (2.7 mg/kg of olive oil), medium (164 mg/kg), or high (366 mg/kg) phenolic content but

were otherwise similar in their composition. The phenolic content provided benefits in a direct dose-dependent manner for plasma lipids and lipid oxidative damage (Covas, 2006b). In a subset of subjects it was shown that all three olive oils caused an increase in plasma and LDL oleic acid content ($P < 0.05$). In addition, olive oils rich in phenolic compounds led to an increase in their concentrations in LDL ($P < 0.005$) in a direct relationship with the phenolic content of oils. This can account for the increased resistance of LDL to oxidation, and the decrease of oxidized LDL, observed within the frame of this clinical trial (Gimeno, 2007). Phenolic content of LDL was correlated with concentrations of HOTYR in plasma (Covas, 2006b) and its presence in LDL has been demonstrated later (de la Torre-Carbot, 2007).

Overall results of sustained doses olive oil intervention studies in humans (summarized in details in *Supplementary Table II*) have provided evidence of:

(i) the *in vivo* protective role of olive oil phenolic compounds on lipid cardiovascular risk factors, including lipid oxidative damage, in humans at real-life olive oil dosage;

(ii) the fact that olive oil phenolics contribute to health benefits of olive oil and therefore this food cannot longer be considered only as a source of MUFA fat.

Previous studies have not been able to demonstrate such findings due to several deficiencies in the design summarized as follows (Covas, 2007):

(i) the experimental design of studies (present/absent wash-out[5], period of intervention);

(ii) control and type of diet applied (diet compliance biomarkers, the amount of polyphenols consumed, type of olive oil pattern);

(iii) population sample (size and homogeneity);

(iv) physiological characteristics of the participants (age, sex and oxidative status, etc.);

(v) the sensitivity and the specificity of the oxidative stress biomarkers evaluated.

The balance in pro-oxidant and antioxidant reactions is well regulated in the body and, therefore, the interventions with antioxidant-rich compounds at dietary doses exert only marginal effects in healthy volunteers. In addition, the detection of these effects is challenged due to the current state of the art of the oxidative biomarkers (Giustarini, 2009). In fact, the protective effect of olive oil phenolic compounds on oxidative damage in humans was better displayed in participants with a compromised oxidative status (males, males submitted to a low antioxidant diet, post-menopausal females) or in patients with high oxidative stress status (hyperlypemic, coronary heart disease, hypercholesteromic, ect) (see ***Supplemental Table II***).

[5] Wash-out periods is the minimum number of days between administrations of olive oil polyphenols needed to avoid influence of the previous administration on the plasma and urinary concentration levels of these polyphenols.

3. Bioactive compounds of virgin olive oil: phenolic compounds

3.1. Diversity of olive oil

Olive oil is graded in six categories: extra virgin olive oil, virgin olive oil, refined olive oil, olive oil, refined residue oil, and olive residue oil. They differ in three main aspects:

(i) the acidity[6]: extra virgin olive oil (EVOO) (acidity up to 0.8% as oleic acid), virgin olive oil (acidity up to 2.0%), olive oil (a mixture of refined and virgin olive oil), and olive residue oil (a blend of refined residue oil and virgin olive oil);

(ii) the fact that they have been obtained by different physical or chemical means. Virgin (VOO) means that the olive oil was produced by the use of physical means and no chemical treatment. Refined olive oil (ROO) means that the oil has been chemically treated to neutralize strong tastes (characterized as defects) and neutralize the acid content (free fatty acids), but its lipid composition is the same as for VOO;

(iii) the microconstituents and micronutrients content: phenols, α-tocopherol and squalene, etc. (Boskou, 2006). Differences in oxidative stability between virgin and refined olive oils bring to discovery of olive oils

[6] Acidity of oil's is defined as the percent, measured by weight, of free oleic acid it contains. This is a measure of the oil's chemical degradation; as the oil degrades, more fatty acids are freed from the glycerides, increasing the level of free acidity and thereby increasing rancidity. Another measure of the oil's chemical degradation is the organic peroxide level, which measures the degree to which the oil is oxidized, another cause of rancidity.

antioxidants about a half a century ago. Later specific systematic studies show the peculiar composition of VOO in terms of phenolic antioxidants that cannot be found in any other vegetable oils (Servili, 2004).

3.2. Olive oil derived phenolic antioxidants

The chemical composition of VOO consists of major and minor components. The major components, that include glycerols, represent more than 98% of the total oil weight and non-glycerol or unsaponifiable fraction consists of 0.4–5 % (Servili, 2004; Tripoli, 2005). Olive oil glycerol content is composed mainly of the mixed triglyceride esters of oleic acid and palmitic acid and of other fatty acids (***Table 2***). Oleic acid, a MUFA (18:1n-9), represents 70–80% of the fatty acids present in olive oil (Abia, 1999).

Minor components, that are present in a very low amount (about 2% of oil weight), include more than 230 chemical compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants (Servili, 2004).

The main antioxidants of VOO are carotenes and phenolic compounds that include lipophilic and hydrophilic phenols (Boskou, 1996). While the lipophilic phenols, among which tocopherols can be found in other vegetable oils, some hydrophilic phenols of VOO (including phenolic acids, polyphenols, secoiridoid compounds and derivatives) are not generally present in other oils and fats. Moreover, the hydrophilic phenols of VOO constitute a group of secondary *Olea europaea L.* plant metabolites (Jensen, 2002) that show peculiar sensory and healthy properties (Servili, 2004).

Table 2 *Chemical composition of olive oil (Escrich, 2007).*

Saponifiable fraction (98–99%)	**Unsaponifiable fraction (about 2%)**
Main fatty acids present in triacylglycerols: Oleic acid (18:1n-9) Palmitic acid (16:0) Linoleic acid (18:2n-6) Estearic acid (18:0) Palmitoleic acid (16:1n-9) Linolenic acid (18:3n-3) Miristic acid (14:0)	Non-glyceride esters (alcoholic and sterol compounds, waxes) Aliphatic alcohols Triterpene alcohols Sterols (B-sitosterol, campesterol, estigmasterol,…) Hydrocarbons (squalene, B-carotene, lycopene,…) Pigments (chlorophylls,…) Lipophilic phenolics (tocopherols and tocotrienols) Hydrophilic phenolics (phenolic acids, phenolic alcohols, secoiridoids, lignans and flavones) Volatile compounds

There are at least thirty-six structurally distinct olive oil phenolics that have been identified (Cicerale, 2009). They can be grouped according to their similar chemical structure in the following groups:

(i) phenolic acids (three sub-groups: benzoic acid derivatives, cinnamic acid derivatives and other phenolic acids and derivatives) (Carrasco-Pancorbo, 2005a);

(ii) phenolic alcohols (compounds with hydroxyl group attached to an aromatic hydrocarbon group);

(iii) secoiridoids (characterized by the presence of either elenoic acid (EA) or EA derivatives in their molecular structure) (Carrasco-Pancorbo, 2005a);

(iv) hydroxyl-isocromans (3,4-dihydroxy-1H-benzopyran derivatives) (Bianco, 2001);

(v) flavonoids (compounds containing two benzene rings joined by a linear three carbon chain, two sub-groups: flavones and flavanols);

(vi) lignans (compounds which structure based on the condensation of aromatic aldehydes) (Carrasco-Pancorbo, 2006).

Among vegetable oils, virgin olive oil (VOO) has nutritional and sensory characteristics that make it unique and a basic component of the Mediterranean diet. Sensory properties of VOO are largely affected by phenolic and volatile compounds. Volatiles are mainly responsible for the aroma of VOO, especially for the green sensory notes of high-quality VOO, whereas compounds with a phenolic structure affect both the taste, in particular the positive bitterness organoleptic attribute, and the oxidative stability of the VOO (Gutiérrez-Rosales, 2003; Andrewes, 2003; Busch, 2006). Phenolics and volatiles are therefore the compounds chiefly responsible for the flavour of EVOOs.

The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by agronomic and technological conditions of olives production: cultivar, fruit ripening, climatic conditions of production, and some agronomic techniques such as the irrigation (Gómez-Rico, 2006; Soler-Rivas, 2000; Servili, 2003; Kalua, 2006). Crushing (Soler-Rivas, 2000) and malaxation (Kalua, 2006) are the most important critical points of the oil mechanical extraction process. Secoiridoid aglycons are originated, during crushing, by the hydrolysis of their glucosides. Extraction systems, such as pressure and centrifugation, play an important role in the oil phenolic composition. Oil obtained by pressure systems that does not require addition of water shows higher phenolic concentration in comparison to the one obtained by the traditional centrifugation (Kaula, 2006).

Due to these and other agronomic and technological aspects of olive oil production, that strongly affect their occurrence, the definition of the average concentration of hydrophilic phenols in VOO is rather difficult (Cicerale, 2009). The effect of storage time and conditions on the reduction of initial total phenolic content as well has been noted in a number of studies (Brenes, 2001; Okogeri, 2002). In general, the concentration of phenols in olive oils may range between 40 and 900 mg/kg or up to 1000 mg/kg (Montedoro, 1992).

There are several experimental approaches to report food phenolic content. It is common to report the total content of polyphenols, expressed as gallic acic equivalents (GAE) using the Folin-Ciocalteu reagent (Singleton, 1999). Other phenols are used for reporting total phenolic content as caffeic acid, and therefore results are reported as caffeic acid equivalents (CAE). Alternatively, phenolic compounds can be quantitated separately by chromatographic methods (Suarez, 2008).

3.3. HOTYR and TYR secoiridoids as main polar phenolic compounds present in olive oil

As it was mentioned earlier, VOO contains different classes of phenolic compounds (Carasco-Pancorbo, 2006; Gómez-Alonso, 2002; Servili, 2002, 2004). The type of phenols in EVOO differs from those of the olive tree and fruit. Oleuropein (OLE), demethyl-oleuropein, ligstroside (LGS) and nüzhenide are the most abundant secoiridoid glycosides over all olive tree and fruit (peel, pulp and seed). Phenolic acids (benzoic acids and cinnamic acids) were also found in olive fruits by different authors, while the phenolic acids,

phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families. Secoiridoids, on the contrary, are primary chemotaxons of O*leaceae,* which includes *Olea europaea L.*, *Gentianales* and *Cornales* (Jensen, 2002; Soler-Rivas, 2000). Olives and VOO are the only edible products that contain secoiridoids obtained from these species extensively used in the human nutrition; in addition the secoiridoids are the most prevalent phenols of VOO.

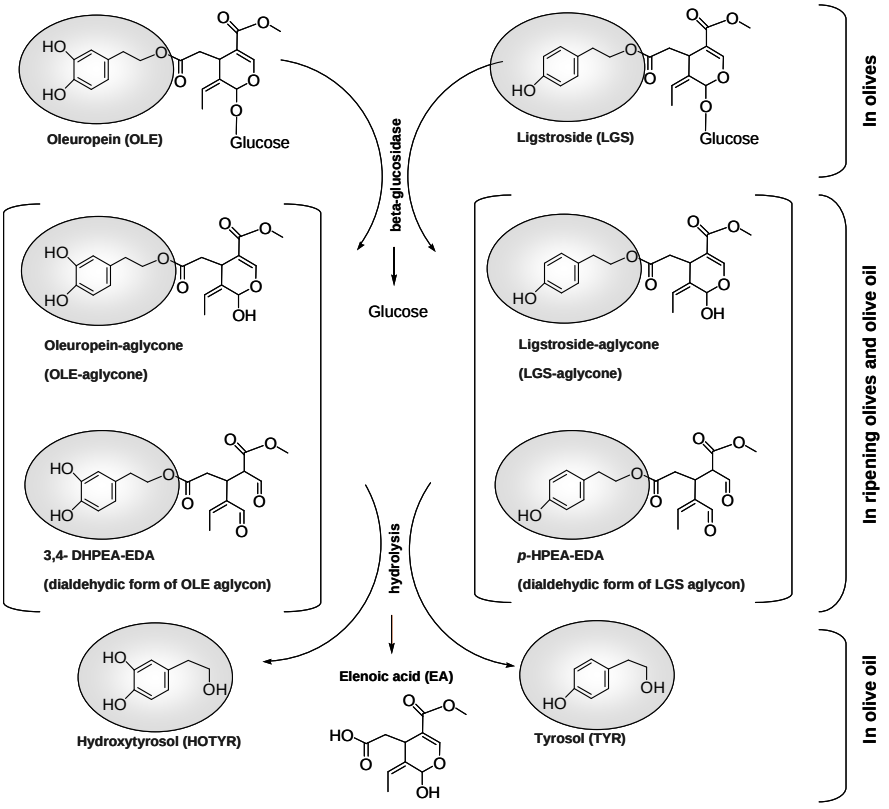


Figure 9 *Chemical structures of the secoiridoid derivatives and phenolic alcohols present in olives and olive oil.*

The phenolic compounds classified as secoiridoids are characterized by the presence of either elenolic acid (EA) or elenolic acid derivatives in their molecular structure. The most abundant secoiridoids of VOO are 3,4-DHPEA-EDA and *p*-HPEA-EDA, and OLE-aglycon (3,4-DHPEA-EA) (Gomez-Alonso, 2002) (***Fig. 9***). These compounds are intermediate structures of the biochemical transformation of secoiridoid glucosides of olive fruit (OLE and LGS) in the final aglycon derivatives: 3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively (***Fig. 9***). OLE-aglycon is the ester of EA with 3,4-dihydroxyphenylethanol (3,4-DHPEA or hydroxytyrosol), and LGS-aglycon is the ester of EA with 4-hydroxyphenylethanol (*p*-HPEA or tyrosol).

The hydroxytyrosol (HOTYR) and tyrosol (TYR) are the main phenolic alcohols of VOO (Gomez-Alonso, 2002). Their concentration is very low in fresh oils, but increases during oil storage due to the hydrolysis of VOO secoiridoids that contain them in their molecular structures (Brenes, 2001) (***Fig. 9)***.

4. Molecular mechanisms of action of HOTYR and TYR

4.1. Antioxidant activities of olive oil phenolic compounds: HOTYR and TYR

There are two main directions in the investigation of antioxidant activities of olive oil phenols:

(i) the evaluation of the effect of phenols on the stability of the oil preventing autoxidation that has purely technological character;

(ii) the evaluation of their biological effects and its application in human health protection.

The antioxidant activity of hydrophilic phenols of VOO has been extensively studied. The correlation among total phenols, their antioxidant activity and the shelf life of olive oil, behaviour during frying and other cooking processes was recently confirmed. The data showed that the natural antioxidants present in olive oil and especially HOTYR and its derivatives can extend the olive oil shelf life and protect it from decomposition occurring during thermal treatment (Velasco, 2002; Carrasco-Pancorbo, 2007; Hrncirik, 2005).

Phenolic compounds can inhibit oil's oxidation by three main mechanisms: radical scavenging, hydrogen atom transfer, and metal chelating. In addition, the antioxidant activity of phenols can be enhanced by the presence of tocopherols in olive oil (Mateos, 2003). The components which are mainly responsible for the remarkable resistance of olive oil to oxidation are the HOTYR-containing compounds (HOTYR, 3,4-DHPEA-EDA, OLE-aglycon); on the contrary, TYR, lignans and LGS-derivatives seems to exert much weaker antioxidants activities against olive oil fat oxidation (Carrasco-Pancorbo, 2005b, 2007).

The biologically relevant activities of olive oil phenols and possible mechanisms underlying these properties will be discussed in detail below, since it takes special place in the scope of this thesis.

4.1.1. Primary and secondary antioxidant activities of HOTYR and TYR

As it was previously explained, an antioxidant eliminates potential initiators of oxidation and thus prevents or stops a reaction of oxidation. Antioxidant activity of any primary antioxidant is implemented by the donation of an electron or hydrogen atom to a radical derivative, whereas secondary antioxidants remove the component initiating and stimulating a free radical chain reaction, therefore, thus preventing the initiation of oxidation.

A primary antioxidant can be effective if it is able to donate an electron (or hydrogen atom) rapidly to a radical molecule and itself becomes more stable then the original radical (***Fig. 10***).

A secondary antioxidant can prevent reaction from taking place by absorbing ultraviolet light, scavenging oxygen, chelating transition metals, or inhibiting enzymes involved in the formation of reactive oxygen species, for example, NADPH oxidase and xanthine oxidase, dopamine-β-hydroxylase, lipoxygenases, etc.

Depending on the specific set of conditions, antioxidants being oxidized can act as pro-oxidants, chemicals that induce oxidative stress, either through creating reactive species or inhibiting antioxidant systems (Puglia, 1984). The importance of the antioxidant and pro-oxidant activities of antioxidants is still under investigation (Halliwell, 2008).

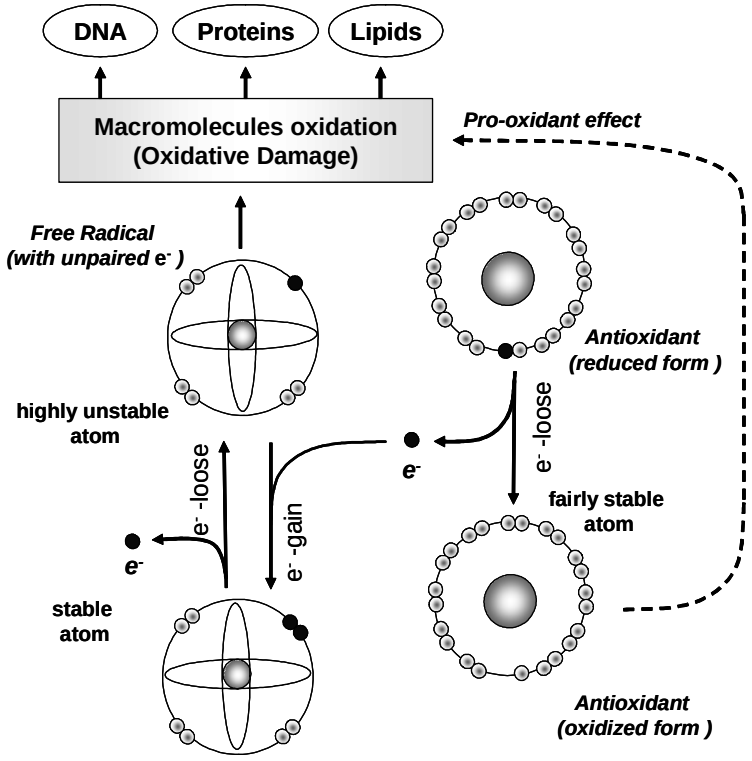


Figure 10 *Exchange reaction generated between free radical as an oxidizing agent and antioxidant as reducing agent and its potential pro-oxidant activity.*

Many different substrates, system compositions and analytical methods are employed in the evaluation of the effectiveness of antioxidants against biologically relevant reactive species (for their detailed description refer to ***Supplemental Table I***). Antioxidant effectiveness is measured by monitoring the inhibition of the oxidation of a suitable substrate under standard conditions using chemical, instrumental or sensory methods. In practice there is no gold standard for any of the methods, therefore, the interpretation of the results should be careful, based on the essential features of the test (according to the suitability of substrate, an oxidation

initiator and an appropriate measure of the end-point) (Sánchez-Moreno, 2002; Huang, 2005; Frankel, 2000).

There is a plethora of studies with respect to the potential of olive oil phenols to scavenge synthetic radicals, superperoxide radicals, and peroxyl radicals or neutralize reactive species and reduced damages caused by hydrogen peroxide and peroxynitrite ion (reviewed by Visioli, 2002a, b; Boskou 2009). Some of them will be underlined in the following paragraphs due to their relevance to the objectives of this thesis.

4.1.1.1. Chemical properties of HOTYR and TYR related to their antioxidant activities

The function of antioxidants is to intercept and react with RS at a faster rate than substrate and, since a variety of RS are able to attack a variety of targets including lipids, nucleic acids, and proteins, the chemical and physical properties of antioxidants could define the success of this protection. There are several chemical mechanisms for oxidation in which olive oil antioxidants can play a preventive role and which depend on chemistry and structural properties of phenolic compounds:

(i) H-atom transfer;
(ii) Electron transfer;
(iii) Metal chelation;

H-atom transfer (HAT) is one of the principal mechanisms in oxidation. The role of phenolic antioxidant (ArOH, since it contains at least one hydroxyl group attached to benzyl ring) is to interrupt the reaction by donation of an H-atom. To be effective $ArO^{\cdot}$ must

be a relatively stable free radical (FR), so that it reacts slowly with substrate of oxidation but rapidly with a FR (***Fig. 10***) oxidizing it (e.g. peroxyl, alkoxyl, alkyl, and superoxide radicals). The rate of the reaction for the substrates with a FR depends on the energetic barrier height for transfer of an H-atom from the substrate (or ArOH in case with antioxidant). As the reaction with FR and ArOH becomes more exotermic, the barrier should decrease and the antioxidant will react faster with the FR, thus preventing reaction with substrate. Therefore, Bond Dissociation Enthalpy (BDE) in ArOH will be an important factor in determining the efficacy of an antioxidant, since the weaker the OH bond the faster will be the reaction with FR.

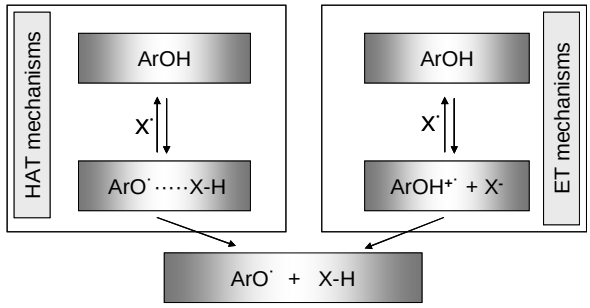


Figure 11 *HAT and ET mechanism of FR deactivation.*

Electron transfer (ET, e^--transfer) is another mechanism by which an antioxidant can deactivate a FR. In this reaction a radical cation is formed first followed by a rapid and reversible deprotonation in solution. The net result is the same as in the HAT mechanism (***Fig. 11***). However, if the radical cation $ArOH^+$ has sufficient lifetime it can attack suitable substrates: DNA, protein and lipids, etc, therefore, it can behave as pro-oxidant (***Fig. 10***). The Ionization

Potential (IP) of a molecule define its electron transfer capacity (Nenandis, 2005).

Metal-chelation is a property of solely catechol containing compounds. Such compounds can form stable complexes with various di- and trivalent metal ions, the complexes with trivalent ions being the most stable. Due to the high stability of catechol-metal complexes, compounds containing the catechol group can sequester metals from other complexes, thus preventing metals from undergoing redox reactions (Schweigert, 2001).

Theoretical investigations

Both the HAT and ET mechanisms must always occur in parallel, but with different rates. Evaluating antioxidants from a theoretical perspective, it is desirable to determine accurately both BDE and IP, the former relevant to the atom-transfer mechanism ($ArOH \rightarrow ArO^{\cdot}$) and the latter relevant to electron transfer ($ArOH \rightarrow ArOH^{+}$). The reduction reaction via donation of an electron is typical for phenolic antioxidants. Antioxidants that are reducing agents can also act as pro-oxidants. For example, a polyphenol has antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide, however, it will also reduce metal ions that generate free radicals through the Fenton reaction. It is worth noting that when the BDE or IP become too low, the compound can act as a pro-oxidant rather than as an antioxidant (Nenadis, 2005).

Theoretical BDE and IP values were applied for a prediction of the radical scavenging potential of phenolic compounds of olives and olive leaves (Nenadis, 2005). Thus, catechol-containing compounds (HOTYR and its secoiridoid derivatives) appeared to

have the lowest BDE values, whereas monophenols such as TYR had much higher BDE values (a lower potential for radical scavenging) and HVAlc, a methyl conjugate of HOTYR, being intermediate. In real systems, however, the activity of these compounds may vary due to differences in their lipophilicity and the composition of the system where they act (Nenadis, 2005).

Synthetic radical scavenging activities

Different synthetic radicals were used not only for evaluating the total antioxidant activity of olive oil (Valavanidis, 2004; Gorinstein, 2003), olive oil fractions (Lee, 2008, 2009) but also for individual phenols (Briante, 2003; Carrasco-Pancorbo, 2005b). Scavenging effects of HOTYR, TYR and other individual olive oil phenols were evaluated mainly using ABST (Paiva-Martins, 2003), DPPH (Roche, 2005) and DMPD (Briante, 2003) synthetic radical decolourization assays[7]. The reduction of these long-living radicals, which is recorded spectrophotometrically, requires transfer of hydrogen form tested compounds to the synthetic radical, which mimics *in vivo* radical species. The results of these assays showed that HOTYR and its derivatives (all sharing *o*-diphenolic structure) are the most potent radical scavengers of olive oil origin, whereas monophenols as TYR are quite weak. Moreover, it was shown that activities of HOTYR found in these tests are higher than to those reported for well-known natural antioxidants as α-tocopherol, trolox (a water soluble analogue of tocopherol) or ascorbic acid (Nenadis, 2002; Visioli, 1998). Few and controversial data are available on

[7] The results of radical scavenging assays are usually expressed as EC_{50} or Trolox equivalents characterize the ability of compounds to donate hydrogen, in addition, amount of hydrogen atoms donated to radical could be evaluated stoichiometrically.

the radical scavenging activities of HVAlc (Tuck, 2002; Grasso, 2007).

Specific radicals scavenging activities

The scavenging capacity of olive oil phenols (HOTYR, TYR and their secoiridoid derivatives) towards most important biological reactive species were studied by using a variety of *in vitro* and *ex vivo* methods, and the reports on some of them are summarized in ***Supplementary Table III***.

The reported activities of olive oil phenols in scavenging biological radicals are very dependent on the methods used in the evaluation. There are two possible mechanisms of antioxidant behaviour (and two ways of its detection): indirect by suppression of the radical generation or other way of interaction on the extent of oxidation (measuring levels of the damage done by RS), and direct by scavenging of the generated radicals (measure the levels of the trapped molecules) (Halliwell, 2004). Independently on the mechanism, in the majority of cases, olive oil *o*-diphenolics (HOTYR and its derivatives) behaved as potent antioxidants, whereas the monophenolic compound TYR was poorly active. The HVAlc was studied in several studies and was shown to be partially active against reactive species in several detection systems (see ***Supplementary Table III***).

Transition metal chelation and reduction

In the presence of transition metal[8] ions, both radical scavenging and metal chelation contribute to the antioxidant effects of phenols. Transition metals are strongly implicated in the production of highly reactive hydroxyl radicals by the superoxide driven Fenton reaction[9] as well as in the direct reductive decomposition of lipid hydroperoxides to provide alkoxyl and lipid peroxyl radicals as propagation radicals (Haliwell, 1995) (***Fig. 12***). Catechol containing phenols may chelate transition metal ions, hence reducing metal-induced oxidative reaction, but they also reduce transition metals.

One of the potent inhibitory effects of olive oil phenols bearing a catechol on lipid peroxidation may be related to the formation of Cu(II)-oxygen chelate. Therefore, antioxidant effects of HOTYR and its secoiridoid derivatives due to the metal chelation were studied in several studies (Paiva-Martins, 2005; Briante, 2003). The protonated catechol group is not a particularly good ligand for metal cations, but once deprotonated can chelate metals at physiological pH (Hider, 2001; Paiva-Martins, 2005). Thus it was shown that olive oil 3,4-dihydroxyphenols, including HOTYR, can form complexes with Cu(II), however these compounds with catechol structures were susceptible to oxidation (Briante, 2003). The ability of HOTYR to chelate transition metals can be related to the high activity of both hydrogen atoms of its catechol group (Erkoç, 2003).

[8] Metals that have an incomplete inner electron shell and that serve as transitional links between the most and the least electropositive in a series of elements.

[9] Ferrous Iron(II) is oxidized by hydrogen peroxide to ferric iron(III), a hydroxyl radical and a hydroxyl anion. Iron(III) is then reduced back to iron(II), a peroxide radical and a proton by the same hydrogen peroxide: (1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ and (2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\cdot} + H^{+}$.Then, the generated radicals are engage in secondary reactions.

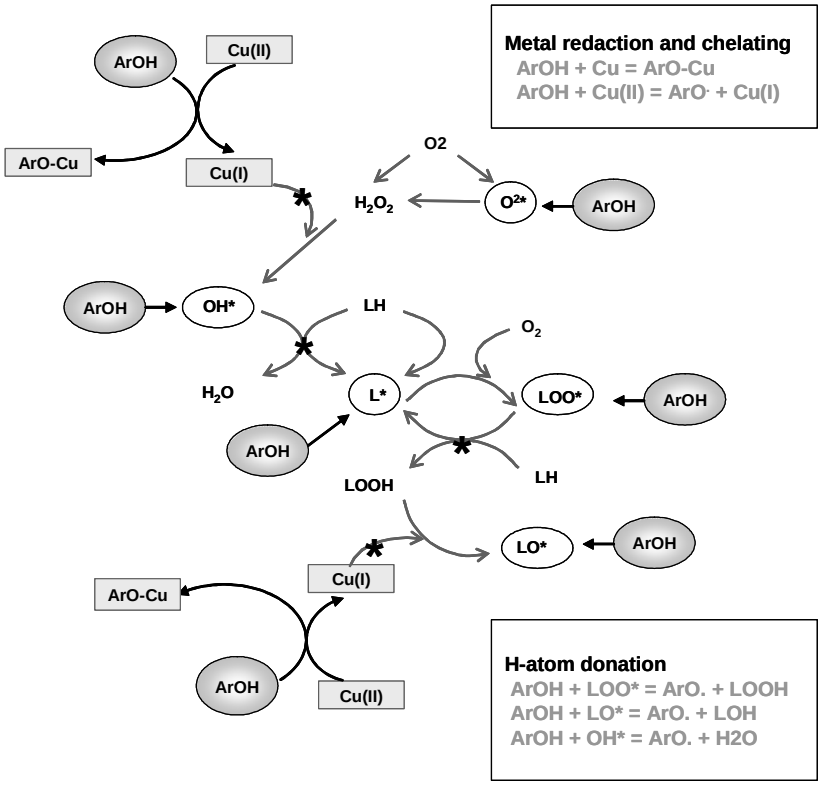


Figure 12 *Lipid peroxidation involving Cu as a transition metal and role of the olive oil antioxidants preventing such reaction.*
ArOH – is reduced form of oxidant; ArO· – is oxidized form of antioxidant; Cu(II) and Cu(I) – are oxidized and reduced forms of Cu, respectively; OH - hydroperoxide radical; O2* - superoxide radical; LH - lipid molecule; LOOH – lipoperoxide; LOO* - lipid peroxide radical; LO* - lipid oxide radical; L* - lipid radical; asterisks – sites where olive oil phenols could interrupted oxidation process.*

Since reduced transition metals are more active that non-reduced at catalyzing the decomposition of hydroperoxides into free radicals (***Fig. 12***), metal-reducing properties of polyphenols can increase oxidative reactions. Transition metal reductions by olive oil phenols and phenols bearing structural similarity (methylcatechols, catechols and monohydroxy compounds) have been recorded in

several experimental studies (Briante, 2003; Paiva-Martins, 2005; Aguiar, 2007; Manna, 2002; Mazziotti, 2006). It was shown that the reducing capacity is connected to the presence of a specific ligand of the reduced ion (Briante, 2003). In general, *o*-diphenols (as are olive oil HOTYR and its secoiridoid derivatives) can reduce substantial amounts of transition ions (Cu^{2+} and Fe^{3+}), whereas monohydroxy compounds (as TYR) did not reduce (Manna, 2002), probably because they cannot form quinones. Although the methoxyl group does not oxidize during reaction, it is an electron donor stimulating the reactivity of the vicinal hydroxyl group by induction, therefore HVAlc can actively reduce transition metals (Aguiar, 2007). Undergoing redox reactions, catechol (as HOTYR) (Roche, 2005) and methoxycatechols (Fujisawa, 2005) (which are structurally similar to HVAlc) can cycle between themselves, often producing redox active polymers (Hotta, 2002).

Regeneration of other antioxidants via their reduction.
Antioxidant synergism

In nature, antioxidants exist in combination and these antioxidants may act additively or even synergistically against oxidation. A combination of different antioxidants can be superior to the action of single antioxidants in protecting LDL lipid and protein moiety against oxidation (Yeomans, 2005).

Few studies evaluated the interaction of olive oil phenols with other antioxidants. Some evidence of a higher antioxidant effect of olive oil phenols in combinations with tocopherol have been provided in a study with liposomes (Paiva-Martins, 2003). Also mixtures of biophenols were more active than individual biophenols as antiproliferative agents, particularly it was observed for a mixture of

hydroxytyrosol/caffeic acid in protecting DNA from oxidative damage and inhibiting the growth of cancer cells (Obied, 2009). The mechanisms of such interactions are not clear. Probably, the regeneration of antioxidants from their electron-oxidized form by olive oil phenols could take place in analogy to the well-known synergism between tocopherol and ascorbate (Buettner, 1993).

4.1.1.2. Physical properties of HOTYR and TYR contributing to their antioxidant activities

Other factors that may contribute to the overall performance of the compounds in real systems are: (i) the molecule size and (ii) its lipophilicity.

Olive oil phenol alcohols seem to be quite similar in molecule size, although do not exhibit close similarity in their structures: HOTYR is a catechol, TYR is a phenol and HVAlc is a methoxyphenol. The three-dimensional configuration of compounds is expected to moderate penetration into membranes and thus affect antioxidant performance in biological systems (Nenadis, 2003; Paiva-Martins, 2003).

Lipophilicity/polarity plays an important role in cell-uptake, receptor binding and other properties influencing the biological activity of a compound. Lipophilicity of molecules is evaluated by measuring the partitioning coefficient between an organic phase and aqueous phase. It was reported (Paiva-Martins, 2003) that HOTYR, together with its acetate, aldehyde of OLE-aglycone cannot penetrate membranes, as a consequence of their hydrophilic properties and their non-planar structures defining their conformational mobility.

Therefore, their effectiveness as antioxidants were associated with their interaction with the surface of the phospholipids bilayer, where they are supposed to act as scavengers of aqueous peroxyl radicals but not as scavengers of chain propagation lipid peroxyl radicals within membrane (Paiva-Martins, 2003). Thus, the high polarity of HOTYR results in a small concentration of the phenol in the lipid phase, that also directs the distribution of its activities within biological systems.

4.1.1.3. CVD related antioxidant properties of HOTYR and TYR: *in vivo* and *in vitro* studies

The principal antioxidant properties of olive oil phenols described on chemical and physical models could explain to some extent the mechanisms underlying their antioxidant activities in more complex biological models and systems. A set of experiments, reviewed below, showed that olive oil phenols protect various macromolecules from their oxidation, and could participate in the protection of cell and whole organism against oxidative processes primary involved in CVDs development and progression.

Protection against oxidative damage of macromolecules

Mixtures of olive phenols are able to reduce hydrogen peroxide (H_2O_2)-induced DNA damage in cells (Fabiani, 2008; Nousis, 2005) as well as individual olive oil phenols as HOTYR and caffeic acid, and to a lesser extent HVAlc (Grasso, 2007; Fabiani, 2008; Nousis, 2005; Quiles, 2002). The activity of TYR against hydroperoxyde induced DNA damage remains unclear: it is able to reduce DNA oxidation only at high doses in oxidative-stress-sensitive cells

(Quiles, 2002), and did not exert any protection activity in hydrogen peroxide exposed Jurkat cells (Nousis, 2005), nevertheless, in a study with activated monocytes it was reported to be more effective than HOTYR (Fabiani, 2008). It is worth noting that complex mixtures of olive phenols could exert DNA damaging effects by themselves in the absence of any hydrogen peroxide (Nousis, 2005). HOTYR was also highly protective against the peroxynitrite-dependent nitration of tyrosine and DNA damage *in vitro* (Deiana, 1999).

The role of phenolic compounds on *in vivo* DNA oxidative damage after olive oil consumption in humans remains unclear. The protective role was observed against of DNA oxidative damage taking place in peripheral blood mononuclear cells or lymphocytes (Weinbrenner, 2004a; Salvini, 2006), but not on the whole body DNA oxidation (Machowetz, 2007; Hillestrøm, 2006) measured by accumulation of DNA oxidative products in urine (Poulsen, 2005), where it was lowered irrespectively to amount of phenols by any type of olive oil (Machowetz, 2007).

LDL-oxidation

Among the various substrates which can be oxidized by free-radical-mediated reactions is LDL. Several *in vitro* system have been developed to mimic the reactions occurring *in vivo*, among them the susceptibility of isolated LDL and of lipid models (micelles, vesicles, emulsions and liposomes) to oxidation are the most common (Cheng, 2003; Frankel, 2000; Paiva-Martins, 2006; Saija, 1998). The experimental set-up involves either free radicals or transition metal ions induced lipid oxidation (Esterbauer, 1989), and the inhibitory effect of variety of lipid-soluble and water soluble

antioxidants, and complex mixtures containing them on LDL oxidation (Briante, 2004, Bagnati, 1999; Leene, 2002; Fitó, 2000, Visioli, 1995, Caruso, 1999).

HOTYR and OLE were reported to inhibit the radical induced lipid peroxidation of fatty acids in lipid model systems (micelles, vesicles and liposomes), but not TYR (Roche, 2005; Paiva-Martins, 2003; Saija, 1998). They act rather as retardants, reducing the initiating hydrophilic peroxyl radicals in aqueous phase, than as chain breakers like α-tocopherol. The long lasting antioxidant effect was explained by the residual activity of some of their oxidation products (Roche, 2005). In addition, their antioxidant activity in lipid models depended on their location and orientation in the system, where HOTYR and its secoiridoid derivatives scavenge aqueous peroxyl radicals near the membrane surface (Saija, 1998; Paiva-Martins, 2003).

The protective effect of olive oil phenols on oxidation of human LDL *in vivo* has been observed in several clinical and intervention studies, earlier discussed in this chapter. In contrast to their mono-hydroxy counterparts (TYR and hydroxyphenylacetic acid), the *o*-diphenols (HOTYR, dihydroxyphenylacetic acid and OLE-aglycone) were reported to efficiently increase the *in vitro* resistance to oxidation of LDL isolated after being plasma pre-incubated with tested compounds (Leenen, 2002) (***Fig. 13***). Both HOTYR and OLE potently and dose dependently inhibit *in vitro* peroxyl radical–dependent and metal-induced oxidation of LDL isolated from plasma (Fitó, 2000; Visioli, 1995). In addition, OLE together with TYR were shown to prevent cholesterol oxide formation and the apoproteic moiety modification formed during LDL photo-oxidation

by UV light (Caruso, 1999). The macrophage-like cell-mediated oxidation of LDL was inhibited by HOTYR and TYR, although to different extent (100% HOTYR and 40% TYR), after a pre-incubating cell lines with the tested compounds (Di Benedetto, 2006).

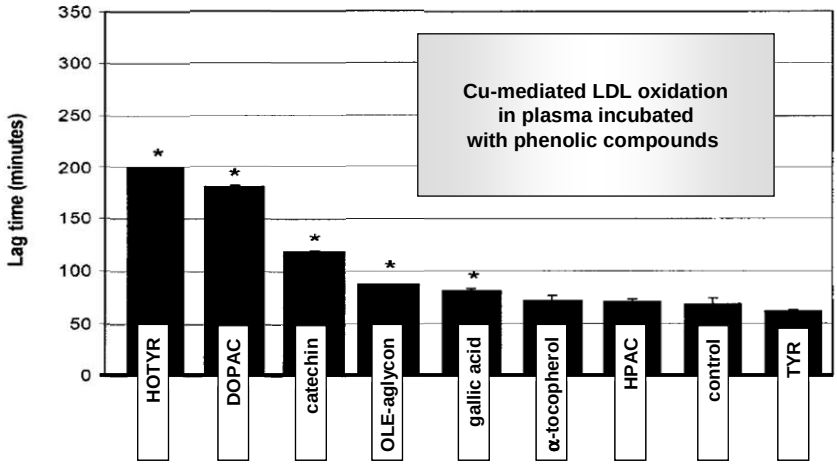


Figure 13 *Inhibition of Cu-mediated LDL oxidation by olive oil phenols and other antioxidants (catechol and α-tocopherol) preincubated with plasma at 1 mM concentration, where HOTYR – hydroxytyrosol; DOPAC – dihydroxyphenylacetic acid; OLE-aglycon – oleuropein aglycon; HPAC – hydroxyphenylacetic acid; TYR – tyrosol; control – no compound was added (adapted form Leenen, 2002).*

The phenolic content of olive oil provided benefits in a direct dose-dependent manner for plasma lipids and lipid oxidative damage in humans (Covas, 2006b). In a subset of subjects it was shown that olive oil caused an increase in plasma and LDL oleic acid content. In addition, olive oil rich in phenolics led to an increase in their concentrations in LDL in a direct relationship with the phenolic content of oils. This can account for the increased resistance of LDL to oxidation, and the decrease of oxidized LDL, observed within the frame of this clinical trial (Gimeno, 2007). Phenolic

content of LDL was correlated with plasma concentrations of HOTYR in plasma (Covas, 2006b) and its presence in LDL has been demonstrated (de la Torre-Carbot, 2007).

Several studies applying cell culture and animal models reinforce the data on the protective role of main olive oil phenols (HOTYR, OLE and to some extent TYR) against differentially induced LDL oxidation and will be discussed later.

Antioxidant function of olive oil phenols in cell and animal models

In parallel to studies with humans, several animal studies have demonstrated that the degree of oxLDL *in vivo* decreases as the phenolic content in the administrated olive oil increases (Ochoa, 2002; Wiseman, 1996). A number of in vitro studies support these findings (Franconi, 2006; Masella, 2004; Visioli, 1995). Also, positive changes in the plasma antioxidant activity and lipid metabolism were attributed to the phenolic content in a study with rats adapted for cholesterol-free and cholesterol-containing diets (Gorinstein, 2002; Krzeminski, 2003). The consequences of smoke-induced oxidative stress were reduced in rats by administration olive mill waste water rich in HOTYR (Visioli, 2000a).

Supplementation with individual olive oil phenolics also improve the atherogenic status in animal models via: (i) increasing the ability of LDL to resists oxidation and at the same time reducing the plasmatic levels of total, free and esterified cholesterol (Coni, 2000); (ii) a direct protection against the post-ischemic oxidative burst in the isolated rat heart (Manna, 2004); (iii) improving blood lipid profile and antioxidant status in hyperlipemic rabbits

(Gonzales-Santiago, 2006); and (iv) significantly lowering serum total- and LDL-cholesterol levels, whereas increased HDL-cholesterol levels and retarded the lipid peroxidation processes (Fki, 2007).

Cell cultures experiments with HOTYR and its secoiridoids (3,4-DHPEA-EDA, OLE and 3,4-DHPEA-EA) have shown that these phenolics: (i) positively affect the antioxidant defence system of hepatic cells, favouring their cell integrity and resistance to oxidative stress (Goya, 2007); (ii) significantly protect red blood cells from oxidative damage (Paiva-Martins, 2009) and against peroxide-induced cytotoxicity (Manna, 1999). HOTYR and its metabolite HVAlc were able to prevent the lipid peroxidation process in renal cells (Deiana, 2008) whereas OLE completely prevented the LDL oxidation mediated macrophage-like cells (Masella, 2004). The oxidized LDL-induced alterations in Caco-2 cells were almost completely prevented by pre-treatment with TYR (Giovannini, 1999).

4.2. Non-antioxidant activities of olive oil phenols

Dietary polyphenols can potentially influence normal and pathological cellular processes through modulation of intracellular signaling pathways (Santangelo, 2007). Olive oil dietary phenols exhibit several biological activities that are not directly related to their antioxidant properties. The parent compounds and/or their metabolites:

(i) have impact on cellular signaling pathways;

(ii) influence the expression of certain genes;

(iii) act as inhibitors/activators of regulatory enzymes.

In these ways they reveal additional biological effects which might be of importance in the context of CVD prevention related and the consumption of a diet rich in antioxidants (Giovannini, 2007).

Enzymes inhibition/activation

Olive oil dietary phenols activities on enzymes potentially sensitive to phenolic compounds have been tested in a variety of cellular models: platelets, leukocytes, macrophages, etc.

Olive extract strongly inhibited lipoxygenase activities of rat platelets and polymorphonuclear leukocytes and HOTYR was identified as a potent specific inhibitor of arachidonate lipoxygenase activities (Kohyama, 1997). HOTYR is able to modulate several enzymatic activities linked to CVD: inhibit the pro-inflammatory 5-lipoxygenase activity in leukocytes (de la Puerta, 1999) and the expression of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in cells treated with lipopolysaccharide (Zhang, 2009). However in human endothelial cells under non-inflammatory conditions, HOTYR was not able to modulate the eNOS enzyme neither at the level of its expression nor its activity (Schmitt, 2007). OLE also was suspected to cause some increase in expression of iNOS cellular expression (de la Puerta, 2001). An inhibition of cAMP-phosphodiesterase was proposed to be one of the mechanisms through which olive oil phenols inhibit platelet aggregation (Dell'Agli, 2008). OLE was able to restore glutathione reductase (GS-R) and peroxidase (GSH-Px) activities in LDL-challenged macrophage-like cells (Masella, 2004) and, in contrast to HOTYR, was found to strongly inhibit CYP3A following an mechanism based inhibition and weakly CYP1A2

(Stupans, 2001), which could partially explain its in vivo protective effect against LDL oxidation (Coni, 2000).

Modulation of signaling pathways and gene expression

The response of cells to oxidative stress is very complex and modulated by a variety of regulators, some of the main signaling pathways involved in cellular response to OS are present in ***Fig. 14*** (Selfried, 2007; Valko, 2007). Increasing evidences demonstrate that oxidants and antioxidants can influence important signal cascades, such as mitogen-activated protein kinases (MAPKs), which control proliferation and apoptosis; protein tyrosine phosphatases (PTPs) and tyrosine kinases which regulate the phosphorylation state of many important signalling molecules implicated in regulation of many cellular processes, kinase protein kinase C (PKC-α), involved in signal transduction to various effector pathways that regulate transcription and cell cycle control, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a multiprotein complex known to activate genes involved in the early cellular defence reaction (Genestra, 2007).

The interaction of olive oil phenols with cell signaling systems and their influence on gene expression is on the preliminary stages of investigation. Many of these studies are dedicated to the analysis of their interaction with well characterized signaling pathways mainly involved in carcinogenesis (Menendez, 2009; Corona, 2007). Nevertheless, some interesting data has been also obtained on their interaction with cellular processes involved in development and progression of CVD.

Recently it was shown, that protective action of HOTYR and HVAlc against peroxide-induced injury in renal epithelium was linked to their potential to modulate the activation of ERK, Akt and JNK and interaction with some of the apoptosis-related signalling pathways (Incani, 2009). Concerning the influence of olive oil phenols on the expression of CVD inflammatory related proteins, it has been described that some phenolic compounds may inhibit cytokine and eicosanoid production by inhibiting IL-1β mRNA and protein expression and COX-2 activity and transcription (Carluccio, 2003; Petroni, 1997; de la Puerta, 1999). These interactions may contribute to the anti-atherogenic properties ascribed to EVOO. OLE-glycoside inhibits the production of IL-6 or TNF-α (Miles, 2005a), but both OLE and TYR were unable to decrease IFN-gamma production or IL-2 or IL-4 concentrations in stimulated human whole blood cultures (Miles, 2005b).

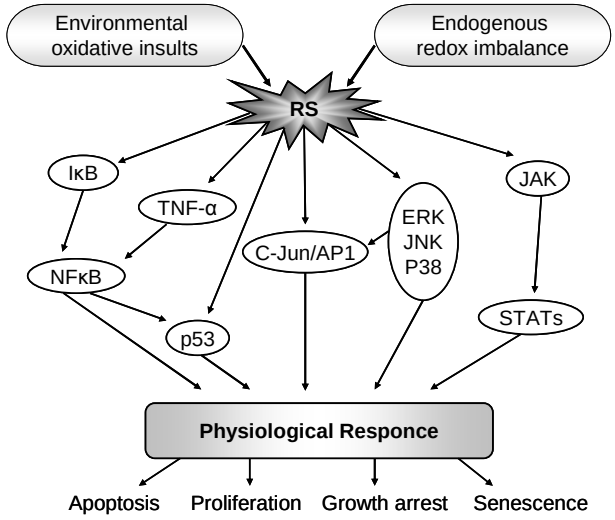


Figure 14 *Main routes of cellular response to oxidative stress (adapted form Seifried, 2007).*

Monocyte adhesion to endothelial cells can also be modulated by VOO phenolic compounds. Already at physiologically relevant concentrations of phenolics, an extract from EVOO was reported to strongly reduce cell surface expression of ICAM-1 and VCAM-1, adhesion molecules involved in early steps of atherosclerosis, linked to a reduction in mRNA levels. OLE and HOTYR appeared to be the main components responsible for these effects (Dell´Agli, 2006). Furthermore, olive oil individual phenols as OLE, TYR, HOTYR, and HVAlc were reported to significantly reduce the secretion of these adhesion molecules, in addition to protect against cytotoxic effects of hydrogen peroxide and oxidized LDL on cells (Turner, 2005). The involvement of transcription factors NF-κB and AP-1 in mediating VCAM-1 transcriptional inhibition by phenolic compounds, among which were present OLE-aglycon and HOTYR, was reported (Carluccio, 2003).

HOTYR in a concentration-dependent manner could inhibit the expression of iNOS and COX-2 and also significantly attenuate the LPS-induced transcription of TNF-alpha in THP-1 cells (Zhang, 2009). HOTYR inhibits *in vitro* the formation of thromboxane B2 (TXB(2)) by stimulated platelets and the accumulation of TXB(2) and eicosatetraenoid production in serum (Petroni, 1995). In addition, HOTYR in a dose-related manner inhibits the production of leukotriene B4 (LTB(4)), the main arachidonic acid metabolite synthesized by stimulated polymorphonuclear leukocytes responsible for chemotaxis, aggregation and degranulation of these cells (Petroni, 1997).

Relationship between expression of redox-homeostasis related proteins and olive oil phenols was investigated in few studies,

mainly concentrated in glutathione system functioning. Thus, it was shown that at low concentrations HOTYR can decrease mRNA levels of GSH-Px and phospholipid hydroperoxide, whereas TYR increased these levels at high concentration (Quiles, 2002). OLE was able to restore the expression of several genes of GSH-related enzymes in LDL challenged macrophage-like cells, as a result, it was suggested that an activation of mRNA transcription of these enzymes represents an important mechanism in olive oil phenolic antioxidant action (Masella, 2008).

4.3. Biological activities of olive oil phenolic compounds HOTYR and TYR

Antioxidant and non-antioxidant biological actions of olive oil phenolic compounds have been intensively studied regarding their relevance to human health. Some of the mechanisms underlying the health beneficial properties of olive oil phenols (OOPh) have been reported based on the conducted *in vivo* and *in vitro* and *ex vivo* experiments summarized in ***Table 3***:

Table 3 *Biological activities of HOTYR and TYR which might underline health beneficial properties of olive oil.*

Targets	Main mechanisms	Ref
	Antiatherogenic properties	
LDL	Inhibition of LDL oxidation: - preserving the endogenous LDL antioxidant pool; - scavenging lipid radicals; - chelating metals inducing oxidation;	Visioli, 1995, 2000a; Masella, 1999 ;
Cholesterol	Decrease in blood levels of cholesterol: - inhibition of HMG-CoA reductase, a principal enzyme in cholesterol synthesis	Benkhalti, 2002;
	Anti-thrombotic activities	
Platelets	Inhibition of platelets aggregation:	López-

Cells interactions	- decrease in platelet thromboxane synthesis; - increase in leukocyte nitric oxide production; Inhibition of monocytes adhesion to endothelial cells: - alteration in expressions of adhesion molecules (ICAM-1 and VCAM-1);	Miranda 2007; González-Correa, 2008; Carluccio, 2003; Turner, 2005; Manna, 2009;
Anti-tumorogenic activities		
Cell death and proliferation	- modulation of signal transduction pathway, enzymes activities and protein expression; - inhibition of cell cycle progression; - induction of apoptosis;	Corona, 2009; Han, 2009; Fabiani, 2002;
Neuroprotective action		
Neuronal cells	Neurons cytoprotective effect: - modification of thrombogenic processes; - diminishing platelet aggregation; - reducing oxidative stress; - cytoprotection; - enhance resistance to oxidative stress; - protecting from hypoxia-reoxygenation effects	González-Correa, 2007, 2008; Schaffer, 2007; Young, 2008; Hashimoto, 2004;
Anti-inflammatory effects		
Inflammation	Immunomodulation: - inhibitory action on pro-inflammatory enzymes (COX, lipoxygenase, myeloperoxidase); - reduce formation of pro-inflammatory molecules: TXB(2) and LTB(4);	de la Puerta, 2000; Petroni, 1997; Martinez-Domínguez, 2001;
Anti-microbial, anti-fungal and anti-viral activity		
Pathogen	Inhibition of viral and bacterial growth and activity: - protein denaturants; - inhibitors of principal pathways; - modulators of oxidative stress.	Kubo, 1985 ; Konno, 1999; Lee-Huang, 2007a, 2007b

5. Olive oil as functional food modifying transcriptome of genes related to CVD

It is recognized that understanding the effect of diet on health requires the study of the mechanisms of nutrients and other

bioactive food constituents at the molecular level (Scalbert, 2008; Hocquette, 2009; Garcia-Cañas, 2010). It has been demonstrated in studies with humans, animals and cell cultures studies that different food components can modulate gene expression (GE) in diverse ways. These observations are in the basis of a new field that focuses on the study of the interaction between nutrition and human genome: Nutritional Genomics (Nutrigenomics).

There is a dynamic, two-way interaction between nutrition and the human genome. This interaction determines gene expression and the metabolic response, which ultimately affects an individual's health status and/or predisposition to disease (***Fig. 15***) (Roche, 2004).

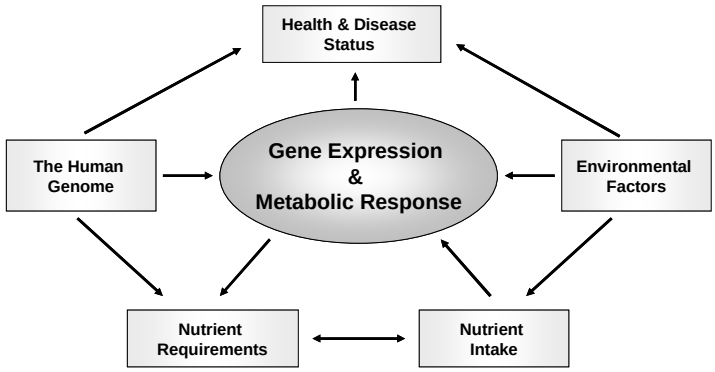


Figure 15 *Interaction between nutrition and the human genome (adapted from Roche, 2004).*

Olive oil is composed by diverse constituents, many of which have specific biological activities, altogether contributing to health benefits of this dietary product. Therefore, pioneering nutrigenomic research was focused mainly in two principal olive oil features: its intrinsic monounsaturated fatty acids composition (Escrich 2006,

2007; Menendez, 2006) and its specific microconstituents content (Acín, 2007; Carluccio, 2003; Menendez, 2008), where a central place was dedicated to the redox-active phenolic compounds. Until now few studies have studied the interaction of these olive oil components with the expression of genes, and all of them followed a hypothesis driven approach. The majority of olive oil nutrigenomic studies were conducted in cancer research (Escrich, 2007, 2008; Menendez, 2006, 2008) and very few of them, discussed below, are concerned with that interaction in respect to CVD.

5.1. Olive oil lipids and gene expression

Dietary fatty acids interact with multiple nutrient-sensitive transcription factors. Some of them can explain the molecular basis of several health effects associated with altered dietary fatty acid composition (Roche, 2004). The effect of dietary lipids on GE can be indirect, via changes in cell membranes and signal transduction pathways to the nucleus, and direct, when effects of fatty acids or their metabolites may be directly mediated by binding to various nuclear receptors and activating their transcription factor action (Escrich, 2007). It has been shown, that dietary lipids and their metabolites modify the expression of genes which can be potentially involved in development and progression of AT processes or CVD, and related to them metabolic pathologies as diabetes and obesity (Li, 2005; Raclot, 1997; Landschulz, 1994; Ren, 1997). Recent experimental evidences show that exist specific receptors for fatty acids or their metabolites that are able to regulate gene expression and co-ordinately affect metabolic or

signalling pathways associated with CVD (Vanden Heuvel, 2009; Weaver, 2009).

There are a few experimental studies addressing the role of olive oil fatty acids transcriptome activity with respect to CVD related genes. Thus, it was described that some of the monounsaturated fatty acids typical for olive oil (mainly oleic, linoleic and linolenic fatty acids) can interact with AT-related genes (Toborek, 2002). Such studies have been mainly conducted in cell cultures or animal models (Osada, 1991), and to a lesser extent in humans (Bellido, 2004).

Nutrigenomic experiments, focusing in more specific and close to real *in vivo* situation, started to be performed just recently, promoted by methodological developments, as high-throughput technologies. Recently it has been demonstrated in an *in vitro* model that different fatty acid composition of triglyceride-rich lipoproteins (TRL) is capable of differentially modifying gene expression in human coronary artery smooth muscle cells (Bermudez, 2008). In this study the ingestion of meals enriched with different sources of fatty acids (refined olive oil, butter and or a mixture of vegetable and fish oils), was studied and results show that TRL-refined olive oil promoted a less atherogenic gene profile than the other two treatments.

5.2. Olive oil phenolics and gene expression

For many years, dietary polyphenols were thought to protect cell constituents against oxidative damage through scavenging of free

radicals. However, nowadays this concept appears to be an oversimplified view of their mode of action (Scalbert, 2005). It was shown that the expression of various genes can be effected by a variety of phytochemicals, especially those exhibiting antioxidants properties (de Kok, 2009; Nair, 2007). Genes involved in the physiopathological processes leading to the CVDs as well can be affected as it has been shown in different experimental studies (Yeh, 2009; Nicholson, 2008).

Olive oil phenols have been acknowledged for their array of biological activities, where anti-atherogenic activities play a central role, as discussed earlier in this chapter. Although, the antioxidant properties of olive oil phenols have been extensively studied, it is still unclear whether and how dietary antioxidants contribute to the *in vivo* cellular antioxidant defense. In addition, there are many uncertainties regarding the bioavailability of olive oil phenols and, therefore, their access to intracellular processes and signaling pathways (in this regard see chapter II). Nowadays, we are starting to acknowledge that olive oil phenolics may influence human physiology through cell-mediated effects (e.g. via induction of transcription factors), rather than by directly interacting with free radicals or with some key enzymes as often thought, as it has been shown in several cell cultures and animal models studies, (see Non-antioxidant activities of olive oil phenols), however, no data are available about their *in vivo* transcriptome activities in humans.

5.3. Olive oil as a complex trascriptome active food

In nutrigenomic sense, olive oil bioactive constituents can be referred to as signals that are detected by cellular sensor systems and affect the expression of the genome at several levels (mRNA and proteins) and subsequently, the production of metabolites (***Fig. 16***). Being composed by a number of different chemical molecules, olive oil behaves as a complex dietary product, wherein all bioactive compounds interact each other, altogether making their impact on biological system even more diverse. Olive oil as a complex food can have a number of direct (interaction with a number of transcription factors responsible for up- and down-regulation of gene expression) and indirect effects (via metabolism related interaction with cell signaling cascades which then alter gene transcription) on gene expression (Roche, 2004; Müller, 2003; Santangelo, 2007).

The development of new methodological approaches in the field of genomics facilitates the study of nutritional-genomic interactions on all impacted by nutrition factor levels, among which transcription is recognized as a principal one. New high-throughput technologies in transcriptome analysis make possible to assess the effect of a specific diet or nutrient on the expression of a large proportion of the whole genome (Garcia-Cañas, 2010). The monitored gene-expression profiling can facilitates the information about the mechanism underlying the beneficial or adverse effects of a certain nutrient or diet, help to identify important genes, proteins or metabolites that might, act as ‘molecular biomarkers’, and help to

characterize the basic molecular pathways of gene regulation by nutrients at a more basic level (***Fig. 16***).

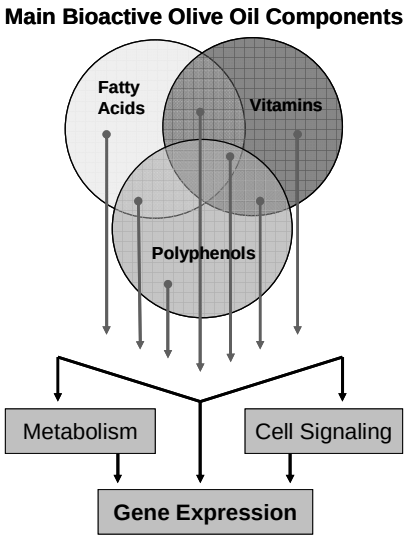


Figure 16 *Complexity of olive oil and direct and indirect cellular transcriptome response.*

Thus, nutrigenomic effects of olive oil on the development of AT were analyzed in series of experiments on genetically modified mice that spontaneously develop atherosclerosis (Acín, 2007; Alemany, 2009; Arbones-Mainar, 2007). Despite limitations of this animal model associated with morphological and physiological differences with humans, partially minimized by the similarity of the two genomes, several genes have been identified as responders to olive oil consumption (Guillén, 2009).

No data on *in vivo* olive oil-genome interaction are available in humans.

5.4. Olive oil nutrigenomics: limitations and perspectives

The use of cell culture or animal models is relevant in terms of understanding the interactions of olive oil components and gene expression. However, these experimental models are limited either by the use of doses/concentrations higher than those encountered in the diet or the use of simulated to real life dietary interventions conditions. This originates difficulties in extrapolating data to humans and clinical practice. The direct, definitive information on the effects of olive oil, whether they are nutritional or non-nutritional components, on human health can only be obtained through investigation in human subjects. An interaction between olive oil and human genome is essential in gaining the mechanistic insights on its health beneficial actions.

The application of gene expression profiling technologies in nutrition studies has the potential of providing highly detailed qualitative and quantitative descriptions of the molecular alterations in biological processes taking place in the human organism. Nevertheless, gene transcription analyses in studies involving human subjects are challenged due to a number of specific ethical and practical limitations:

(i) restriction in biological samples volume that can be collected (blood, saliva, body fluids, etc.);
(ii) wide genetic diversity between individuals;
(iii) high physiologic variability within the subjects participating in the study;
(iv) environmental conditions could be only partially controlled;

(v) weak influence of dietary factors, especially when the dose corresponds to the real-life applied doses, on the genome (e.g. transcriptome, proteome and metabolome);

(vi) only early or short-lasting effects could be monitored precisely;

(vii) uncontrolled confounding factors;

(viii) particular technical/methodological limitations, etc.

To achieve reliable results in nutrigenomic studies in humans, it is critical to define the main factors influencing gene expression variations and keep them minimized. This will reduce the influence of multifactorial system, defined by subject-environmental interaction, on the variability of gene expression system.

Two different, but complementary, strategies are settled in molecular nutrition research (Müller, 2003) (***Fig. 17***), which could be successfully applied to olive oil nutrigenomics research:

(i) the traditional hypothesis driven approach;

Using this approach, the specific genes and proteins, the expression of which is suspected to be influenced by olive oil could be identified using genomics tools - such as transcriptomics, proteomics and metabolomics. Subsequently, this allows identification of the regulatory pathways through which olive oil influences human homeostasis.

(ii) the system biology approach;

The signature of gene, protein and metabolites associated with olive oil intake could be catalogued, and might provide 'early warning' molecular biomarkers for nutrient-induced changes to homeostasis.

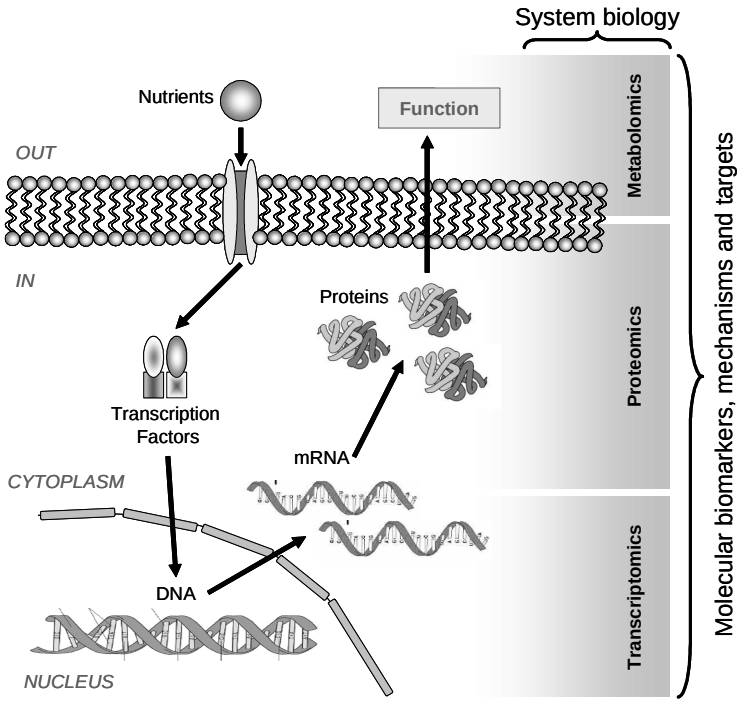


Figure 17 *Strategies in nutrigenomics analysis (adapted from Müller, 2003).*

Since no studies have been done on the *in vivo* olive oil gene-nutrition interaction in humans, one of the first steps should be to investigate:

(i) whether olive oil as a complex foodstuff consumed <u>at real-life dietary doses</u> can alter human transcriptome;

(ii) whether this interaction could be quantitatively monitored and analyzed, and, subsequently, used in extrapolation to some of the olive oil human health beneficial activities.

CHAPTER II

METABOLISM AND DISPOSITION OF OLIVE OIL PHENOLIC COMPOUNDS HOTYR AND TYR

1. Intake of HOTYR and TYR according to the dietary ingestion of olive oil

Intake of olive oil in the Mediterranean countries is estimated to be 30-50 g/day per capita (Boskou, 2000). Wide ranges (50-1000 mg/kg) have been reported for the amounts of total polar phenols in olive oils (Montedoro, 1992). Usual values range between 100 and 300 mg/kg (Boskou, 2006). Due to natural variability and many other factors (climate, area of growth, index of maturation, extraction, processing, storage etc. discussed in previous chapter), it is difficult to establish concentration levels of individual phenols. Despite of that, phenolic alcohols, phenolic acids, and secoiridoids were reported to be the most prevalent classes of hydrophilic phenols found in VOO (Servili, 2002, 2004) among which the most abundant are secoiridoid aglycons (Selvaggini, 2006; Gómez-Alonso, 2002), free HOTYR and TYR were found only in trace amounts (less than 10 mg/kg oil) (Servili, 2002; Christophoridou, 2009; Gómez-Alonso, 2002).

Based on a 50 g daily consumption of olive oil with an average concentration of polyphenols of 180 mg/kg, dietary intake of olive oil polyphenols has been estimated to be around 9 mg/day (Vissers, 2004). It was supposed that around 1 mg of these

polyphenols, which is equivalent to 6 μmol[10], is derived from free HOTYR and TYR, and 8 mg (23 μmol) is related to their EA esters: OLE- and LGS-aglycons (Vissers, 2004) (***Fig. 18***). The ingestion of HOTYR and TYR as EA-linked derivatives is probably the highest, given that they are broken down in gastrointestinal (GI) tract into HOTYR, TYR and EA, as will be discussed later in this chapter.

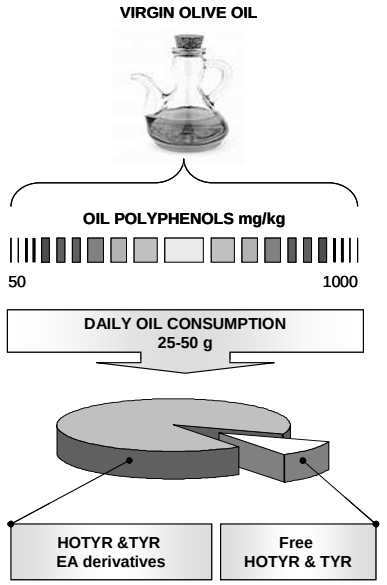


Figure 18 *Olive oil polyphenols dietary consumption.*

Several clinical and animal studies have provided evidence that HOTYR and TYR compounds are absorbed and exert their biological effects in a dose-dependent manner (Visioli, 2001; Weinbrenner, 2004a, b). However, some authors caution that the attained concentrations after their ingestion are too low to explain

[10] The amount of dietary intake in moles gets more insights into the potential of the antioxidants rather than milligrams, because the antioxidant activity depends on the number of reactive OH groups (Vissers, 2004).

the observed biological activities in *in vitro* and *in vivo* models at higher doses/concentrations (Vissers, 2004). In addition, the effect of any dietary compound is influenced by the active bioavailable dose rather than the dose ingested. Depending on the individual predisposition, including genetics and medication, a bioavailable dose may cause different magnitudes of effects in different people (Holst, 2008).

Beside being reported to be VOO polyphenols, HOTYR and TYR have been detected also in various food stuffs and beverages (Duncan, 1984; Rodríguez Madrera, 2006; Cartoni, 1997; Romero , 2004). In addition, they were reported to be present in red and white wines (di Tommaso, 1998; de la Torre, 2006).

2. HOTYR and TYR bioavailability studies

The data collected in a number of clinical intervention studies on the effects of olive oil phenols rise up a lot of questions, among which the most intriguing ones are:

(i) how these compounds behave within human body?

(ii) what are their mechanisms of action?

The answers to these and some other questions are directly related to the rate and extent to which the active olive oil polyphenols are absorbed from dietary and supplementary products and become available at the site of their action. In other words, to their bioavailability.

After oral consumption, the uptake of olive oil phenolic compounds into the body is not absolute, and a certain percentage is not

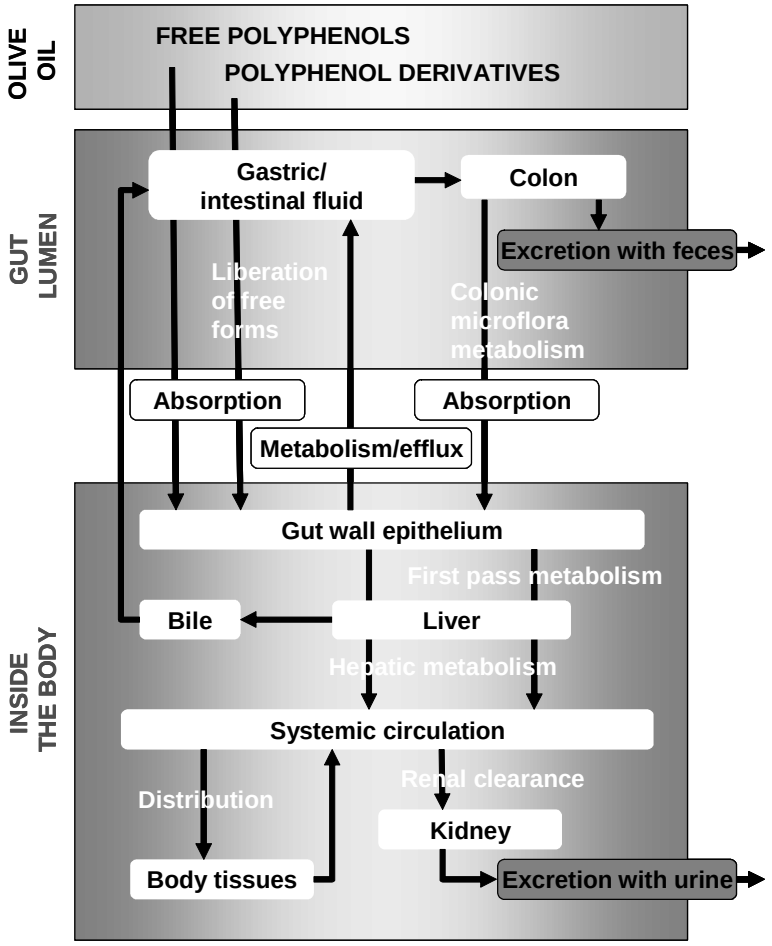


Figure 19 *Basic events describing the fate of olive oil nutrients/phenolic compounds within organism and following it their bioavailability (adapted from Holst, 2008a).*

absorbed. According to the classical pharmacological approach (Holst, 2008), the bioavailability of HOTYR and TYR (***Fig. 19***), specifically,

- their absorption (the diffusion or transport of a compound from the site of administration into the systemic circulation),

including their liberation (processes involved in the release of a compound from the site of administration),

- distribution (the diffusion or transportation of a compound from the systemic circulation to the body tissue),
- metabolism (the biochemical conversion or biotransformation of a compound) and
- excretion (the elimination of a compound, or its metabolite, from the body via renal, biliary or pulmonary processes)

was studied in different experimental models and linked to the data collected in clinical studies.

2.1. Analysis of olive oil polyphenols in biological samples

All evidence on bioavailability of olive oil phenolic compounds has been obtained by measuring the concentration of olive oil specific phenols and their metabolites in different samples (biofluids and tissue extracts). Such measurements require controlled dosing of olive oil or supplements with known content of polyphenols and sensitive analytical techniques for their analysis in biological samples. Different separation and detection systems have been applied to the analysis of olive oil polyphenols. These determinations refer mainly to the key olive oil polyphenols TYR, HOTYR and HVAlc as its main metabolite, and to lesser extend to other important olive oil related phenols as OLE aglycon and glycoside, HOTYR glycoside, HOTYR acetate (HOTYRAc) and metabolites, as HVA. Altogether, methodological approaches in olive oil phenolic compounds analysis in biological fluids could be divided into two main groups according to their objectives:

(i) methods for their quantification (a quantitative analysis);

(ii) exploratory methods (a descriptive analysis).

Short description of main methodological achievements in analysis of olive oil phenols bioavailability are presented in ***Supplementary Table IV***.

Quantitative methods in analysis of olive oil polyphenols bioavailability

The first reported analysis of HOTYR plasma concentration was done in rats by Japanese investigators using synthetic HOTYR for oral intervention in rats (Bai, 1998). To determine HOTYR plasma concentrations, they applied a liquid-liquid extraction (LLE) and used BSTFA for the derivatization of alcohol groups in order to make such polar compound suitable for gas-chromatographic separation[11]. Finally mass-detection was used in selective ion monitoring mode (SIM) for identification of derivatized compounds. As a result, for the first time plasma kinetics parameters of free HOTYR were reported. After the administration of 10 mg per rat of synthetic HOTYR, a fast and short onset of relatively low HOTYR plasmatic concentrations were noticed with a high variability between animals suggesting a quite poor bioavailability.

[11] GC is a technique for separation of volatile compounds which are thermally stable. Unfortunately, not always compounds of biomedical and environmental interest, particularly for those of containing polar functional groups, as in case with HOTYR, are suitable for it. These groups are difficult to analyze by GC either because they are not sufficiently volatile, tail badly, are too strongly attracted to the stationary phase, thermally unstable or even decomposed. Therefore, chemical derivatization prior to analysis is generally done. Therefore this increase their volatility and decrease the polarity of compounds, reduce thermal degradation of samples by increasing their thermal stability, increase detector response by incorporating functional groups which lead to higher detector signals, and improve separation.

Italian investigators developed a GC-MS approach for the detection of two main olive oil polyphenols HOTYR and TYR in postprandial urine of volunteers administered with different amounts of olive oil phenolic extracts (OOPhEx) (Visioli, 2000b). The method was based on Bai´s methodological approach, they analyzed however, both free and glucuroconjugated HOTYR and TYR. The glucuronides were identified indirectly using an enzymatic digestion of the sample with β-glucuronidase[12]. This GC-MS methodology was afterwards used for the identification of HVAlc and HVA as putative HOTYR methyl-conjugated metabolites (Caruso, 2001). Later it was adapted for the quantification of HVAlc, as a principal methyl conjugated metabolite of HOTYR (Visioli, 2003). Although previously used in studies with high dosage of OOPhEx (Visioli, 2000b; Caruso, 2001), this method was sensitive enough to analyse urine samples collected in human volunteers after interventions with dietary dose of EVOO (Visioli, 2003).

Another GC-MS method, combining LLE sample preparation and alcohol groups derivatization was developed by Miró-Casas and colleagues (Miró-Casas, 2001a, b) and was successfully applied for analysis of urinary concentrations and excretion rates of HOTYR and TYR after a dietary dose of VOO. In theses studies, to control the amount of administered polyphenols, the VOO was subjected to experimental hydrolytic conditions imitating gastro-

[12] The phase II conjugates of olive oil phenols, as a rule, are more polar than their precursor and they are even less suitable for GC analysis. Therefore, these metabolites should be deconjugated prior to GC analysis using different hydrolytic techniques. The most widely used are chemical and enzymatic hydrolysis. Two possible chemical deconjugation techniques could be applied: alkaline and acidic hydrolyses; and, usually, they are unspecific to the type of conjugation. Enzymatic deconjugation is more specific and requires particular type of hydrolysing enzyme to deconjugate the corresponding metabolite: glucuronase, sulfatase, etc.

intestinal digestion (pH, temperature and incubation time) and characterized using analogous GC-MS analysis for determining the content of free forms of phenols. In this way, it was discovered that in olive oil there are many other then secoiridoid precursors of HOTYR and TYR which might significantly contribute to their bioavailability. Acidic hydrolysis[12] applied to postprandial urine samples showed that HOTYR and TYR are excreted in urine mainly as conjugated metabolite and just a small amount of them (about 6% for HOTYR and about 12% for TYR) was present as intact polyphenols. Good recovery and a high sensitivity of the developed method allowed them for the first time to detect HOTYR and TYR basal urinary concentrations after wash-out periods low in polyphenols, therefore, confirming that there are other than VOO sources of HOTYR and maybe for TYR as well. Using this method it was estimated that 24-h urinary levels of both phenols, regardless of high inter-individual variability in their excretion rates, were good biomarkers of VOO intake, both for a single and for sustained moderate dietary doses (Miró-Casas, 2003b). Therefore, this methodology was successfully applied and is currently in use in several VOO intervention studies (Covas, 2006b; Fitó, 2007, 2008) in order to supervise the diet compliance of participating subjects.

The developed methodology by Miró-Casas and colleagues was further adjusted for the simultaneous identification of HOTYR and its methyl-conjugated metabolite HVAlc (Miró-Casas, 2003a). Adequate selectivity and sensitivity of the analytical method allowed to determine HOTYR and HVAlc concentrations in postprandial plasma, and, therefore, for the first time the disposition of one of the main olive oil antioxidant HOTYR in humans after dietary dosage intervention with VOO was reported (Miró-Casas,

2003a). In contrast to urine, phenolics in their free forms could not be detected in plasma samples due to their very low concentrations. Using two different approaches for indirect identification of HOTYR and HVAlc metabolites, enzymatic (with β-glucuronidase) and acidic (HCl) hydrolysis, the authors tried to identify types of conjugation involved in their metabolism. According to the type of hydrolysis, liberated HOTYR and HVAlc would come either from their glucuronoconjugates (specific enzymatic hydrolysis with β-glucuronidase) or from a pool of different conjugates (unspecific chemical hydrolysis with acid). Overall results obtained by this group demonstrated that following dietary ingestion olive oil polyphenols are available within human body mainly as phase II metabolites. In view of these results, it was proposed that the biological activity of HOTYR most probably derives from its metabolites rather than from intact HOTYR even not-detectable in plasma. This concept was supported by an Australian group (Tuck, 2002), where one of the HOTYR metabolites, 3´-*O*-glucuronide, isolated from rat urine, was reported to be more active as scavenger of radicals than HOTYR itself.

While the main evidence of olive oil polyphenols bioavailability in humans was obtained using GC-MS methodologies, there were several attempts to develop LC methods for their analysis. HPLC coupled to spectrophotometric[13] detectors were the first choice in

[13] Due to the presence of phenol ring in the structure of the olive oil polyphenols, they could be easily detected by the spectophotometry. Phenols absorb in the ultraviolet (UV) region, the presence of aromatic ring results in effective absorbance of the UV between 240 and 315 nm. Nevertheless, this type of absorption is unspecific one, generating certain low selectivity in UV analysis of complex mixture where olive oil polyphenols could be present along with other numerous aromatic ring containing compounds.

olive oil phenols analysis due to their wide spread accessibility and simplicity in application. In addition, there was no need for specific sample derivatization prior to analysis, as it was in case with GC. These LC methods were mainly oriented to the direct detection of the free forms of the polyphenolic compounds (Ruiz-Gutierrez, 2000; Tsarbopoulos, 2003; Tan, 2003; Grizis, 2003). None of them allowed the detection of conjugated metabolites. As a result they were mainly used in intervention studies where high dosage of olive oil polyphenols should be administrated to subjects in order to reach a suitable limit of detection (LOD) for the plasmatic and urinary concentrations of intact olive oil polyphenols (HOTYR, TYR, OLE, etc.)

Although certain HPLC-UV/FP methodologies appears to be more sensitive than others, and despite the advantages of the low cost of the analysis and ease of operation, these techniques suffer from low sensitivity and poor selectivity. In this regard, HPLC separation techniques were coupled to mass spectrometry detector and adjusted for the analysis of olive oil phenols in urine and plasma from both human and animal studies. Thus, Del Boccio and colleagues (Del Boccio, 2003) reported a HPLC-MS method optimized for the simultaneous examination of OLE and HOTYR in biological fluids. This MS method had superior sensitivity compared to UV methods and was able to detect both compounds in urine and plasma in the nanogram range. The method was used for analysis of plasma and urine of rats fed with a single oral dose of oleuropein (100 mg/kg). Enzymatic treatment of plasma did not revealed glucuronides of OLE, whereas in 24-h urine more than 90% of both OLE and HOTYR were present as glucuronides.

A novel approach using GC-MS for the simultaneous detection of HOTYR, TYR and EA in rat urine was proposed by Bazoti and colleges (Bazoti, 2005). In order to increase method sensitivity and selectivity, they applied tandem mass spectrometry (MS-MS) for detection of derivatized compounds instead of previously used selective ion monitoring (SIM mode). Therefore, they planed to detect and quantify EA along with TYR and HOTYR in urine samples of rats fed with OLE and EVOO. Although, the LOD and LOQ (limit of quantification) of this method were extremely low (picogram concentrations) for all compounds, they could not detect EA in rat urines after sustained oral intake of OLE or EVOO as a dietary supplement. Regarding phenol metabolites, the method discriminated only glucuronides of HOTYR and TYR using enzyme-mediated hydrolysis. Neither sulfate- no methyl-conjugates were taken into consideration within this study.

Recently, the olive oil phenols and their phase II conjugated metabolites were reported to be detected in human LDL particles using specific SPE-UPLC method for their isolation and separation. The metabolites recognition was based on theoretically predicted MS-MS fragmentations of conjugates (de la Torre-Carbot, 2006, 2007). Thus, TYR, HOTYR and HVA monosulfates and monoglucuronidates were detected in human LDL samples. Nonetheless, the HOTYR glucuronide isomers could not be well separated and properly identified due to the identical mass-spectrophotometric behavior. Using as standards principal phenols HOTYR and HVA, the method was validated for qualitative and quantitative analysis of the olive oil phenols phase II metabolites in human LDL particles for routine analysis in clinical and intervention studies, however, its application has not been reported yet.

A similar methodological approach (UPLC with a selective reaction monitoring using theoretically predicted MS-fragmentation) was applied for identification and quantification of various olive oil phenolics (including HOTYR, TYR, HVA, 3,4-DHPEA-EDA and p-HPEA-EDA) and their phase II metabolites in plasma (Suarez, 2009). The developed SPE-UPLC–ESI-MS/MS method was validated at μM range using parent compounds. The metabolites were tentatively quantified by using the calibration curves corresponding to their phenolic compounds. Its applicability was tested on several samples of plasma from human subjects intervened with VOO at dietary doses (30 mL), however no clinical application was reported.

Exploratory methods in studying olive oil phenols bioavailability

In parallel to human studies, Australian researchers (Tuck, 2001, 2002) were studying different aspects of bioavailability of main olive oil polyphenols using *in vivo* animal models. To estimate the absorption and excretion of tritium labeled HOTYR and TYR administrated to rats they applied HPLC-UV coupled to radiometric analyzer for their separation and identification in urines of animals. In addition to the demonstration that the vehicle and the route of administration of polyphenols could have impact on their bioavailability, the authors for the first time have identified via specific enzyme-mediated hydrolysis (Tuck, 2001) their main urinary phase II metabolites in rats: sulfates and glucuronides. Lately, using the same exploratory methodology, plus applying MS/MS and NMR spectra analysis to chromatographically isolated compounds, they structurally characterized main phase II

metabolites of HOTYR: its 3´-*O*-glucuronide, *O*-sulfate and, although incompletely, its methyl-conjugate (Tuck, 2002).

A similar methodological approaches (intervention with radiolabel HOTYR, following HPLC-UV-radioactivity detection in biological samples and enzymatic hydrolysis metabolites identification) were used in another *in vivo* study investigating HOTYR tissue distribution and metabolism in rats (D´Angelo, 2001). In this way, by means of radioactivity and correspondence to reference standard, MOPET, DOPAL, DOPAC and HVA were identified as dopamine related metabolites of endogenously administrated HOTYR. The formation of their sulfoconjugated but not glucuronidated derivatives was acknowledged.

Using enzymatic hydrolysis and HPLC-UV methodology, the presence of only glucuro- and methyl-conjugated metabolites of HOTYR were identified in experiments as with intestine epithelia (Caco2) so with hepatic (HepG2) cells cultures models (Mateo, 2005; Corona, 2006).

The prevalent presence of olive oil phenols in the form of phase II metabolites within the organism following olive oil phenolics ingestion (e.g. as olive oil, OOPhEx and pure olive oil phenols) was acknowledged in many studies, however no direct methods for their identification and quantification in biological fluids was reported. The main drawback for that was the absence of corresponding standards. Therefore, olive oil phenol metabolites have not been quantified accurately as well as their metabolic rates.

2.2. Absorption in gastrointestinal tract

A prerequisite for the bioavailability of any compound is it bioaccessibility in the gut, defined as the amount that is potentially absorbable from the lumen. Olive oil antioxidants are by nature and function subject to oxidation (this issue was discussed in chapter I), which limits their stability in the product during storage, food processing and digestion, and thus their bioaccessibility. Many factors affect the bioavailability of HOTYR and TYR. These factors can be defined into two main groups (Holst, 2008): (i) exogenous (a complexity of the food matrix, the chemical form, structure and amount of ingested antioxidant) and (ii) endogenous (mucosal mass, intestinal transit time, rate of emptying, metabolism and extent of conjugation, and protein binding in blood and tissue, etc.).

The majority of HOTYR and TYR is present in the food as precursors, some of these precursors are glycosides, but are predominantly absorbed as aglycones. Although the gastro-intestinal conditions *in vivo* are complex with the food matrix affecting the precise pH, the incubation of polyphenols at gastric and intestinal pH can give us information about the stability of polyphenols in the gastro-intestinal tract environment. After ingestion, olive oil polyphenols pass through some kind of gastro-intestinal dissolution, where their absorption could be affected by pH, presence of enzymes, motility and interaction with substances, microbiome, etc. Some *in vitro* studies mimic gastric and intestinal conditions, using appropriate pH solutions (Miró-Casas, 2001a, b, 2003a; Corona, 2006). Others, incubate polyphenols in gastric and duodenal juices collected from human volunteers (Vissers, 2002). The results of such studies indicate that once olive oil was

ingested, HOTYR, TYR and their OLE and LST aglycones, underwent rapid non-enzymatic hydrolysis under gastric conditions, resulting in liberation and significant increases in the amount of free HOTYR and TYR entering the small intestine (Corona, 2006). After HOTYR and TYR are readily absorbed in small intestine while non modified previously in the GI tract EA derivatives (OLE and LGS) are likely to reach colon and, therefore, to be exposed to metabolic activities of intestinal microflora. As a result, their bioavailability and bioavailability of the derived HOTYR and TYR will be to some extent regulated through their degradation by colonic microflora. This assumption was investigated using an *in vitro* colonic microflora incubation approach (Corona, 2006). In general, studies performed in rats suggest that the absorption of HOTYR is almost complete while TYR is absorbed to bit lesser extend (about 75%) (Tuck, 2001). It is worth noting, that there are many variables which can affect olive oil polyphenols bioavailability at different levels, contributing to its high intra- and inter-individual variability.

In a pioneering experiment on the bioavailability and disposition of olive oil phenolic compounds in humans (Visioli, 2000b), HOTYR and TYR were spiked to a poor-phenolic content olive oil and administered to healthy volunteers. Preliminary conclusions were that phenolic compounds, namely HOTYR and TYR, are dose-dependently (at least at the doses employed in this study) absorbed in humans after ingestion and that their bioavailability is extremely poor, most compounds being recovered in biological fluids as conjugates.

In vitro models have shown that both HOTYR and TYR are able to cross human Caco-2 cell monolayers via a bidirectional passive

diffusion mechanism (Manna, 2000; Corona, 2006). The occurrence of a passive basolateral-apical intestinal transport of small olive oil phenols was confirmed *in vivo*, where some of the radioactivity of HOTYR and/or its metabolites was detected in faeces and intestinal tracts after intravenous injection of radiolabelled HOTYR to rats (D´Angelo, 2001). Absorption studies on the isolated rat small intestine model showed that the bulk of administered HOTYR and TYR is absorbed, and undergoes phase-II (HOTYR conversion to HVAlc, and TYR, HVAlc and HOTYR glucuronidation) biotransformation in small intestine (Corona, 2006) which was in agreement with *in vivo* data showing the presence of glucuronides in urine following the ingestion of olive oil polyphenols (Visioli, 2000; Caruso, 2001; Miro-Casas, 2001a, 2003). A study involving ileostomy subjects (Vissers, 2002) confirmed conclusively that the main site of absorption for free forms of HOTYR and TYR in humans is the small intestine.

The absorption of OLE was studied *in situ* using an intestinal perfusion technique (Edgecombe, 2000). Although it was shown that OLE can be absorbed, albeit poorly, from isolated perfused rat intestin, the mechanisms remains to be unclear. Therefore, it is possible that OLE exerts its biological activities through its conversion to HOTYR because a poor absorption at the GI tract (Edgecombe, 2000). Bioavailability studies in rats support this notion as peak plasma concentrations reached after high doses of OLE (100 mg/kg) are in the nanogram range suggesting its conversion to HOTYR at the GI tract and a poor absorption of OLE itself (Del Boccio, 2003; Bazoti, 2005). These observations have been further confirmed in rat models and humans (Vissers, 2002; Visioli, 2003) where high, but not related to ingestion of its free

form, levels of total HOTYR excretion in urine were detected and related to OLE administered. Additionally, in contrast to HOTYR and TYR, OLE was not absorbed through isolated segments of rat small intestine neither was able to cross human Caco-2 cell monolayers (Corona, 2006). Being stable under acidic conditions in stomach and relatively stable in duodenal fluid (Vissers, 2002) it is likely that OLE can reach the large intestine, where it may be subjected to a rapid degradation by the colonic microflora. Furthermore it was shown that one of the major OLE degradation products by colonic microflora during *in vitro* incubation was identified as being HOTYR (Corona, 2006). Therefore, as previously demonstrated for various phenolic acids (Rechner, 2004), the microflora-dependent hydrolysis of OLE may consequently increase the bioavailability of OLE-derived HOTYR via the uptake of HOTYR through the large intestine (Corona, 2006). No such experiments were reported for LGS-derived TYR.

Several authors have performed experiments where polyphenols were administered in different vehicles (oil-, water- and food-component based matrices) and via different routes (oral vs. intravenous) (Tuck, 2001; Visioli, 2003). The purpose of these studies was to have a better understanding of the relevance of the biological matrix surrounding phenol compounds in terms of favouring/disfavouring its absorption. Oral bioavailability estimates of HOTYR and TYR were 25% higher when administered in an olive oil solution compared to an aqueous solution. For both compounds intravenously and orally administrated oil-based dosing resulted in significantly greater absorption and elimination of the phenolics in urine within 24 h than the oral, aqueous dosing method. There were no significant differences in the amount of

phenolic compounds eliminated in urine between the intravenous and the oral oil-based dosing methods for either TYR or HOTYR (Tuck, 2003). These results were further confirmed in humans where HOTYR bioavailability was compared by administering this compound in different matrices (olive oil, spiked refined oil, or low-fat yogurt). It was found that HOTYR recovery (measured as urinary HOTYR) was much higher after its administration as a natural component of VOO (44.2% of HOTYR administered) than after its addition to refined olive oil (23% of HOTYR administered), or yogurt (5.8% of dose or approximately 13% of that recorded after VOO intake) (Visioli, 2003a). Factors leading to an improved absorption of HOTYR and TYR in these cases can be modified by the presence of fat, proteins, carbohydrates, an aqueous components, and/or emulsifiers in food matrices. Although olive oil polyphenols were shown to have relatively weak phenol–protein binding for the different food proteins and low oil–water partition coefficients (Pripp, 2005), food matrices co-ingested with polyphenols may have a significant impact on HOTYR and TYR bioaccesibility as it was seen for other phenolics (Scholz, 2007). The lack of systematic information on the effects of other components on the bioavailability of olive oil polyphenols needs to be addressed, and more human studies should be conducted in this field to establish general principles affecting HOTYR and TYR absorption *in vivo*. Information derived from such experiments could be useful for the optimal design of future bioefficacy studies.

2.3. HOTYR and TYR metabolism and distribution

2.3.1. Metabolic pathways and metabolic disposition of phenolic compounds

During absorption, phenols bioavailability is decreased by extensive phase II biotransformation reactions that produce conjugates and metabolites. Once absorbed, olive oil polyphenols are subject to 3 main types of conjugation: methylation, sulfation, and glucuronidation. The resulting water soluble and stable conjugates are rapidly excreted by the body (Holst, 2008).

Methylation

Catechol-*O*-methyl transferase (COMT) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to polyphenols having an *o*-diphenolic (catechol) moiety such as for HOTYR (***Fig. 20***). The methylation generally occurs predominantly in the 3´-position of the catechol, but a minor proportion of 4´-*O*-methylated product might be also formed. COMT is present in a wide range of tissues. Its activity is the highest in the liver and the kidneys although significant methylation can occurs in the small intestine as it was reported for HOTYR in rat intestine (Corona, 2006).

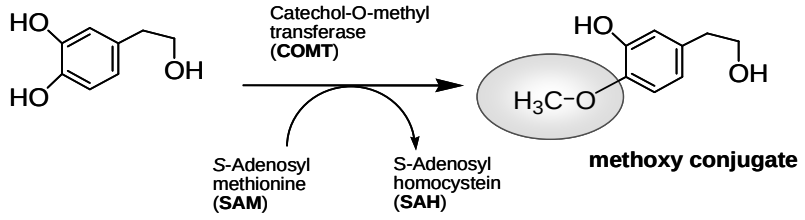


Figure 20 *Mechanism of HOTYR methylation by COMT and its conversion to HVAlc.*

Sulfate conjugation

Sulfotransferases (SULTs) catalyze the transfer of a sulfate moiety from 3´-phosphoadenosine-5´-phosphosulfate (PAPS) to a hydroxyl group on various substrates, including polyphenols (***Fig. 21***). Neither the isoforms that are specifically involved in the

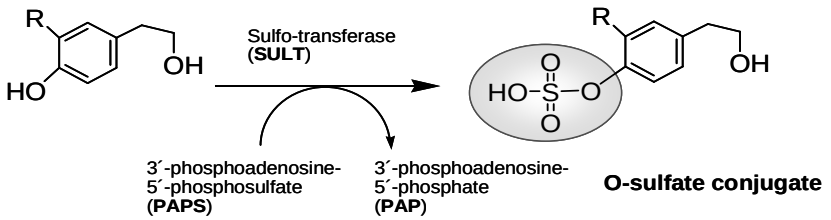


Figure 21 *Mechanism of sulfation by SULT.*

conjugation of polyphenols nor the position of sulfation for the various polyphenols have yet been clearly identified, but sulfation clearly occurs mainly in the liver (Falany, 1997). In most cases, the addition of a sulfate moiety to a compound increases its water solubility and decreases its biological activity. However, many of these enzymes are also capable of bioactivating procarcinogens to reactive electrophiles.

Glucuronide conjugation

UDP-glucuronosyl transferases (UDPGT) are membrane-bound enzymes that are located in the endoplasmic reticulum in many tissues and that catalyze the transfer of a glucuronic acid moiety from UDP-glucuronic acid to many drugs and dietary derived compounds (***Fig. 22***). The presence of glucuronoconjugated metabolites after HOTYR perfusion in the small intestine of rats shows that glucuronidation of polyphenols first occurs in the enterocytes (Corona, 2006) before passing through the liver. This

is probably the case in humans as well, because in humans the *in vitro* glucuronidation of polyphenols by microsomes from the intestine is as much intensive as by microsomes from the liver (Antonio, 2003). About 15 isoforms of UDPGT have been identified in humans, and these isoforms have broad and overlapping substrate specificities and different tissue distribution (Fisher, 2001). The subfamily of UDPGT called UGT1A that is localized in the intestine probably plays a major role in the first-pass metabolism of simple polyphenols, especially catechols (Antonio, 2003). The specificity of the active isoenzyme of the 1A class seems to differ according to the polyphenol considered (Antonio, 2002). UDPGT isoenzymes have a wide polymorphic expression pattern that could results in a high interindividual variability in polyphenol glucuronidation.

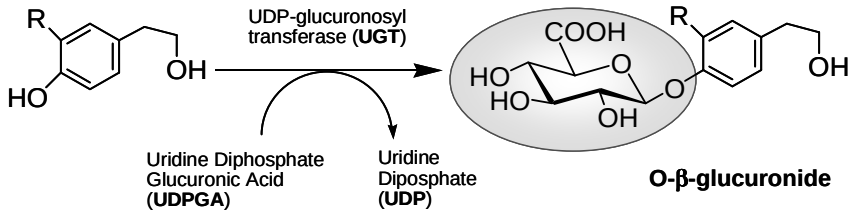


Figure 22 *Mechanism of glucuronidation by UGTs.*

HOTYR and TYR were shown to be dose-dependently absorbed in humans after olive oil ingestion and recovered in biological fluids as glucuronoconjugates (Visioli, 2000b). An increase in the dose of administered phenolics increased the proportion of their conjugation with glucuronic acid. Several human and animal studies have confirmed that over 90% of urinary metabolites of HOTYR and TYR were mainly glucuronide metabolites (Visioli, 2000, 2003; Caruso,

2001; Vissers, 2002; Miró-Casas, 2001b, 2003a, b), yet free phenols and methylconjugates, not glucuronoconjugated, were also excreted in urine. Sulfoconjugates of HOTYR, TYR, or their metabolites (methyl or glucuronide conjugates) have been detected in animal experiments (Tuck, 2002; D´Angelo, 2001) and recently indirectly in humans (Gonzalez-Santiago, 2010).

The relative importance of the 3 types of conjugation (methylation, sulfation, and glucuronidation) appears to vary according to the nature of the substrate and the dose ingested. Sulfation is generally a higher-affinity, lower-capacity pathway than is glucuronidation, so that when the ingested dose increases, a shift from sulfation toward glucuronidation occurs (Koster, 1981). In general the capacity of all three conjugation reactions is high, resulting in very low concentrations of free aglycones in plasma and urine after the intake of a nutritional dose. Saturation of the conjugation processes for both HOTYR and TYR were not studied in neither experimental nor human studies. Competitive inhibition of conjugation could occur in the presence of various polyphenols and xenobiotics in the intestine, but it has never been studied. In these conditions, significant amounts of free aglycones could circulate in blood.

Most bioavailability studies on olive oil phenols have measured total HOTYR and TYR concentrations in blood and/or urine after acidic or enzymatic treatment of the samples.

There is a lack of studies in which glucuronide and sulfates, as well as other possible conjugates, of HOTYR and TYR in biological samples were measured directly. This identification must include not only the nature and number of the conjugating group but also the position of these groups on the polyphenol chemical structure because these positions can affect the biological activities of conjugates.

2.3.2. First pass metabolism

The liver was originally considered to be the major site of xenobiotic metabolism, however, small intestine enterocytes express a significant capacity for phase I and phase II metabolism and drug transport. Therefore, small intestine metabolism can significantly limit the uptake of compounds. Because of the difficulty in accessing the small intestine as a site of absorption and first pass metabolism (only human *in situ* perfusion or studies in ileostomized subjects allow *in vivo* data to be obtained), the pathways are mainly studied on cell cultures and in animal models (Ferrec, 2001). In the process of crossing epithelial cells of the GI tract, polyphenolic compounds from olive oil are subject to a classic phase I/II biotransformation, and therefore, subjected to an important first pass metabolism.

It was shown that at the end of transepithelial transport through Caco-2 cell about 10% of HOTYR was converted to HVAlc, the metabolic product of COMT (Manna, 2000). Further on, in a number of animal and human studies it was confirmed that HVAlc,

together with HVA (a further oxidized form of the aliphatic hydroxyl residue HVAlc), were the main methylated metabolites of HOTYR detected in plasma and in urine (D´Angelo, 2001; Caruso, 2001). Corona and co-workers (Corona, 2006) in Caco-cell, an enterocytes transport model, in addition to the *O*-methylated derivatives of HOTYR found a novel glutathionylated conjugate of HOTYR as its main first pass metabolite, no further reported in other HOTYR bioavailability studies both *in vivo* and *in vitro*. Although Caco-2 cell cultures are widely used in absorption, passage and transport studies as the model system of the human intestinal epithelium, they have a number of limitations related to the first pass metabolism (Ferrec, 2001). Therefore, an extrapolation of the data from first pass metabolism obtained on this model should be made carefully, since these cell lines are originated from tumours and lack several important phase I and II enzymes, and, therefore, could give an inaccurate representation of the first pass metabolism of a given compound.

HOTYR, TYR, HVAlc and their glucuronides were detected in rat small intestine model, which partially confirm findings in Caco-2 cells, as no glucuronides were detected in the cellular model (Corona, 2006). This is presumably because this cell culture do not posses UDPGT activity due to their colonic origin (Ferrec, 2001). The rat small intestine model shows no HVA and no sulfate-conjugated metabolites, earlier reported to be found in rat urine after HOTYR intake (Tuck, 2002) suggesting that these metabolites are formed after transport across the small intestine, most probably in the liver (Corona, 2006). Unfortunately, the most relevant to *in vivo* first pass metabolism experiment involving ileostomy human subjects (Vissers, 2002), could not distinguish between free and

conjugated forms of HOTYR and TYR to describe their biotransformation at the level of small intestine.

2.3.3. Hepatic metabolism

The hepatic metabolism of olive oil phenols (HOTYR, HOTYRAc - hydroxytyrosol acetate, and TYR), has been studied in human hepatoma HepG2 cells as a model system of the human liver (Mateos, 2005). The main metabolites produced by these cells were *O*-glucuronides, *O*-methyl-*O*-glucuronides, and *O*-methyl conjugates, whereas no sulfate conjugates of any of the assayed phenols could be detected. HOTYR metabolites exceeded 75% of the analyzed phenols (32% glucuronoconjugated, 26% methylated and 18% methylated and glucuronoconjugated), with 25% of free, non-metabolized HOTYR, whereas TYR was poorly metabolized, with less than 10% of the phenol glucuronidated. These results suggest that extensive phase II metabolism of olive oil phenols also takes place in the liver.

Sulfate conjugates of HOTYR and TYR, as products of hepatic metabolism, were detected and identified in urine only in animal models (rats) after both intravenous and oral olive oil phenols administration (Tuck, 2002, 2001). The pharmacokinetics of HOTYR intravenously administered to rats indicates a fast and extensive uptake of the molecule by the organs and tissues, with a preferential renal uptake (D´Angelo, 2001). HOTYR is metabolized to four oxidized and/or methylated derivatives. A significant fraction of total HOTYR recovered is associated with the sulfoconjugated forms, which also represent the major urinary excretion products.

The recovery of HOTYR in urine is about 6% of the dose administered, 0.3% is recovered as HVAlc, 12.3% as DOPAC (3,4-dihydroxyphenylacetic acid), 23.6% as HVA (3-methyl-4-hydroxyphenylacetic acid) and 26% as DOPAL (3,4-dihydroxyphenylacetaldehyde) (D'Angelo, 2001). On the basis of reported data, an intracellular metabolic pathway of exogenously administrated HOTYR implies the involvement of COMT, alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), and SULT (D´Angelo, 2001). On the contrary, in majority of *in vivo* studies involving human subjects only methylated, glucuronoconjugated (Miró-Casas, 2003a) and just to some extent oxidized methyl metabolites (HVA) (Caruso, 2001) have been reported in plasma and urine samples, claiming that phase II methyl- and glucuro-conjugating pathways are the principal metabolic pathways for olive oil phenols in humans.

Some authors (Visioli, 2003) question the rat model as a good one for this type of studies as it displays an extremely high HOTYR basal metabolism (30 folds over humans). Investigators suggest that differences might be due to the absence of a gall bladder in rats, which results in the presentation of lipid-soluble or amphiphilic molecules such as HOTYR to the intestinal flora. In addition, the disposition of exogenous HOTYR maybe be cross contaminated with catecholamines disposition pathways (Visioli, 2003). The absence of the glucuronides of HOTYR and its oxidized and/or methylated metabolites reported by others (D´Angelo, 2001) is probably due to the administration route used. As stated earlier, the HOTYR administered by the oral route is the subject of an extensive first-pass metabolism where the contribution of intestinal metabolism is quite relevant while when HOTYR is administered

intravenously, only the hepatic contribution to its disposition is seen (Tuck, 2002).

2.3.4. Plasma transport, binding to lipoproteins and tissue uptake/distribution

The degree of binding to albumin, a primary protein responsible for the binding in plasma, may have consequences for the rate of clearance of both olive oil phenols and their metabolites, and for their delivery to cells and tissues. The conventional view is that cellular uptake is proportional to the unbound concentration of metabolites. No data is available for HOTYR and TYR and other olive oil phenols.

The partitioning of polyphenols and their metabolites between aqueous and lipid phases is largely in favour of the aqueous phase because of their hydrophilicity (discussed in the previous chapter). At physiologic pH most polyphenols interact with the polar head groups of phospholipids at the membrane surface via the formation of hydrogen bonds that involve the hydroxyl group of the polyphenols (Manach, 2004). This adsorption of polyphenols probably limits the access of aqueous oxidants to the membrane surface and their initial interaction on that surface.

LDL is made up of lipophilic structures that, once oxidized, participate in the aetiology of atherosclerosis (discussed in chapter I). Several studies have shown that olive oil polyphenols have the ability to protect LDL from oxidation (Wiseman, 1996; Stupans, 2002). TYR and HOTYR were recovered in all lipoprotein structures, except in VLDL, with concentrations peaking between 1

and 2 h after olive oil ingestion (Bonanome, 2000). In recent studies not only TYR and HOTYR, but also several metabolites were identified in LDL: HOTYR glucuronide and sulfate, TYR glucuronide and sulfate, and homovanillic acid sulfate (de la Torre-Carbot, 2006, 2007). In addition, the concentration of total phenolic compounds in LDL has been shown to be directly correlated with the phenolic concentration of olive oils and with the resistance of LDL to their *in vitro* oxidation (Gimeno, 2007). At postprandial state, after ingestion of VOO with a high phenolic content (366 mg/kg of olive oil), the phenolic content of LDL directly correlates with the plasma concentration of TYR and HOTYR (Covas, 2006a). The nature of the bond between LDL and phenolic compounds, including olive oil phenolic compounds and their metabolites deserves further investigation due to the physiopathological implications involved. Only a small proportion of plasma polyphenols are in fact associated with the LDL fraction, and, most probably, due to ionic interactions with charged residues on the surface of the particles. Therefore, protection probably occurs at the interface between lipophilic and hydrophilic phases.

Determination of the actual bioavailability of olive oil derived HOTYR and TYR and their metabolites in tissues may be much more important than their plasma concentrations. Data are still very scarce, even in animals. It is still difficult to say whether some polyphenols accumulate in specific target organs. The nature of the tissular metabolites may be different from that of blood metabolites because of the specific uptake or elimination of some of the tissular metabolites or because of intracellular metabolism.

When single dose of the radiolabelled HOTYR was intravenously injected, the pharmacokinetic analysis indicates a fast and extensive uptake of the molecule by the organs and tissues investigated, with a preferential renal uptake. The time-course analysis indicates that the highest radioactivity was monitored at first 2 minutes in blood, associated with both plasma and blood cells, and at first 5 min in different organs/tissues, mainly in skeletal muscles, in kidney, in liver, in heart and lung, and to some extent in brain (D´Angelo, 2001). The intracellular metabolic pathway of exogenously administered HOTYR, implying the involvement of COMT, alcohol dehydrogenase, aldehyde dehydrogenase, and SULT, has been proposed.

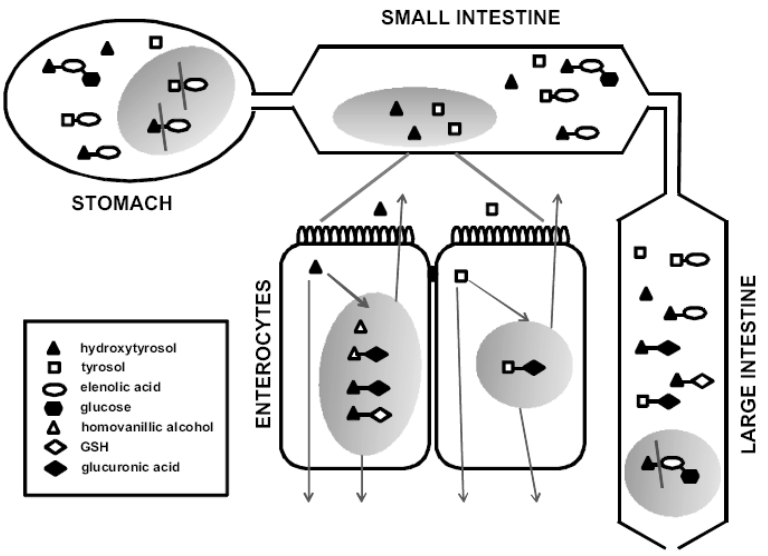


Figure 23 *Schematic representation of GI absorption and metabolism of olive oil phenols (Corona, 2009b).*

Following ingestion, modified and unmodified (by acidic hydrolysis in stomach) olive oil phenols and their metabolites (excreted into

gut lumen via basolateral transport after first path metabolism) are principally concentrated in gastrointestinal tract (***Fig. 23***). Although afterwards, small phenols undergo rapid absorption in small intestine, some poorely absorbed forms such as secoiridoids and glycosides proceed to large intestine, where they can undergo rapid degradation by the colonic microflora to smaller functional phenolic compounds (Corona, 2006). As results of dietary consumption of olive oil, the phenolics and their intestinal derived metabolites are distributed within gastrointestinal tract at higher levels then compared to other organs and tissues. Therefore, the gastrointestinal tract is considered one of the organ/tissue target where olive oil phenols can exert their biological activities (Corona, 2009b).

2.4. Excretion

Parent olive oil phenols and metabolites may follow 2 pathways of excretion: (i) the biliary and (ii) the urinary.

Large, extensively conjugated metabolite are more likely to be eliminated in the bile, whereas small conjugates such as sulfates are preferentially excreted in urine. Biliary excretion of polyphenols in humans may differ greatly from that in rats because of the existence of the gall bladder in humans. Intestinal bacteria possess β-glucuronidases that are able to release free aglycones from conjugated metabolites secreted in bile. Aglycones can be re-absorbed which results in the enterohepatic cycling (***Fig. 24***).

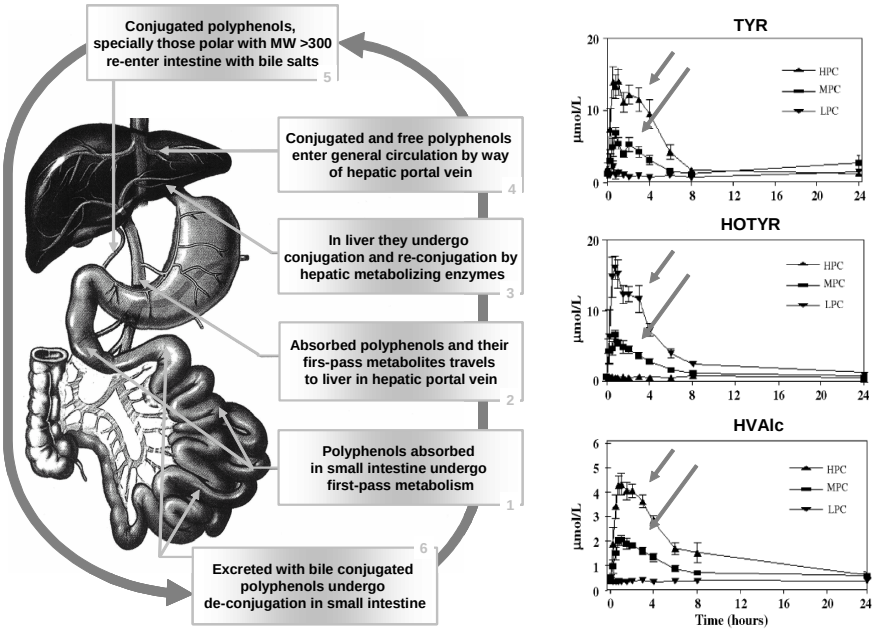


Figure 24 *Route of enterohepatic cycling of olive oil phenols (see second peak in plasma concentrations marked with grey arrows)*

TYR, HOTYR, and HVAlc after ingestion of 40 mL of olive oil with high (HPC), medium (MPC), and low (LPC) phenolic content (Covas, 2006b).

A second plasma peak for HOTYR, TYR and HVAlc was observed in plasma in humans volunteers between the 1st and 4th hour after ingestion of olive oil rich in phenolic compounds (***Fig. 24***) (Covas, 2006b), indicating that some of the phase II metabolites, most probably glucuronides due to their molecular mass, of HOTYR and HVAlc undergoes enterohepatic cycling.

Urinary excretion studies. The total amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations

(***Fig. 25***), however a high inter-individual variability in the rates of excretion are always observed (Miró-Casas 2001a, b, 2003a, b).

90% of an intravenous dose of HOTYR administrated to rats was recovered in urine (rat models) indicating that renal excretion represents the preferential elimination route of HOTYR and/or its metabolites. Less than 9% is excreted via intestine, where a basolateral-apical intestinal transport of HOTYR and/or its metabolites can take place (D´Angelo, 2001).

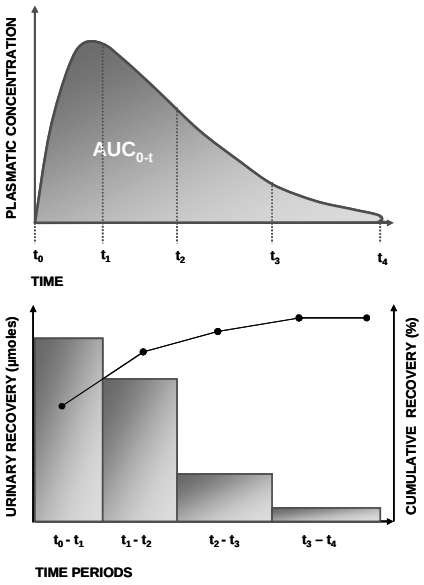


Figure 25 *Urinary recovery within the plasmatic and urinary levels of concentrations, where t_0 to t_4 represent different time points*

Total excretion of olive oil phenols orally administered to humans is quite modest, mainly due to their incomplete absorption. The specific structural characteristics of olive oil phenols (small polar planar molecules) influence not only their absorption, but also on their extensive metabolism and distribution within the human organism. It is worth noting that the estimated values for olive oil phenol recoveries do not account for certain metabolites which

cannot be identified as a results of analytical difficulties. So far, glucuronides were acknowledged as the major metabolites recovered in urine of humans after consumption of olive oil rich in phenols, as for HOTYR so for TYR and HVAlc (Miró-Casas, 2003a, b), whereas in animals, significant amounts of sulfated metabolites were detected in urine along with glucuronides (D´Angelo, 2001).

According to the experimental data, the rate of excretion of olive oil phenols via urine is quite fast: almost total elimination of compounds from the body could be achieved within 24 hours, with predominant excretion within first 4-6 hours after ingestion (Miró-Casas, 2001a, b, 2003a, b). This suggests that maintenance of high concentrations of olive oil metabolites in plasma, which is equal to their distribution within human body, could be achieved only with regular and very frequent consumption of olive oil rich in phenolics. The repeated intakes of these compounds must be very close together in time to obtain an accumulation of metabolites in plasma; otherwise, plasma concentrations regularly fluctuate after repeated ingestions, and accumulation/steady state concentration can occur only at very low concentration

3. Bioavailability and metabolic disposition in humans

In a pioneering experiment on the bioavailability and disposition of olive oil phenolic compounds in humans (Visioli, 2000b), HOTYR and TYR were spiked to a poor-phenolic content olive oil and administered to healthy volunteers. Preliminary conclusions, later confirmed, were that phenolic compounds are dose-dependently absorbed in humans after olive oil ingestion and that their

bioavailability is extremely poor, most phenolic compounds being recovered in biological fluids as conjugates. An increase in the dose of administered phenolics increases the proportion of their conjugation with glucuronic acid.

Further studies on olive oil phenolic compounds bioavailability were performed with EVOO (Miró-Casas, 2001a, b, 2003a, b). After administration of 25 mL of EVOO (with an estimated content of HOTYR of 49.3 mg/L or 1.2 mg administrated), HOTYR plasma concentrations peaked at 30 min and those of HVAlc at 50 min, with concentrations about 25 ng/mL and 4 ng/mL for HOTYR and HVAlc, respectively. The estimated half-life for HOTYR was 3 h after fitting plasma concentration with a mono-compartimental model. Plasma concentrations declined, most probably following a bi-compartimental model (some missing data points prevented the application of this model), and at 8 h HOTYR concentrations could not be distinguished from background (***Fig. 26***). It cannot be discarded, as discussed earlier, that there is a partial enterohepatic recirculation of HOTYR conjugates.

HOTYR and HVAlc were analyzed in their free and conjugated forms (both in plasma and urine), and it was estimated that more than 98% of each compound were in their conjugated forms, mainly glucuronides, confirming previous findings. In urine, HOTYR and HVAlc concentrations peaked in the collection period 0-2 h (Miró-Casa, 2003a). In a second experiment, EVOO (25 mL) with three increasing concentrations of polyphenols – high (486 mg/kg of olive oil), moderate (133 mg/kg), and low (10 mg/kg) – were administrated on 4 consecutive days. Plasma and urinary

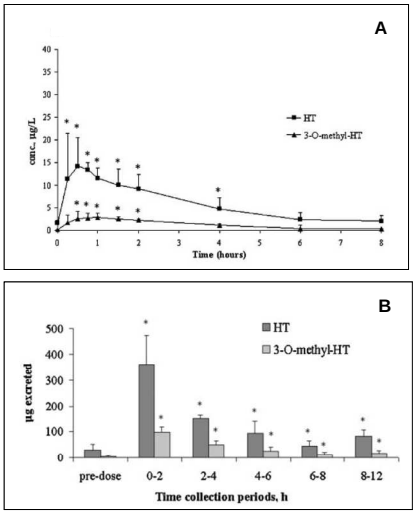


Figure 26 *HOTYR and HVAlc concentration in plasma (A) and urine (B) after acute olive oil ingestion (Miró-Casas, 2003a).*

concentrations of HOTYR, TYR and HVAlc increased significantly in a dose-dependent manner. An increase in plasma concentrations of HOTYR and TYR was observed from day 1 and 4, mainly at postprandial state, which could reflect an increased "pool" of phenolic compounds (Weinbrenner, 2004b). This observation was reproduced in a clinical trail where healthy volunteers were administrated with a single dose of 50 mL EVOO, and later with repeated doses of 25 mL of the same oil during a 1-week period. The mean recovery values for HOTYR after sustained doses were 1.5-fold higher than those obtained after a single 50-mL dose (Miró-Casas, 2003b).

Most bioavailability studies on olive oil phenols have measured total HOTYR and TYR concentrations in blood or urine after acidic or enzymatic treatment of the samples. There is a lack of studies in which glucuronide and sulfate conjugates of HOTYR and TYR in biological samples were measured.

4. Biomarkers of olive oil ingestion

The fact that HOTYR and TYR urinary recoveries are dependent on the phenolic content of olive oil administrated, after doses compatible with dietary habits, confirms the usefulness of these compounds as biomarkers in clinical trials. With regards to the dose-effect relationship, 24-h urinary TYR seems to be a better biomarker of sustained and moderate doses of VOO consumption than HOTYR (Miró-Casas, 2003b). This is mainly due to the cross-metabolism between HOTYR and dopamine. Both HOTYR and TYR urinary concentrations have been used, and are currently in use, in nutritional intervention studies as biomarkers of treatment compliance (Covas, 2006a, b; Fitó, 2007) (***Fig. 27***).

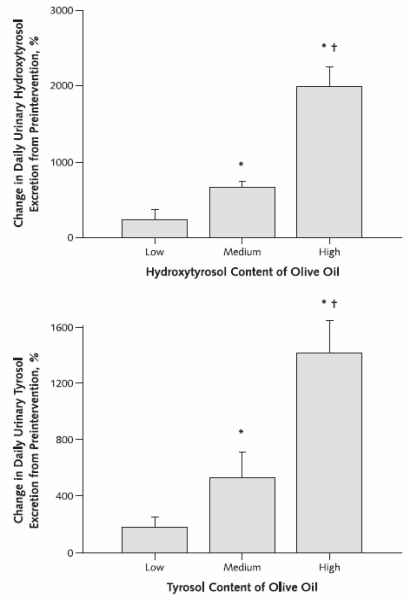


Figure 27 *Changes from preintervention in urinary HOTYR and TYR excretion periods as the function of the phenolic content of the olive oil administered (low vs. medium vs. high) (Covas, 2006b).*

5. Endogenous HOTYR

The recovery of radiolabelled HOTYR in rat urine after its intravenous ingestion was reported to be about 6% of the dose

administered. Other radiolabelled metabolites of HOTYR detected in urine were identified as: HVAlc (MOPET, 3-hydroxy-4-methoxyphenylethanol), DOPAC (3,4-dihydroxyphenylacetic acid), HVA (3-methyl-4-hydroxyphenylacetic acid) and DOPAL (3,4-dihydroxyphenylacetaldehyde) (D'Angelo, 2001). Interestingly, all of the reported metabolites of HOTYR are common to dopamine metabolism (DOPAC, HVA, DOPAL, MOPET), which is not surprising as HOTYR itself can be renamed as DOPET, a well known dopamine metabolite (de la Torre, 2006) (***Fig. 28***).

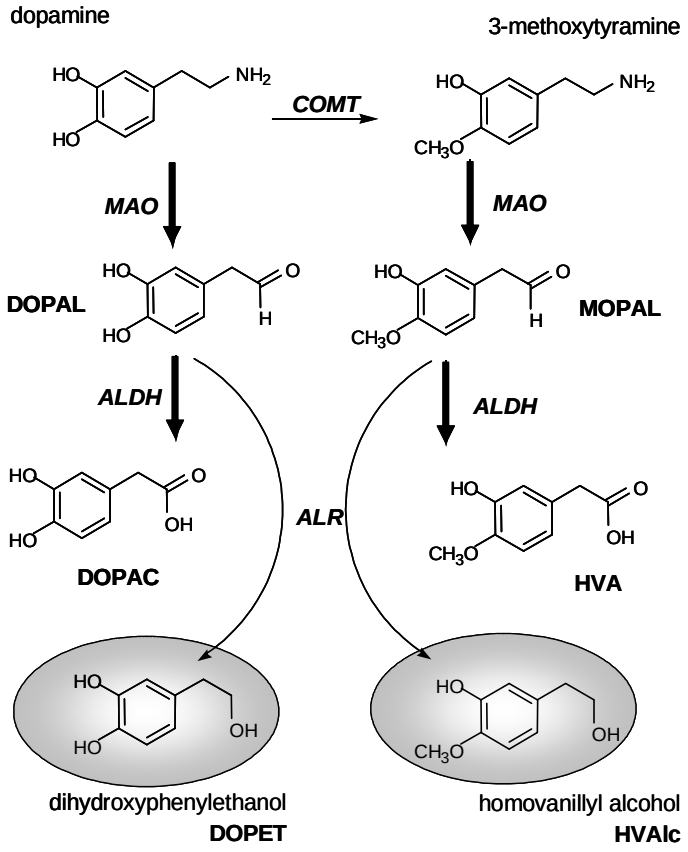


Figure 28 *HOTYR (DOPET) as a minor product of dopamine oxidative degradation (adapted from de la Torre, 2008). COMT – catechol methyltransferase; MAO – monoaminoxidase; ALDH – aldehyde dehydrogenase; ALR – aldehyde/aldose reductase.*

OBJECTIVES

On the basis of the background information provided, the main research objectives were defined as follows:

A) To evaluate the disposition and bioavailability of glucuronoconjugated metabolites of HOTYR, TYR and HVAlc (as methylconjugate of HOTYR) in humans after consumption of olive oil rich in phenolic compounds.

To achieve this goal, the next series of experimental steps have been planed:

1 ***Synthesis of reference compounds of metabolites of HOTYR, TYR and HVAlc, and HOPhPr as an appropriate internal standard for use in analytical, chemical and biological methods;***

2 ***Development and validation of a HPLC-MS detection method for direct quantification of the mentioned conjugated metabolites in urine;***

3 ***Analysis and assessment of HOTYR and TYR metabolism and excretion in human urine after olive oil consumption in samples belonging to a pilot intervention study.***

B) To evaluate the antioxidant properties of HOTYR and TYR glucuronoconjugated metabolites vs. their parent compounds against oxidative stress.

To achieve the aim we have planned the following tests to be performed:

1 ***A chemical test to evaluate the antioxidant potential of conjugated metabolites in comparison to parent compounds;***

2 ***An in vitro experiment for the evaluation of their antioxidant activities in biological systems.***

C) To evaluate biological activities of olive oil in human organism mediated by transcriptome response to dietary intervention and to estimate its possible impact on human health.

To accomplish this objective we have planned the following experiments:

1 ***Evaluation of methods for the total RNA isolation from human total blood and mononuclear cells;***

2 ***Estimation of the variables that might influence gene expression in human subjects;***

3 ***Selection of gene-responders to dietary administration of phenol rich olive oil (mid term intervention) in human subjects.***

METODOLOGICAL APPROACHES

1. Experimental design

To achieve the objectives of the thesis the experimental work was presented in two blocks: preparative and experimental studies (***Fig. 29***).

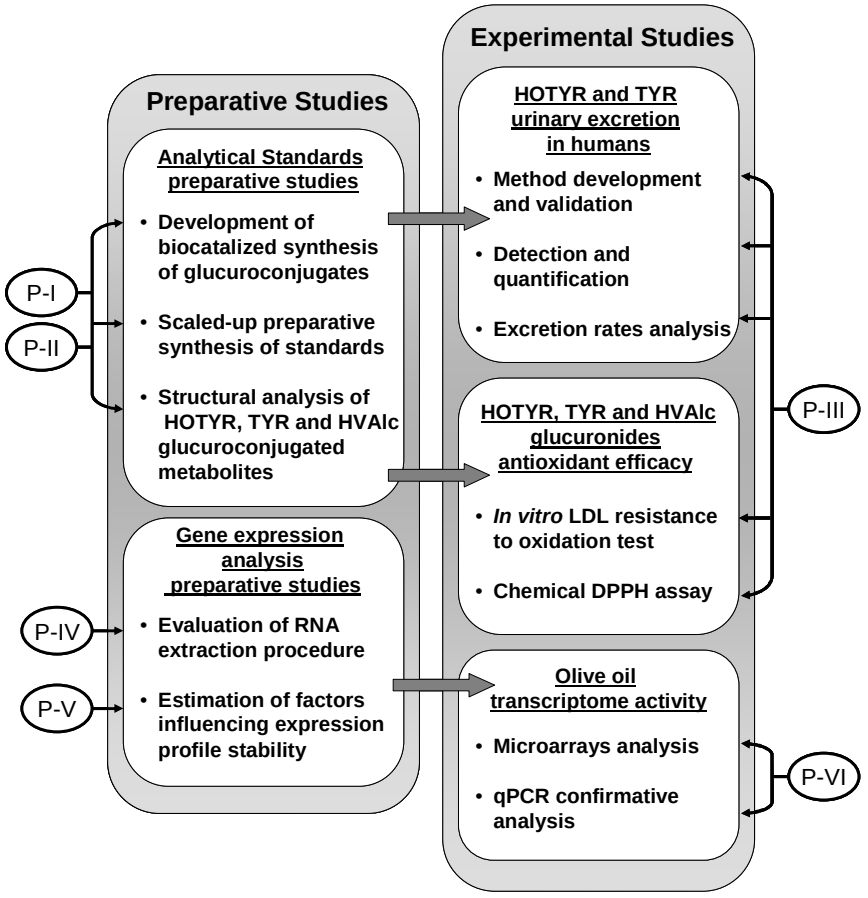


Figure 29 *Scheme representing experimental studies and methodological approaches and corresponding publication in this dissertation.*

The argumentations of choice and the explanations of principles for the methods used in this thesis are grouped by the area of investigation according to the enclosed scheme and are

summarized in this section. Some parts of the work were performed in collaboration with different research groups belonging to different institutions, which are specified in the text. More detailed descriptions of methods (chemicals, procedures, instrumentation, analysis, etc.) can be found in the original publications (**P-I** – **P-VI**) (see Results and Discussions).

2. Glucuronidated metabolites of olive oil phenols analysis

2.1. Preparative studies

Phase II metabolites are needed as reference substances for analytical studies on the bioavailability of olive oil phenols and in general in olive oil research. However, they are not commercially available. Despite of being the major metabolites, only small amounts of glucuronide conjugates can be isolated from urine or tissues after administration of the olive oil to humans or laboratory animals. In addition, this method is very laborious given the small amount of purified compound obtained, and the purity could be doubtful.

Therefore, to achieve the main objectives of this thesis, the preparation of glucuronidated metabolites of olive oil phenols (HOTYR, TYR and HVAlc) was planned in collaboration with laboratory of Instituto de Química Avanzada de Cataluña, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona under the supervision of Dr. Jesús Joglar. The workflow of preparative experiment is present on the ***Fig. 30***.

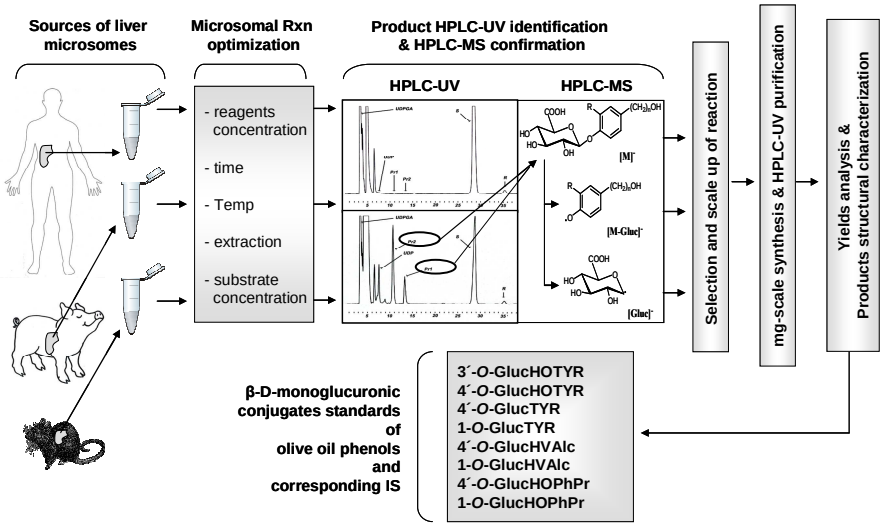


Figure 30 *A workflow for the preparation of glucuronidated standards of olive oil phenols (HOTYR, TYR and HVAlv) and the corresponding putative internal standard (HOPhPr glucuronide)*

2.1.1. Biocatalized synthesis of glucuronidated metabolites

The standards of glucuronide conjugated metabolites of olive oil phenols, in particular of HOTYR, TYR and HVAlc, as well as their corresponding internal standards for use in analytical method, were planed to be enzymatically synthesized. Different kinds of enzyme preparations have been used, such as rat, porcine and human liver microsomes[14], to adjust the synthesis close to real biotransformations taking place in humans (***Fig. 30***). Enzyme-

[14] Microsomes are vesicle-like artifacts formed from the endoplasmic reticulum (ER) when eukaryotic cells are broken-up in the laboratory. Being of ER origin, they content variety of ER membrane bound enzymes, between them UGTs, therefore can be used as a source of different enzymes in the compounds transformation.

assisted synthesis produces mainly β-anomers of mono-glucuronides with a proper regio- or stereoselectivity, the latest one is an important advantage for HOTYR glucuronidated isoforms synthesis.

The comparison of different sources of microsomes and the detailed description of the established method of biocatalized synthesis of glucuronidated metabolites of mentioned olive oil phenols is described in following original publications: **P-I** and **P-II**.

2.1.2. Preparative synthesis of glucuronoconjugates

The reaction of biotransformation was scaled up for preparative synthesis of glucuroconjugates in a milligram range using porcine liver microsomes, as easily accessible and plentiful source of UGT-rich microsomes, able to transform the olive oil phenols in the biologically relevant glucuronidated metabolites. A simple and straightforward HPLC method with UV detection has been successfully developed for products isolation and purification. Glucuronides were lyophilized, weighted and their purity was also evaluated by HPLC-UV. The total workflow in preparation of glucuronidated metabolites of olive oil phenols is presented in ***Fig. 30.***

The detailed description of the products purification and output analysis of the established preparative synthesis are described in the original publications **P-I** and **P-II**.

2.1.3. Structural characterization of synthesized glucuronidated metabolites

Two complementary methods were used in the structural identification and characterization of synthesized glucuronidated metabolites of olive oil phenols: mass spectrometry (MS) and nuclear magnetic resonance (NMR) (***Fig. 31***). First, MS was used to identify glucuronides already on the stage of method establishment. The advantage of the MS technique is that only a very small quantity of compound is required to obtain accurate tandem mass (MS/MS)[15] spectra. Using negative MS/MS, the typical fragmentation pattern for the metabolites has been searched: characteristic fragment ions from the glucuronide moiety at m/z^- 175 $[C_6H_7O_6]^-$ and m/z^- 113 $[C_6H_7O_6–CO_2–H_2O]^-$ (Levsen, 2005) and for core compound fragment ions of relevance were m/z^- 153, 137, 167 and 151 for HOTYR, TYR, HVAlc and HOPhPr, respectively.

The determination of the conjugation site in HOTYR was not possible because the glucuronide isomers are similar in MS/MS spectra. Therefore, NMR[16] analysis was required for a detailed structural characterization of HOTYR glucuronides. The site of glucuronidation can be identified by comparing the chemical shifts

[15] Tandem mass spectrometry, also known as MS/MS, involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring in between the stages.

[16] NMR, nuclear magnetic resonance spectroscopy is a technique which exploits the magnetic properties of certain nuclei. When placed in a magnetic field, NMR active nuclei (such as ^1H or ^{13}C) absorb at a frequency characteristic of the isotope. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field, and, therefore, could be monitored, giving information (chemical shift and J-coupling) about the structural disposition of NMR active nuclei in the molecule.

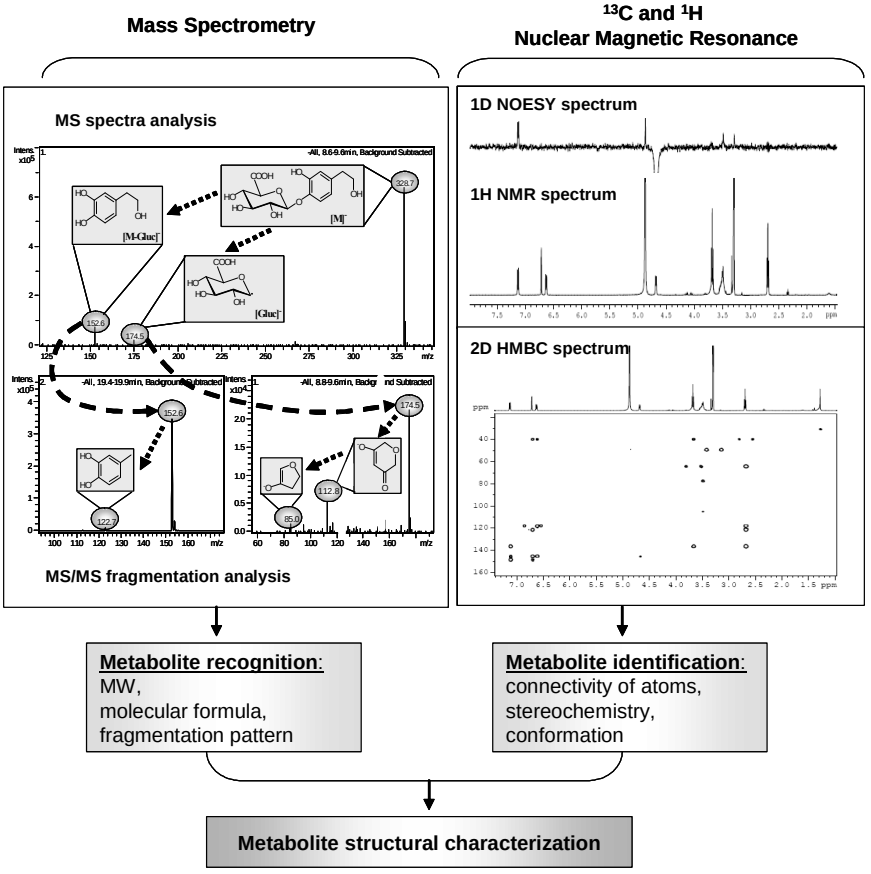


Figure 31 *Identification and structural characterization of glucuronidated metabolites (as an example 4´-O-HOTYRGluc for illustration) of olive oil phenols using MS/MS fragmentation in combination with NMR analysis.*

and spin-to-spin coupling (J-coupling) of the glucuronide to those of the aglycone. The largest changes in these parameters upon glucuronidation are in the atoms located near the conjugation site. Therefore, carbon (^{13}C) and proton (^{1}H) NMR studies were performed in the Instituto de Química Avanzada de Cataluña, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona under the supervision of Dr. Jesús Joglar, Dr. Pere Clapés and in collaboration with Dr. Teodor Parella (Servei de

Ressonància Magnètica Nuclear, Universitat Autònoma de Barcelona). Although these studies required a sufficient amount of compound, in the milligram range, they could be successfully carried out, since the developed semi-preparative synthesis could cover this range.

The MS/MS fragmentation pattern and NMR spectra of parent compounds were established using commercially available standards of HOTYR, TYR, HVAlc and HOPhPr. For detailed description of MS/MS and NMR instrumentation and analysis of synthesized mono-glucuronidated metabolites of live oil phenols should be referred to original publication **P-I**.

2.2. Glucuronidated metabolites study

Direct analysis of glucuroconjugated metabolites is not straightforward. Glucuronides are thermolabile, highly polar and non-volatile compounds present in different type of biological matrices. Due to the complexity of biological samples the compounds should be well resolved from matrix background using separation techniques such as chromatography. Techniques, such as gas chromatography (GC) in which high temperatures are used are inappropriate for their direct analysis because glucuronides are thermolabile and non-volatile. The primary technique for the direct determination of non-volatile, polar and water-soluble compounds, as are olive oil phenols and their phase II metabolites (glucuronides and sulfates) is liquid chromatography (LC).

2.2.1. Analytical methods for qualitative determination and preparative separation of olive oil phenols glucuronidated metabolites

LC with ultraviolet (UV) detection is the first choice in analysis of olive oil phenols in biological fluids (for detailed review of methods see chapter II). However, the lack of standards for the olive oil phenol metabolites and low sensitivity to this type of compounds limited their direct analysis in biological fluids using this detection system (UV). Nonetheless, this approach is often applied in analysis of olive oil phenols metabolism (see chapter II).

The main challenge in chromatographic analysis of glucuronides of olive oil phenols studied in this thesis was related to the separation of two isomers of HOTYR: 3´-*O*-glucuronide and 4´-*O*-glucuronide. Being very similar in their structure (***Fig. 32***), these two isomers were suspected to have identical UV absorption spectrum and very similar chromatographic behaviour. To carry out a preparative study of both standards, a well resolved chromatographic separation of both isomers was needed. In general, separation of highly polar compounds (as are phase II metabolite, e.g. glucuronides) is challenging due to their weak retention by any type of chromatographic column, and even more complicated if they are structurally similar as are isomers. Therefore, three types of chromatographic columns of various parameters (diameter and particle size) and different in filling chemistry specialized in retention of polar compounds were tested (see ***Table 4***) at chromatographic elution conditions reported for HPLC-MS analysis

of nitrocatechol glucuronides (Keski-Hynnilä, 2000) but compatible with preparative chromatography[17] UV-detection (at 215 nm[18]).

The column which separates both products of HOTYR glucuronidation, and resolves it from other biosynthetic reaction components, was Atlantis dC18 5 µm 150 x 4.6 mm (Waters). This column and its prototype for semi-preparative chromatographic separation (Atlantis C18, 5 µm, 150 x 10 mm) were used in biosynthesis analysis and preparation of all glucuronide metabolites presented in this thesis. A detailed description of the HPLC-UV chromatographic conditions for each type of glucuronides in the biosynthetic reaction and its analysis is available in the publication **P-I**.

MS provides both qualitative and quantitative information on the analytes and has been widely used for the identification of metabolites. In combination with liquid chromatographic retention parameters, tandem mass spectra (MS/MS) additionally offers a possibility of structural identification of metabolites (Levsen, 2005), providing additional sensitivity and increased selectivity for the analysis of olive oil phenols conjugates. Therefore, LC−MS is routinely applied in metabolic studies for identification and/or characterisation of metabolites. The HPLC-MS method, applied in MS-identification and confirmation of glucuronidation products of

[17] The microsomal glucuronidation reaction (on the stage of method setup) was used for testing and adjusting the chromatographic separation conditions for both glucuronidated forms of HOTYR.

[18] 215 nm represents absorption wavelength for the majority of components of the biosynthetic reaction: proteins of microsomal fraction, UDPGA, UDP, benzoic ring of phenols (HOTYR, TYR, HVAlc and HOPhPr) and corresponding products of glucuronidation

microsomal biosynthesis in this study, make use of parameters established in the analytical HPLC-UV method.

Table 4 *Column tested in the study for the separation of HOTYR glucuronidated isomers.*

Column	Parameters	Filling & Mode	Specific application
Synergy POLAR-RP 4 µm	80 Å 150 x 2.0 mm	Ether-linked phenyl with polar endcapping; Reverse Phase (RP)[19] mode	For extreme retention of polar and aromatic compounds and operation in 100% aqueous mobile phases[20].
Atlantis T3 3 µm	100Å , 150 x 2.1 mm	silica-based C18 line; Reverse Phase (RP)[19] mode	For retention of polar compounds and operation in 100% aqueous mobile phases[20].
Atlantis® dC_{18} 5 µm	100Å , 150 x 4.6 mm		

Theoretically predicted MS and MS/MS spectra for olive oil phenols glucuronidated metabolites were used for identification and confirmation of products formation in microsomal glucuronidation reactions (***Fig. 31***).

Both, HPLC-UV and HPLC-MS (MS/MS) approaches were used for detection of the products of glucuronidation in biocatalized

[19] Reversed-phase (RP) chromatography uses a non-polar stationary phase (the most popular column is a octadecyl carbon chain (C18) bonded silica) for compounds separation. Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a RP column. where polar compounds are eluted first while non-polar compounds are retained

[20] Mobile phase is a carrier for solutes through the stationary phase and used to adjust the chromatographic separation and retention of analytes. In RP-LC the combination of organic and aqueous solvents are used. Polar compounds are less retained on the column and, therefore, less organic solvents needed for their elution. Sometimes for highly polar compounds (as are phase II metabolites) up to that totally 100% aqueous mobile phase could be required

synthesis. In addition, on the basis of HPLC-UV method, a semi-preparative HPLC-UV methodology was developed. For detailed description of all these methodological approaches refer to the publication **P-I**.

2.2.2. Direct quantification of glucuronidated metabolites

To evaluate the contribution of glucuronidation to the metabolic disposition of olive oil phenols (HOTYR, TYR and HVAlc) in humans, a direct LC-MS detection method was developed and validated for their quantification in human urine using synthesized standards for glucuronidated metabolites.

Four basic points were essential in the development of a direct LC-MS analysis of olive oil phenolic glucuronides and their parent compounds:

(i) the selection of appropriated internals standards;
(ii) the development of a sample preparation procedure;
(iii) the optimization of chromatographic separation;
(iv) the optimization of mass spectral analysis.

To minimize major sources of inaccuracy starting from sample manipulation and ending by instrumental analysis, and also to improve precision of detection for both groups of compounds of interest, two types of internal standards were used: I.S.1, a newly synthesised glucuronide of HOPhPr (4´-*O*-HOPhPrGluc) and I.S.2, HOPhPr for the analysis of glucuronides and their parent compounds, respectively (***Fig. 32***). Both compounds have fulfilled all criteria required for I.S. (refer to original publication **P-III)**: these

compounds are not normally present in the biological samples (based on the screening of a number of samples); they are chemically related analytes (based on preliminary structure analysis); they are chromatographically eluted similarly to analytes under investigation (based on preliminary studies); and, finally, they have an analogous MS/MS behaviour to the analyzed compounds (based on previously done MS fragmentation analysis).

Urine, as a biological matrix in which glucuronides will be analysed, contains interfering compounds at high concentrations, which may alter the chromatographic separation or suppress the ionisation process in mass spectrometry. Disturbing matrix compounds must be removed in sample pre-treatment to improve the selectivity, accuracy, reliability, and repeatability of analyses. In addition, glucuronides should be concentrated in the pre-treatment procedure in order to improve method sensitivity. SPE[21] has achieved the widest acceptance among pre-treatment methods, owing to the easy manipulation, high analyte recovery, extraction reproducibility, capacity for increasing selectively the analyte concentration. For the pre-treatment of urines, Oasis HLB cartridges (Waters), containing a polymeric water-wettable reversed-phase sorbent, were chosen due to their capacity to retain a wide range of polar compounds using a simple generic extraction procedure. The clean up of samples, elution and filtering were optimized for the simultaneous extraction from urine of three

[21] SPE, a solid phase extraction, is a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. SPE uses the affinity of solutes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components.

types of polar compounds: extremely polar glucuronidated metabolites (all glucuroconjugates, including 4´-*O*-HOPhPrGluc as I.S.1), highly polar catechol-containing HOTYR and compounds with relatively lower polarity (TYR, HVAlc and HOPhPr as I.S.2). The final protocol of SPE urine extraction for the aforementioned compounds is presented in the publication **P-III**.

A preliminary developed HPLC-MS assay for the analysis of glucuronides from the biocatalytic reaction of synthesis (**P-III**) was optimized for a simultaneous detection of all compounds of interest (***Fig. 32***) and transferred to a Waters ACQUITY UPLC™ system. Theoretically, the time of chromatographic analysis was reduced almost 7 times (from 45 min in conventional HPLC-MS analysis to 6.5 min in UPLC-MS analysis), therefore, higher sample analysis throughput, lower consumption of mobile phase, better assay reproducibility and sensitivity could be achieved. UPLC provides faster analyses through the use of a novel separation material with a very small particle size (ACQUITY BEH columns 1.7 µm, 100 mm × 2.1 mm) and unique core chemistry (Bridged Ethyl Hybrid particles), which should be operated at higher pressures (up to 15,000 psi), injects samples into a smaller system dwell volume, and captures detector signals at high data rates for fast eluting peaks. During the course of optimization of the UPLC method for simultaneous detection of all types of analytes, glucuronides and their parent compounds in human urine samples, the following fine-tunings were made: flow rate vs. percentage of organic solvent in mobile phase, gradient elution, strength and pH of aqueous phase. The final chromatographic method for analysis of the aforementioned compounds in human urine is presented in the publication **P-III**.

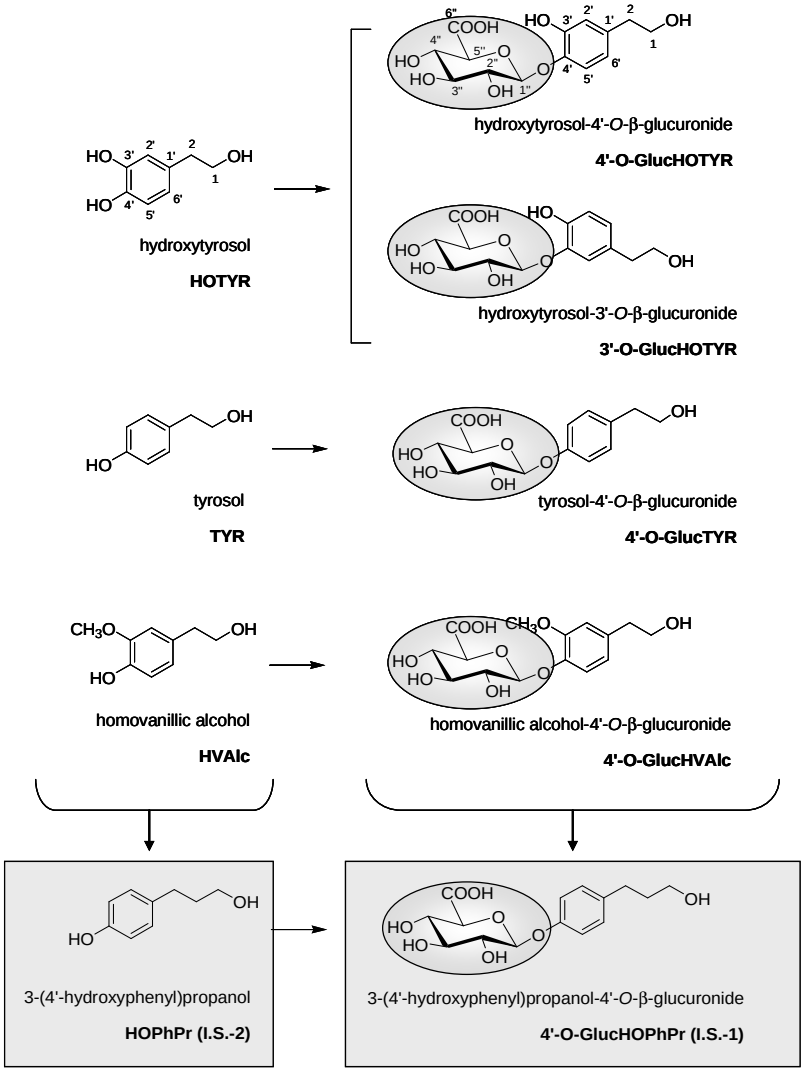


Figure 32 *Compounds analyzed in this study: olive oil phenols parent compounds and their glucuronides, and their corresponding internal standards*

In this work, the SPE-UPLC-MRM method was developed for the identification and quantification of olive oil phenols metabolites, using synthesized and well characterized standards of glucuronide conjugates. This direct method was optimized for detection and

quantification of both glucuronides and their parent compounds in human urine. Method was validated according to FDA/ICH requirements. Detailed description of the developed method and its validation parameters are presented in original publication **P-III**.

2.2.3. Determination of glucuronide metabolites excretion rates

Urinary excretion studies measure the cumulative amount of olive oil polyphenols excreted in the urine. These studies are base on the premise that urinary excretion of the polyphenols is directly proportional to the plasma concentration of total compound. Thus, the total quantity of olive oil polyphenols excreted in the urine is a reflection of the quantity of polyphenols absorbed from the gastrointestinal tract.

The contribution of glucuronide conjugation reactions to human disposition of olive oil phenols was investigated by analysing the urinary recovery of HOTYR, TYR and HVAlc glucuronides. Urine samples were generated in a pilot intervention study with VOO in human healthy volunteers (Apendix B, GEpilot study) (***Fig. 33***).

The concentration of metabolites was planned to be estimated in urines collected at three time points: prior to intervention after wash-out period (basal concentration of conjugates), 6 h after acute ingestion of 50 mL of VOO and 24 h after ingestion. The calculation of excretion rates was related to those amounts of compounds (HOTYR and TYR) detected after acidic hydrolysis, trying to mimic gastrointestinal hydrolysis, of VOO. Detailed description of analysis (UPLC-MRM) and calculation of excretion

rates for olive oil phenols glucuronides and also of their parent compounds are presented in the original publication P-III.

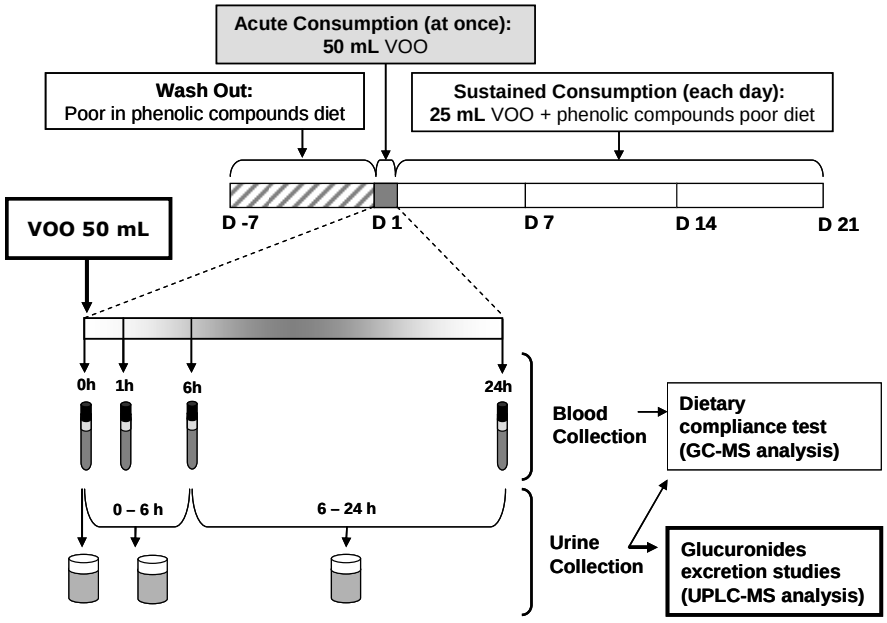


Figure 33 *Study design for the evaluation of the metabolic disposition of olive oil phenols following the GEpilot study protocol (Appendix B).*

2.3. Assessing antioxidant efficiency of olive oil phenols and their glucuronidated metabolites

Several studies (reviewed in chapter I) have shown that olive oil phenolics may act as inhibitors of *in vitro* and *in vivo* LDL oxidation. A number of different mechanisms, including scavenging of free radicals and reactive species, metal chelation, protecting or regenerating α-tocopherol present in LDL, and binding with proteins, could be involved (Burkit, 2001). Following ingestion of

olive oil, phenols are distributed within the body and can be detected not only in plasma and urine, but also in LDL particles (this is discussed in details in chapter II), confirming the hypothesis that LD particle can be target of their action. Nevertheless, due to the extend metabolism of phenols, within LD particle they were detected as phase II metabolites (de la Torre-Carbot, 2006, 2007). Therefore, it is of great interest to know whether the phase II metabolites exert the same antioxidant activities as their parent compounds.

The oxidation targets several sites of LDL (ApoB, cholesterol, triglycerides, fatty acids etc.) among which lipid peroxidation is a key process of oxidation (see chapter I). The oxidation of LDL (its polyunsaturated fatty acids) is prevented by proportional concentration of water-soluble antioxidants surrounding them and liposoluble antioxidants incorporated within the LDL lipid bilayer. Peroxidation of polyunsaturated fatty acids, once initiated, involves a free radical chain reaction and, as a result, a variety of degradation products is generated, among which conjugated dienes (CD) are primary products (***Fig. 34***). The formation of conjugated dienes occurs when free radicals attack the hydrogen atoms of methylene groups between double bonds, leading to the rearrangement of bonds (Recknagel, 1984). The properties of cell-oxidized LDLs are very similar if not identical to LDL oxidized in cell-free medium (Steinbrecher, 1985). Therefore, the *in vitro* and *ex vivo* LDL oxidation models are of special interest for researchers due to their relatively straightforward experimental performance and convenient extrapolation of results to *in vivo* data.

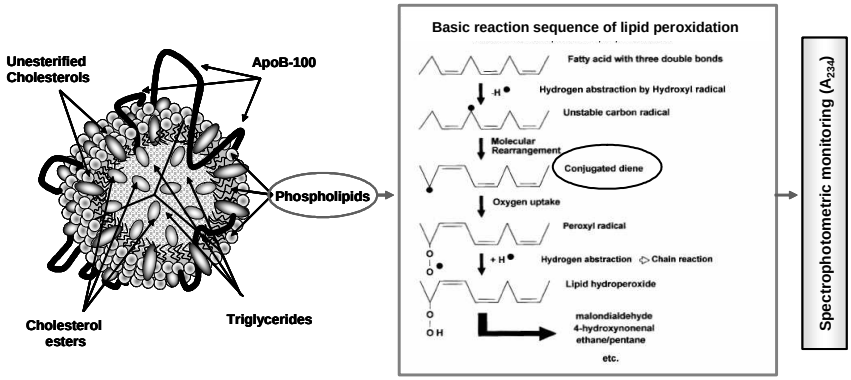


Figure 34 *LDL particle composition and phospholipids as a main site for monitoring of LDL oxidation.*

The scavenging of free radicals by hydrogen-atom donation is considered to be a basic mechanism of action of olive oil phenolic antioxidants against LDL oxidation, although other mechanisms may be involved (see chapter I). Therefore, those methodologies which estimate the scavenging of free radicals by the phenolic antioxidants are quite useful. Among the different published methodologies (Huang, 2005) for determining the antiradical activity of both isolated compounds and complex mixtures of antiradicals, the DPPH assay, initially developed by Blois (Blois, 1958) and more recently adapted by Brand-Williams (Brand-Williams, 1995) is the most widely used because its simplicity. This test has been used for many decades to study the mechanism of H-atom donation to free radical from certain substrates. DPPH does not dimerize[22], exhibit a stable absorbance over a wide range of pH, resist oxidation, reaction conditions are mild and, as discussed earlier, provides basic information on the reactivity of compounds with regard to their structure (Son, 2002). All these characteristics explain the increasing popularity of DPPH test for

[22] In solutions this radical remain in its monomeric form.

applying in screening of antioxidant potency or to show up the mechanism of reaction with the ArOH.

To compare the antioxidant activities of glucuronidated metabolites to their parent compounds, olive oil phenols, the LDL resistance to oxidation test and the DPPH test were carried out. Modifications were made in both methods in order to adjust the methodologies to:

(i) a specific concentration range (close to that recorded in pharmacological studies);
(ii) minimize the amounts of standards (since just limited amounts of synthesized compounds could be available);
(iii) perform measurements in a small reaction volume (to reduce amount of material used in the analysis, e.g. isolated LDL and DPPH solution);
(iv) test simultaneously all compounds (to reduce batch-to-batch differences);
(v) introducing a probe compound, Trolox (to check the success of the experiment and for comparison purposes).

2.3.1. LDL resistance to oxidation test

Formation of conjugated dienes in the LDL particle can be measured directly by monitoring an absorbance at a wavelength of 234 nm (Esterbauer, 1989). The kinetics of the diene formation i.e. the change of the absorbance *vs.* time can be clearly divided into three phases (***Fig. 35***):

(i) Lag-phase during which the dienes formation is very slow;

(ii) Propagation phase when the dienes are very rapidly formed to a maximal value;

(iii) Decomposition phase during which the dienes decrease again.

During the lag-phase (or induction) phase, the lipophilic antioxidants protect the polyunsaturated fatty acids against oxidation and thus prevent the lipid peroxidation process entering into the propagation chain reaction. The oxidation of LDL starts after consumption and/or inactivation of its antioxidants, among which α-tocopherol is one of the most abundant.

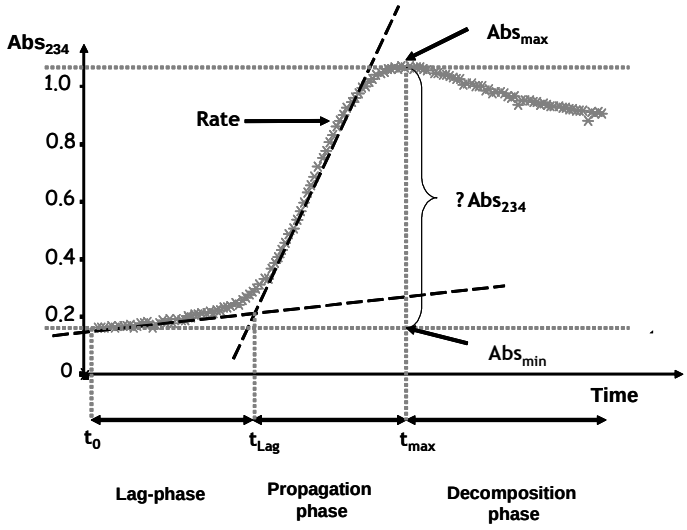


Figure 35 *Kinetics of LDL oxidation by monitoring the change in the absorbance at 234 nm*

If the LDL particle is depleted of its antioxidants, the lipid peroxidation process enters the propagation phase in which the polyunsaturated fatty acids are rapidly converted to conjugated lipid hydroperoxides as indicated by the increase of the 234 nm absorbance. The transition from lag-phase to propagation is

continuous, nevertheless, the rate of the diene formation during the two phases differ widely enough to obtain from the curve the length of the lag-phase. The end of the lag-phase is defined as the intersection of the two straight lines as shown on the picture (***Fig. 35***). In addition to the length of the lag-phase the curve also allows to determine the maximum rate of oxidation and the maximum amount of conjugated dienes formed in the LDL. After reaching its maximum value, the 234 nm absorbance slowly decreases again. This is because lipid peroxides are labile and decompose in a number of consecutive reactions to a variety of products (Esterbauer, 1989).

The vulnerability to oxidative modification has traditionally been estimated *ex vivo* by challenging LDL particles with strong pro-oxidants: either metal ion-dependent (iron and copper ions) or independent (for example, AAPH-induced) oxidation processe. Some studies indicate that both types of pro-oxidants generate different mechanism of oxidation in LDL particles (Frei, 1993). Although it is debated whether copper is a suitable initiator for LDL oxidation *in vivo*, copper accelerated oxidation of human LDL is the most extensively studied *in vitro* mechanism. This exudation requires both binding of Cu^{2+} ions by apolipoprotein B (ApoB) and reduction of cooper by LDL (Kuzuya, 1992). Some compounds can prolong lag-phase in LDL oxidation and therefore retard its oxidation most likely via reactivation of vitamin E (Niki, 1987). Therefore, the measurement of the lag-phase by monitoring LDL oxidation offers the possibility to study the complex antioxidant effects of olive oil phenols metabolites and to compare them to those of their parent compounds.

The specific protocol of the *in vitro* analysis on the LDL resistance to oxidation was developed to test the activities of olive oil phenols (HOTYR, Tyr and HVAlc) and their glucuronides (3´- and 4´-*O*-GlucHOTYR, 4´-*O*-GlucTYR and 4´-*O*-GlucHVAlc). The analysis was performed in the laboratory of the Oxidative Stress and Nutrition Research Group at IMIM-Hospital del Mar under the supervision of Dr. Montserrat Fitó. Detailed description of the methodology is provided within Material and Methods part of **P-III** publication.

2.3.2. DPPH assay

This method is based on the reduction of free stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), which strongly absorbs at 515 nm, to the corresponding hydrazine, which is almost transparent at this wavelength, by the transfer of hydrogen atoms from the antiradical (***Fig. 36***). Hence, the time evolution of the absorbance, subsequently converted to DPPH concentration, is the parameter monitored. The overall stoichiometry[23] of the reaction is the number of molecules of DPPH reduced (decolourized) by one molecule of the reductant (antioxidant). This reaction is intended to provide the link with the reactions taking place in an oxidizing system, such as the autoxidation of a lipid or other unsaturated substances. The DPPH radical is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance ArOH.

[23] Stoichiometry is the calculation of quantitative (measurable) relationships of the reactants and products in a balanced chemical reaction. It can be used to calculate quantities such as the amount of products that can be produced with the given reactants and percent yield.

The reaction between the DPPH and the substrate may be expected to be stoichiometric; the end-point may then be represented in terms of n_{DPPH}, the number of DPPH molecules reduced by one molecule of the substrate. The overall stoichiometry is not necessarily a whole number such as 1 or 2, due to the complexity of the reaction between DPPH and the reductant. An EC_{50} value (otherwise called the IC50 value) has been introduced for the interpretation of the results of the DPPH method. It is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour). In the original method a reaction time of 30 minutes was recommended, however, in view of the fact that the rate of reaction varies widely among substrates the best practice seems to be to follow the reaction until it has gone to completion ("plateau") (***Fig. 36***).

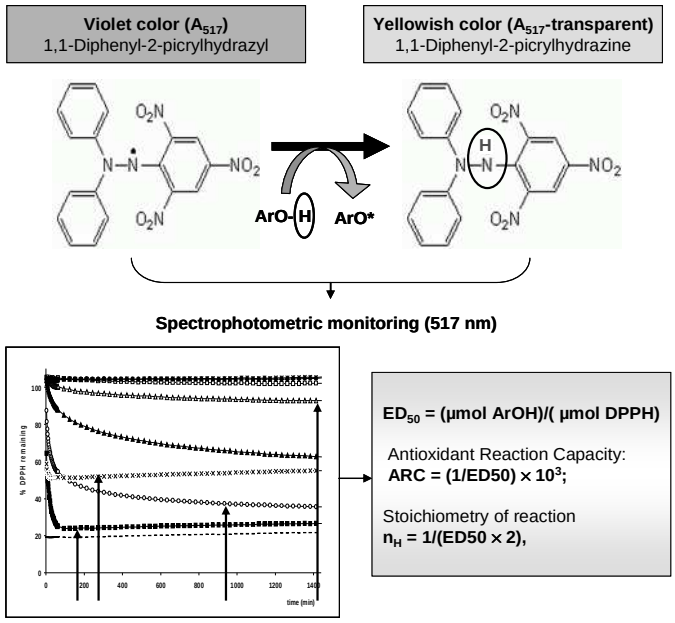


Figure 36 *DPPH reduction mechanism and it monitoring.*

The analysis of radical scavenging activities using the DPPH test was performed for the same compounds tested for LDL resistance to oxidation test. The method development and validation was performed in collaboration with the group of Instituto de Química Avanzada de Cataluña, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona (Dr. José Lluis Torres) under the supervision of Dr. Sonia Touriño. Due to the chemical variety of tested in our study compounds, the DPPH scavenging activities were estimated by both end point reading (at different time intervals) and kinetic behaviour (see original publication **P-III** for details).

3. Gene expression studies on olive oil transcriptome activity

3.1. Preparative methodology studies for gene expression analysis

Due to the high sensitivity of the techniques studying gene expression, researchers must take into account all sources of variation not attributable to the experimental design and interventions. Sources of variation frequently observed in any experiment, including gene expression studies, can be split into two main groups: biological variability and technical variability (Bustin, 2010). Technical variability refers to a noise introduced into the measurement system. Biological variability refers to natural heterogeneity among individuals, due to differences in their genetic background, developmental or physiological stages, environmental factors and gender, among others. No matter the cause, high

variability often prevents the detection of true differential expression patterns, as it decreases the power of the statistical test translating to relatively high false-negative rates (Steibel, 2005). Therefore, prior to conduct any experiment on gene expression, especially those on such complex subjects as humans, both types of factors should be acknowledged and their influence should be either well controlled or reduced to a minimum.

Because the procurement of tissues is invasive and not justified on ethical grounds, gene expression studies in humans are performed in a surrogate peripheral tissue such as blood (Burczynski, 2006). Two type of blood samples are commonly used in clinical intervention and epidemiologic studies: total blood (the whole blood as it is plus anticoagulant to preserve cellular integrity) and cellular fractions (buffy coat[24] and mononuclear cell fraction, etc). Subsequently, these samples constitute a source of RNA in nutrigenomics experiments. While total blood is the most frequent sample collected in these studies, this type of sample brings many challenges into RNA extraction and later on in gene expression analysis due to the “dirtiness” and the complexity of this tissue (Feezor, 2004). Previous extraction of RNA-informative[25] blood cells can overcome some of these problems in both sample preparation and gene expression analysis (Debey, 2004). Although, specific blood cells can be used for RNA extraction and gene expression analysis, the outcomes of the gene-nutrition interactions may depend upon the type of cell used. Therefore, it

[24] It is an enriched leukocyte blood fraction obtained by the sedimental separation of leukoid cells from erytroid cells and plasma.

[25] Within whole spectra of blood cells, only 0.1%, mainly represented by leukocytes, are carrier of RNA and, therefore, are transcriptome active cells. Other cells as erythrocytes do not have RNA.

was of our interest to evaluate whether the differences in cell specific genomic behaviour could have impact on the experiments, planed for our olive oil nutrigenomics studies.

Working with low-quality RNA may strongly compromise the experimental results of downstream applications which are often labour-intensive, time-consuming, and highly expensive. Pure and integral RNA is a key element for the successful application of modern molecular biological methods, like quantitative RT-PCR and microarray analysis (Rainen, 2002; Fleige, 2006; Kiewe, 2009). Therefore, several total RNA[26] extraction procedures were evaluated in order to select the most appropriate for their application in future nutrigenomics studies.

Because the study design and the organization of the collection of samples could have a strong impact on the gene expression variability, we looked at several physiological parameters, which could be to some extent modified within or controlled over clinical nutrigenomic studies: gender status, diurnal variation, menstrual cycle (women).

3.1.1. Evaluation of RNA extraction procedure

In the evaluation of the total RNA extraction procedures two group of parameters influencing gene expression analysis in variety of downstream applications (Bustin, 2009) should be considered:

[26] Total RNA refer to the whole pool of RNA molecules obtained by corresponding extraction procedure from samples, and can combine different classes of RNA molecules, including tRNA (transport RNA), rRNA (ribsosomal RNA), mRNA (messenger RNA), etc. From this pool only mRNA molecules are used in gene expression analysis, since they represent the transcriptome activity of genome.

qualitative (purity and integrity) and quantitative (concentration and recovery):

(i) Total RNA purity, is evaluated by the presence of protein and salts and other impurities (e.g. ethanol) in the extract of total RNA. The commonly used method for the evaluation of RNA purity is based on the spectrophometric estimation of absorbance at three wavelengths: 230 nm (specific for ethanol and salts), 260 nm (specific for nucleic acid) and 280 nm (specific for proteins). Ratios A_{260}/A_{280} and A_{260}/A_{280}, represents the relative abundance of some impurities and proteins in the RNA sample. Elevated concentrations of these impurities in the RNA samples can interfere with the downstream application and also can challenge the stability of samples. Commonly accepted ranges (of ratios) are within 1.6-2.0, but sometime it is strictly dictated by the following application, as in case with microarrays where it should be relatively pure – above 1.9. In addition, it should be insured that there are no significant DNA traces in the isolated RNA sample, since it can interfere with both estimation of RNA concentration and with downstream applications (Naderi, 2004).

(ii) Total RNA concentration is calculated on the basis of the absorbance at 260 nm by nucleic acids using the Lambert-Beer law, which predicts a linear change in absorbance *vs.* concentration. There could be special requirements on the concentration of RNA in samples for downstream application. For examples, microarray experiments require quite concentrated samples, whereas quantitative PCR can be performed with quite diluted ones. In addition, qualitative and quantitative analysis of

extracted RNA can be restricted to the specific concentration ranges of the samples.

(iii) Total RNA recovery refers to how much of total RNA could be extracted form the unit of samples by the mean of different techniques. It is important since the amount of RNA in blood samples is very limited. The higher recovery of the RNA increases the efficiency of an application (for sometimes a unique and very precious clinical sample).

(iv) Total RNA integrity assessment is a critical step in obtaining meaningful gene expression data. Typically total RNA integrity is estimated by the evaluation of the integrity of ribosomal RNA sub-units bands (ration between 28s and 18s rRNA) co-extracted and co-existing in the sample of total RNA along with mRNA, the target of gene expression studies. To verify RNA integrity a commercially available automated capillary-electrophoresis system, for example 2100 Bioanalyzer (Agilent Technologies), could be applied. The electrophoretic profiles allow a visual inspection of RNA integrity, and estimate an approximate ratio between the mass of ribosomal sub-units, however this value was claimed to be imprecise regarding to the integrity of mRNA in the samples of total RNA (Imbeaud, 2005; Schroeder, 2006). In addition to this visual estimation of the RNA integrity (ribosomal RNA ratio), the 2100 Bioanalyzer software estimates the RNA quality by calculating the RNA integrity number (RIN). Using this software tool, sample integrity is determined for the entire electrophoretic trace of the RNA sample. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments is ensured. The assigned RIN is claimed to be

independent of sample concentration, instrument and analyst therefore becoming a *de facto* standard parameter for RNA integrity.

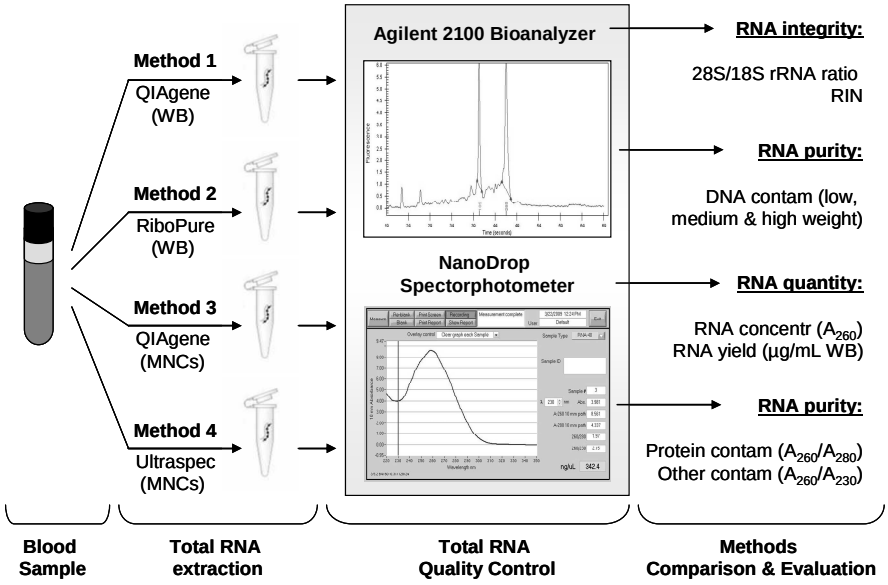


Figure 37 *Scheme of evaluation of several methods for total RNA extraction procedures from whole blood (WB) and isolated mononuclear cells (MNCs) for application in nutrigenomics studies.*

Two different blood sample types were evaluated for total RNA extraction: whole blood (WB) and mononuclear cells (MNCs) extracts. The collection of samples was performed within the RNACLIN study protocol (Appendix A). Four different extraction procedures: two using WB samples (QIAgene and RiboPure) and two extracted MNCs samples (Ultraspec and QIAgene) were evaluated as presented on the ***Fig. 37***.

A detailed evaluation protocol procedure as well as analysis performed in the study can be found in original publication **P-IV** (presented within Results and Discussions section of this thesis).

3.1.2. Estimation of factors influencing gene expression profile stability

The simplest gene expression experiment looks for changes in expression of genes across a single factor of interest, as could be intervention with VOO. Often human nutrigenomics experiments are conducted in heterogeneous groups of individuals and these studies are extended over the time according to the type of intervention applied and/or to the outcomes researchers are interested in. As a result a number of factors influencing gene expression could have an impact on the final results. Therefore, we were interested in determining several factors we thought may contribute significantly to variability in human nutrigenomics studies.

Two types of factors we felt may modify the experimental design of nutrigenomic studies: (i) gender and in particular the impact of the menstrual cycle in gene expression; and (ii) time-dependent gene expression variation. To evaluate the role of these variables, a controlled clinical study was conducted according to the RNACLIN protocol (Appendix A) (***Fig. 38***). One of the objectives of the study was to evaluate the stability of *SOD1* and *SOD2* expression, a pair of genes which expression is directly related to the stability of the redox system of total blood and, in particular, white blood cells. In addition, these genes were supposed to be direct and indirect

targets of future nutrigenomics studies such as those planned with VOO.

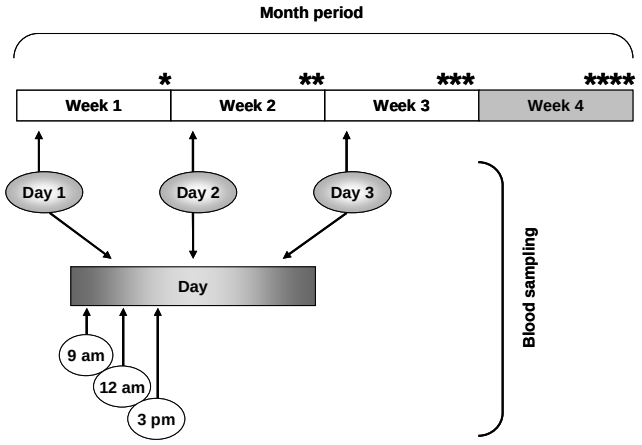


Figure 38 *Study design on gene expression stability. Asterisk represents the phases of female menstrual cycle: * - follicular phase; ** - ovulation; *** - luteal phase; **** - menses week.*

A detailed description of the study is presented in original publication **P-V**. The RNACLIN study protocol should be found in Appendix A. A summary of study is shown in the following scheme (***Fig. 38***).

3.2. Gene expression experimental studies

The analysis of changes in mRNA expression induced by nutrients and bioactive food constituents is often the first step to study the flow of molecular information from the genome to the proteome and metabolome and one of the main goals in nutrigenomics research (Müller, 2003). Different approaches are used in gene expression analysis (Garcia-Cañas, 2010; Knasmüller, 2008). There are two main applications of them: screening of a global gene expression

profile and a targeted analysis of the expression level of genes of interest.

The microarray analysis of changes induced by VOO ingestion at global gene expression level offers opportunities to identify the effect of this food component on metabolic pathways and homeostatic control. A DNA microarray is a collection of oligonucleotides or probes, representing thousands of genes, attached to a solid surface, at predefined locations within a grid pattern. This technique is based on complementary nucleic acids hybridization and it can be used to measure the relative quantities of specific mRNAs in samples for thousands of genes simultaneously. There are many different microarray platforms available for gene expression analysis. They mainly differ in the procedure and the chemistry of the labelling and hybridization processes, which to some extend could have an influence on gene expression changes detection (Garcia-Cañas, 2010; Muyal, 2008).

The final output of generated results is a long list of differentially expressed genes pending of a further biological interpretation. Public databases are used for the systematic analysis of results in order to assemble a summary of the most enriched and significant biological aspects. The principle behind enrichment analysis is that if a certain biological process is occurring in a given study, the co-functioning genes involved should have a higher (enriched) potential to be selected as a relevant group by high-throughput screening technologies. This approach increases the probability to identify the correct biological processes most pertinent to the biological mechanism under study (Huang, 2009). There is a variety of bioinformatics resources (DAVID, Onto-Express, FatiGO,

GOminer, etc.) for the biological interpretation of gene expression microarrays data. They help in identifying the enriched biological processes, functions and components represented in the lists of differentially expressed genes statistically comparing them to the annotations in control samples. Enrichment analysis is possible thanks to appropriately structured databases such as Gene Ontology (http://www.geneontology.org), which provide a systematic and controlled language, or ontology, for the consistent description of attributes of genes and gene products, in three key biological domains that are shared by all organisms: molecular function, biological process and cellular component.

In addition to GO pattern analysis of differentially expressed genes, the selection could be performed according to particular research interests in the differentially expressed genes: for example, their relation to the specific biological processes or their involvement in any type of pathology. Therefore, after identification of a profile of differentially expressed genes, the selection of individual genes could be done by looking at their application in and/or relevance to specific process based on reported data.

Although microarray platforms are claimed to be highly sensitive and reproducible the results on differential gene expression should be confirmed by more precise and sensitive methodologies (Rockett, 2004). Quantitative PCR (qPCR) is a commonly used validation tool for confirming gene expression results obtained from microarray analysis. In addition to microarray confirmation analysis, qPCR is widely used in direct gene expression analysis. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to additional reference genes) of

one or more specific sequences in a sample. This technology may be used in determining how expression of a particular gene changes over time, such as in the response of tissue and cells to the exposure to environmental conditions and xenobiotics, including dietary food components such as VOO.

In qPCR analysis, mRNA previously converted into its cDNA in a reverse transcription (RT)[27] reaction (first strength cDNA synthesis) is amplified in a PCR by specific primers. The amounts of amplified products are visualized (fluorescent dye or any type of probes). The expression level of the target gene is computed relative to the expression level of one or more reference genes[28], often named as housekeeping genes (Nolan, 2006). Real-time qPCR monitors the amount of amplicon generated as the reaction occurs. The amount of product is directly related to the fluorescence of a reporter dye. Because it detects the amount of product as the reaction progresses, Real-Time PCR provides a wide linear dynamic range, demonstrates high sensitivity, and allows quantification (Kubista, 2006).

3.2.1. Microarray experiment

In the present study the microarray experiment was applied to evaluate *in vivo* MNCs gene response to the nutritional intervention

[27] RT (reverse transcription) reaction replicate single stranded DNA from an RNA template by a reverse transcriptase, also known as RNA-dependent DNA polymerase. It is used to apply the polymerase chain reaction technique to RNA The classical PCR technique can be applied only to DNA strands, but, with the help of RT, RNA can be transcribed into DNA, thus making PCR analysis of RNA molecules possible.

[28] Reference gene is typically a constitutive gene that is transcribed at a relatively constant level, therefore, are used as internal standards in qPCR since it is generally assumed that their expression is unaffected by experimental conditions

with VOO. The intervention study, and samples collection and preparation were performed at IMIM-Hospital del Mar, whereas the microarray experiment was performed using a microarray Service (National Centre of Cardiovascular Investigation, CNIC, www.cnic.es) and data processing was supported by Integromics (www.integromics.com). Final data analysis and data mining were carried out at IMIM-Hospital del Mar (***Fig. 39***). Detailed description of the work performed at IMIM-Hospital del Mar is described in the Materials and Methods section of original paper **P-VI**. Samples were originated within the GEpilot study protocol (see Appendix B).

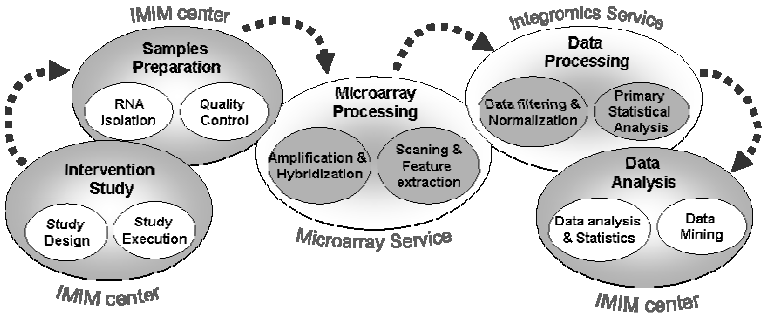


Figure 39 *Workflow on microarray experiment applied in this study*

The microarray platform used in the present study was Human Genome Survey Microarray V2.0, of Applied Biosystems, which interrogates about 30,000 genes including 8,000 that are not available in public databases and are derived from the non-public Celera database. This platform corresponds to single-channel microarray[29] and based on specifically long oligonucleotide probes

[29] In a single-channel microarrays or one-color microarrays, the arrays provide intensity data for each probe or probe set indicating a relative level of hybridization with the labelled target. However, they do not truly indicate abundance levels of a gene but rather relative abundance when

attached to a nylon surface, and on using chemiluminescence for the labelling and detection. The strengths of the single-dye system lie in the fact that an aberrant sample cannot affect the raw data derived from other samples, because each array chip is exposed to only one sample and that data are more easily compared to arrays from different experiments. In addition, a high specificity of hybridization and a low detection limit of the system allows the data analysis be more sensitive compared to other detection systems (Grewal, 2007). Therefore, the AB Human Genome Survey Microarray platform was found to be very advantageous for the nutrigenomics pilot experiment (GEpilot, for detailed study protocol see Appendix B). Two conditions were planed to be compared: mid/long term effects after virgin olive oil ingestion and baseline (after a wash-out period with controlled diet). The response of biological systems was expected to be very weak, almost close to the “normal” variability, especially in healthy volunteers, since the intervention with virgin olive oil was at doses compatible with its dietary intake in the context of the Mediterranean diet.

The probes of Human Genome Survey Microarray are identified following an Applied Biosystems codification system, which can link them to the corresponding gene expression assay, provided by the same company and used in microarry validation: TaqMan® Gene Expression Assays. In addition, integration with the Applied Biosystems PANTHER™ Classification System provides valuable information on molecular function and biological process of microarrays probes, allowing direct online (PANTHER Software:

compared to other samples or conditions when processed in the same experiment.

http://www.pantherdb.org/) GO enrichment analysis of microarray experimental data.

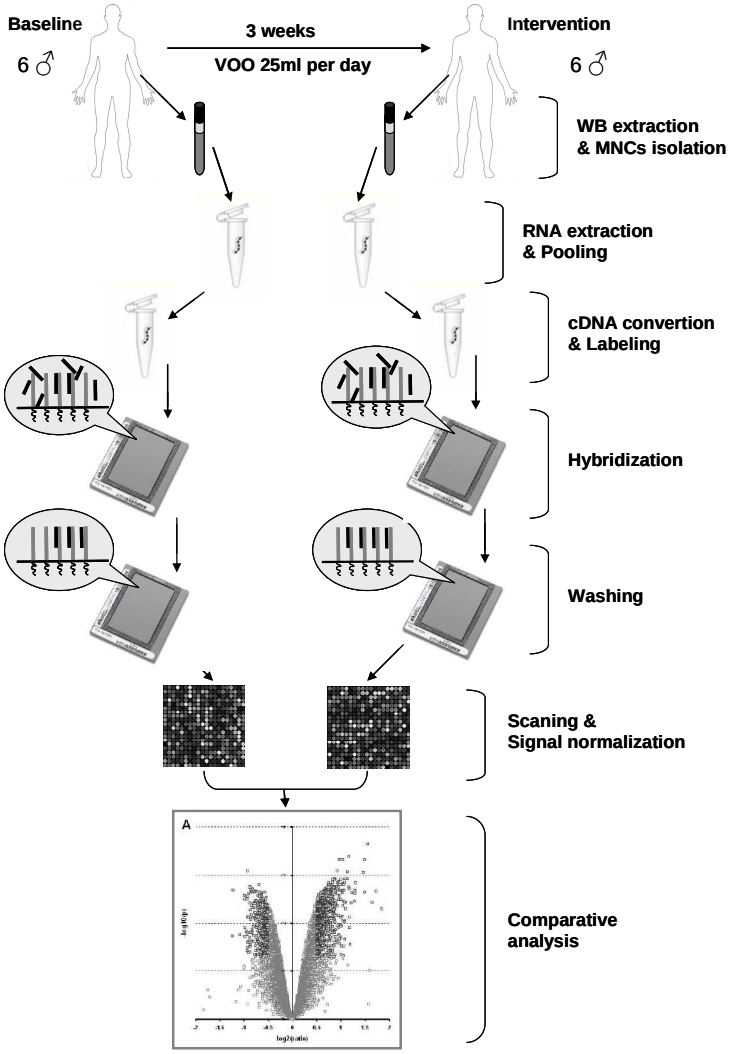


Figure 40 *Gene expression experiment using AB Human Genome Survey Microarray platform: RNA was extracted from two different samples of MNCs (baseline vs. intervention with VOO), converted into cDNA and labelled. Samples were hybridized to the two arrays, further washed and scanned. Differences in gene expression were revealed by comparison of chemiluminescent patterns of both arrays.*

Details on sample preparation, the protocol on microarray experiments, data analysis and the algorithm for gene selection are described within Material and Methods in original paper **P-VI** (see also Results and Discussions section of this dissertation) and also on ***Fig. 40***.

3.2.2. Real-Time qPCR

For confirmation of microarray results and for validation of the stability of gene expression either during the RNA extraction procedure or in the analysis of physiological factors influencing the gene expression, TaqMan Real Time PCR, assays based on 5' nuclease chemistry using TaqMan® MGB (minor groove binder), probes was applied. This technology was selected as a Gold Standard in the MicroArray Quality Control (MAQC) Project, due to its high specificity, high sensitivity and large dynamic range of any gene expression technology (Canales, 2006).

Two TaqMan Real Time PCR approaches were applied in this work (***Fig. 41***): (i) individual TaqMan® Gene Expression Assay and (ii) a TaqMan® Custom Array using Micro Fluidity Cards[30]. The choice of the approach depended on the amounts of the genes to be analyzed according to the driven objectives. Thus in evaluation studies, where only 2 target genes (*SOD1* and *SOD2*) and one housekeeping/reference gene (β-actin) were chosen, the individual

[30] The TaqMan® Custom Array is a 384-well Micro Fluidic Card that enables performing of 384 simultaneous real-time PCR reactions without the need to use liquid-handling robots or multi-channel pipettors to fill the card. Thus, this medium-throughput array allows for 8 samples to be run in parallel against 24 TaqMan® Gene Expression Assay targets that were pre-loaded into each of the wells on the card.

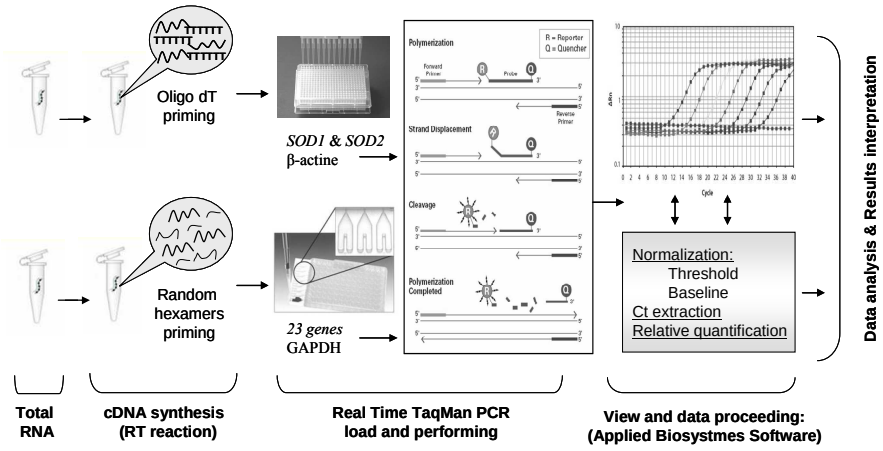


Figure 41 *Real Time qPCR: principles of TaqMan qPCR chemistry, analysis and its applications in the present study using either individual Gene Expression Arrays for two SOD genes or a Custom Micro Fluidic Cards for microarray confirmation analysis for a group of 23 genes.*

qPCR gene assay was applied to oligo-dT reverse-transcribed[31] cDNA samples. In the microarrays confirmation study, the Micro Fluidity Cards were used, because of their advantages in sample manipulation and the simultaneous analysis of expression for 24 genes (23 of interest and 1 housekeeping gene, *GAPDH*). Prior to PCR quantification, total RNA of samples corresponding to microarray study were converted to cDNA using random primers[32] as it was required by manufacturer´ established protocol.

[31] Oligo dT primer is used as a primer for first strand cDNA synthesis with reverse transcriptase. The primer hybridizes to the poly(A) tail of mRNA, therefore, poly(A) containing mRNAs are reverse transcribed.

[32] Random Primers consist of a mixture of short oligonucleotides representing all possible short sequences on RNA molecules, therefore, virtually all types of RNA molecules are reverse transcribed.

The corresponding RT reactions (first strength cDNA synthesis) and Real Time PCR were performed according to the manufacturer instructions and are described in detail within the Material and Methods section of corresponding papers: for single gene Real Time PCR see original publication **P-IV** and **P-V**; and for microarray confirmation original publication **P-VI** (see Results and Discussions of this thesis, respectively).

RESULTS AND DISCUSSIONS

Following original publications represent a section of Results and corresponding to them Discussions:

Publication I (**P-I**) – **Khymenets O**, Joglar J, Clapés P, Covas MI, de la Torre R.
Biocatalyzed synthesis and structural characterization of monoglucuronides of hydroxytyrosol, tyrosol, homovanillic alcohol, and 3-(4 -Hydroxyphenyl).
Adv Synth Catal 2006; 348 (15): 2155-2162.

Publication II (**P-II**) – **Khymenets O**, Clapés P, Parella T, Covas MI, de la Torre R, Joglar J.
Biocatalyzed synthesis of monoglucuronides of Hydroxytyrosol, Tyrosol, Homovanillic Alcohol, and 3-(4'-Hydroxyphenyl)propanol using liver cells microsomal fractions.
En: Whittall J, Sutton P, eds. Practical Methods for Biocatalysis and Biotransformations. : John Wiley & Sons, Ltd, 2009: 245-250.

Publication III (**P-III**) – **Khymenets O**, Fitó M, Touriño S, Muñoz-Aguayo D, Pujadas M, Torres JL, Joglar J, Farré M, Covas MI, de la Torre R.
Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion.
Submitted to Drug Metab & Dispos (2010).

Publication IV (**P-IV**) – **Khymenets O**, Ortuño J, Fitó M, Covas MI, Farré M, de la Torre R.

Evaluation of RNA isolation procedures from human blood and its application for gene expression studies (*Sod-1, Sod-2*).
Anal Biochem 2005; 347: 156-158.

Publication V (**P-V**) – **Khymenets O**, Covas MI, Farré M, Langohr K, Fitó M, de la Torre R.
Role of sex and time of blood sampling in *SOD1* and *SOD2* expression variability.
Clin Biochem 2008; 41(16-17): 1348-1354.

Publication VI (**P-VI**) – **Khymenets O**, Fitó M, Covas MI, Farré M, Pujadas-Bastardes M, Muñoz D, Konstantinidou V, de la Torre R.
Mononuclear cell transcriptome response after sustained virgin olive oil consumption in humans: an exploratory nutrigenomics study.
OMICS 2009; 13(1): 7-19.

Khymenets O, Joglar J, Clapés P, Parella T, Covas MI, de la Torre R. Biocatalyzed synthesis and structural characterization of monoglucuronides of hydroxytyrosol, tyrosol, homovanillic alcohol, and 3-(4'-hydroxyphenyl)propanol. Adv Synth Catal. 2006; 348(15): 2155-62.

Khymenets O, Clapés P, Parella T, Covas MA, de la Torre R, Joglar J. Biocatalysed synthesis of monoglucuronides of hidroxytyrosol, tyrosol, homovanillic alcohol and 3-(40-hydroxyphenyl)propanol using liver cell microsomal fractions. Dins de: Whittall J, Sutton P (eds) Practical methods for biocatalasis and biotransformations. Chichester : Wiley, 2010. p. 245-50.

Publication III (P-III) and corresponding Supplementary Material

Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion

Olha Khymenets, Montserat Fitó, Sonia Touriño, Daniel Muñoz-Aguayo, Mitona Pujadas, Josep Lluis Torres, Jesús Joglar, Magí Farré, Maria-Isabel Covas, Rafael de la Torre

Human Pharmacology and Clinical Neurosciences Research Group, Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar), Barcelona, Spain (OKH, MP, MFa,RdlT)

CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Santiago de Compostela, Spain (OKH, MFi, MP, MIC, RdlT)

Cardiovascular Risk and Nutrition Research Group, IMIM-Hospital del Mar, Barcelona, Spain (MFi, DMA, MIC)

Instituto de Química Avanzada de Cataluña, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona, Spain (ST, JLT, JJ)

Universitat Autónoma de Barcelona (UAB), Barcelona, Spain (MFa)

Universitat Pompeu Fabra (CEXS-UPF), Barcelona, Spain (RdlT)

Running title: glucuronides of olive oil phenols and antioxidant activity (44 characters)

Correspondence should be addressed to:

Rafael de la Torre, PharmD, PhD,

Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Hospital del Mar. C/Dr.Aiguader 88, Barcelona 08003, Spain.

Tel.: +34 93 316 04 84; fax: +34 93 316 04 60,

e-mail: rtorre@imim.es

Number of text pages: 24

Number of tables: 2

Number of figures: 6

Number of references: 39

Words in Abstract: 198

Words in Introduction: 670

Words in Discussion: 1520

Key words: olive oil, phenols, metabolism, glucuronides, DPPH, LDL oxidation, hydroxytyrosol

List of Abbreviations: ACN, acetonitrile; ARC, antiradical capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; HOPhPr, 3-(4-hydroxyphenyl)propanol; HOTYR, hydroxytyrosol; HVA, homovanillic acid; HVAlc, homovanillyl alcohol; I.S., internal Standard; LDL, low density lipoproteins; MeOH, methanol; 4´-*O*-Gluc-HOTYR, 4´-*O*-hydroxytyrosol glucuronide; 3´-*O*-Gluc-HOTYR, 3´-*O*-hydroxytyrysol glucuronide; 4´-*O*-Gluc-TYR, 4´-*O*-tyrosol glucuronide; 4´-*O*-Gluc-HVAlc, 4´-*O*-homovanillyl alcohol glucuronide; 4´-*O*-Gluc-HOPhPr, 4´-*O*-hydroxyphenylpropanol glucuronide; OR

oxidation rate; TYR, tyrosol; UDPGT, uridine diphosphate glucuronyltransferase; VOO, virgen olive oil.

Abstract

Hydroxytyrosol (HOTYR) and tyrosol (TYR), main phenolic compounds of olive oil have been reported to contribute to the prevention of age-related diseases due to their antioxidant activities. Their bioavailability in humans is poor and they are found in biological fluids mainly as conjugated metabolites. Free phenols concentrations are low and unlikely to explain biological activities seen in humans after olive oil intake. In this context antioxidant activities of conjugated metabolites in a range of concentrations compatible with their dietary consumption were evaluated. A high performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-MS) method was developed for the simultaneous analysis of 3- and 4-*O*-HOTYR-glucuronides, and 4-*O*-glucuronides of TYR and HValc (homovanillyl alcohol) in human urine. Concentrations of metabolites and their core compounds were estimated in an intervention study with 11 healthy volunteers supplemented with 50 mL virgin olive oil. After 24 hours about 10% of the dose was recovered as free phenols (less than 5% of the total recovery) and as glucuronides. Glucuronides and core compounds were tested for their chemical (hydrogen donation by DPPH test, range 0.06-1mM) and *in vitro* biological (inhibition of Cu-mediated LDL oxidation, range 0.01-10 μM) antioxidant activities. None of the glucuronides displayed significant antioxidant activities.

1. Introduction

A number of epidemiological studies have provided evidence of the health benefits derived from the Mediterranean diet against cancer and cardiovascular diseases [1, 2]. The main characteristics of such a diet are its richness in natural vitamins and antioxidants, from vegetables and fruits, and a high content of monounsaturated fatty acids, olive oil being the main source of fat (Trichopolou et al., 2003).

The biological benefits of olive oil consumption are not only limited to its high content of monounsaturated fat, olive oil minor components also display bioactive properties (Covas et al., 2006). Phenolic compounds, the most studied olive oil minor components, belong to the hydrosoluble fraction of olive oil. Some of the most representative phenolic compounds in olive oil are hydroxytyrosol (HOTYR) and tyrosol (TYR) and their respective secoiridoid derivatives, oleuropein and ligstroside (Servili and Montedoro, 2002). They have been shown to exhibit strong antioxidant properties that contribute to the protection of olive oil against lipid rancidity (Servili and Montedoro, 2002). Recent intervention clinical trials have provided evidence that the phenolic content of an olive oil contributes to the protection in humans against lipid oxidative damage in a dose dependent manner (Covas et al., 2002).

One of the first steps in linking the biological activities of phenol compounds of dietary origin to health benefits in humans is to demonstrate their bioavailability from diet. Several intervention studies in human and animal models have reported that phenolic compounds are rapidly absorbed in a dose dependent manner with the phenolic content of the olive oil administered (Weinbrenner et al.,2004; Visioli et al.,2000). Olive oil phenolics are extensively metabolized in the gut and liver

and, thus, in biological fluids they are found mainly as phase II metabolites (e.g. glucuronides and sulfates) of HOTYR, TYR and *O*-methylconjugate of HOTYR (homovanillyl alcohol, HVAlc) (Miro-Casas et al., 2003). In rats administered with HOTYR, either orally or intravenously, both glucuronide and sulfate conjugates of HOTYR and its *O*-methylated metabolite HVAlc were detected in different biological matrices and tissues (D'Angelo et al., 2001; Tuck et al., 2002). Despite inter-species differences (Visioli et al., 2003), the first pass intestinal and hepatic metabolism plays an important role in the bioavailability and disposition of olive oil phenolics, to such an extent that their free forms are present only at very low concentrations in biological fluids (Miro-Casas et al., 2003). In this context, the study of the bioactivity of the biological metabolites of olive oil phenolic compounds is a crucial step in order to understand the beneficial health effects observed after the administration of olive oil with different amounts of phenols (Covas et al., 2006; Weinbrenner et al., 2004).

The transformation of HOTYR and TYR into their phase II metabolites was predicted to negatively influence their activities as antioxidants (Nenadis et al., 2005). Phase II metabolites are generally considered to be pharmacologically inactive and targets for excretion. However, some phase II metabolites (e.g. glucuronides) of food derived antioxidants, such as catechins, have been reported to be biologically active (Lu et al., 2003). Moreover, in a study in rats (Tuck et al., 2002) it was reported that the urinary excreted 3´-*O*-HOTYR-glucuronide, but not its 3´-*O*-sulfate conjugate, was a more potent antioxidant (by DPPH test) than its parent compound HOTYR.

In the majority of the studies, the concentrations of phenolic compounds used in *in vitro* and *in vivo* experiments to obtain biological effects have a greater

disparity than those which are present in the human body after real-life doses of phenolic-rich foods. It is, therefore, mandatory to have a proper estimation of the expected concentrations of phenols, and their main metabolites, in humans after phenolic-rich food consumption. These data are required in order to establish the range of concentrations to be tested *in vitro* and *in vivo* in the evaluation of their biological activities.

The aim of the present study was to assess the antioxidant capacity of the hydroxytyrosol glucuronides, within a range of biologically relevant concentrations. The range of concentrations tested was derived from those detected in human urine after a dietary dose of virgin olive oil.

2. Methods and Materials

2.1. Reagents and chemicals

Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) (HOTYR) and tyrosol (4-hydroxyphenylethanol) (TYR) were purchased from Extrasynthese (Extrasynthèse, Lyon, France). Homovanillyl alcohol (HVAlc), 3-(4-hydroxyphenyl)propanol (HOPhPr) (used as internal standard, I.S.2), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) (95%), and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) were supplied by Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO). Synthetic urine UriSub® (CST Technologies Inc., Great Neck, NY) was used for UPLC-MRM assay validation. Methanol (MeOH) and acetonitrile (ACN) were of analytical grade from Scharlau (Scharlau Chemie, Barcelona, Spain). Mobile phase was filtered with 0.22 µm nylon filter (Whatman, England). Sodium bisulfite, acetic acid, ammonium hydroxide, hypochloric and phosphoric acid were supplied

by Merck (LiChrosolv®, Barcelona, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France).

Glucuronides: 4´-*O*-hydroxytyrosol (4´-*O*-Gluc-HOTYR) and 3´-*O*-hydroxytyrysol (3´-*O*-Gluc-HOTYR) glucuronides, 4´-*O*-tyrosol glucuronide (4´-*O*-Gluc-TYR), 4´-*O*-homovanillyl alcohol (4´-*O*-Gluc-HVAlc), and 4´-*O*-hydroxyphenylpropanol (4´-*O*-Gluc-HOPhPr) (used as internal standard, I.S.1) (Fig 1) glucuronides were synthesized according to the method described by Khymenets *et al.* (Khymenets et al., 2006).

2.2. Study design, diet, and sample collection

2.2.1. Human subjects

Six healthy male (aged 22 to 28) and five female (aged 20 to 44) volunteers were recruited. The institutional ethics' committee (CEIC-IMAS) approved the protocol and the participants signed an informed consent. The study was performed in accordance with ethical standards formulated in the Helsinki Declaration of 1975 (revised 1999). All volunteers were healthy on the basis of a physical and medical examination and standard biochemical and hematological tests.

2.2.2. Characteristic of virgin olive oil (VOO) used for intervention

The VOO used in this investigation was of Spanish origin and had been utilized in former studies (Cicero et al., 2008). The amount of total HOTYR, HVAlc, and TYR in VOO was determined as previously described (Miro-Casas et al., 2003b).

2.2.3. General procedure

Prior to the dietary intervention, volunteers followed a one week washout period in which sunflower oil was provided as a source of fat for all purposes. During the first four days of the washout period, participants were asked to follow an antioxidant-controlled diet consisting of no more than 2 pieces of fruit, 2 servings of vegetables or legumes, 2 cups of tea or coffee per day, and the total avoidance of wine, beer, and olive oil. During the last 3 days of the washout period, and on the intervention day, the volunteers followed a strict low-phenolic compound diet. Phenolic-rich foods (vegetables, legumes, fruit, juice, wine, coffee, tea, caffeine-containing soft drinks, beer, cocoa, marmalade, olive oil, and olives) were totally excluded from the participants´ diet. On the intervention day, at fasting state, 50 mL (44 g) of VOO were administered in a single dose with bread (200 g).

Spot urine was collected at 8 a.m. at fasting state prior to VOO administration and from 0-6 h and 6-24 h after VOO consumption on the intervention day. Urine samples were preserved with sodium bisulfite (1 mM final concentration) at acidic conditions (0.24 M HCl final concentration) and stored at -20ºC prior to use.

Blood samples were collected in EDTA-containing tubes immediately before (0h) and 1h, 6h, and 24h after VOO intervention. Plasma samples were obtained by centrifugation of whole blood directly after being drawn and were preserved at -20ºC prior to use.

2.3. Analysis of free and glucuroconjugated HOTYR, TYR, and HVAlc in urine samples by SPE-UPLC-MRM

2.3.1. Sample preparation

Prior to analysis a mix of internal standards was added to each tube (final concentration of 500 ng/mL I.S.-1 and 1000 ng/mL I.S.-2.) and dried under nitrogen (25ºC, 10-15 psi, 1 min). After thawing at room temperature, aliquots of 1 mL of urine were distributed to tubes. Samples were diluted 1:1 with 4% H_3PO_4 and applied to pre-conditioned with 2mL of methanol and equilibrated with 2 mL of water Oasis® HLB 3cc (60 mg) cartridges (Waters Corporation, Ireland). Samples were washed with 2 mL of water, extracted with 3 mL of methanol which was evaporated under nitrogen (25ºC, 10-15 psi), and the extracts were reconstituted in 200 µL either of 0.5% acetic acid (for synthetic urine) or of solvent [A] of mobile phase (real urine samples). This generic method of extraction demonstrated optimum recovery values for the simultaneous extraction of glucuronides and their parent compounds in both synthetic and real urine samples (data not shown). Finally, samples were filtered using Spin-X® Centrifuge 2-mL polypropylene tubes with 0.22 µm nylon filter (Corning®, Corning Incorporated, NY, USA) and analyzed by UPLC-MS as described below.

2.3.2. LC-MS conditions

Analysis was performed using a Waters Acquity Ultra Performance LC system (Waters, Milford, MA, USA) coupled to a triple quadrupole (Quattro Premier XE) mass spectrometer provided with an orthogonal Z-spray–electrospray interface (ESI) (Waters Associates, Milford, MA, USA). Gradient chromatographic separation of HOTYR, TYR, HVAlc and their glucuronides was performed on an Acquity UPLC™ BEH C_{18} column (100 mm × 2.1 mm, i.d., 1.7 µm particle size) (Waters Corporation®, Ireland). For gradient elution, mobile phase [A] 1 mM ammonium acetate at pH 5, and phase [B] 100% ACN, were applied. The following elution conditions were used: 0 – 2 min linear gradient at 3% [B]; 2 – 2.2

min gradient to 10 % [B]; 2.2 – 3.0 min linear; 3.0 – 3.2 min gradient to 20 % [B]; 3.2 – 4.8 min linear; 4.8 – 5.2 gradient to 3 % [B]; 5.2 – 6.5 linear at 3 % [B] for column equilibration. UPLC operating conditions were as follows: column temperature, 40ºC; flow rate, 0.4 mL/min; injection volume, 10 µL.

Ionization was performed in the negative mode. The following inlet conditions were applied: drying gas, nitrogen (1000 L/h, 400ºC); capillary voltage, 3.0 kV; cone voltage, 25.0 V. All compounds were monitored in the multiple reaction monitoring (MRM) mode. Based on the characteristic spectrum of collision energy induced fragmentations, identified using reference standards, the specific MS transmission pairs (under optimum collision energy, eV) were chosen for their discriminatory identification (see Supplemental Table 1). All transitions were monitored within 0.04 sec dwell time with 0.02 sec of delay in three consecutive MS segments: 1) 0 – 2.4 min for all glucuronides and I.S.-1; 2) 2.4 – 3.2 for HOTYR only; and 3) 3.2 – 6.5 for TYR, HVAlc and I.S.-2.

2.3.3. Quantification procedure

Analytes were quantified by comparison of their peak area ratios (analyte versus the corresponding I.S.) with calibration curves in which the peak area ratios of spiked calibration standards had been plotted versus their concentrations using a weighted ($1/\chi^2$) calibration model. Integrated peak area ratios of HOTYR, TYR, and HVAlc were compared with I.S.-2 (HOPhPr) and their glucuronides with I.S.-1 (*4´-O*-Gluc-HOPhPr), respectively.

2.3.4. SPE-UPLC-MRM assay validation

Within the framework of the development and validation of the analytical method, we should take into consideration that all the tested compounds were either endogenous (HOTYR and HVAlc are dopamine metabolites) or

contaminants from unknown components of diet (HOTYR- and TYR-related compounds). As a result, there were no biological fluids (urine and plasma) completely free from these substances. For this reason synthetic urine (UriSub) was used as a surrogate matrix for method validation. Prior to the application of the method to real urine samples, an intra- and inter-assay validation protocol was carried out in synthetic urine.

Standard stock solutions of the analytes (HOTYR, 4´-*O*-Gluc-HOTYR, 3´-*O*-Gluc-HOTYR, TYR, 4´-*O*-Gluc-TYR, HVAlc, and 4´-*O*-Gluc-HVAlc) and I.S.s (HOPhPr and 4´-*O*-Gluc-HOPhPr) were prepared in MeOH at a concentration of 1 mg/mL each. Mixtures of the analytes (glucuronides and parent compounds) at concentrations of 1, 10, and 100 µg/mL and of the I.S.s containing 100 µg/mL of I.S.-2 (HOPhPr) and 50 µg/mL of I.S.-1 (HOPhPr-Gluc4) were prepared by dilution/combination of the aforementioned stock solutions in MeOH for the UPLC-MS/MS analysis. All methanol solutions of compounds were kept in dark-colored, well-sealed vials at -20ºC and were seen to be stable for more than a 1 year period.

Calibration curves were prepared in synthetic urine at concentrations of 20, 100, 500, 1000, and 2000 ng/mL for all analytes. Internal standards I.S.-1 and I.S.-2 were prepared at concentrations of 500 and 1000 ng/mL. Control samples containing appropriate amounts of analytes at three different concentrations: low (40 ng/mL), medium (250 ng/mL), and high (1500 ng/mL) were used. Four replicate analyses were performed with synthetic urine samples corresponding to the first level of concentrations (20 ng/mL) of the calibration curve. Additionally, 3 and 10 standard deviations (SD) of the calculated concentrations at this calibration level were used in order to estimate the limits of detection (LODs) and

quantification (LOQs), respectively. Precision was calculated as the relative standard deviation (RSD) of the quality control sample concentrations. Accuracy was expressed as the relative error of the calculated concentrations.

Recovery was determined by comparing the absolute peak areas of standards and I.S spiked into synthetic urine and extracted by SPE with those spiked post extractions at three different concentrations: 20, 500, and 2000 ng/mL (the lowest, the medium, and the highest curve points). The extraction yield compared between synthetic and real urines was shown to be similar for all tested compounds and I.S.s (data not shown). A standard curve prepared in 5 different basal urines (collected in the study) was used in the quantitative analysis of analytes in the urine samples of the study.

2.4. GC-MS plasma and urine sample analysis

The urine and plasma samples collected in this study were analyzed in parallel for total and free HOTYR, TYR, and HVAlc as previously described (Miro-Casas et al., 2003; Miro-Casas et al., 2003b).

2.5. Evaluation of antioxidant activities

2.5.1. LDL resistance to oxidation assay

LDL isolation was performed by sequential flotation ultracentrifugation (Havel et al., 1955). Native LDL was dialyzed by molecular size exclusion chromatography in a G25 Sephadex column (Pharmacia, Uppsala, Sweden), with 2.7 mL phosphate buffered saline (PBS) 10 mM pH 7.4, under gravity feed at 4^{o} C. Apolipoprotein-B was determined by immunoturbidimetry (ABX Diagnostics, Montpellier, France). Conjugated diene formation after copper-mediated LDL

oxidation was assessed as previously described (Fito et al., 2000) with some modification for testing in small samples. Briefly, dialyzed LDL (final concentration 0.05 g of Apo-B/L) in PBS at a final volume of 160 µl was incubated with 10 uL of methanol in the presence or absence (control) of the analytes. In continuation, 10 µL of a 100 µM cupric sulfate (final concentration 0.625 µmol) solution was added to a 96-well half-area flat bottom UV-transparent microplate (Corning®). To minimize evaporation during prolonged incubation time, 10 µl of mineral oil (Sigma-Aldrich) was layered over the reaction mixture and the plate was covered with an adhesive optical transparent film. Absorbance at 234 nm was continuously monitored at 15 min intervals for 24 h at 36ºC in an Infinite M200 lector (TECAN IBERICA, Männedorf, Switzerland). Controls and samples with the concentration of all tested compounds at 0.01, 0.1, 1, 5, and 10 µM were evaluated in the same run in duplicate and each experiment was repeated three times. For data presentation, the x-axis value corresponding to the intercept of the propagation phase tangent with the extrapolated line for the slow propagation reaction was calculated (Lag time). The diene versus time profile can be divided into three consecutive phases: slow phase, propagation phase, and decomposition phase. The length of the lag phase was determined as the intercept of the propagation phase tangent with the extrapolated line for the slow reaction. In addition, oxidation rate (OR) and maximum diene formation were calculated from the slope of the propagation phase tangent and by the maximum increase of the absorbance at 234 nm, respectively, using the molar absorbance ε234nm for conjugated dienes (29,500 $L \cdot mol^{-1} \cdot cm^{-1}$). All variables (Lag-time, OR and Maximum diene formation) were calculated as ratios between values observed for each compounds vs. those corresponding to the control LDL oxidation reaction.

The interseries CV(%) were 9.4, 6.7, and 10.4 for the lag time, maximal rate, and maximal amount of dienes, respectively.

2.5.2. DPPH assay

The antiradical capacity of all the tested compounds was evaluated by the DPPH stable radical method as described earlier (Touriño et al., 2005) with some modifications for testing in small reaction volumes. Briefly, the compounds (MeOH solutions, 5 µL) were added to aliquots (250 µL) of a 60 µM DPPH solution made up in degassed MeOH, in a 96-well transparent flat-bottom microplate (Greiner Bio-One GmbH, Frickenhausen, Germany), covered by an adhesive ViewSeal transparent film (Greiner Bio One). The mixture was analyzed for absorbance at 517 nm spectrophotometrically (Infinite 200™ Multi-mode Microplate Reader, TECAN IBERICA, Männedorf, Switzerland) every 2 min starting at minute 3 (plate preparation time) up to 1h and subsequentially every 30 min over the following 23 h at 25ºC in the dark. Each calibration curve and the five different concentrations of the tested compounds (0.062, 0.125, 0.25, 0.5, and 1 mM) had been previously freshly prepared and acquired in duplicate. The experiments were repeated three times. The percentage of remaining DPPH was calculated according to the calibration curves made separately for each experiment and read within the same plate and same time points of incubation together with the analyzed samples. The method was validated by confirming that within plate and between plate CV% of slopes for calibration curves, as well as intraday and interday performance, did not exceed 10% for the 24-h period of kinetic measurements (data not shown). The experiments were performed with an excess of DPPH radical in order to exhaust the H-donating capacity of the polyphenols. The compounds were characterized by their capacity to reduce DPPH as monitored by the decrease of its absorbance

at 517 nm. The radical scavenging activities of the tested compounds were compared on the basis of their ED_{50} (the concentration able to consume half the initial amount of free radical, expressed as the molar ratio compound to radical). The results were expressed as antiradical capacity (ARC), which is the inverse of ED_{50}. The total stoichiometry of the reaction was evaluated from the spectrophotometric data with different antioxidant concentrations at three points: 30min, 4h, and 24h. It was expressed as amount of hydrogen atoms transferred from the analyte to the DPPH radical according to the following formula: $n_H = 1/(ED_{50} \times 2)$.

2.6. Data evaluation, quantification, and statistical analysis

Normality of continuous variables was assessed by Kolmogorov–Levene test. For data comparison a paired Student t-test was employed. A least-squares ($1/\chi^2$) regression analysis was used to obtain correlation coefficients and slopes. Linear regression test was applied for correlation estimations. The data from LDL resistance to oxidation test were treated using one-way ANOVA where concentration and compounds were defined as factors. To check for a type of trend in response across the concentration, the polynomial contrast was applied.

Statistical analyses were performed with SPSS for Windows (version 12.0) and significance was defined as $P < 0.05$. Data expressed are either Mean (SD), Mean (CV%), or Mean ($CI_{95\%}$) as specified.

3. Results

3.1. HOTYR and TYR content in VOO and volunteer dietary compliance.

The concentrations of HOTYR and TYR, estimated as a fraction combining free forms of HOTYR and TYR and those resulting from the acidic hydrolysis of their secoiridoid derivatives present in olive oil, were 67.6 µg/mL and 42.0 µg/mL, respectively. Therefore, the total amounts of HOTYR and TYR consumed with 50 mL of VOO were 22.0 ± 1.4 µmoles (3.38 ± 0.22 mg) and 15.2 ± 0.9 µmoles (2.10 ± 0.12 mg), respectively (Table 1).

The compliance with the dietary recommendations during the wash-out period was controlled by analyzing the plasma and urine concentrations of TYR, HOTYR, and HVAlc (Miro-Casas et al., 2003; Miro-Casas et al., 2003b) prior to the nutritional intervention. The same analytical methodology was applied over 24 h after VOO intervention (Fig 2 (A) and (B)). The volunteers' urinary and plasma concentrations of HOTYR, TYR, and HVAlc, which were monitored during the study, are shown in Fig 2 (B).

3.2. UPLC-MRM method development and validation

To understand the impact of glucuronidation (a presumed bioactivation pathway of one of the predominant metabolic pathways of olive oil phenols) a direct SPE-UPLC-MRM method for the simultaneous determination of TYR, HOTYR, and HVAlc and their corresponding *O*-glucuronides (Khymenets et al., 2006) (Fig 1) was developed.

HOPhPr was used as an internal standard (I.S.-2) for parent compound (HOTYR, TYR and HVAlc) quantification since they share structural similarities. It was also used in GC-MS sample analysis as previously reported (Miro-Casas et al., 2003; Miro-Casas et al., 2003b)]. With regard to glucuronides, the 4´-*O*-

glucuronide of HOPhPr was predicted to be the most appropriate I.S. for such a type of metabolite and was, therefore, synthesized according to the earlier established methodology (Khymenets et al., 2006). Both I.S.s exhibited analogous extraction recoveries (94.8 ± 7.1 % (7.5%) for I.S.-2, 93.6 ± 7.2 (7.7%) for I.S.-1) in a similar manner to analytes, chromatographic behavior, and fragmentation pattern (see Supplemental Table 1). The optimal resolution of the tested compounds, in particular the glucuronides of HOTYR, was obtained in synthetic urine samples under reported chromatographic conditions within 4.5 min (total analysis run time of 6.5 min). Analysis of basal and post-prandrial urines revealed that there were no co-eluting compounds with the proposed I.S.s and that they were well separated from other peaks with identical MS fragmentation (see Fig 3). Basal urine samples, despite being collected after a wash out period, still contained visible traces of almost all the tested compounds (data not shown).

Good linearity for the developed assay (r^2 > 0.99) was found within the investigated calibration range of 20 – 2000 ng/mL in synthetic urine, for all compounds. Recoveries within 89 % - 94 % and 92 % - 97 % were observed for glucuronides and parent compounds tested, respectively (Supplemental Table 2). LODs and LOQs for all compounds were not higher than the lower calibrator (20 ng/mL), except for HOTYR. Intra- and inter-day precision and accuracy results reported for all compounds were according to the requirements for method validation criteria (RSD % and ERR% were ≤ 20 % for CI and ≤ 15 % for CM and CS for both intra- and inter-day experiments), except those of HOTYR particularly at the lowest concentration tested (Supplemental Table 2).

3.3. Identification of parents and their glucuronide metabolites in urine samples from the intervention study. Agreement between GC-MS and UPLC-MRM methods.

Because of a prominent matrix effect from the biological samples, a final quantification of compounds in urines from the intervention study was performed with average curves built in real urines. The achieved chromatographic separation in synthetic urine for the analytes (especially for the glucuronidated isoforms of HOTYR) was reproduced in real urine. The elution time of the analytes was slightly delayed in urine matrix compared to synthetic urine (data not shown) due to the extra load of co-extracted compounds.

Fig 3 represents the MRM extracted chromatograms of urine samples collected from a healthy subject: baseline, from 0 to 6 h, and from 6 to 24 h after a single dose of VOO (50 mL). Urinary concentrations for all tested compounds were calculated before and after olive oil consumption. Some analyte traces were identified in the basal urines of all the study participants. The intact TYR and HOTYR, as well as the methylated metabolite of the later, HVAlc, were detected at significant concentrations (328.1 ± 157.9 ng/mL, 282.0 ± 172.7 ng/mL and 269.6 ± 160.9 ng/mL, respectively) in all urines collected 6h after VOO acute intake. Concentrations of *O*-glucuronide metabolites were substantially higher in 6h postprandial urines when compared to their parent compounds (2087.8 ± 914.6 ng/mL, 876.7 ± 390.2 ng/mL, 1255.3 ± 554.1 ng/mL and 797.7 ± 451.0 ng/mL for 4´-*O*-GlucTYR, 4´-*O*-GlucHOTYR, 3´-*O*-GlucHOTYR, and 4´-*O*-GlucHVAlc, respectively).

The recently developed UPLC-MRM method was compared with the earlier GC-MS method routinely used in our laboratory for the identification of free forms of HOTYR, TYR, and HVAlc within the working range of the LC method (20 - 2000 ng/mL). Despite the fact that HOTYR was unstable in synthetic urine and only slightly stable in real urine samples (data not shown), its quantification correlated well between methods ($r = 0.902$, $p < 0.000$). Both free HVAlc and TYR quantifications correlated strongly between methods ($r = 0.975$ and $r = 0.954$ with $p < 0.001$ for both analytes, respectively). Therefore, in spite of some concern regarding the stability of HOTYR at low concentrations, the quantification of free forms of olive oil phenols by the new LC/MS/MS method could be suitably used for the estimation of their urinary excretion rates.

3.4. Postprandial 24-h urinary excretion studies

The excretion rates of phenols and their glucuronides were evaluated in 24-h postprandial urines of healthy volunteers (n = 11) administered with a single dose of 50 mL virgin olive oil.

3.4.1. Excretion of free and glucuronconjugated HOTYR, TYR, and HVAlc

Table 1 shows the excretion rates of the parent phenols (TYR, HOTYR, and HVAlc) and their glucuronides in the collected fractions of urines: at 0 - 6 h and at 6 - 24 h. A maximum excretion for both free and glucuronides forms of phenols was detected in urine within the first 6 hours after VOO ingestion.

After VOO intake, 3.3 % of TYR and 1.8 % of HOTYR were excreted in urine in its free form (see Fig 4). About 4.5% of ingested HOTYR was excreted as the *O*-methylated metabolite HVAlc, two thirds of which (approximately 70% of the total excreted) was 4´-*O*-Glucuronidated. It is noteworthy that the recovery of

phenols as glucuronides accounts for about 10% of the dose consumed (10.5% for HOTYR and HVAlc glucuronides together, and 9.6% for TYR glucuronide). Therefore, for both HOTYR and TYR around 13% of the dose consumed was recovered (free forms + glucuronides) 24 h after VOO ingestion.

After VOO ingestion HOTYR, TYR, and HVAlc and their glucuronides were rapidly eliminated in urine. In the first 6 h post intake, 10.9 % of the consumed HOTYR (7.5 % as HOTYR free and glucuronides and 3.4% for HVAlc and glucuronide) and 8.4 % of the TYR consumed were excreted. These recoveries correspond to 83 % (HOTYR), 75 % (HValc), and 91 % (TYR) of the total phenols excreted within 24 h. In the time period from 6 to 24 h, the rate of phenols excreted was low, with only 1 - 2 % of the compounds consumed being eliminated in urine. A figure that corresponds to 10 - 25 % of the total excreted in urine within 24 h.

3.4. Assessment of the antioxidant activities of phenolic compounds and their glucuronides

The antioxidant activities of the major phase II metabolites, *O*-glucuronides of olive oil polyphenols TYR, HOTYR, and HVAlc were compared with those of their parent compounds using LDL resistance to oxidation and standard DPPH radical scavenging tests. Both tests were adjusted for small reaction volumes and validated for continuous use during a 24-h period in order to monitor the kinetics of the reactions simultaneously for study compounds. Trolox was evaluated in both tests as a reference antioxidant.

3.4.1. LDL oxidation test

The LDL oxidation test was performed at different compound concentrations, covering all biologically relevant ranges: 0.01 – 0.1 (mainly referring to plasmatic

levels reported earlier) and 1 – 5 – 10 μM (urinary levels estimated within this study) ranges. As in the case of DPPH radical scavenging, the main protagonists concerning the protection of LDL against Cu-induced oxidation were HOTYR, trolox, and HVAlc (Fig 5). No data could be generated for HVAlc at concentrations of 5 and 10 μM due to a regular deterioration in the monitored diene conjugate formation, the cause of which was unknown to us. As a result, HVAlc was only evaluated at three concentrations: 0.01, 0.1, and 1 uM. Both HOTYR and its methylated metabolite HVAlc expressed much stronger protective effects than trolox against LD oxidation by prolonging almost twice the lag time of diene formation at the concentration of 1 μM (Fig 5).In contrast to HOTYR and HVAlc, TYR appeared to demonstrate only some minute inhibition effect on LDL oxidation at the highest concentration tested (10 μM) (Fig 5). Neither HVAlc nor TYR glucuronides exhibited antioxidant activity in this test.

According to our data, however, glucuronides of HOTYR appeared to maintain some residual activity in protecting LDL from Cu-mediated oxidation (Fig 5), in particular at the highest concentration tested (5 and 10 uM). The 4´-*O*-glucuronide of HOTYR appeared to retain more activity against LDL oxidation than the 3´-*O*-glucuronide. In addition to delaying (Lag time) the formation of conjugated dienes, HOTYR, TYR, and HVAlc and both glucuronides of HOTYR appeared to alter the maximal rate of reaction in a dose dependent manner. The maximum rate of reaction was decreased with increasing compound concentrations (linear trend at $p < 0.001$), although with different amplitude (data not shown). In contrast, and in agreement with earlier reported data (Albertini et al., 1999), the rate of reaction was not affected by the increase of trolox concentrations in the reaction. No dose-dependent changes were observed in the

maximum amount of dienes formed during Cu-mediated LDL oxidation for all the compounds at the concentrations tested.

3.4.2. DPPH test

The kinetics of DPPH radical scavenging (% of remaining DPPH) of parent compounds and glucuronides are shown in Fig 6 (A) and (B). In comparison with the other tested compounds HOTYR and trolox reacted rapidly with DPPH. After the first minutes of reaction, HOTYR appeared to be a stronger antiradical scavenger than trolox at the same concentrations (Fig 6 (A)). The trolox reaction reached its plateau within the first 5 minutes of incubation whilst HOTYR induced a first decay in absorbance within the first 3 minutes, followed by a slower slope (up to 60 min) and only afterwards did the reaction reach its plateau. This biphasic kinetic behavior is well known for catechol antioxidants (Goupyi et al., 2003), including HOTYR (Gordon et al., 2001), and can be explained by the degradation products of HOTYR which are involved in the reaction until equilibrium is reached. In comparison to HOTYR, the reaction of HVAlc with DPPH was slower and the equilibrium could be reached only at a time closer to the end of the 24-h incubation period. Nevertheless, at 2 h a more reduced DPPH could be detected in the reaction with HVAlc than with trolox, and at 24 h it was close to the amount reduced by HOTYR (Fig 6). Some long term residual activities in DPPH radical scavenging were observed for both HOTYR glucuronides, but not for HVAlc glucuronide. TYR and its glucuronide, 4´-*O*-GlucTYR, exhibited no radical scavenging activities against DPPH over the whole 24-h period of reaction. Due to the variation in the kinetic behavior of the tested compounds, their total stoichiometry of reaction was estimated at three points of readings: 30 min, 4 h, and 8 h (data shown in Table 2).

4. Discussion

The aim of this study was to study olive oil simple phenols metabolic disposition and to assess the antioxidant potential of their glucuronides. To the best of our knowledge this is the first time that both, an analytical method for the direct HOTYR glucuronides determination and their antioxidant capacity at concentrations relevant to real-life doses of olive oil, are reported.

Previous data in animal models suggested that the radical scavenging capacity of HOTYR glucuronides was even superior than the one displayed by HOTYR (Tuck et al., 2002). Because the lack of reference standards and analytical methodology to quantify HOTYR glucuronides (and related compounds), the first step was to develop an analytical method for their direct detection and quantification in human urine, using well characterized standards (Khymenets et al., 2006). This allowed us to estimate the role of glucuronidation in the disposition of phenols (HOTYR and TYR) in humans following a dietary consumption of olive oil. The *in vitro* LDL resistance to oxidation test was applied to all glucuronides in order to estimate to what extent metabolism could modify the antioxidant capacity of the main olive oil phenols in this model of lipid oxidation. In addition, the radical scavenging activities of the parent compounds and their glucuronides were compared in the standard DPPH test.

The proposed UPLC-MRM method involves the direct measurement of TYR, HOTYR, its methylated metabolite HVAlc and their glucuronides. This method is a step forward in our understanding of the metabolic fate of phenols as compared with previous reports where indirect approaches were used (Visioli et al., 2000;

Miro-Casas et al., 2003; Visioli et al., 2003; Miro-Casas et al., 2003b; Caruso et al., 2001).

Studies performed with radiolabeled HOTYR administered intravenously (D´Angelo et al., 2001) and orally to rats (Tuck et al., 2002) have shown that HVAlc and HVA are methylated metabolites of exogenous HOTYR. An observation supported by a number of studies in cell cultures (Manna et al., 2000; Mateos et al., 2005), animal models (Tuck et al., 2002), and in humans (Miro-Casas et al., 2003; Visioli et al., 2003). The olive oil phenol, HOTYR is also an endogenous compound, one of the oxidative by-products of dopamine oxidative metabolism, known as DOPET (de la Torre et al., 2006). DOPET and its metabolites (methylated, phase II conjugated, etc.) are present in virtually all biological matrices of human and animal origin. Concentrations of HOTYR and TYR can be lowered after strict dietary restrictions (e.g. one week wash-out period) such as those applied in the present study. In spite of this, practically all basal urines collected contained detectable concentrations of phenolic metabolites. In humans, HVA detected in biological fluids combines the exogenous HOTYR contribution and endogenous dopamine metabolism (Caruso et al., 2001). Therefore HVA was not included in our analyses because the difficulty in discriminating both sources, although the contribution of olive oil to HVA body concentrations should not be ignored. As a result of the crossed metabolism between dopamine and HOTYR, our analytical methodology had to be validated in synthetic urine.

In the present study the maximum rates of HOTYR and TYR excretion correspond to the first 6 hours after VOO administration, which is in agreement with earlier reported data (Miro-Casas et al., 2003). This indicates that the olive oil

phenols undergo rapid absorption and quick metabolic and renal clearance. Only a small fraction of the free form of phenols is recovered in urine.

Glucuronides are present in the small intestine of rats after HOTYR perfusion suggesting that glucuronidation first occurs in the enterocytes (Corona et al., 2006) before further conjugation in the liver. This may also be the case in humans as the *in vitro* glucuronidation of polyphenols by microsomes from the intestine is as extensive as the hepatic one (Antonio et al., 2003). In the present study, sulfate conjugates were not analyzed due to the absence of specific standards (they were detected but not quantified).

LDL oxidation is one of the key steps in the initiation and progression of atherosclerosis and its inhibition by olive oil polyphenols has been reported in several intervention studies (Marrugat et al., 2004; Covas et al., 2006)] and *in vitro* studies (Fito et al., 2000; Visioli et al., 1995). A dose-dependent decrease of oxidized LDL was associated with the phenol content of the administered olive oil, in a randomized crossover controlled trial in humans (Gimeno et al., 2007).

The impact of glucuronidation on the antioxidant activity of olive oil phenols was evaluated by determining their protective effect against Cu-induced oxidation of LDL. Oxidation was potently inhibited by HOTYR to a similar extent as the reference compound trolox, in agreement with previous reports (Stupans et al., 2002; Rietjens et al., 2007). HVAlc also inhibited lipid oxidation at low concentrations (0.01, 0.1 and 1 uM) in agreement with previous reports (Turner et al., 2005; Deiana et al., 2008). The glucuronidation of HVAlc entails the loss of activity against lipid oxidation, whereas some residual activities could be observed for both HOTYR glucuronides. In contrast to HOTYR, TYR has very weak activity in this model even at the highest concentration, in agreement with an earlier report

(Briante al., 2003) and not surprisingly, the glucuronide was totally inactive. Overall glucuronidation of phenols greatly lessen the protective activities of olive oil phenols against Cu-mediated LDL oxidation.

The radical scavenging activities of phenols and glucuronides using, the DPPH assay was also assessed. Among the parent olive oil phenols HOTYR possessed excellent radical scavenging activities, in agreement with previous reports (Briante al., 2003; Roche al., 2005; Carrasco-Pancorbo et al., 2005; Erkoç et al., 2003) while TYR was found to be totally inactive. HVAlc displays some scavenging activity, but lower than for HOTYR with respect to kinetics and stoichiometry of reaction.

Overall results demonstrate that HOTYR and HVAlc glucuronides do not display the antiradical activities present in their parent compounds. Nonetheless, glucuronides of HOTYR, unlike that of HVAlc, appear to have some scavenging capacity over extended time periods (up to 24h). The fact that the phenolic group is free in the glucuronide while it is methylated in HVAlc may explain such observation. Previous observations suggesting a radical scavenging activity of glucuronides higher than for HOTYR itself do not agree with present results (Tuck et al., 2002). The fact that all our studies were performed using well characterized, pure compounds (Khymenets et al., 2006), instead of being extracted from urine is a strength of our work. In addition, our results confirm previously theoretically studies where bond dissociation enthalpy of phenolic hydroxyl groups was used to predict the H-atom-donating of HOTYR and its phase II metabolites glucuronides and sulfates (Nenadis et al., 2005).

According to our data, the isomeric conjugation with glucuronic acid displays dissimilar effects on the residual hydrogen-donating activities of HOTYR

glucuronides regarding the inhibition of Cu-mediated LDL oxidation. It appears that the 3´-*O*-GlucHOTYR retains more residual proton donating activities than the 4´-*O*-GlucHOTYR. Theoretical calculations on HOTYR radical activities (Erkoç et al., 2003) estimated that from all the carbon atoms the upper (4´-located) oxygen on the ring of HOTYR has the largest positive excess charge and is, therefore, the first to be donated, primarily during the radical scavenging reaction. This reactivity moderately persists in the methylated metabolite HVAlc, supported by the presence of a strong electron-donating methoxy group. The extremely slow, but significant DPPH antiradical activities of HOTYR glucuronidated metabolites (stoichiometric number less than 1) could be explained only by the remaining hydrogen donation activities on the left hydroxyl group. Therefore, the 3´-*O*-glucuronide (free 4´-hydroxyl group) appeared to be more active than the 4´-*O*-conjugate (free 3´-*O*-hydroxyl group). The 4´-glucuronide of HVAlc did not show residual activities since its hydrogen donating hydroxyl group on the benzyl ring was substituted by a glucuronic residue. In the case of TYR, the energy of the single benzyl-ring hydroxyl group is not sufficient for hydrogen donation (Nenadis et al., 2005). Free radicals have short half-lives *in vivo*, and that the fast period of reaction with the DPPH radical might be crucial in antiradical protection. Nevertheless, both fast and slow mechanisms of scavenging could be involved in the biological activities of polyphenols against free radicals, as was seen in the LDL oxidation test.

In summary, HOTYR and TYR, the main simple phenols derived from olive oil, are extensively metabolized in gut and liver. Because low concentrations of free phenols are recovered in biological fluids, the antioxidant activity of their glucuronides was examined as an alternative hypothesis that could explain the

beneficial health effects derived from its consumption from olive oil (Covas et al 2006). Two glucuronides of HOTYR and one for TYR and HVAlc, were detected in human urine after a single dose of 50 mL virgin olive oil. Whilst olive oil polyphenols display a different magnitude in protective activity against Cu-induced LDL oxidation and DPPH test (from the most potent HOTYR and HVAlc, to the low activity of TYR), their glucuronides do not. Overall, the lack of antioxidant activity of glucuronides, compared with that of the parent compounds suggest that they are not chief contributors for explaining the antioxidant effects provided by olive oils rich in phenolic compounds seen in humans. Further mechanisms should be examined to solve this enigma.

Acknowledgements: This study has been supported by the MICINN (AGL2009-13517-CO3-O1) and 2005SGR00156 from Generalitat de Catalunya-Comissió Interdepartamental de Recerca i Innovació Tecnològica, Barcelona, Spain. We would like to thank the volunteers who participated in this study and Esther Menoyo, RN, for her valuable assistance in dealing with them. The authors thank the Fundación Patrimonio Comunal Olivarero and Hojiblanca SA, for donating the olive oil. The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain.

References

1. Albertini R and Abuja PM (1999) Prooxidant and antioxidant properties of Trolox C, analogue of vitamin E, in oxidation of low-density lipoprotein. *Free Radic Res*. **30**:181-188.
2. Antonio L, Xu J, Little JM, Burchell B, Magdalou J, and Radominska-Pandya A (2003) Glucuronidation of catechols by human hepatic, gastric, and intestinal microsomal UDP-glucuronosyltransferases (UGT) and recombinant UGT1A6, UGT1A9, and UGT2B7. *Arch Biochem Biophys*. **411**:251-261
3. Bonanome A, Pagnan A, Caruso D, Toia A Xamin A, Fedeli E, Berra B, Zamburlini A, Ursini F, and Galli G (2000) Evidence of postprandial absorption of olive oil phenols in humans. *Nutr Metab Cardiovasc Dis*. **10**:111-120
4. Briante R, Febbraio F, and Nucci R (2003) Antioxidant properties of low molecular weight phenols present in the Mediterranean diet. *J Agric Food Chem*. **51**:6975-6981
5. Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Del Carlo M Gallina-Toschi T, Lercker G, Compagnone D, and Fernández-Gutiérrez A (2005) Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *J Agric Food Chem*. **53**:8918-8925
6. Caruso D, Visioli F, Patelli R, Galli C, and Galli G (2001) Urinary excretion of olive oil phenols and their metabolites in humans. *Metabolism*. **50**:1426-1428
7. Cicero AF, Nascetti S López-Sabater MC, Elosua R, Salonen JT, Nyyssönen K, Poulsen HE, Zunft H Kiesewetter H, de la Torre K, Covas MI, Kaikkonen J, Mursu J Koenbick C, Bäumler H, Gaddi AV, and 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. *J Am Coll Nutr.* **27**:314-320

8. Corona G, Tzounis X, Assunta Dessì M, Deiana M, Debnam ES, Visioli F, and Spencer JP (2006) The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. *Free Radic Res.* **40**:647-658
9. Covas MI, de la Torre K, Farré-Albaladejo M, Kaikkonen J, Fitó M, López-Sabater C Pujadas-Bastardes MA, Joglar J, Weinbrenner T Lamuela-Raventós RM, and de la Torre R (2006) Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. *Free Radic Biol Med.* **15**;40:608-616; 2006.
10. Covas MI, Nyyssönen, K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, Gaddi A, de la Torre R, Mursu J, Bäumler H Nascetti S, Salonen JT, Fitó M, Virtanen J Marrugat J, and EUROLIVE Study Group (2006) The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Ann Intern Med.* **145**:333-341; 2006.
11. Covas MI, Ruiz-Gutiérrez V de la Torre R, Kafatos A, Lamuela-Raventós RM, Osada J, Owen RW, and Visioli F (2006) Minor components of olive oil: evidence to date of health benefits in humans. *Nutrition Reviews* **64**:20-30
12. D'Angelo S, Manna C, Migliardi V, Mazzoni O, Morrica P, Capasso G, Pontoni G, Galletti, P, and Zappia V (2001) Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metab Dispos.* **29**:1492-1498; 2001.
13. de la Torre R, Covas MI, Pujadas MA, Fitó M and Farré M (2006) Is dopamine behind the health benefits of red wine? *Eur J Nutr.* **45**:307-310

14. Deiana M, Incani A, Rosa A, Corona G, Atzeri A. Loru D, Paola Melis M, and Assunta Dessì M (2008) Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H(2)O(2) induced lipid peroxidation in renal tubular epithelial cells. *Food Chem Toxicol*. **46**:2984-2990
15. Erkoç F, Keskin N, and Erkoç Ş (2003) Theoretical investigation of hydroxytyrosol and its radicals. *J of Molec Structure (Theochem)*. **625**:87-94
16. Fitó M, Covas MI, Lamuela-Raventós RM, Vila J, Torrents L, de la Torre C, and Marrugat J (2000) Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids*. **35**:633-638
17. Gimeno E, de la Torre-Carbot K, Lamuela-Raventós RM, Castellote AI, Fitó M, de la Torre R, Covas MI, and López-Sabater MC (2007) Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial. *Br J Nutr*. **98**:1243-1250 .
18. Goupy P, Dufour C, Loonis M, and Dangles O (2003) Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J Agric Food Chem*. **51**:615-622.
19. Havel RJ, Eder HA, and Bragdon JH (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Inves*. **34**:1345–1349
20. Khymenets O, Joglar J, Clapes P, Parella T, Covas MI, and 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

21. Lu H, Meng X, Li C, Sang S, Patten C, Sheng S, Hong J, Bai N, Winnik B, Ho CT and Yang CS (2003) Glucuronides of tea catechins: enzymology of biosynthesis and biological activities. *Drug Metab Dispos.* **31**:452-461

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

23. Marrugat J, Covas MI, Fito M, Schröder H, Miró-Casas E, Gimeno E, López-Sabater MC, de la Torre R, Farré M, and SOLOS Investigators (2004). Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation--a randomized controlled trial. *Eur J Nutr.* **43**:140-147

24. Mateos R, Goya L, and Bravo L (2005) Metabolism of the olive oil phenols hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate by human hepatoma HepG2 cells. *J Agric Food Chem.* **53**:9897-905

25. Miro-Casas E, Covas MI, Farre M, Fito M, Ortuño J, Weinbrenner T, Roset P, and de la Torre R (2003a) Hydroxytyrosol disposition in humans. *Clin Chem.* **49**:945-952

26. Miró-Casas E, Covas MI, Fitó M, Farré-Albadalejo M, Marrugat J, and de la Torre R (2003b) Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. *Eur J Clin Nutr.* **57**:186-190

27. Nenadis N, Wang LF, Tsimidou MZ, and Zhang HY (2005) Radical scavenging potential of phenolic compounds encountered in O. europaea products as indicated by calculation of bond dissociation enthalpy and ionization potential values. *J Agric Food Chem.* **53**:295-299

28. Rietjens SJ, Bast A, and Haenen GR (2007) New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. *J Agric Food Chem*. **55**:7609-7614
29. Roche M, Dufour C, Mora N, and Dangles O (2005) Antioxidant activity of olive phenols: mechanistic investigation and characterization of oxidation products by mass spectrometry. *Org Biomol Chem*. 3:423-430
30. Servili M and Montedoro GF (2002) Contribution of phenolic compounds to virgin olive oil quality. *Eur. J. Lipid Sci. Technol*. 104:602–613 .
31. Stupans I, Kirlich A, Tuck KL, and Hayball PJ (2002) Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants. *J Agric Food Chem*. 50:2464-2469
32. Touriño S, Selga A, Jiménez A, Juliá L, Lozano C, Lizárraga D, Cascante M, and Torres JL (2005) Procyanidin fractions from pine (Pinus pinaster) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J Agric Food Chem*. 53:4728-4735
33. Trichopoulou A, Costacou T, Bamia C, and Trichopoulos D (2003) Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med*. **348**:2599- 2608
34. Tuck KL, Hayball PJ, and Stupans I (2002) Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. *J Agric Food Chem*. **50**:2404-2409
35. Turner R, Etienne N, Alonso MG, de Pascual-Teresa S, Minihane AM, Weinberg PD, and Rimbach G (2005) Antioxidant and anti-atherogenic activities of olive oil phenolics. *Int J Vitam Nutr Res*. **75**:61-70

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

37. Visioli F, Galli C, Bornet F, Mattei A, Patelli R, Galli G, and Caruso D (2000) Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett.* **468**:159-160

38. Visioli F, Galli C, Grande S, Colonnelli K, Patelli C, Galli G, and Caruso D (2003) Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. *J Nutr.* **133**:2612-2615

39. Weinbrenner T, Fitó M, de la Torre R, Saez GT, Rijken P, Tormos C, Coolen S, Farré M, Abanades S, Schroder H, Marrugat J, and Covas MI (2004) Olive oils high in phenolic compounds modulate oxidative/ antioxidative status in men. *J Nutr.* **134**:2314-2321.

Figure Legends

Fig 1: Structures of core compounds and their glucuronoconjugated metabolites and internal standards, I.S.-1 and I.S.-2, used in the UPLC-SPE-MRM method.

Fig 2: Plasma concentrations vs. time curve (**A**) and urinary concentrations (**B**) of total TYR, HOTYR and HVAlc for 24 h after single dose (50 mL) ingestion of VOO measured by GC-MS method (see section Materials and Methods). Values are present as Mean (SD).

Fig 3 **:** Extracted ion chromatograms of the identified phenolic (HOTYR, TYR and HVAlc) compounds and their glucuronides by SPE-UPLC-MS/MS in following samples: **Basal** - urine after wash out period prior VOO administration; **0 – 6 h** – urine collected over first 6 h after 50 mL of VOO ingestion; **6 – 24 h** – urine collected after 6 till 24 h after intervention with VOO. In all cases 500 ng/mL and 1000 ng/mL of I.S.-1 (4´-O-GlucHOPhPr) and of I.S.-2 (HOPhPr), respectively, were added to samples.

Fig 4: 24-hour urine recoveries of TYR, HOTYR and HVAlc, as a main methylconjugated metabolite of HOTYR, in their free (white part of bar) and glucuroconjugated (3´-*O*-Glucuronides (present only in HOTYR) as a dark grey part of bar and 4´-*O*-Glucuronides as a light grey part of bar) forms in healthy volunteers after ingestion of VOO. Circles represent total amount (as 100%) of compounds (HOTYR and TYR) consumed with 50 mL of VOO, where as black

segments represent a % of excreted in respect to the amount of compound consumed. HVAlc* - the % of HVAlc generated from HOTYR.

Fig 5: The 24-h (A) and 1-h (B) kinetics of remaining $DPPH^{\bullet}$ (%) in reaction between 60 μM DPPH radical and tested compounds at concentration 0.025 mM/rxn (see Materials and Methods section for explanations).

Fig 6: Changes in Lag time ratio of *in vitro* Cu-induced LDL oxidation in the presence of different HOTYR, TYR, HVAlc and their glucuronides in comparison to trolox as a reference compound. Changes are expressed as a ratio between Lag time generated in the presence of compounds to the Lag time of the control LDL oxidation reaction (see Material and Methods). * - significance at $p < 0.0001$ level for linear trend in response.

Table 1.

Compound	Dose fed	Excreted *µmol*		
	µmol [a]	in 0 – 6 h [b]	in 6 – 24 h	in 0 - 24 h [c]
HOTYR total[d]	21.96 (1.41)	1.67 (1.20 – 2.90)	0.34 (0.17 – 0. 83)	2.01 (31.26%)
HOTYR free		0.36 (0.06 – 0. 74)	0.03 (0 - 0.12)	0.39 (48.83%)
4´-*O*-GlucHOTYR		0.53 (0.33 – 0.81)	0.15 (0.05 – 0.33)	0.67 (28.99%)
3´-*O*-GlucHOTYR		0.77 (0.55 – 1.34)	0.17 (0.07 – 0.37)	0.95 (29.45%)
TYR total	15.20 (0.86)	1.79 (1.1 – 3.04)	0.19 (0.01 – 0.36)	1.96 (31.12%)
TYR free		0.47 (0.3 - 0.85)	0.03 (0 – 0.1)	0.50 (33.41%)
4´-*O*-GlucTYR		1.32 (0.71 – 2.19)	0.16 (0.02 – 0.29)	1.49 (32.28%)
HVAlc total	0.27 (0.01)	0.75 (0.54 – 1.26)	0.24 (0 – 0.76)	0.99 (22.85%)
HVAlc free		0.31 (0.2 – 0.6)	0.004 (0 – 0.02)	0.31 (37.35%)
4´-*O*-GlucHVAlc		0.44 (0.24 – 0.67)	0.24 (0 – 0.76)	0.63 (17.97%)

[a] The amounts of HOTYR, TYR and HVAlc administrated with 50 mL of VOO as a single dose (for a dietary intervention see Materials and Methods) are presented as Mean (SD).
[b] Data are presented as Mean (Min - Max) measured in 11 volunteers participating study.
[c] Data are presented as Mean (CV%) measured in 11 volunteers participating study.
[d] Here "total" refers to the amount of core compound detected both in free and conjugated with glucuronic acid.

Table 2.

Compound	DPPH radical scavenging								
	30 min			4 h			24 h		
	ED_{50}[e], *min*	*ARC*[f]	n_H	ED_{50}, *min*	*ARC*	n_H[g]	ED_{50}, *min*	*ARC*	n_H
HOTYR	0.20	5.12	2.56	0.14	7.17	3.58	0.12	8.28	4.14
	(0.02)	(0.52)	(0.26)	(0.01)	(0.51)	(0.26)	(0.02)	(1.52)	(0.76)
4´-*O*-GlucHOTYR	4.81	0.22	0.11	2.19	0.47	0.23	1.58	0.64	0.32
	(1.4)	(0.08)	(0.04)	(0.39)	(0.09)	(0.05)	(0.24)	(0.11)	(0.05)
3´-*O*-GlucHOTYR	2.00	0.3	0.59	1.04	0.55	1.10	0.72	0.69	1.39
	(0.31)	(0.05)	(0.10)	(0.1)	(0.08)	(0.15)	(0.06)	(0.06)	(0.11)
TYR	7.02	0.15	0.07	5.76	0.18	0.09	3.89	0.26	0.13
	(1.41)	(0.03)	(0.01)	(0.95)	(0.03)	(0.01)	(0.25)	(0.02)	(0.01)
4´-*O*-GlucTYR	NA	NA	0	NA	NA	0	NA	NA	0
HVAlc	0.4	2.54	1.27	0.25	4.02	2.01	0.22	4.55	2.28
	(0.05)	(0.29)	(0.14)	(0.02)	(0.32)	(0.16)	(0.01)	(0.21)	(0.10)
4´-*O*-GlucHVAlc	NA	NA	0	NA	NA	0	NA	NA	0
Trolox	0.26	3.8	1.9	0.28	3.57	1.79	0.32	3.15	1.58
	(0.01)	(0.17)	(0.09)	(0.01)	(0.13)	(0.06)	(0.06)	(0.51)	(0.26)

[e] - ED_{50} = (µmol of compound)/(µmol of DPPH)
[f] - ARC = $(1/ED_{50}) \times 10^3$ - Antioxidant Reaction Capacity;
[g] - n_H – number of hydrogen atoms donated to DPPH radical by compounds, calculated as n = $1/(ED_{50} \times 2)$,
NA – no activity

Fig 1

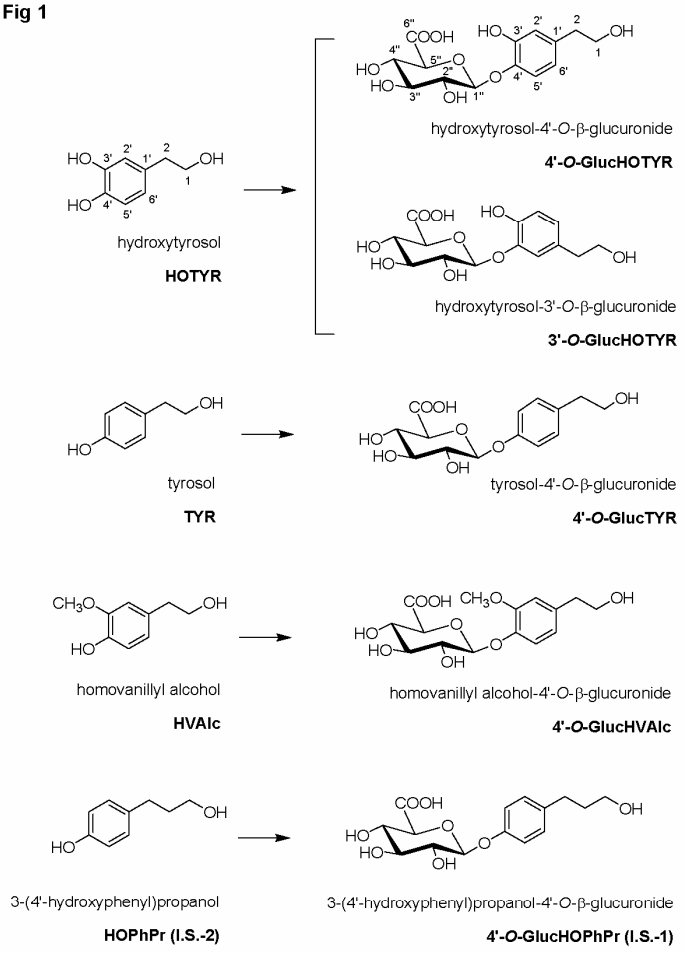

hydroxytyrosol
HOTYR
hydroxytyrosol-4'-O-β-glucuronide
4'-O-GlucHOTYR
hydroxytyrosol-3'-O-β-glucuronide
3'-O-GlucHOTYR
tyrosol
TYR
tyrosol-4'-O-β-glucuronide
4'-O-GlucTYR
homovanillyl alcohol
HVAlc
homovanillyl alcohol-4'-O-β-glucuronide
4'-O-GlucHVAlc
3-(4'-hydroxyphenyl)propanol
HOPhPr (I.S.-2)
3-(4'-hydroxyphenyl)propanol-4'-O-β-glucuronide
4'-O-GlucHOPhPr (I.S.-1)

Fig 2

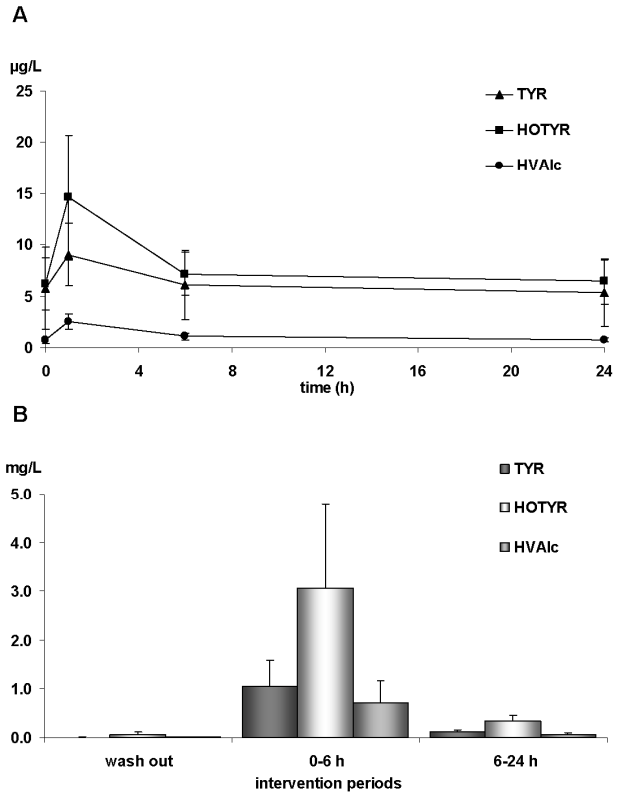

A
µg/L
25
20
15
10
5
0
0
4
8
12
16
20
24
time (h)
TYR
HOTYR
HVAlc
B
mg/L
5.0
4.0
3.0
2.0
1.0
0.0
wash out
0-6 h
6-24 h
intervention periods
TYR
HOTYR
HVAlc

Fig 3

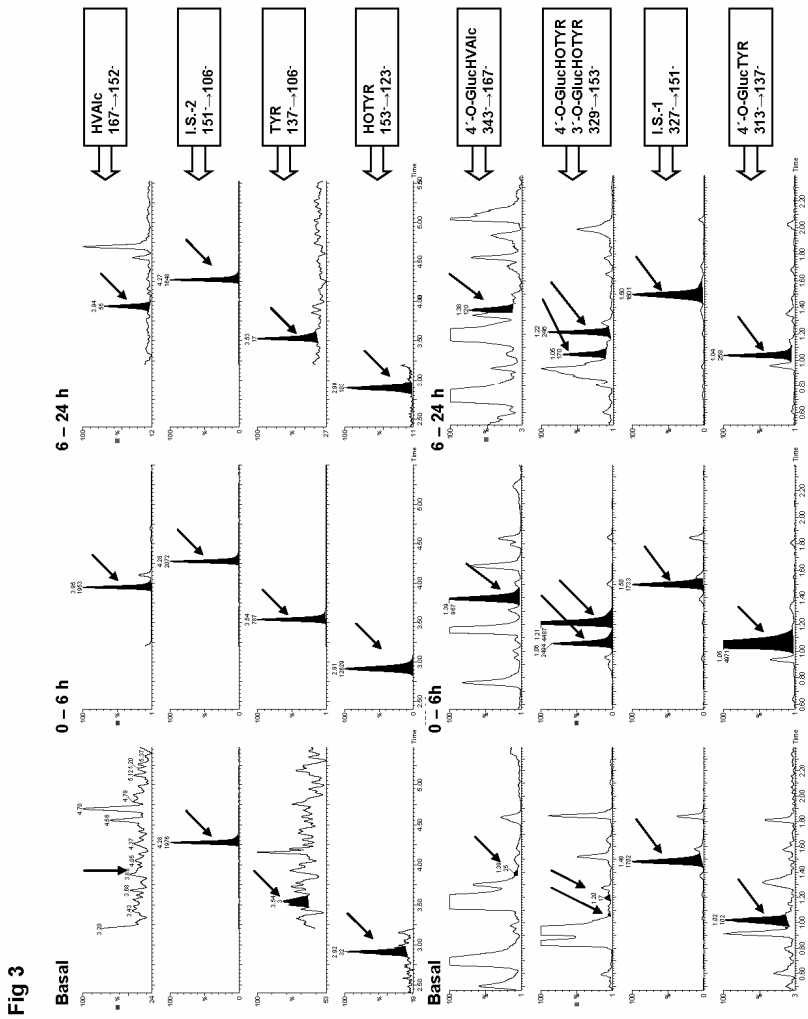

Basal
0 – 6 h
6 – 24 h
HVAlc
167→152-
I.S.-2
151→106-
TYR
137→106-
HOTYR
153→123-
Basal
0 – 6h
6 – 24 h
4´-O-GlucHVAlc
343→167-
4´-O-GlucHOTYR
3´-O-GlucHOTYR
329→153-
I.S.-1
327→151-
4´-O-GlucTYR
313→137-

Fig 4

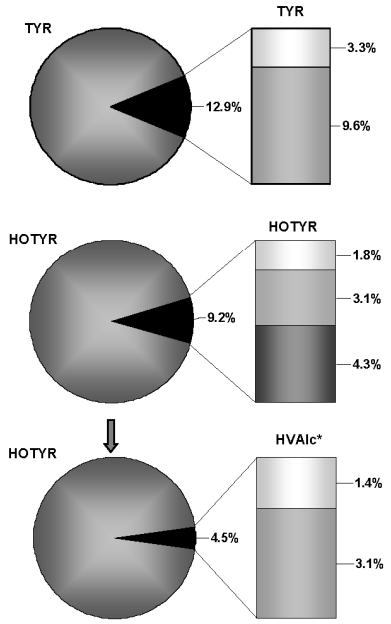


Fig 5

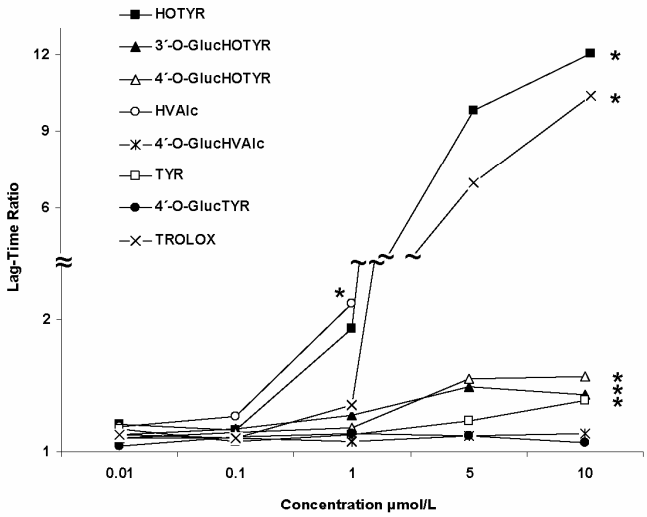
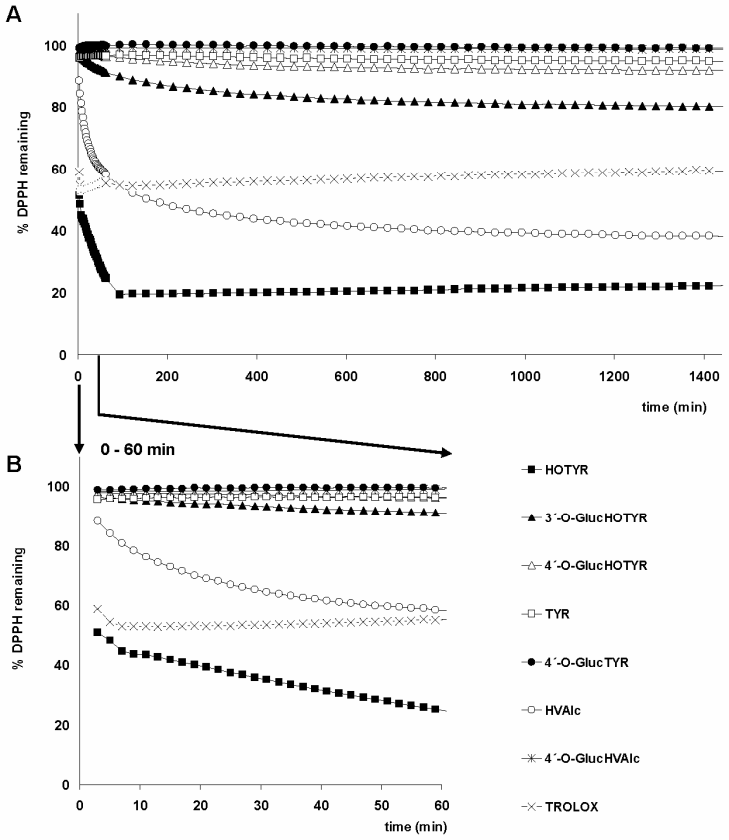

HOTYR
3´-O-GlucHOTYR
4´-O-GlucHOTYR
HVAlc
4´-O-GlucHVAlc
TYR
4´-O-GlucTYR
TROLOX
Lag-Time Ratio
12
9
6
2
1
0.01
0.1
1
5
10
Concentration µmol/L

Fig 6


A
% DPPH remaining
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
time (min)
0 - 60 min
B
% DPPH remaining
100
80
60
40
20
0
0
10
20
30
40
50
60
time (min)
HOTYR
3´-O-GlucHOTYR
4´-O-GlucHOTYR
TYR
4´-O-GlucTYR
HVAlc
4´-O-GlucHVAlc
TROLOX

Supplemental Table 1: Fragmentation pattern of studied compounds and MRM transitions selected for qualitative analysis performed in negative mode.

Compounds (MW)	Molecular ion $[M-H]^-$ [a], *m/z*$^-$	Main fragment ions		Characteristic MS pair (at its optimum CE, e*V*)
		m/z$^-$	Fragment description	
HOTYR	153	123$^-$	$[M-H-H_2CO(30)]^-$	153→123 (15)
(154)				
4´-*O*-GlucHOTYR	329	153$^-$	$[M-H-Gluc(176)]^-$	329→153$^-$(20)
(330)		175$^-$	$[Gluc-H]^-$ [b]	
		113$^-$	$[Gluc-H-CO_2-H_2O]^-$ [c]	
3´-*O*-GlucHOTYR	329	153$^-$	$[M-H-Gluc(176)]^-$	329→153$^-$(20)
(330)		175$^-$	$[Gluc-H]^-$ [b]	
		113$^-$	$[Gluc-H-CO_2-H_2O]^-$ [c]	
TYR	137	106$^-$	$[M-H-CH_3O^{\cdot}(31)]^-$	137→106 (15)
(138)		93$^-$	$[M-H-C_2H_4OH(44)]^-$	
4´-*O*-GlucTYR	313	137$^-$	$[M-H-Gluc(176)]^-$	313→137$^-$(25)
(314)		175$^-$	$[Gluc-H]^-$ [b]	
		113$^-$	$[Gluc-H-CO_2-H_2O]^-$ [c]	
HVAlc	168	152$^-$	$[M-H-CH_3(15)]^-$	167→152 (15)
(168)		122$^-$	$[M-H-CH_3(15)-H_2CO(30)]^-$	
4´-*O*-GlucHVAlc	343	167$^-$	$[M-H-Gluc(176)]^-$	343→167 (20)
(344)		175$^-$	$[Gluc-H]^-$ [b]	
		113$^-$	$[Gluc-H-CO_2-H_2O]^-$ [c]	
HOPhPr,	151$^-$	121$^-$	$[M-H-H_2CO(30)]^-$	151→106(15)
I.S.-2 (152)		106$^-$	$[M-H-C_2H_5O^{\cdot}(45)]$	
4´-*O*-GlucHOPhPr	327$^-$	151$^-$	$[M-H-Gluc(176)]^-$	327→151$^-$(25)
I.S.-1 (328)		175$^-$	$[Gluc-H]^-$ [b]	
		113$^-$	$[Gluc-H-CO_2-H_2O]^-$ [c]	

[a] $[M-H]^-$ - deprotonated molecular ion
[b] $[Gluc-H]^-$ - anhydroglucuronic acid residue, *m/z* 175
[c] $[Gluc-H-CO_2-H_2O]^-$ - secondary fragment ion at *m/z* 113 (loss of CO_2 and water from *m/z* 175)

Supplemental Table 2. Summary for the UPLC-SPE-MRM assay pre-validation for simultaneous determination and quantification of free HOTYR, TYR and HVAlc and their glucuronidated conjugates in SU as surrogate matrix.

Compound	LOD,	LOQ,	Recovery,	Intra-day[2]		Inter-day[3]	
	ng/mL	*ng/mL*	*%*[1]	*RSD %*[4]	*ERR %*[5]	*RSD %*	*ERR %*
4´-*O*-GlucTYR	7.2	20	89.3	8.1	20.1	8.7	20.0
			(13.6)	12.7	19.4	10.9	18.9
				4.6	18.1	5.6	14.0
4´-*O*-GlucHOTYR	4.5	13.6	93.7	8.2	7.6	6.8	6.4
			(11.5)	11.0	10.1	11.2	9.6
				2.5	9.3	5.4	12.6
3´-*O*-GlucHOTYR	2.5	7.6	94.5	12.4	20.5	10.3	8.6
			(11)	4.4	3.7	9.9	7.9
				3.6	10.5	6.4	6.5
4´-*O*-GlucHVAlc	4.1	12.3	92.7	7.1	6.4	10.3	8.6
			(11)	3.1	2.9	9.9	7.9
				3.0	3.6	6.4	6.5
HOTYR	*24.7*	*75.06*	92.8	*57.9*	*59.8*	*68.4*	*50.6*
			(*25.2*)	*23.9*	*24.9*	*21.5*	*19.7*
				12.7	9.4	9.7	7.4
TYR	0.62	1.87	97.1	11.9	9.6	11.4	11.0
			(13.7)	9.9	8.21	12.5	12.4
				3.9	6.6	8.2	7.2
HVAlc	2.87	8.7	94.1	6.9	9.0	14.9	12.8
			(15.7)	9.7	7.4	9.4	6.9
				0.5	10.6	8.7	9.7

[1] Mean (CV%), n = 12 (quadruplicates per each of three concentrations: 20, 500 and 2000 ng/mL)
[2] Intra-day: n=3 per each control quality samples: CI, CM, and CS, respectively;
[3] Inter-day: n=9, 3 days per each control quality sample: CI, CM, and CS, respectively;
[4] RSD: relative standard deviation of the control samples concentrations (imprecision)
[5] ERR: relative error of the estimated concentrations (bias)

Khymenets O, Ortuño J, Fitó M, Covas MI, Ferré M, de la Torre R. Evaluation of RNA isolation procedures from human blood and its application for gene expression studies (Sod-1, Sod-2). Anal Biochem. 2005; 347(1): 156-8.

Khymenets O, Covas MI, Farré M, Langohr K, Fitó M, de la Torre R. Role of sex and time of blood sampling in SOD1 and SOD2 expression variability. Clin Biochem. 2008; 41(16-17): 1348-54.

Khymenets O, Fitó M, Covas MI, Farré M, Pujadas MA, Muñoz D, et al. Mononuclear cell trascriptome response after sustained virgin olive oil consumption in Humans: an exploratory nutrigenomics study. OMICS. 2009; 13(1): 7-19.

CONCLUDING REMARKS

In vitro as well as *in vivo* experiments have provided convincing results regarding the potential health benefits of olive oil derived compounds (MUFA, tocopherols, polyphenols as HOTYR and TYR, etc.) commonly called as “bioactive compounds”. They are considered contributory factors for various health-maintaining properties, which altogether give a reason for defining olive oil as a “functional food”. A general theory is emerging that the olive oil “bioactive components” like polyphenols induce metabolic effects functioning as antioxidants and/or being regulators of genome activities, and, thus, deliver a health benefit beyond basic nutrition.

In recent years, olive oil phenols, where the most abundant are HOTYR and TYR and their seicoiroid derivatives, were thoroughly investigated for their human health-maintaining properties at different stages. Results confirmed the potency of these bioactive compounds. They have been subjected to investigation on their potential antioxidant, anticancer, and anti-inflammatory activities using different *in vitro* and *in vivo* models as well as in clinical trials, in order to establish their role in protection against various age-related disease, including cardiovascular diseases (CVDs). Although these secondary plant metabolites were considered to be non-nutritional, they were recognized to be very important ingredients for the maintenance of human cardio-vascular health.

Traditionally, the antioxidant properties of olive oil phenols were considered to be important contributors in CVDs prevention. However, all studies supporting this statement take into account only antioxidant activities of unaltered phenolic compounds without considering the role of metabolic biotransformations they undergo within human organism.

The impact of metabolism on the biological activities of olive oil phenols was not adequately considered until now. The main reason for that was the unavailability of proper standards of metabolic compounds. The only study, where metabolites (HOTYR and HVAlc sulfates and glucuronides) basic antioxidant activities (by the means of DPPH antiradical test) were compared to their parent compounds (HOTYR and HVAlc), was performed on extracted compounds form rat urine (Tuck, 2002). Surprisingly, in this study the glucuroconjugation, but not sulfatation, was shown to enhance significantly the DPPH antiradical activity of parent compounds HOTYR (a quite potent antiradical itself). This observation was, however, in disagreement with the predicted theoretically values pointing out to a loss of antiradical activity (Nenandis, 2005). Despite this inconsistency between theoretical and experimental results, the majority of papers on olive oil phenols research referenced this experimental work in the justification of results and used it as starting point of many hypotheses. Therefore, the investigation of the impact of metabolic transformation (e.g. glucuronidation of HOTYR as a probe example) on the antioxidant activity of the olive oil phenols was one of the goals of this thesis. In addition, studies on the bioavailability of olive oil phenols and their metabolic disposition as glucuronides in humans were planed to re-evaluate biological activities of polyphenols and their metabolites in a range of concentrations biologically meaningful.

To accomplish with these tasks, standards of metabolites were required. Since these standards were not commercially available, their synthesis was undertaken. A methodology has been developed for the biocatalyzed syntheses of *O*-*β*-D-monoglucuronide conjugates of HOTYR, TYR, HVAlc, and HOPhPr

with a single-step product isolation and in high yield. Glucuronides were synthesized using porcine liver microsomes, analyzed and separated by HPLC-UV, identified by HPLC-MS, and their structures unequivocally established by NMR techniques. The outcome of the glucuronidation reaction depends on the structure of the phenolic compounds. Thus, the glucuronidation of HOTYR, biocatalyzed with liver microsomes, proceeded exclusively on the phenol groups. The regioselectivity was similar to that observed for human and rat liver microsomes, the 4´-hydroxy position being more favored than the 3´-hydroxy one. In the case of TYR, HVAlc, and HOPhPr, two products were formed during microsomal glucuronidation: a major one, the phenolic *O*-*β*-D-glucuronidated derivative and, a minor one, the *O*-*β*-D-glucuronidated of the aliphatic alcohol. The results of this work were presented in following publications: original paper **P-I** and methodological publication **P-II**. This method has provided, for the first time, glucuronide metabolites of the antioxidant phenolic compounds present in olive oil in a ratio close to the *in vivo* phase II metabolism in humans.

The purified well characterized standards were applied in the development of a direct analytical method for the quantitative determination of glucuronides in urine. This method allowed us to evaluate the olive oil phenols bioavailability in humans and their metabolic disposition as glucuronides. A high performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-MS) method was developed for the simultaneous analysis of 3´- and 4´-*O*-HOTYR-glucuronides, and 4´-*O*-glucuronides of TYR and HVAlc (homovanyl alcohol) in human urine. This is the first time that a direct method for the quantitative analysis of glucuronidated

metabolites of olive oil phenols HOTYR and TYR in human urines was reported and successfully applied in a human intervention study with VOO administered at dietary doses.

Using this method, concentrations of metabolites and their core compounds were estimated in an intervention study with 11 healthy volunteers supplemented with 50 mL VOO. Thus, after 24 hours about 13% of the dose was recovered. Free phenols were less than 5% of the total recovery. The phenols were mainly recovered as glucuronides. Our results confirm previously reported data about very low bioavailability of OOPh (HOTYR, TYR, HVac).

According to earlier reported data and in agreement with our records (using the newly developed direct method of analysis) free phenols concentrations in biological fluids are low, due to the extensive phase II metabolism (where glucuronidation take primary place). Therefore, on the one hand, the concentrations of free forms of olive oil polyphenols are unlikely to explain biological activities (Vissers, 2006) seen in humans after olive oil intake (Weinbrenner, 2004). On the other hand, an enhanced antiradical activity of glucuronidated metabolite in comparison to the parent compound HOTYR (Tuck, 2002), although contradictory to theoretical predictions (Nenandis, 2005), has been reported and broadly acknowledged. In this context antioxidant activities of conjugated metabolites in a range of concentrations compatible with their dietary consumption (combining previously reported plasmatic concentrations and newly reported urinary concentrations by the direct quantitative method) were evaluated. Previously synthesized and well characterized glucuronides and corresponding to them parent compounds were tested for their

chemical (hydrogen donation by DPPH test) and *in vitro* biological (inhibition of Cu-mediated LDL oxidation) antioxidant activities. The results of these comparative analyses, presented within original publication **P-III** (under submission), showed that none of the olive oil phenols glucuronides displayed relevant antioxidant activities when compared to their parent compounds.

However antioxidant properties of OOPh traditionally recognized as basic in CVDs prevention still are an area under discussion, emerging experimental data suggest that these phenols can also act as potential signals, which influence sensor systems that modify gene expression and subsequently are in charge of maintenance organism homeostasis. In this context just a few investigations were performed both *in vitro* and *in vivo* and fewer in humans. It is believed that the overall effect of olive oil, especially on the entire human organism, cannot be accounted for phenolics or other compounds taken separately from other components of the oil matrix. Therefore, strategies looking into the synergistic effect of the olive oil components could be more appropriate.

Direct, definitive information about the effects of olive oil and its principal components on human cardiovascular health can only be obtained through investigation in human subjects. However, because of ethical and practical limitations, intervention trials in healthy subjects and patients often provide information only on early or short-lasting biological effects of the intervention, typically measured as clinico-chemical and, due to the existing antioxidant theory of atheriosclerosis (AT), chiefly oxidative stress related biomarkers. These and other biomarkers associated with CVDs were explored in a large number of human studies with olive oil.

However, no well-defined mechanisms of the olive oil action, declared to be health beneficial, could be derived from these studies. Therefore, new additional markers of olive oil effects need to be identified and this require a re-examination of their mechanism of action.

Nutrigenomics provides a high throughput genomics tools in nutrition research, which allow increasingly detailed molecular studies of nutrient-genome interaction and, thus, have helped to change the focus of the field (Müller, 2003). These tools are expected to extend understanding of how olive oil as a foodstuff influences metabolic pathways and homeostatic control, and how this regulation could be distributed in the early phase of diet-related CVDs.

Looking for new molecular mechanisms of olive oil action against CVDs development and progression and, therefore, possibly for new molecular biomarkers, a main attention is directed to gene expression activities (transcriptome) as to a principal event in genome response to any factor, including dietary intervention. In search of explanations for a protective role of olive oil in CVDs development and progression, we were interested in events provoked by VOO ingestion on transcriptome level in human MNCs, cells playing a crucial role in AT development and progression. As a result, a design for study investigating gene-VOO interaction in human healthy volunteers was developed. However performance of transcriptome studies in humans is very challenging due to the permanent interaction of such complex organism with environmental variables.

To ensure a high quality of extracted total RNA, a protocol for evaluation of different extraction methods from human blood sample was carried out. The overall results of this validation were presented in original publication **P-IV**. Two main physiological parameters influencing on gene expression, sex and time of sampling, were as well evaluated in our preparative studies. Our results, presented in original publication **P-V**, on the basis of *SOD1* and *SOD2* expressions demonstrate how sex and daytime, and to some extend the period of menstrual cycle in women, deserve being controlled when human gene expression analyses are evaluated, particularly within the framework of clinical trials or cohort studies. The outcomes of these preparative studies: (i) a validated total RNA extraction procedure from MNCs using Ultraspec reagent, (ii) an optimized by time (during day and over month) samples collection and (iii) the know-how on gender contribution to gene expression, were applied in the design of the GEpilot study protocol (a study on VOO-gene expression interaction in human MNCs, see Appendix B).

The objective of the GEpilot exploratory study was to identify the MNC genes that respond to VOO consumption in order to ascertain the molecular mechanisms underlying the beneficial action of VOO in the prevention of AT. Gene expression profiles of MNCs from healthy individuals were examined after 3 weeks of moderate and regular consumption of VOO, as the main fat source in a diet controlled for antioxidant content. The response to VOO consumption was confirmed for 10 up-regulated genes (*ADAM17*, *ALDH1A1*, *BIRC1*, *ERCC5*, *LIAS*, *OGT*, *PPARBP*, *TNFSF10*, *USP48,* and *XRCC5*). Their putative role in the molecular mechanisms involved in AT development and progression was

discussed within original publication **P-VI**, focusing on a possible relationship with VOO consumption. Our data support the hypothesis that 3 weeks of nutritional intervention with VOO supplementation, at doses common in the Mediterranean diet, can alter the expression of genes, among which are genes related to development and progression of atherogenic events. The presented work suggests that VOO may be involved in several molecular pathways involved in antiatherogenic protection in humans *in vivo*. The findings of the GEpilot exploratory study collectively support future longer-term prospective studies in larger cohorts of subjects to discern the molecular genetic signatures underlying the beneficial effects of VOO on atherosclerosis risk. In fact, several presently ongoing research projects, as PREDIMED and PREDIGEN carried out in the Oxidative Stress and Nutrition Research Group of IMIM-Hospital del Mar, were structured on the basis of the mentioned exploratory nutrigenomics study.

CONCLUSIONS

Main achievements of the present research project, obtained according to the determined objectives of the study, are summarized below:

1. The synthesis of reference compounds for HOTYR, TYR and HVAlc glucuronidated metabolites and corresponding to them internal standard (HOPhPr glucuronide) was developed using microsomal synthesis as the most appropriate method to produce standards equivalent to *in vivo* phase II olive oil phenols metabolites.

2. The preparative production of glucuronidated standards was established in milligram range. The synthesized products were successfully separated and purified by semi-preparative chromatography, allowing to obtain a reference standards of grade purity (>95%) and in amounts suitable for application in majority of analytical, biochemical and biological studies.

3. The structure of synthesized metabolites was successfully established and well characterized using MS and NMR techniques. Their correspondence to *in vivo* olive oil derived HOTYR, TYR and HVAlc glucuronidated metabolites in humans was corroborated and, therefore, they were effectively applied in development of a direct LC-MS method for their analysis in human biological fluids.

4. Developed UPLC-MS methodology was successfully validated and applied for direct detection and quantification of HOTYR, TYR and HVAlc glucuronides and their parent compounds in 24-h postprandial urines of volunteers

intervened with single 50 mL dose of VOO. Therefore, for the first time glucuronidated metabolites of HOTYR, TYR and HVAlc were directly identified and their concentration were estimated in human urine samples corresponding to intervention studies with VOO at real life doses.

5. Assessing the concentrations and rates of excretion for HOTYR, TYR and HVAlc glucuronides in 24-h postprandial urine samples belonging to VOO intervention study, the role of glucuronidation in metabolism and excretion of olive oil phenols was estimated. The very low bioavailablity of unconjugated forms of olive oil phenols (accounting only for 3% of totally consumed) was confirmed. The rate of glucuronoconjugation was estimated to be higher than 75% (other Phase II metaboites not measured, not considered) and the recovery as glucuronides was a 10% of consumed olive oil phenols.

6. The antioxidant activities of olive oil phenol derived glucuronides were compared with their parent compounds using *in vitro* Cu-mediated LDL oxidation test at their relevant for *in vivo* concentration ranges (10 μM - 1mM). Therefore, for the first time experimentally was shown that the phase II metabolic transformation (e.g. glucuronoconjugation) of the most important olive oil antioxidants highly reduce their well known inhibition activities against LDL oxidation, a principal process involved in atherogenesis.

7. Basic for antioxidants hydrogen donation properties were assessed by traditional DPPH test using pure *in vivo*-

equivalent metabolites standards. Therefore, for the first time hydrogen donating activities of olive oil derived HOTYR, TYR and HVAlc glucuronides were accurately estimated and the lost of antiradical activities characteristic for their parent compounds was stated.

8. Result from bioavailability and antioxidant properties studies point out that the antioxidant activities could not be chiefly responsible for the beneficial action of olive oil phenolics on human health *in vivo*, mainly due to their extensive phase II biotransformation. There should be other mechanisms which might explain the reported health assistance of olive oil phenols, and nutrigenomics studies (transcriptomics as a principal one), therefore, could facilitate their identification.

9. The protocol for samples collection, accounting for the principal fators influencing on gene expression variability in humans (e.g. sex, diurnal and moth variations, the later one for women), and the protocol for total RNA extraction from blood samples were established according to the performed preparative evaluation studies. They were advantageously applied in the design of a pilot VOO nutrigenomic study involving human subjects.

10. The analysis of transcriptome response to VOO administration was performed using microarray experiments involving pooled samples of MNCs total RNA corresponding to the wash-out and 3-week intervention periods in male subjects. Therefore, for the first time *in vivo* transcriptome response of MNCs, cells involved in primary atherogenic

events, to VOO supplemented at real life dietary doses was reported in humans.

11. 23 genes related to CVDs were selected on the basis of microarray results and their response was revalidated by Real-Time qPCR in individual total RNA samples. Results revealed that 10 atherogenesis related genes could be potential targeted by ingested VOO. Therefore, for the first time several putative sites for the VOO-genome interaction were reported on the base of *in vivo* transcriptome study in human.

BIBLIOGRAPHY

"WHO Disease and injury country estimates". World Health Organization. 2009. Retrieved Nov.11, 2009: http://www.who.int/mediacentre/factsheets/fs317/en/index.html.

Abia R, Perona JS, Pacheco YM, Montero E, Muriana FJ, Ruiz-Gutiérrez V. Postprandial triacylglycerols from dietary virgin olive oil are selectively cleared in humans. J Nutr. ***1999***;129(12):2184-91.

Acín S, Navarro MA, Perona JS, Surra JC, Guillen N, Arnal C, Sarría AJ, Arbonés-Mainar JM, Carnicer R, Ruiz-Gutiérrez V, Osada J. Microarray analysis of hepatic genes differentially expressed in the presence of the unsaponifiable fraction of olive oil in apolipoprotein E-deficient mice. Br J Nutr. ***2007***;97(4):628-38.

Aguiar A, Ferraz A. Fe(3+)- and Cu(2+)-reduction by phenol derivatives associated with Azure B degradation in Fenton-like reactions. Chemosphere. ***2007***;66(5):947-54.

Alemany R, Navarro MA, Vögler O, Perona JS, Osada J, Ruiz-Gutiérrez V. Olive Oils Modulate Fatty Acid Content and Signaling Protein Expression in Apolipoprotein E Knockout Mice Brain. Lipids. ***2009***. [Epub ahead of print].

Alipour A, Elte JW, van Zaanen HC, Rietveld AP, Castro Cabezas M. Novel aspects of postprandial lipemia in relation to atherosclerosis. Atheroscler Suppl. ***2008***;9(2):39-44.

Andrewes P, Busch JL, de Joode T, Groenewegen A, Alexandre H.Sensory properties of virgin olive oil polyphenols: identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. J Agric Food Chem. ***2003***;51(5):1415-20.

Antonio L, Grillasca JP, Taskinen J, Elovaara E, Burchell B, Piet MH, Ethell B, Ouzzine M, Fournel-Gigleux S, Magdalou J. Characterization of catechol glucuronidation in rat liver. Drug Metab Dispos. ***2002***;30(2):199-207.

Antonio L, Xu J, Little JM, Burchell B, Magdalou J, Radominska-Pandya A. Glucuronidation of catechols by human hepatic, gastric, and intestinal microsomal UDP-glucuronosyltransferases (UGT) and recombinant UGT1A6, UGT1A9, and UGT2B7. Arch Biochem Biophys. ***2003***;411(2):251-61.

Arbones-Mainar JM, Ross K, Rucklidge GJ, Reid M, Duncan G, Arthur JR, Horgan GW, Navarro MA, Carnicer R, Arnal C, Osada J, de Roos B. Extra virgin olive oils increase hepatic fat accumulation and hepatic antioxidant protein levels in APOE-/- mice. J Proteome Res. ***2007***;6(10):4041-54.

Bagnati M, Perugini C, Cau C, Bordone R, Albano E, Bellomo G. When and why a water-soluble antioxidant becomes pro-oxidant during copper-induced low-density lipoprotein oxidation: a study using uric acid. Biochem J. ***1999***;340 (Pt 1):143-52.

Bai C, Yan X, Takenaka M, Sekiya K, Nagata T. Determination of synthetic hydroxytyrosol in rat plasma by GC-MS. J Agric Food Chem. ***1998***;46:3998-4001.

Bazoti FN, Gikas E, Puel C, Coxam V, Tsarbopoulos A. Development of a sensitive and specific solid phase extraction--gas chromatography-tandem mass spectrometry method for the determination of elenolic acid, hydroxytyrosol, and tyrosol in rat urine. J Agric Food Chem. ***2005***;53(16):6213-21.

Bellido C, López-Miranda J, Blanco-Colio LM, Pérez-Martínez P, Muriana FJ, Martín-Ventura JL, Marín C, Gómez P, Fuentes F, Egido J, Pérez-Jiménez F. Butter and walnuts, but not olive oil, elicit postprandial activation of nuclear transcription factor kappaB in peripheral blood mononuclear cells from healthy men. Am J Clin Nutr. ***2004***;80(6):1487-91.

Benkhaltia F, J. Prostb, E. Pazc, F. Perez-Jimenezc, C. El modafara and E. El Boustani, a Effects of feeding virgin olive oil or their polyphenols on lipid of rat liver. Nutr Research ***2002;*** 22(9):1067-75.

Bermúdez B, López S, Pacheco YM, Villar J, Muriana FJ, Hoheisel JD, Bauer A, Abia R. Influence of postprandial triglyceride-rich lipoproteins on lipid-mediated gene expression in smooth muscle cells of the human coronary artery. Cardiovasc Res. ***2008***;79(2):294-303.

Bianco A, Coccioli F, Guiso M, Marra C. The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromas. Food Chem, ***2001***;77:450-11.

Bleas G, Vassilakis C, Harizanis C, Tsimidou M, Boskou DG. J. Agric. Food Chem. ***2002***;50: 3688-92.

Blois MS. Antioxidant determinations by the use of a stable free radical. Nature ***1958***;26:1199–200.

Blumberg J. Use of biomarkers of oxidative stress in research studies. J Nutr. ***2004;***134(11):3188S-89S.

Bogani P, Galli C, Villa M, Visioli F. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. Atherosclerosis.

2007;190(1):181-6.

Bonanome A, Pagnan A, Caruso D, Toia A, Xamin A, Fedeli E, Berra B, Zamburlini A, Ursini F, Galli G. Evidence of postprandial absorption of olive oil phenols in humans. Nutr Metab Cardiovasc Dis. ***2000***;10(3):111-20.

Boskou D. Olive oil Chemistry and Technology, AOCS Press, Champaign, IL, USA, ***1996***:52-83.

Boskou D. Olive oil Minor Constituents and Health. CRC Press, Taylor and Francis Group, ***2009***:230pp.

Boskou D. Olive Oil: Chemistry and Technology, Contributor American Oil Chemists Society, AOCS Press, ***2006:***268 pp.

Boskou D: Olive oil. World Rev. Nutr. Diet. ***2000***;87:56–77**.**

Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity, Lebensmittel-Wissenschaft und -Technologie/Food Science and Technology, ***1995***;28:25-30.

Brenes M, Garcia A, Garcia P, Garrido A. Acid hydrolysis of secoiridoids aglycons during storage of virgin olive oil. J Agr Food Chem. ***2001;***49:5609-14.

Brenes M, Garcia A, Garcia P, Rios JJ, Garrido A. Phenolic compounds in Spanish olive oils. J Agric Food Chem. ***1999***;47:3535-40.

Briante R, Febbraio F, Nucci R. Antioxidant properties of low molecular weight phenols present in the mediterranean diet. J Agric Food Chem. ***2003;***51(24):6975-81.

Briante R, Febbraio F, Nucci R. Antioxidant/prooxidant effects of dietary non-flavonoid phenols on the Cu2+-induced oxidation of human low-density lipoprotein (LDL). Chem Biodivers. ***2004***;1(11):1716-29.

Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophys. ***1993***;300(2):535-43.

Burczynski ME, Rockett JC, editors. Surrogate tissue analysis: genomics, proteomics, and metabolomic approaches. FL: Taylor and Francis Group, ***2006***.

Burkitt MJ. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-

tocopherol, thiols, and ceruloplasmin. Arch Biochem Biophys. ***2001***;394(1):117-35.

Busch JL, Hrncirik K, Bulukin E, Boucon C, Mascini M. Biosensor measurements of polar phenolics for the assessment of the bitterness and pungency of virgin olive oil. J Agric Food Chem. ***2006***;54(12):4371-7.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. ***2009***;55(4):611-22.

Bustin SA. Why the need for qPCR publication guidelines?-The case for MIQE. Methods. ***2010.*** [Epub ahead of print].

Canales RD, Luo Y, Willey JC, Austermiller B, Barbacioru CC, Boysen C, Hunkapiller K, Jensen RV, Knight CR, Lee KY, Ma Y, Maqsodi B, Papallo A, Peters EH, Poulter K, Ruppel PL, Samaha RR, Shi L, Yang W, Zhang L, Goodsaid FM. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat Biotechnol. ***2006***;24(9):1115-22.

Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distante A, De Caterina R. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol. ***2003***;23(4):622-9.

Carrasco-Pancorbo A, Arráez-Román D, Segura-Carretero A, Fernández-Gutiérrez A. Capillary electrophoresis-electrospray ionization-mass spectrometry method to determine the phenolic fraction of extra-virgin olive oil. Electrophoresis. ***2006***;27(11):2182-96.

Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Gallina-Toschi T, Fernandez-Gutiérrez A. Analytical determination of polyphenols in olive oils. J Sep Sci. ***2005a;***28(9-10):837-58.

Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Lercker G, Fernández-Gutiérrez A. Evaluation of the influence of thermal oxidation on the phenolic composition and on the antioxidant activity of extra-virgin olive oils. J Agric Food Chem. ***2007***;55(12):4771-80.

Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Del Carlo M, Gallina-Toschi T, Lercker G, Compagnone D, Fernández-Gutiérrez A. Evaluation of the antioxidant capacity of individual

phenolic compounds in virgin olive oil. J Agric Food Chem. ***2005b***;53(23):8918-25.

Cartoni GP, Coccioli F, Spagnoli M. Analysis of ethereal extracts of wines and other alcoholic beverages by high-performance liquid chromatography with microbore columns. J Chromatogr A. ***1997***;782(2):219-26.

Caruso D, Berra B, Giavarini F, Cortesi N, Fedeli E, Galli G. Effect of virgin olive oil phenolic compounds on in vitro oxidation of human low density lipoproteins. Nutr Metab Cardiovasc Dis. ***1999***;9(3):102-7.

Caruso D, Visioli F, Patelli R, Galli C, Galli G. Urinary excretion of olive oil phenols and their metabolites in humans. Metabolism. ***2001***;50(12):1426-8.

Cheng Z, Ren J, Chang W, Chen Z. Study on the multiple mechanisms underlying the reaction between hydroxyl radical and phenolic compounds by quantitative structure and activity relationship. Bioorgan Med Chemistry, ***2002***;10:4067-73.

Cheng Z, Ren J, Li Y, Chang W, Chen Z. Establishment of a quantitative structure-activity relationship model for evaluating and predicting the protective potentials of phenolic antioxidants on lipid peroxidation. J Pharm Sci. ***2003***;92(3):475-84.

Christophoridou S, Dais P. Detection and quantification of phenolic compounds in olive oil by high resolution 1H nuclear magnetic resonance spectroscopy. Anal Chim Acta. ***2009***;633(2):283-92.

Cicerale S, Conlan XA, Sinclair AJ, Keast RS. Chemistry and health of olive oil phenolics. Crit Rev Food Sci Nutr. ***2009***;49(3):218-36.

Cicero AF, Nascetti S, López-Sabater MC, Elosua R, Salonen JT, Nyyssönen K, Poulsen HE, Zunft HJ, Kiesewetter H, de la Torre K, Covas MI, Kaikkonen J, Mursu J, Koenbick C, Bäumler H, Gaddi AV; EUROLIVE Study Group. 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(2):314-20.

Coni E, Di Benedetto R, Di Pasquale M, Masella R, Modesti D, Mattei R, Carlini EA. Protective effect of oleuropein, an olive oil biophenol, on low density lipoprotein oxidizability in rabbits. Lipids. ***2000***;35(1):45-54.

Corona G, Deiana M, Incani A, Vauzour D, Dessì MA, Spencer JP. Hydroxytyrosol inhibits the proliferation of human colon

adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. Mol Nutr Food Res. ***2009a***;53(7):897-903.

Corona G, Deiana M, Incani A, Vauzour D, Dessì MA, Spencer JP. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. Biochem Biophys Res Commun. ***2007***;362(3):606-11.

Corona G, Spencer JP, Dessì MA. Extra virgin olive oil phenolics: absorption, metabolism, and biological activities in the GI tract. Toxicol Ind Health. ***2009b***;25(4-5):285-93.

Corona G, Tzounis X, Assunta Dessì M, Deiana M, Debnam ES, Visioli F, Spencer JP. The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. Free Radic Res. ***2006***;40(6):647-58.

Covas MI, de la Torre K, Farré-Albaladejo M, Kaikkonen J, Fitó M, López-Sabater C, Pujadas-Bastardes MA, Joglar J, Weinbrenner T, Lamuela-Raventós RM, de la Torre R. Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. Free Radic Biol Med. ***2006a***;40(4):608-16.

Covas MI, Nyyssönen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, Gaddi A, de la Torre R, Mursu J, Bäumler H, Nascetti S, Salonen JT, Fitó M, Virtanen J, Marrugat J, EUROLIVE Study Group. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Ann Intern Med. ***2006b***;145(5):333-41.

Covas MI. Olive oil and the cardiovascular system. Pharmacol Res. ***2007***;55(3):175-86.

Cutler RG. Oxidative stress profiling: part I. Its potential importance in the optimization of human health. Ann N Y Acad Sci ***2005a***;1055:93-135.

Cutler RG, Plummer J, Chowdhury K, Heward C. Oxidative stress profiling: part II. Theory, technology, and practice. Ann N Y Acad Sci. ***2005b***;1055:136-58.

Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med. ***2006a***;10(2):389-406.

Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem. ***2006b***;52(4):601-23.

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(11):1492-8.

Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic Biol Med. ***1999***;27(11-12):1151-63.

Davies MJ. The oxidative environment and protein damage. Biochim Biophys Acta. ***2005***;1703(2):93-109.

de Kok TM, de Waard P, Wilms LC, van Breda SG. Antioxidative and antigenotoxic properties of vegetables and dietary phytochemicals: The value of genomics biomarkers in molecular epidemiology. Mol Nutr Food Res. ***2009***. [Epub ahead of print].

de la Puerta R, Martínez Domínguez ME, Ruíz-Gutíerrez V, Flavill JA, Hoult JR. Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission. Life Sci. ***2001***;69(10):1213-22.

de la Puerta R, Martínez-Domínguez E, Ruíz-Gutiérrez V. Effect of minor components of virgin olive oil on topical antiinflammatory assays. Z Naturforsch C. **2000**;55(9-10):814-9.

de la Puerta R, Ruiz Gutierrez V, Hoult JR. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. Biochem Pharmacol. ***1999***;57(4):445-9.

de la Torre R, Covas MI, Pujadas MA, Fitó M, Farré M. Is dopamine behind the health benefits of red wine? Eur J Nutr. ***2006***;45(5):307-10.

de la Torre-Carbot K, Chávez-Servín JL, Jaúregui O, Castellote AI, Lamuela-Raventós RM, Fitó M, Covas MI, Muñoz-Aguayo D, López-Sabater MC. Presence of virgin olive oil phenolic metabolites in human low density lipoprotein fraction: determination by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Anal Chim Acta. ***2007***;583(2):402-10.

de la Torre-Carbot K, Jauregui O, Castellote AI, Lamuela-Raventós RM, Covas MI, Casals I, López-Sabater MC. Rapid high-performance liquid chromatography-electrospray ionization tandem mass spectrometry method for qualitative and quantitative analysis of virgin olive oil phenolic metabolites in human low-density lipoproteins. J Chromatogr A. ***2006***;1116(1-2):69-75.

de Logeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infraction. Final report of the Lyon Diet Heart Study. Circulation, ***1999***;99:779-85.

de Lorgeril M, Salen P. The Mediterranean diet: rationale and evidence for its benefit. Curr Atheroscler Rep. ***2008***;10(6):518-22.

de Lorgeril M, Salen P. The Mediterranean-style diet for the prevention of cardiovascular diseases.Public Health Nutr. ***2006***;9(1A):118-23.

De Rosa S, Cirillo P, Paglia A, Sasso L, Di Palma V, Chiariello M. Reactive Oxygen Species and Antioxidants in the Pathophysiology of Cardiovascular Disease: Does the Actual Knowledge Justify a Clinical Approach? Curr Vasc Pharmacol. ***2010***. [Epub ahead of print].

Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J. **1997**;324 (Pt 1):1-18.

Debey S, Schoenbeck U, Hellmich M, Gathof BS, Pillai R, Zander T, Schultze JL. Comparison of different isolation techniques prior gene expression profiling of blood derived cells: impact on physiological responses, on overall expression and the role of different cell types. Pharmacogenomics J. ***2004***;4(3):193-207.

Deiana M, Aruoma OI, Bianchi ML, Spencer JP, Kaur H, Halliwell B, Aeschbach R, Banni S, Dessi MA, Corongiu FP. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. Free Radic Biol Med. ***1999***;26(5-6):762-9.

Deiana M, Incani A, Rosa A, Corona G, Atzeri A, Loru D, Paola Melis M, Assunta Dessì M. Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H(2)O(2) induced lipid peroxidation in renal tubular epithelial cells. Food Chem Toxicol. ***2008***;46(9):2984-90.

Del Boccio P, Di Deo A, De Curtis A, Celli N, Iacoviello L, Rotilio D. Liquid chromatography-tandem mass spectrometry analysis of oleuropein and its metabolite hydroxytyrosol in rat plasma and urine after oral administration. J Chromatogr B Analyt Technol Biomed Life Sci. ***2003***;785(1):47-56.

Dell'Agli M, Fagnani R, Mitro N, Scurati S, Masciadri M, Mussoni L, Galli GV, Bosisio E, Crestani M, De Fabiani E, Tremoli E, Caruso D. Minor components of olive oil modulate proatherogenic adhesion

molecules involved in endothelial activation. J Agric Food Chem. ***2006***;54(9):3259-64.

Di Benedetto R, Varì R, Scazzocchio B, Filesi C, Santangelo C, Giovannini C, Matarrese P, D'Archivio M, Masella R. Tyrosol, the major extra virgin olive oil compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness. Nutr Metab Cardiovasc Dis. ***2007***;17(7):535-45.

Di Tommaso D, Calabrese R., Rotillo D. Identification and quiantification of hydroxytyrosol in Italian wines. J High Resol Chromatogr. ***1998***;(10):549-53.

Droge W. Free radicals in the physiological control of cell function. Physiol Rev. ***2002***;82:47-95.

Duncan MW, Smythe GA, Nicholson MV, Clezy PS. Comparison of high-performance liquid chromatography with electrochemical detection and gas chromatography-mass fragmentography for the assay of salsolinol, dopamine and dopamine metabolites in food and beverage samples. J Chromatogr. ***1984***;336(1):199-209.

Edgecombe SC, Stretch GL, Hayball PJ. Oleuropein, an antioxidant polyphenol from olive oil, is poorly absorbed from isolated perfused rat intestine. J Nutr. ***2000***;130(12):2996-3002.

Erkoç F, Keskin N, Erkoç Ş. Theoretical investigation of hydroxytyrosol and its radicals. J of Molec Structure (Theochem). ***2003***;625:87-94.

Escrich E, Moral R, Grau L, Costa I, Solanas M. Molecular mechanisms of the effects of olive oil and other dietary lipids on cancer. Mol Nutr Food Res. ***2007***;51(10):1279-92.

Escrich E, Solanas M, Moral R, Costa I, Grau L. Are the olive oil and other dietary lipids related to cancer? Experimental evidence. Clin Transl Oncol. ***2006***;8(12):868-83.

Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Radic Res Commun. ***1989***;6(1):67-75.

Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: induction, repair and significance. Mutat Res. ***2004***;567(1):1-61.

Fabiani R, De Bartolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur

J Cancer Prev. ***2002***;11(4):351-8.

Fabiani R, Rosignoli P, De Bartolomeo A, Fuccelli R, Servili M, Montedoro GF, Morozzi G. Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. J Nutr. ***2008***;138(8):1411-6.

Falany CN. Enzymology of human cytosolic sulfotransferases. FASEB J. ***1997***;11(4):206-16.

Feezor RJ, Baker HV, Mindrinos M, Hayden D, Tannahill CL, Brownstein BH, Fay A, MacMillan S, Laramie J, Xiao W, Moldawer LL, Cobb JP, Laudanski K, Miller-Graziano CL, Maier RV, Schoenfeld D, Davis RW, Tompkins RG; Inflammation and Host Response to Injury, Large-Scale Collaborative Research Program. Whole blood and leukocyte RNA isolation for gene expression analyses. Physiol Genomics. ***2004***;19(3):247-54.

Ferrec E, Chesne C, Artusson P, Brayden D, Fabre G, Gires P, Guillou F, Rousset M, Rubas W, Scarino ML. In vitro models of the intestinal barrier. The report and recommendations of ECVAM Workshop 46. ATLA ***2001***;29:649-68.

Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. ***2000;*** 408(6809):239-47.

Fisher MB, Paine MF, Strelevitz TJ, Wrighton SA. The role of hepatic and extrahepatic UDP-glucuronosyltransferases in human drug metabolism. Drug Metab Rev. ***2001***;33(3-4):273-97.

Fitó M, Cladellas M, de la Torre R, Martí J, Alcántara M, Pujadas-Bastardes M, Marrugat J, Bruguera J, López-Sabater MC, Vila J, Covas MI; The members of the SOLOS Investigators. Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: a randomized, crossover, controlled, clinical trial. Atherosclerosis. ***2005***;181(1):149-58.

Fitó M, Cladellas M, de la Torre R, Martí J, Muñoz D, Schröder H, Alcántara M, Pujadas-Bastardes M, Marrugat J, López-Sabater MC, Bruguera J, Covas MI; SOLOS Investigators. Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. Eur J Clin Nutr. **2008**;62(4):570-4.

Fitó M, Covas MI, Lamuela-Raventós RM, Vila J, Torrents L, de la Torre C, Marrugat J. Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. Lipids.

2000;35(6):633-8.

Fitó M, Gimeno E, Covas MI, Miró E, López-Sabater Mdel C, Farré M, de TR, Marrugat J. Postprandial and short-term effects of dietary virgin olive oil on oxidant/antioxidant status. Lipids. ***2002***;37(3):245-51.

Fitó M, Guxens M, Corella D, Sáez G, Estruch R, de la Torre R, Francés F, Cabezas C, López-Sabater Mdel C, Marrugat J, García-Arellano A, Arós F, Ruiz-Gutierrez V, Ros E, Salas-Salvadó J, Fiol M, Solá R, Covas MI; for the PREDIMED Study Investigators. Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. Arch Intern Med. ***2007***;167(11):1195-203.

Fki I, Sahnoun Z, Sayadi S. Hypocholesterolemic effects of phenolic extracts and purified hydroxytyrosol recovered from olive mill wastewater in rats fed a cholesterol-rich diet. J Agric Food Chem. ***2007***;55(3):624-31.

Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnol Lett. ***2006***;28(19):1601-13.

Fogelman AM. When good cholesterol goes bad. Nat Med. ***2004***;10(9):902-3.

Franconi F, Coinu R, Carta S, Urgeghe PP, Ieri F, Mulinacci N, Romani A. Antioxidant effect of two virgin olive oils depends on the concentration and composition of minor polar compounds. J Agric Food Chem. ***2006***;54(8):3121-5.

Frankel EN, Meyer AS. The problem of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. J Sci Food Agric. ***2000***;80:1925-41.

Frei B, Gaziano JM. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. J Lipid Res. ***1993***;34(12):2135-45.

Fujisawa S, Atsumi T, Murakami Y, Kadoma Y. Dimerization. ROS formation, and biological activity of o-methoxyphenols. Arch Immunol Ther Exp (Warsz). ***2005***;53:28-38.

Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME. Human sulfotransferases and their role in chemical metabolism. Toxicol Sci. ***2006***;90(1):5-22.

García-Cañas V, Simó C, León C, Cifuentes A. Advances in Nutrigenomics research: novel and future analytical approaches to investigate the biological activity of natural compounds and food functions. J Pharm Biomed Anal. ***2010*** Jan 20;51(2):290-304.

Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal. ***2007***;19(9):1807-19.

Gimeno E, de la Torre-Carbot K, Lamuela-Raventós RM, Castellote AI, Fitó M, de la Torre R, Covas MI, López-Sabater MC. Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial. Br J Nutr. ***2007***;98(6):1243-50.

Giovannini C, Scazzocchio B, Varì R, Santangelo C, D'Archivio M, Masella R. Apoptosis in cancer and atherosclerosis: polyphenol activities. Ann Ist Super Sanita. ***2007***;43(4):406-16.

Giovannini C, Straface E, Modesti D, Coni E, Cantafora A, De Vincenzi M, Malorni W, Masella R. Tyrosol, the major olive oil biophenol, protects against oxidized-LDL-induced injury in Caco-2 cells. J Nutr. ***1999***;129(7):1269-77.

Giustarini D, Dalle-Donne I, Tsikas D, Rossi R. Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers. Crit Rev Clin Lab Sci. ***2009***;46(5-6):241-81.

Gómez-Alonso S, Salvador MD, Fregapane G. Phenolic compounds profile of cornicabra virgin olive oil. J Agric Food Chem. ***2002***;50(23):6812-7.

Gómez-Rico A, Salvador MD, La Greca M, Fregapane G. Phenolic and volatile compounds of extra virgin olive oil (Olea europaea L. Cv. Cornicabra) with regard to fruit ripening and irrigation management. J Agric Food Chem. ***2006***;54(19):7130-6.

González-Correa JA, Muñoz-Marín J, Arrebola MM, Guerrero A, Narbona F, López-Villodres JA, De La Cruz JP. Dietary virgin olive oil reduces oxidative stress and cellular damage in rat brain slices subjected to hypoxia-reoxygenation. Lipids. ***2007***;42(10):921-9.

González-Correa JA, Navas MD, Muñoz-Marín J, Trujillo M, Fernández-Bolaños J, de la Cruz JP.Effects of hydroxytyrosol and hydroxytyrosol acetate administration to rats on platelet function compared to acetylsalicylic acid. J Agric Food Chem. **2008;**56(17):7872-6.

González-Santiago M, Fonollá J, Lopez-Huertas E. Human absorption of a supplement containing purified hydroxytyrosol, a natural

antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. Pharmacol Res. ***2010*** [Epub ahead of print].

González-Santiago M, Martín-Bautista E, Carrero JJ, Fonollá J, Baró L, Bartolomé MV, Gil-Loyzaga P, López-Huertas E. One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development. Atherosclerosis. ***2006***;188(1):35-42.

Gorinstein S, Leontowicz H, Lojek A, Leontowicz M, Ciz M, Krzeminski R, Gralak M, Czerwinski J, Jastrzebski Z, Trakhtenberg S, Grigelmo-Miguel N, Soliva-Fortuny R, Martin-Belloso O. Olive oils improve lipid metabolism and increase antioxidant potential in rats fed diets containing cholesterol. J Agric Food Chem. ***2002***;50(21):6102-8.

Gorinstein S, Martin-Belloso O, Katrich E, Lojek A, Cíz M, Gligelmo-Miguel N, Haruenkit R, Park YS, Jung ST, Trakhtenberg S. Comparison of the contents of the main biochemical compounds and the antioxidant activity of some Spanish olive oils as determined by four different radical scavenging tests. J Nutr Biochem. ***2003***;14(3):154-9.

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(2):70-8.

Grasso S, Siracusa L, Spatafora C, Renis M, Tringali C. Hydroxytyrosol lipophilic analogues: enzymatic synthesis, radical scavenging activity and DNA oxidative damage protection. Bioorg Chem. ***2007***;35(2):137-52.

Grewal A, Lambert P, Stockton J. Analysis of expression data: an overview. Curr Protoc Hum Genet. ***2007***;Chapter 11:Unit11.4.

Grizis C, Atta-Politou J, Koupparis M, Simultaneous determination of oleuropein and tyrosol in plasma using high performance liquid chromatography with UV detection. J Liquid Chromatogr Related Technol. ***2003;***26:599-616.

Guillén N, Acín S, Navarro MA, Surra JC, Arnal C, Lou-Bonafonte JM, Muniesa P, Martínez-Gracia MV, Osada J. Knowledge of the biological actions of extra virgin olive oil gained from mice lacking apolipoprotein E. Rev Esp Cardiol. ***2009***;62(3):294-304.

Gutiérrez-Rosales F, Ríos JJ, Gómez-Rey ML. Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electrospray ionization mass spectrometry. J Agric Food Chem. ***2003***;51(20):6021-5.

Halliwell B, Aeschbach R, Löliger J, Aruoma OI. The characterization of antioxidants. Food Chem Toxicol. ***1995***;33(7):601-17.

Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. ***2004***;142(2):231-55.

Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch Biochem Biophys. ***2008***;476(2):107-12.

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

Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. ***2005***;352(16):1685-95.

Hashimoto T, Ibi M, Matsuno K, Nakashima S, Tanigawa T, Yoshikawa T, Yabe-Nishimura C. An endogenous metabolite of dopamine, 3,4-dihydroxyphenylethanol, acts as a unique cytoprotective agent against oxidative stress-induced injury. Free Radic Biol Med. ***2004***;36(5):555-64.

Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest. ***1997***;99(9):2075-81.

Hider RC, Liu ZD, Khodr HH. Metal chelation of polyphenols. Methods Enzymol. ***2001***;335:190-203.

Hillestrøm PR, Covas MI, Poulsen HE. Effect of dietary virgin olive oil on urinary excretion of etheno-DNA adducts. Free Radic Biol Med. ***2006***;41(7):1133-8.

Hocquette JF, Cassar-Malek I, Scalbert A, Guillou F. Contribution of genomics to the understanding of physiological functions. J Physiol Pharmacol. ***2009***;60 Suppl 3:5-16**.**

Holst B, Williamson G. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Curr Opin Biotechnol. ***2008***;19(2):73-82.

Hotta H, Nagano S, Ueda M, Tsujino Y, Koyama J, Osakai T. Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation. Biochim Biophys Acta. ***2002***;1572(1):123-32.

Hrncirik K, Fritsche S. Relation between the endogenous antioxidant system and the quality of extra virgin olive oil under accelerated storage conditions. J Agric Food Chem. ***2005***;53(6):2103-10.

Hu FB, Willett WC. Optimal diets for prevention of coronary heart disease. JAMA. ***2002***;288(20):2569-78.

Hu FB. Diet and lifestyle influences on risk of coronary heart disease. Curr Atheroscler Rep. ***2009***;11(4):257-63.

Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem. ***2005***;53(6):1841-56.

Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. ***2009***;37(1):1-13.

Hyson D, Rutledge JC, Berglund L. Postprandial lipemia and cardiovascular disease. Curr Atheroscler Rep. ***2003***;5(6):437-44.

Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Res. ***2005***;33(6):e56.

Incani A, Deiana M, Corona G, Vafeiadou K, Vauzour D, Dessì MA, Spencer JP. Involvement of ERK, Akt and JNK signalling in H(2)O(2)-induced cell injury and protection by hydroxytyrosol and its metabolite homovanillic alcohol. Mol Nutr Food Res. ***2009*;** [Epub ahead of print].

Jensen SR, Franzyk H, Wallander E. Chemotaxonomy of the Oleaceae: iridoids as taxonomic markers. Phytochemistry. ***2002***;60(3):213-31.

Jessup W, Kritharides L, Stocker R. Lipid oxidation in atherogenesis: an overview. Biochem Soc Trans. ***2004***;32(Pt 1):134-8.

Kalua CM, Bedgood DR Jr, Bishop AG, Prenzler PD. Changes in volatile and phenolic compounds with malaxation time and temperature during virgin olive oil production. J Agric Food Chem. ***2006***;54(20):7641-51.

Katan MB, Zock PL, Mensink RP. Dietery oils, serum lipoproteins, and coronary heart disease. Am J Clin Nutr. ***1995***;61:1368S-73S.

Keski-Hynnilä H, Raana K, Taskinen J, Kostiainen R. Direct analysis of nitrocatechol-type glucuronides in urine by capillary electrophoresis-electrospray ionisation mass spectrometry and tandem mass spectrometry. J Chromatogr B Biomed Sci Appl. ***2000***;749(2):253-63.

Keys A. Seven countries: a multivariate analysis of death and coronary heart disease. London: Harvard University Press, ***1980***.

Kiewe P, Gueller S, Komor M, Stroux A, Thiel E, Hofmann WK. Prediction of qualitative outcome of oligonucleotide microarray hybridization by measurement of RNA integrity using the 2100 Bioanalyzer capillary electrophoresis system. Ann Hematol. ***2009***;88(12):1177-83.

Knasmüller S, Nersesyan A, Misík M, Gerner C, Mikulits W, Ehrlich V, Hoelzl C, Szakmary A, Wagner KH. Use of conventional and -omics based methods for health claims of dietary antioxidants: a critical overview. Br J Nutr. ***2008***;99 E Suppl 1:ES3-52.

Kohyama N, Nagata T, Fujimoto S, Sekiya K. Inhibition of arachidonate lipoxygenase activities by 2-(3,4-dihydroxyphenyl)ethanol, a phenolic compound from olives. Biosci Biotechnol Biochem. ***1997***;61(2):347-50.

Konno K, Hirayama C, Yasui H, Nakamura M. Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree. Proc Natl Acad Sci U S A. ***1999***;96(16):9159-64.

Koster H, Halsema I, Scholtens E, Knippers M, Mulder GJ.Dose-dependent shifts in the sulfation and glucuronidation of phenolic compounds in the rat in vivo and in isolated hepatocytes. The role of saturation of phenolsulfotransferase. Biochem Pharmacol. ***1981***;30(18):2569-75.

Kregel KC, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. Am J Physiol Regul Integr Comp Physiol. ***2007***;292(1):R18-36.

Krzeminski R, Gorinstein S, Leontowicz H, Leontowicz M, Gralak M, Czerwinski J, Lojek A, Cíz M, Martin-Belloso O, Gligelmo-Miguel N, Trakhtenberg S. Effect of different olive oils on bile excretion in rats fed cholesterol-containing and cholesterol-free diets. J Agric Food

Chem. ***2003***;51(19):5774-9.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction. Mol Aspects Med. ***2006***;27(2-3):95-125.

Kubo I, Matsumoto A, Takase I. A multichemical defense mechanism of bitter olive *Olea europea* (Oleaceae). Is Oleuropein a phytoalexin precursor? J Chem Ecol ***1985;***11:251-263.

Kuzuya M, Yamada K, Hayashi T, Funaki C, Naito M, Asai K, Kuzuya F. Role of lipoprotein-copper complex in copper catalyzed-peroxidation of low-density lipoprotein. Biochim Biophys Acta. ***1992***;1123(3):334-41.

Landschulz KT, Jump DB, MacDougald OA, Lane MD. Transcriptional control of the stearoyl-CoA desaturase-1 gene by polyunsaturated fatty acids. Biochem Biophys Res Commun. ***1994***;200(2):763-8.

Lee OH, Lee BY, Kim YC, Shetty K, Kim YC. Radical scavenging-linked antioxidant activity of ethanolic extracts of diverse types of extra virgin olive oils. J Food Sci. ***2008***;73(7):C519-25.

Lee OH, Lee BY, Lee J, Lee HB, Son JY, Park CS, Shetty K, Kim YC. Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. Bioresour Technol. ***2009***;100(23):6107-13.

Lee-Huang S, Huang PL, Zhang D, Lee JW, Bao J, Sun Y, Chang YT, Zhang J, Huang PL Discovery of small-molecule HIV-1 fusion and integrase inhibitors oleuropein and hydroxytyrosol: Part I. fusion [corrected] inhibition. Biochem Biophys Res Commun. ***2007***;354(4):872-8.

Lee-Huang S, Huang PL, Zhang D, Lee JW, Bao J, Sun Y, Chang YT, Zhang J, Huang PL. Discovery of small-molecule HIV-1 fusion and integrase inhibitors oleuropein and hydroxytyrosol: part II. integrase inhibition. Biochem Biophys Res Commun. ***2007***;354(4):879-84.

Leenen R, Roodenburg AJ, Vissers MN, Schuurbiers JA, van Putte KP, Wiseman SA, van de Put FH. Supplementation of plasma with olive oil phenols and extracts: influence on LDL oxidation. J Agric Food Chem. ***2002***;50(5):1290-7.

Levenson JW, Skerrett PJ, Gaziano JM. Reducing the global burden of cardiovascular disease: the role of risk factors. Prev Cardiol. ***2002***;5(4):188-99.

Levine RL, Stadtman ER. Oxidative modification of proteins during aging. Exp Gerontol. ***2001***;36(9):1495-502.

Levsen K, Schiebel HM, Behnke B, Dötzer R, Dreher W, Elend M, Thiele H. Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. J Chromatogr A. ***2005***;1067(1-2):55-72.

Li Y, Hou MJ, Ma J, Tang ZH, Zhu HL, Ling WH. Dietary fatty acids regulate cholesterol induction of liver CYP7alpha1 expression and bile acid production. Lipids. ***2005***;40(5):455-62.

López-Miranda J, Delgado-Lista J, Perez-Martinez P, Jimenez-Gómez Y, Fuentes F, Ruano J, Marin C.Olive oil and the haemostatic system. Mol Nutr Food Res. ***2007***;51(10):1249-59.

Machowetz A, Poulsen HE, Gruendel S, Weimann A, Fitó M, Marrugat J, de la Torre R, Salonen JT, Nyyssönen K, Mursu J, Nascetti S, Gaddi A, Kiesewetter H, Bäumler H, Selmi H, Kaikkonen J, Zunft HJ, Covas MI, Koebnick C. Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans. FASEB J. ***2007***;21(1):45-52.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr. ***2004***;79(5):727-47.

Manna C, D'Angelo S, Migliardi V, Loffredi E, Mazzoni O, Morrica P, Galletti P, Zappia V. Protective effect of the phenolic fraction from virgin olive oils against oxidative stress in human cells. J Agric Food Chem. ***2002***;50(22):6521-6.

Manna C, Galletti P, Cucciolla V, Montedoro G, Zappia V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. J Nutr Biochem. ***1999***;10(3):159-65.

Manna C, Galletti P, Maisto G, Cucciolla V, D'Angelo S, Zappia V. Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. FEBS Lett. ***2000***;470(3):341-4.

Manna C, Migliardi V, Golino P, Scognamiglio A, Galletti P, Chiariello M, Zappia V. Oleuropein prevents oxidative myocardial injury induced by ischemia and reperfusion. J Nutr Biochem. ***2004***;15(8):461-6.

Manna C, Napoli D, Cacciapuoti G, Porcelli M, Zappia V. Olive oil phenolic compounds inhibit homocysteine-induced endothelial cell adhesion regardless of their different antioxidant activity. J Agric Food Chem. ***2009***;57(9):3478-82.

Marnett LJ, Plastaras JP. Endogenous DNA damage and mutation. Trends Genet. ***2001***;17(4):214-21.

Marrugat J, Covas MI, Fitó M, Schröder H, Miró-Casas E, Gimeno E, López-Sabater MC, de la Torre R, Farré M; SOLOS Investigators. Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation--a randomized controlled trial. Eur J Nutr. ***2004***;43(3):140-7.

Martínez-Domínguez E, de la Puerta R, Ruiz-Gutiérrez V. Protective effects upon experimental inflammation models of a polyphenol-supplemented virgin olive oil diet. Inflamm Res. ***2001***;50(2):102-6.

Masella R, Cantafora A, Modesti D, Cardilli A, Gennaro L, Bocca A, Coni E. Antioxidant activity of 3,4-DHPEA-EA and protocatechuic acid: a comparative assessment with other olive oil biophenols. Redox Rep. ***1999***;4(3):113-21.

Masella R, Varì R, D'Archivio M, Di Benedetto R, Matarrese P, Malorni W, Scazzocchio B, Giovannini C. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J Nutr. ***2004***;134(4):785-91.

Mateos R, Domínguez MM, Espartero JL, Cert A. Antioxidant effect of phenolic compounds, alpha-tocopherol, and other minor components in virgin olive oil. J Agric Food Chem. ***2003***;51(24):7170-5.

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(26):9897-905.

Mazziotti A, Mazzotti F, Pantusa M, Sportelli L, Sindona G. Pro-oxidant activity of oleuropein determined in vitro by electron spin resonance spin-trapping methodology. J Agric Food Chem. ***2006***;54(20):7444-9**.**

Menendez JA, Lupu R. Mediterranean dietary traditions for the molecular treatment of human cancer: anti-oncogenic actions of the main olive oil's monounsaturated fatty acid oleic acid (18:1n-9). Curr Pharm Biotechnol. ***2006***;7(6):495-502.

Menendez JA, Vazquez-Martin A, Oliveras-Ferraros C, Garcia-Villalba R, Carrasco-Pancorbo A, Fernandez-Gutierrez A, Segura-Carretero A. Extra-virgin olive oil polyphenols inhibit HER2 (erbB-2)-induced malignant transformation in human breast epithelial cells: relationship between the chemical structures of extra-virgin olive oil

secoiridoids and lignans and their inhibitory activities on the tyrosine kinase activity of HER2. Int J Oncol. ***2009***;34(1):43-51.

Menendez JA, Vazquez-Martin A, Oliveras-Ferraros C, Garcia-Villalba R, Carrasco-Pancorbo A, Fernandez-Gutierrez A, Segura-Carretero A. Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. Int J Mol Med. ***2008***;22(4):433-9.

Miles EA, Zoubouli P, Calder PC. Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. Nutrition. ***2005a***;21(3):389-94.

Miles EA, Zoubouli P, Calder PC. Effects of polyphenols on human Th1 and Th2 cytokine production. Clin Nutr. ***2005b***;24(5):780-4.

Miró-Casas E, Covas MI, Farre M, Fitó M, Ortuño J, Weinbrenner T, Roset P, de la Torre R. Hydroxytyrosol disposition in humans. Clin Chem. ***2003a***;49(6 Pt 1):945-52.

Miró-Casas E, Covas MI, Fitó M, Farré-Albadalejo M, Marrugat J, de la Torre R. Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. Eur J Clin Nutr. ***2003***;57(1):186-90.

Miró-Casas E, Farré Albadalejo M, Covas Planells MI, Fitó Colomer M, Lamuela Raventós RM, de la Torre Fornell R. Tyrosol bioavailability in humans after ingestion of virgin olive oil. Clin Chem. ***2001***;47(2):341-3.

Miró-Casas E, Farré Albaladejo M, Covas MI, Rodriguez JO, Menoyo Colomer E, Lamuela Raventós RM, de la Torre R. Capillary gas chromatography-mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake. Anal Biochem. ***2001b***;294(1):63-72.

Montedoro G, Servili M, Baldioli M, Miniati E. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. J. Agric. Food Chem. ***1992;***40(9):1571–1576.

Moschandreas J, Vissers MN, Wiseman S, van Putte KP, Kafatos A. Extra virgin olive oil phenols and markers of oxidation in Greek smokers: a randomized cross-over study. Eur J Clin Nutr. ***2002***;56(10):1024-9.

Müller M, Kersten S. Nutrigenomics: goals and strategies. Nat Rev Genet. ***2003***;4(4):315-22.

Muyal JP, Singh SK, Fehrenbach H. DNA-microarray technology: comparison of methodological factors of recent technique towards gene expression profiling. Crit Rev Biotechnol. ***2008***;28(4):239-51.

Naderi A, Ahmed AA, Barbosa-Morais NL, Aparicio S, Brenton JD, Caldas C. Expression microarray reproducibility is improved by optimising purification steps in RNA amplification and labelling. BMC Genomics. ***2004***;5(1):9.

Nair S, Li W, Kong AN. Natural dietary anti-cancer chemopreventive compounds: redox-mediated differential signaling mechanisms in cytoprotection of normal cells versus cytotoxicity in tumor cells. Acta Pharmacol Sin. ***2007***;28(4):459-72.

Nenadis N, Tsimidou M. Observation of the estimation of scavenging activity of phenolic compounds using rapid 1,1-diphenyl-2-picrylhydrazyl (DPPH) tests. JAOCS. ***2002***;79(12):1191-5.

Nenadis N, Wang LF, Tsimidou MZ, Zhang HY. Radical scavenging potential of phenolic compounds encountered in O. europaea products as indicated by calculation of bond dissociation enthalpy and ionization potential values. J Agric Food Chem. ***2005***;53(2):295-9.

Nenadis N, Zafiropoulou I, Tsimidou M. Commonly used food antioxidants: a comparative study in dispersed systems. Food Chem ***2003***;82(3):403-7.

Nicholson SK, Tucker GA, Brameld JM. Effects of dietary polyphenols on gene expression in human vascular endothelial cells. Proc Nutr Soc. ***2008***;67(1):42-7.

Nicolaïew N, Lemort N, Adorni L, Berra B, Montorfano G, Rapelli S, Cortesi N, Jacotot B. Comparison between extra virgin olive oil and oleic acid rich sunflower oil: effects on postprandial lipemia and LDL susceptibility to oxidation. Ann Nutr Metab. ***1998***;42(5):251-60.

Niki E. Antioxidants in relation to lipid peroxidation. Chem Phys Lipids. ***1987***;44(2-4):227-53.

Niki E. Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med. ***2009***;47(5):469-84.

Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. ***2006***;1(3):1559-82.

Nousis L, Doulias PT, Aligiannis N, Bazios D, Agalias A, Galaris D, Mitakou S. DNA protecting and genotoxic effects of olive oil related components in cells exposed to hydrogen peroxide. Free Radic Res. ***2005***;39(7):787-95.

Obied HK, Prenzler PD, Konczak I, Rehman AU, Robards K. Chemistry and bioactivity of olive biophenols in some antioxidant and antiproliferative in vitro bioassays. Chem Res Toxicol. ***2009***;22(1):227-34.

Ochoa JJ, Quiles JL, Ramírez-Tortosa MC, Mataix J, Huertas JR. Dietary oils high in oleic acid but with different unsaponifiable fraction contents have different effects in fatty acid composition and peroxidation in rabbit LDL. Nutrition. ***2002***;18(1):60-5.

O'Dowd Y, Driss F, Dang PM, Elbim C, Gougerot-Pocidalo MA, Pasquier C, El-Benna J. Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: scavenging of hydrogen peroxide but not superoxide anion produced by human neutrophils. Biochem Pharmacol. ***2004***;68(10):2003-8.

O'Keefe JH, Bell DS. Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor. Am J Cardiol. ***2007***;100(5):899-904.

Okogeri O, Tasioula-Margari M. Changes occurring in phenolic compounds and alpha-tocopherol of virgin olive oil during storage. J Agricul and Food Chem, ***2002;***50:1077-80.

Ortega RM. Importance of functional foods in the Mediterranean diet. Public Health Nutr. ***2006***;9(8A):1136-40.

Osada J, Fernandez-Sanchez A, Diaz-Morillo JL, Aylagas H, Miro-Obradors MJ, Ordovas JM, Palacios-Alaiz E. Hepatic expression of apolipoprotein A-I gene in rats is upregulated by monounsaturated fatty acid diet. Biochem Biophys Res Commun. ***1991***;180(1):162-8.

Owen RW, Mier W, Giacosa A, Hull WE, Spigelhalder B, Bartsch H. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoroids, lignans and squalene. Food Chem Toxicol. ***2000***;38:647-659.

Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol. ***2002***;64(5-6):765-70.

Paiva-Martins F, Fernandes J, Rocha S, Nascimento H, Vitorino R, Amado F, Borges F, Belo L, Santos-Silva A. Effects of olive oil polyphenols on erythrocyte oxidative damage. Mol Nutr Food Res.

2009;53(5):609-16.

Paiva-Martins F, Gordon MH, Gameiro P. Activity and location of olive oil phenolic antioxidants in liposomes. Chem Phys Lipids. ***2003***;124(1):23-36.

Paiva-Martins F, Gordon MH. Interactions of ferric ions with olive oil phenolic compounds. J Agric Food Chem. ***2005***;53(7):2704-9.

Paiva-Martins F, Santos V, Mangericão H, Gordon MH. Effects of copper on the antioxidant activity of olive polyphenols in bulk oil and oil-in-water emulsions. J Agric Food Chem. ***2006***;54(10):3738-43.

Parthasarathy S, Raghavamenon A, Garelnabi MO, Santanam N. Oxidized low-density lipoprotein. Methods Mol Biol. ***2010***;610:403-17.

Petroni A, Blasevich M, Papini N, Salami M, Sala A, Galli C. Inhibition of leukocyte leukotriene B4 production by an olive oil-derived phenol identified by mass-spectrometry. Thromb Res. ***1997***;87(3):315-22.

Petroni A, Blasevich M, Salami M, Papini N, Montedoro GF, Galli C Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil.Thromb Res. ***1995***;78(2):151-60.

Poulsen HE, Prieme H, Loft S. Role of oxidative DNA damage in cancer initiation and promotion. Eur J Cancer Prev. ***1998***;7(1):9-16.

Poulsen HE. Oxidative DNA modifications. Exp Toxicol Pathol. ***2005***;57(Suppl)1:161-9.

Pripp AH, Vreeker R, van Duynhoven J. Binding of olive oil phenolics to food proteins. J Sci Food Agric ***2005;***85:354-62.

Puglia CD, Powell SR. Inhibition of cellular antioxidants: a possible mechanism of toxic cell injury. Environ. Health Perspect. ***1984***;57:307–11.

Quiles JL, Farquharson AJ, Simpson DK, Grant I, Wahle KW. Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. Br J Nutr. ***2002***;88(3):225-34.

Raclot T, Groscolas R, Langin D, Ferré P Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. J Lipid Res. ***1997***;38(10):1963-72.

Rainen L, Oelmueller U, Jurgensen S, Wyrich R, Ballas C, Schram J, Herdman C, Bankaitis-Davis D, Nicholls N, Trollinger D, Tryon V. Stabilization of mRNA expression in whole blood samples. Clin

Chem. ***2002***;48(11):1883-90.

Ramirez-Tortosa MC, Urbano G, López-Jurado M, Nestares T, Gomez MC, Mir A, Ros E, Mataix J, Gil A. Extra-virgin olive oil increases the resistance of LDL to oxidation more than refined olive oil in free-living men with peripheral vascular disease. J Nutr. ***1999***;129(12):2177-83.

Rechner AR, Smith MA, Kuhnle G, Gibson GR, Debnam ES, Srai SK, Moore KP, Rice-Evans CA. Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. Free Radic Biol Med. ***2004***;36(2):212-25**.**

Recknagel RO, Glende EA. Spectrophotometric detection of lipid conjugated dienes. Methods Enzymol. ***1984***;105:331-7.

Ren B, Thelen AP, Peters JM, Gonzalez FJ, Jump DB. Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor alpha. J Biol Chem. ***1997***;272(43):26827-32.

Rietjens SJ, Bast A, Haenen GR. New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. J Agric Food Chem. ***2007***;55(18):7609-14.

Roche HM, Gibney MJ. The impact of postprandial lipemia in accelerating atherothrombosis. J Cardiovasc Risk. ***2000***;7(5):317-24.

Roche HM. Dietary lipids and gene expression. Biochem Soc Trans. ***2004***;32(Pt 6):999-1002.

Roche M, Dufour C, Mora N, Dangles O. Antioxidant activity of olive phenols: mechanistic investigation and characterization of oxidation products by mass spectrometry. Org Biomol Chem. ***2005***;3(3):423-30.

Rockett JC, Hellmann GM. Confirming microarray data--is it really necessary? Genomics. ***2004***;83(4):541-9.

Rodríguez Madrera R, Picinelli Lobo A, Suárez Valles B. Phenolic profile of Asturian (Spain) natural cider. J Agric Food Chem. ***2006***;54(1):120-4.

Romero C, Brenes M, Yousfi K, García P, García A, Garrido A. Effect of cultivar and processing method on the contents of polyphenols in table olives. J Agric Food Chem. ***2004***;52(3):479-84.

Rometo C, Brenes M, Garcia P, Garrido A. Hydroxytyrosol 4-b-glucoside, an important phenolic compound in olive fruits and derived

products. J Agric Food Chem. ***2002***;50:3835-39.

Ruano J, López-Miranda J, de la Torre R, Delgado-Lista J, Fernández J, Caballero J, Covas MI, Jiménez Y, Pérez-Martínez P, Marín C, Fuentes F, Pérez-Jiménez F. Intake of phenol-rich virgin olive oil improves the postprandial prothrombotic profile in hypercholesterolemic patients. Am J Clin Nutr. ***2007***;86(2):341-6.

Ruano J, Lopez-Miranda J, Fuentes F, Moreno JA, Bellido C, Perez-Martinez P, Lozano A, Gómez P, Jiménez Y, Pérez Jiménez F. Phenolic content of virgin olive oil improves ischemic reactive hyperemia in hypercholesterolemic patients. J Am Coll Cardiol. ***2005***;46(10):1864-8.

Ruiz-Gutiérrez V, Juan ME, Cert A, Planas JM. Determination of hydroxytyrosol in plasma by HPLC. Anal Chem. ***2000***;72(18):4458-61.

Sachidanandam K, Fagan SC, Ergul A. Oxidative stress and cardiovascular disease: antioxidants and unresolved issues. Cardiovasc Drug Rev. ***2005***;23(2):115-32.

Saija A, Trombetta DA, Tomaino A, Lo Cascio R, Princi P, Uccella N, Bonina F, Castelli F. In vitro evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. Int. J. Pharm. ***1998***;166:123–33.

Salvini S, Sera F, Caruso D, Giovannelli L, Visioli F, Saieva C, Masala G, Ceroti M, Giovacchini V, Pitozzi V, Galli C, Romani A, Mulinacci N, Bortolomeazzi R, Dolara P, Palli D. Daily consumption of a high-phenol extra-virgin olive oil reduces oxidative DNA damage in postmenopausal women. Br J Nutr. ***2006***;95(4):742-51.

Sánchez-Moreno C. Review: methods used to evaluate the free radical scavenging activity in food and biological systems. Food Sci Tech Int. ***2002***;8(3):121-37.

Santangelo C, Varì R, Scazzocchio B, Di Benedetto R, Filesi C, Masella R. Polyphenols, intracellular signalling and inflammation. Ann Ist Super Sanita. ***2007***;43(4):394-405.

Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: antioxidants and beyond. Am J Clin Nutr. ***2005***;81(1 Suppl):215S-7S.

Scalbert A, Knasmüller S. Genomic effects of phytochemicals and their implication in the maintenance of health. Br J Nutr. **2008**;99 E Suppl 1:ES1-2.

Schaffer S, Podstawa M, Visioli F, Bogani P, Müller WE, Eckert GP. Hydroxytyrosol-rich olive mill wastewater extract protects brain cells in vitro and ex vivo. J Agric Food Chem. ***2007***;55(13):5043-9.

Schmitt CA, Handler N, Heiss EH, Erker T, Dirsch VM. No evidence for modulation of endothelial nitric oxide synthase by the olive oil polyphenol hydroxytyrosol in human endothelial cells. Atherosclerosis. ***2007***;195(1):e58-64.

Scholz S, Williamson G. Interactions affecting the bioavailability of dietary polyphenols in vivo. Int J Vitam Nutr Res. ***2007***;77(3):224-35

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol. ***2006***;7:3.

Schweigert N, Zehnder AJ, Eggen RI. Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. Environ Microbiol. ***2001*** Feb;3(2):81-91.

Seifried HE, Anderson DE, Fisher EI, Milner JA. A review of the interaction among dietary antioxidants and reactive oxygen species. J Nutr Biochem. ***2007***;18(9):567-79.

Selvaggini R, Servili M, Urbani S, Esposto S, Taticchi A, Montedoro G. Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography with fluorometric detection.J Agric Food Chem. ***2006***;54(8):2832-8.

Servili M, Montedoro G. Contribution of phenolic compounds to virgin olive oil quality. Eur J Lipid Sci Technol, ***2002;***104:602-13.

Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro G, Morozzi G. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. J Chromatogr A. ***2004***;1054(1-2):113-27.

Servili M, Selvaggini R, Taticchi A, Esposto S, Montedoro G. Volatile compounds and phenolic composition of virgin olive oil: optimization of temperature and time of exposure of olive pastes to air contact during the mechanical extraction process. J Agric Food Chem. ***2003***;51(27):7980-8.

Sies H, Stahl W, Sevanian A. Nutritional, dietary and postprandial oxidative stress. J Nutr. ***2005***;135(5):969-72.

Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol. ***1997***;82(2):291-5.

Singh U, Jialal I. Oxidative stress and atherosclerosis. Pathophysiology. ***2006***;13(3):129-42.

Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. Meth in Enzymol ***1999***;299:152-78.

Soler-Rivas C, Espín JC, Wichers HJ. Oleuropein and related compounds. J Sci Food Agric. ***2000;***80:1013-23.

Son S, Lewis BA. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure-activity relationship. J Agric Food Chem. ***2002***;50(3):468-72.

Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. Chem Res Toxicol. ***1997***;10(5):485-94.

Steibel JP, Suchyta SP, Rosa GJM. Tackling high variability in gene expression studies. Drug Discovery & Development (online magazine), ***2005***, January 01.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL.Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med. ***1989***;320(14):915-24.

Steinberg D. Low density lipoprotein oxidation and its pathobiological significance.J Biol Chem. ***1997***;272(34):20963-6.

Steinbrecher UP, Witztum JL, Parthasarathy S, Steinberg D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. Arteriosclerosis. ***1987***;7(2):135-43.

Stephens JW, Khanolkar MP, Bain SC. The biological relevance and measurement of plasma markers of oxidative stress in diabetes and cardiovascular disease. Atherosclerosis. ***2009***;202(2):321-9.

Stupans I, Kirlich A, Tuck KL, Hayball PJ. Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants. J Agric Food Chem. ***2002***;50(8):2464-9.

Stupans I, Murray M, Kirlich A, Tuck KL, Hayball PJ. Inactivation of cytochrome P450 by the food-derived complex phenol oleuropein. Food Chem Toxicol. ***2001***;39(11):1119-24.

Suárez M, Macià A, Romero MP, Motilva MJ. Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. J Chromatogr A. ***2008***;1214(1-2):90-9.

Suárez M, Romero MP, Macià A, Valls RM, Fernández S, Solà R, Motilva MJ. 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. J Chromatogr B Analyt Technol Biomed Life Sci. ***2009***;877(32):4097-106.

Tan HW, Tuck KL, Stupans I, Hayball PJ. Simultaneous determination of oleuropein and hydroxytyrosol in rat plasma using liquid chromatography with fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci. ***2003***;785(1):187-91.

Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B. Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. Am J Clin Nutr. ***2002***;75(1):119-25.

Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med. ***2003***;348:2599–608.

Trichopoulou A, Kouris-Blazos A, Wahlovist ML, Gnardellis C, Lagiou P, Polychronopoulos E, et al. Diet and overall survival in elderly people. BMJ ***1995***;311:1457–60.

Trichopoulou A, Lagiou P, Kuper H, Trichopoulos D. Cancer and Mediterranean dietary traditions. Cancer Epidemiol Biomarkers Prev. ***2000***;9(9):869-73.

Trichopoulou A. Mediterranean diet: the past and the present. Nutr Metab Cardiovasc Dis. ***2001***;11(4 Suppl):1-4.

Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S, La Guardia M. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutr Res Rev. ***2005***;18(1):98-112.

Tsarbopoulos A, Gikas E, Papadopoulos N, Aligiannis N, Kafatos A. Simultaneous determination of oleuropein and its metabolites in plasma by high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. ***2003***;785(1):157-64.

Tsimidou M. Polyphenols and quality of virgin olive oil in retrospect. Ital J Food Sci, ***1998***;2:99-116.

Tuck KL, Freeman MP, Hayball PJ, Stretch GL, Stupans I. The in vivo fate of hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and oral dosing of labeled compounds to rats. J Nutr. ***2001***;131(7):1993-6.

Tuck KL, Hayball PJ, Stupans I. Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. J Agric Food Chem. ***2002***;50(8):2404-9.

Turner R, Etienne N, Alonso MG, de Pascual-Teresa S, Minihane AM, Weinberg PD, Rimbach G. Antioxidant and antiatherogenic activities of olive oil phenolics. Int J Vitam Res. ***2005***;75(1):61-70.

Valavanidis A, Nisiotou C, Papageorgiou Y, Kremli I, Satravelas N, Zinieris N, Zygalaki H. Comparison of the radical scavenging potential of polar and lipidic fractions of olive oil and other vegetable oils under normal conditions and after thermal treatment. J Agric Food Chem. ***2004***;52(8):2358-65.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. ***2007***;39(1):44-84.

Vanden Heuvel JP. Cardiovascular disease-related genes and regulation by diet. Curr Atheroscler Rep. ***2009***;11(6):448-55.

Velasco J, Dobarganes C. Oxidative stability of virgen olive oil. Eur J of Lip Sci ***2002***;104(9-10):661-78.

Victor VM, Rocha M, Solá E, Bañuls C, Garcia-Malpartida K, Hernández-Mijares A.Oxidative stress, endothelial dysfunction and atherosclerosis. Curr Pharm Des. ***2009***;15(26):2988-3002.

Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochem Biophys Res Commun. ***1998***;247(1):60-4.

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

Visioli F, Bogani P, Grande S, Galli C. Olive oil and oxidative stress. Grasas Aceites. ***2004***;55:66-75.

Visioli F, Caruso D, Galli C, Viappiani S, Galli G, Sala A. Olive oils rich in natural catecholic phenols decrease isoprostane excretion in humans. Biochem Biophys Res Commun. ***2000***;278(3):797-9.

Visioli F, Caruso D, Grande S, Bosisio R, Villa M, Galli G, Sirtori C, Galli C. Virgin Olive Oil Study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. Eur J Nutr. ***2005***;44(2):121-7.

Visioli F, Caruso D, Plasmati E, Patelli R, Mulinacci N, Romani A, Galli G, Galli C. Hydroxytyrosol, as a component of olive mill waste water, is dose- dependently absorbed and increases the antioxidant capacity of rat plasma. Free Radic Res. ***2001***;34(3):301-5.

Visioli F, Galli C, Bornet F, Mattei A, Patelli R, Galli G, Caruso D. Olive oil phenolics are dose-dependently absorbed in humans. FEBS Lett. ***2000b***;468(2-3):159-60.

Visioli F, Galli C, Galli G, Caruso D. Biological activities and metabolic fate of olive oil phenols. Eur J Lipid Sci Technol. ***2002***;104:677-84.

Visioli F, Galli C, Grande S, Colonnelli K, Patelli C, Galli G, Caruso D. Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. J Nutr. ***2003***;133(8):2612-5.

Visioli F, Galli C. Olive oil phenols and their potential effects on human health. J. Agric. Food Chem. ***1998***;46:4292-6.

Visioli F, Poli A, Galli C. Antioxidant and other biological activities of phenols from olives and olive oil. Medicinal Research Reviews ***2002***;22:65-75.

Vissers MN, Zock PL, Katan MB. Bioavailability and antioxidant effects of olive oil phenols in humans: a review. Eur J Clin Nutr. ***2004***;58(6):955-65.

Vissers MN, Zock PL, Roodenburg AJ, Leenen R, Katan MB. Olive oil phenols are absorbed in humans. J Nutr. ***2002***;132(3):409-17.

Vissers MN, Zock PL, Wiseman SA, Meyboom S, Katan MB. Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers. Eur J Clin Nutr. ***2001***;55(5):334-41.

Weaver KL, Ivester P, Seeds M, Case LD, Arm JP, Chilton FH. Effect of dietary fatty acids on inflammatory gene expression in healthy humans. J Biol Chem. ***2009***;284(23):15400-7.

Wei YH. Oxidative stress and mitochondrial DNA mutations in human aging. Proc Soc Exp Biol Med. ***1998***;217(1):53-63.

Weinbrenner T, Fitó M, de la Torre R, Saez GT, Rijken P, Tormos C, Coolen S, Albaladejo MF, Abanades S, Schroder H, Marrugat J,

Covas MI. Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. J Nutr. ***2004a***;134(9):2314-21.

Weinbrenner T, Fitó M, Farré Albaladejo M, Saez GT, Rijken P, Tormos C, Coolen S, De La Torre R, Covas MI. Bioavailability of phenolic compounds from olive oil and oxidative/antioxidant status at postprandial state in healthy humans. Drugs Exp Clin Res. ***2004b***;30(5-6):207-12.

Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Crit Rev Food Sci Nutr. ***2004***;44(4):275-95.

Willett WC, *Sacks* F, Trichopoulou A, Drescher G, Ferro-Luzzi A, Helsing E, Trichopoulos D Mediterranean diet pyramid: a cultural model for healthy eating. Am J Clin Nutr. 1995;61(Suppl.6):1402S-6S.

Wiseman SA, Mathot JN, de Fouw NJ, Tijburg LB. Dietary non-tocopherol antioxidants present in extra virgin olive oil increase the resistance of low density lipoproteins to oxidation in rabbits. Atherosclerosis. ***1996***;120(1-2):15-23.

Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet. ***1994***;344(8925):793-5.

Yeh CT, Ching LC, Yen GC. Inducing gene expression of cardiac antioxidant enzymes by dietary phenolic acids in rats. J Nutr Biochem. ***2009***;20(3):163-71.

Yeomans VC, Linseisen J, Wolfram G. Interactive effects of polyphenols, tocopherol and ascorbic acid on the Cu2+-mediated oxidative modification of human low density lipoproteins. Eur J Nutr. ***2005***;44(7):422-8.

Young J, Wahle KW, Boyle SP. Cytoprotective effects of phenolic antioxidants and essential fatty acids in human blood monocyte and neuroblastoma cell lines: surrogates for neurological damage in vivo. Prostaglandins Leukot Essent Fatty Acids. ***2008***;78(1):45-59.

Zhang X, Cao J, Zhong L. Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. Naunyn Schmiedebergs Arch Pharmacol. ***2009***;379(6):581-6.

SUPPLEMENTARY MATERIAL

Supplemental Table I

Main reactive species (RS), their role in biological processes and potential targets of their oxidative activity.

Nomenclature of RS		Biological processes	Potential targets
Free Radicals	**Non-Radicals (Oxidants)**		
Reactive Oxygen Species **(ROS)**			
Superoxide, $O_2^{\bullet-}$ Hydroxyl, $OH^{\bullet}$ Hydroperoxyl, $HO_2^{\bullet}$ Carbonate, $CO_3^{\bullet-}$ Peroxyl, $RO_2^{\bullet}$ Alkoxyl, $RO^{\bullet}$ Carbon dioxide radical, $CO_2^{\bullet-}$ Singlet $O_2{}^1\Sigma g^+$	H_2O_2 Hypobromous acid, HOBr Hypochlorous acid, HOCl Ozone, O_3 Singlet oxygen ($O_2{}^1\Delta g$) Organic peroxides, ROOH Peroxynitrite, $ONOO^-$ Peroxynitrate, O_2NOO^- Peroxynitrous acid, ONOOH Peroxomonocarbonate, $HOOCO_2^-$	(i) roles in apoptosis (programmed cell death); (ii) induction of host defence genes; (iii) mobilisation of ion transport systems; (iv) roles in redox signaling or oxidative signaling	***Oxidative damage to***: (i) DNA and RNA; (ii) poly-unsaturated fatty acids (lipids); (iii) amino acids; (iv) inactivation of specific enzymes by co-factors oxidation; They are species causing ***oxidative stress***
Reactive Chlorine Species **(RCIS)**			
Atomic chlorine, $Cl^{\bullet}$	Hypochlorous acid, HOCl Nitryl chloride, NO_2Cl Chloramines Chlorine gas (Cl_2) Bromine chloride (BrCl) Chlorine dioxide (ClO_2)	(i) is generated during neutrophils activation; (ii) contributes to the destruction of bacteria	React with: (i) nucleic acids; (ii) fatty acid groups; (iii) cholesterol; (iv) proteins, etc.
Reactive Nitrogen Species **(RNS)**			
Nitric oxide, $NO^{\bullet}$ Nitrogen dioxide, $NO_2^{\bullet}$ Nitrate radical, $NO_3^{\bullet}$	Nitrous acid, HNO_2 Nitrosyl cation, NO^+ Nitroxyl anion, NO^- Dinitrogen tetroxide, N_2O_4 Dinitrogen trioxide, N_2O_3 Peroxynitrite, $ONOO^-$ Peroxynitrate, O_2NOO^- Peroxynitrous acid, ONOOH Alkyl peroxy-nitrites and -nitrates, ROONO RO_2ONO	(i) produced via the enzymatic activity of inducible nitric oxide synthase; (ii) are cellular or intracellular signaling molecule; (iii) in vasodilation; (iv) immune response;	***Nitrosative damage to***: (i) proteins; (ii) fatty acid of lipids; (iii) thiol modification; (iv) enzymes inhibition , etc. They are species causing ***nitrosative stress;***

Supplemental Table II

Randomized, crossover, controlled studies on the effects of phenolic compounds from olive oil on blood lipids, oxidation, inflammation, and oxidative damage (adapted from Covas, 2007 and Cicerale, 2009)

Treatment	Subjects (n, sex, status)	Phenols concentration	Monitored markers and parameters	Findings	Ref
Acute intervention studies					
EVOO[a] vs. OO[b] vs. CO[c] 50 ml	n = 12 (♂) healthy	607 mg/kg vs. 16 mg/kg vs. 0 mg/kg	Oxidative: urinary hydrogen peroxide levels; serum antioxidant capacity; Inflammatory: plasma TXB(2)[d] and LTB(4)[e]; Serum lipids: TC[f], HDL-C[g], LDL-C[h], TG[i], total plasma FA[j]	↓ in TXB_2 and LTB_4 with ↑ in phenolic content in olive oil and; ↑ plasma antioxidant capacity with ↑ in phenolic content in olive oil.	Bogani, 2007
High vs. Moderate vs. Low content of phenols in similar olive oils 40 ml	n = 12 ♂ healthy	366 mg/kg vs. 164 mg/kg vs. 2.7 mg/kg	Oxidative: 8-epi-$F_{2\alpha}$[k] and oxLDL[l] in plasma; phenolic compounds, lipid composition and 3-CT in LDL; Inflammatory: plasma TXB(2), LTB(4); Serum lipids: TC, HDL-C, TG, LDL-C;	↑ postprandial oxidative stress (by ↑ levels of $F_{2\alpha}$-isoprostanes) for all oils tested; ↓ the degree of LDL oxidation with ↑ of phenolic content in oils;	Covas, 2006a
VOO vs. OO 40 ml	n = 21 5 ♂ and 16 ♀ ***hypercholesterolemic***	400 mg/kg vs. 80 mg/kg	Oxidative: plasma 8-epi-$F_{2\alpha}$, LPO, $NO_{(x)}$; Serum lipids: TC, HDL-C, TG, LDL-C, TRL; Thrombogenic state: PAI-1[m], FVIIa[n].	↓ in LPO , ↓in 8-epi-$F_{2\alpha}$ and ↑ $NO_{(x)}$ production with ↑ phenolic compounds in breackfast meal; ↑ of phenolic compounds in breackfast meal is associated to ↓ postprandial thrombogenic state	Ruano, 2005 and 2007
Phenol-rich vs. Phenols poor olive oil 100 g,	n = 14 healthy ♂	303 mg/kg vs. 0.3 mg/kg	Oxidative: serum antioxidant capacity; antioxidant content of LDL and HDL;	↑ plasma antioxidant capacity due to the olive oil phenol content at 2h postprandial;	Bonanome, 2000
Different concentration of phenolics in olive oil of the same composition 50 ml	n = 6 ♂ healthy	1950 mg/kg vs. 1462.5 mg/kg vs. 975 mg/kg vs. 487.5 mg/kg	Oxidative: urinary 8-epi-$F_{2\alpha}$;	↓ in urinary 8-epi-$F_{2\alpha}$ dose dependent on the phenolic concentration in olive oils;	Visioli, 2000a
Short term intervention studies (up to 1 week)					
High vs. Moderate vs. Low content of phenols in similar olive oils 25 ml/day, 4 days	n = 12 healthy ♂	486 mg/kg vs. 133 mg/kg vs. 10 mg/kg	Oxidative: GSH-Px[o], GS-R[p] and oxLDL in plasma; 8-oxo-dG[q] in urine and mitDNA[r] of MNCs[s]; MDA and and 8-epi-$F_{2\alpha}$ in urine; Serum lipids: TC, HDL-C, TG, LDL-C;	↓plasma oxLDL, ↓ urinary 8-oxo-dG and ↑plasma HDL-C and ↑GSH-Px in doses dependent manner with ↑ of phenolic oils content	Weinbrenner, 2004a

Mid and long term intervention studies					
Phenol-rich vs. Phenols poor olive oil 50 g/day, 4 weeks	n = 14 healthy ♂	303 mg/kg vs. 0.3 mg/kg	Oxidative: oxLDL in plasma; antioxidant content of LDL and HDL; LDL resistance to oxidation;	No changes in LDL oxidability due to the phenol content of olive oil consumed	Bonanome, 2000
VOO vs. ROO[1] 50 ml/day, 3 weeks	n = 40 ***coronary heart disease*** ♂	161 mg/kg vs. 14.7 mg/kg	Oxidative: oxLDL, LPO, GSH-Px and GS-R in plasma; LDL resistance to oxidation; Serum lipids: TC, HDL-C, TG, LDL-C;	↓ plasma oxLDL and ↓ LPO with ↑ of oils phenolic content; ↑ GSH-Px with ↑ of oils phenolic content;	Fito, 2005
VOO vs. ROO 50 ml/day, 3 weeks	n = 28 ***stable coronary heart disease*** ♂	161 mg/kg vs. 14.7 mg/kg	Inflammatory: plasma IL-6, sICAM-1 and sVCAM-1, CRP[u] Serum lipids: TC, HDL-C, TG, LDL-C;	↓ plasma IL-6 and CRP with ↑ of oils phenolic content; No changes for sVCAM-1 and s-ICAM-1	Fito, 2008
High vs. Moderate vs. Low content of phenols in similar olive oils 25 ml/day, 3 weeks	n = 30 healthy ♂	150 mg/kg vs. 68 mg/kg vs. 0 mg/kg	Oxidative: oxLDL, in plasma; LDL resistance to oxidation; Serum lipids: TC, HDL-C, TG, LDL-C;	↑ HDL-C with ↑ of oils phenolic content; ↑ LDL resistance to oxidation with ↑ of oils phenolic content;	Marrugat, 2004
High vs. Low phenols content olive oils 70 g/day, 3 weeks	n = 25 healthy 11 ♂ and 14 ♀	308 mg/kg vs. 43 mg/kg	Oxidative: plasma antioxidant capacity; oxLDL;	No significant changes in oxLDL and antioxidant capacity of plasma;	Moschandreas, 2002
EVOO vs. ROO X ml/day, 12 weeks	n = 24 ♂ ***peripheral vascular disease***	800 mg/kg vs. 60 mg/kg	Oxidative: oxLDL in plasma; Serum lipids: TC, HDL-C, TG, LDL-C;	↓ plasma oxLDL with ↑ of oils phenolic content; No changes in plasma lipids.	Ramirez-Tortosa, 1999
EVOO high phenol vs. EVOO low phenol contents 50 g/day, 8 weeks	n = 10 healthy ♀ post-menopausal	592 mg/kg vs. 147 mg/kg	Oxidative: plasma antioxidant capacity; DNA oxidation (by Comet assay) Serum lipids: TC, HDL-C, TG, LDL-C;	↓ of DNA damage (by Comet assay) with ↑ of oils phenolic content; No changes in plasma antioxidant capacity	Salvini, 2006
EVOO vs. ROO 40 ml/day, 7 weeks	n = 22 ***mildy dyslipidemic*** 12 ♂ and 10 ♀	166 mg/kg vs. 2 mg/kg	Oxidative: plasma antioxidant capacity; urinary 8-epi-$F_{2\alpha}$; Inflammatory: plasma TXB(2) Serum lipids: TC, HDL-C, TG, LDL-C;	↓ plasma TXB(2) with ↑ of oils phenolic content; ↑ plasma antioxidant capacity with ↑ of oils phenolic content; No effects on urinary 8-epi-$F_{2\alpha}$	Visioli, 2005
VOO vs. ROO	n = 46 healthy	308 mg/kg vs. 43 mg/kg	Oxidative: oxLDL, LDL and HDL resistance to oxidation;	No effects were noted for all parameters tested in this study	Vissers, 2001

Supplemental Table II

69 g/day, 3 weeks	15 ♂ and 31 ♀		MDA, Protein carbonyls, LPO and antioxidant capacity of plasma (by FRAP); Serum lipids: TC, HDL-C, TG, LDL-C;		
VOO vs. Oleic acid-rich SO[v] X g/day, 3 weeks	n = 10 healthy ♂	? mg/kg vs. ? mg/kg	Oxidative: LDL resistance to oxidation; Serum lipids: TC, HDL-C, TG, LDL-C;	↑ LDL resistance to oxidation with ↑ of oils phenolic content;	Nicolaïew, 1998
VOO vs. COO vs. ROO 25 ml/day, 3 weeks	n = 28 healthy ♂	366 mg/kg vs. 164 mg/kg vs. 2.7 mg/kg	Oxidative: etheno-DNA adducts; Serum lipids: TC, HDL-C, TG, LDL-C;	No effects were noted for all parameters tested in this study	Hillestrøm, 2006
High vs. Moderate vs. Low content of phenols in similar olive oils 25 ml/day, 3 weeks EUROLIVE study	n = 200 healthy ♂	366 mg/kg vs. 164 mg/kg vs. 2.7 mg/kg	Oxidative: DNA and RNA oxidation markers in urine; LDL fatty acid composition; oxLDL and 8-epi-$F_{2\alpha}$, GSH and GSSG in plasma; ; LDL resistance to oxidation; Serum lipids: TC, HDL-C, TG, LDL-C;	↓ of DNA and RNA oxidative markers non-related with olive oil phenolics ↑ liner for HDL-C and HDL-C/TC with ↑ of oils phenolic content; ↓ liner for plasma oxLDL and oxidative stress markers (by GSH and GSSG) with ↑ of oils phenolic content; ↓ TG levels for all oils; ↑ oleic acid and ↓ linoleic and arachidonic acid concentration in LDL	Machowetz, 2007; Covas, 2006b; Cicero, 2008

[a] EVOO and VOO – extra virgin and virgin olive oil, respectively;
[b] OO – olive oil;
[c] CO – corn oil;
[d] TBX(2) – thromboxane B_2;
[e] LTB(4) – leukotriene B_4;
[f] TC – total cholesterol;
[g] HDL-C – high density lipoprotein cholestarol
[h] LDL-C – low density lipoprotein colesterol;
[i] TG – triglyceride;
[j] FA – fatty acid;
[k] 8-epi-$F_{2\alpha}$ – 8-epi prostaglandin-$F_{2\alpha}$, $F_{2\alpha}$-isoprostane;
[l] oxLDL – oxidated low density lipoproteins;
[m] PAI-1 – plasminogen activator inhibitor-1;
[n] FVIIa – activated factor VII;
[o] GSH-Px – gluctathione peroxidise;
[p] GS-R – gluctathione reductase;
[q] 8-OH-dG – 8-hydroxy-7,8-dihydroxy-2´-deoxyguanosine;
[r] mitDNA – mitochondrial DNA;
[s] MNCs – mononuclear cells;
[t] ROO – refine olive oil;
[u] CRP – C-reactive protein;
[v] SO – sunflower oli;

Supplemental Table III

Biologically relevant reactive species and activities of olive oil phenols in respect to them.

RS	Assay	Olive oil phenols	Effect	Ref
Superoxide radicals ($O2^{\cdot-}$)	*In vitro* xantine oxidase system;	HOTYR, OLE	Potent direct scavenger Potent direct scavenger	Visioli, 1998
	In vitro xantine oxidase system;	HOTYR, OLE, HVAlc , TYR	Potent direct scavenger Potent direct scavenger Poor scavenger Poor scavenger	Rietjens, 2007
Nitric oxide (NO)	decomposition of sodium nitroprusside;	HOTYR, OLE, CA, TYR	Potent scavenger Potent scavenger Potent scavenger Poor scavenger	De la Puerta, 2001
Peroxinitrile (ONOOH)	protection against ONOOH induced DHR-123 oxidation;	HOTYR, OLE, HVAlc , TYR	Potent direct scavenger Potent direct scavenger Good scavenger Poor scavenger	Rietjens, 2007
	α-antiproteinase inactivation assay;	HOTYR, OLE, CA TYR	Potent scavenger Potent scavenger Potent scavenger Good scavenger	De la Puerta, 2001
Hypochlorous acid (HOCl)	HOCl-mediated catalase inactivation;	HOTYR, OLE	Potent direct scavenger Potent direct scavenger	Visioli, 1998
	inhibition of 5-thio-2-nitrobenzoic acid oxidation;	HOTYR, OLE, HVAlc , TYR	Very poor scavenger Very poor scavenger Very poor scavenger Very poor scavenger	Rietjens, 2007
Hydroxyl radical ($OH^{\cdot}$)	deoxyribose method;	HOTYR, OLE, HVAlc , TYR	Potent direct scavenger Potent direct scavenger Good scavenger Poor scavenger	Rietjens, 2007
	ESR spin trapping;	HOTYR,	Limited and most probably indirect scavenging activity	Hashimoto, 2004
H_2O_2	UV spectra monitoring;	HOTYR, OLE, HVAlc , TYR	Very poor scavenger Very poor scavenger Very poor scavenger Very poor scavenger	Rietjens, 2007
	H_2O_2-HRPO cell free chemilumi-niscence;	HOTYR,	Potent direct scavenger	O´Dowd, 2004

Supplemental Table IV

Methodological approaches in quantitative and exploratory analysis of olive oil polyphenols (HOTYR, TYR, OLE and their metabolites) in biological fluids and their applications in bioavailability studies in humans and animals models.

Subjects and intervention	Biofluid	Validation criteria	Chromato graphy	Extraction	Detection		Application	Ref
					Compound	Identification		
Quantitative analyse of olive oil phenols in biological fluids								
Rats, HOTYR synthetic (10mg/mL, 1 mL)	Plasma (1 mL)	Recovery: 97-104 % LOD 10 ng/mL LOQ: NR	**GC**	LLE[i]; ×6.6 CF[ii] I.S.: nonadecanoic acid (10 µg/mL)	Free HOTYR	**MS** (SIM) for BSTFA[iii] derivatization;	Plasma pharmacokinetics of free HOTYR	Bai, 1998
Humans (♂), OA[iv] 50 mL OPhEx[v] in oil solution: (487.5-1960 mg/mL phenols) HOTYR 20-84mg/mL TYR 36-140mg/mL	24-h urine (1 mL)	Recovery: NR[vi] % LOD NR ng/mL LOQ: 10 ng/mL	**Gas Chromatography**	LLE; CF/DF[vii] NR I.S.: α-naphtol (1 µg/mL)	Free and liberated form glucoronides HOTYR and TYR;	**MS** (SIM) for BSTFA derivatization; Enzymatic hydrolysis;	Urinary excretions: Glucuronides + free HOTYR and TYR; Excretion of glucuronides/ingested phenols; Dose dependent absorption	Visioli, 2000
Humans(♂), OA 50 mL OOPhEx in oil solution: HOTYR 7-23.2 mg/50mL		Recovery: NR % LOD NR ng/mL LOQ: 10 ng/mL HOTYR and 0.5 ug HVAlc			Free and liberated form glucuronides and sulfates HOTYR, HVA and HVAlc		HOTYR metabolic convertion to HVAlc; Substrate induced enhancement of HVA; HOTYR, TYR and HVAlc dose dependent urinary excretion as free and librated from metabolites	Caruso, 2001
Humans and Rats OA: (1) EVOO (OLE 200 mg/L and HOTYR 19 mg/L); (2) OOPhEx in oil and yogurt (2 mg free and 7 mg of total[viii] HOTYR)					Free and liberated form glucuronides and sulfates HOTYR and HVAlc		Comparison of urinary excretions and metabolic rates for HOTYR and HVAlc in humans and rats; Bioavailability of HOTYR according to vehicle of administration	Visioli, 2003
Rats, OA: HOTYR aqueous solution 20 mg/kg	Plasma (1 mL)	Recovery: ~98% LOD 37 ng/mL LOQ: 96 ng/mL	RP[ix] **LC**[x] Gradient elution	SPE[xi] ×5 CF; I.S.: catechol (10 µg/mL)	Free HOTYR	**UV** (280 nm)	Plasmatic concentration of free HOTYR	Ruiz-Gutierrez, 2000

Humans OA: 50 mL EVOO (free TYR 216 µg and 1650 µg as total TYR)	Urine (2.5 mL)	Recovery: 80 % LOD 1.9 ng/mL LOQ: 10 ng/mL	**Gas Chromatography**	SPE-LLE: ×25CF I.S.: hydoxyphenyl-propanol (1 µg/2.5mL)	Free and liberated form conjugates TYR	**MS** (SIM) for MSTFA[xii] derivitization Acidic hydrolysis (HCl concentrated)	TYR 24h - urinary recovery TYR excretion as free and conjugated forms	Miro-Casas, 2001a
Humans OA: 50 mL EVOO (1055 µg and 655 µg of total HOTYR and TYR, respectively)		Recovery: 85 %TYR, 60 % HOTYR; LOD 0.6 ng/ml TYR 1.6 ng/mL HOTYR; LOQ:1.7 ng/mL TYR 4.8 ng/mL HOTYR		LLE ×25CF I.S.: hydoxyphenyl-propanol (200 ng/2.5 mL)	Free and liberated from conjugates HOTYR, and TYR		Excreted in urine free forms of HOTYR and TYR Urinary excretion levels and 24h-recoveries of HOTYR and TYR	Miro-Casas, 2001b
Humans OA: 25 mL EVOO	Plasma (1 mL) Urine (2.5 mL)	Plasma Recovery: >42 % LOD 0.46 ng/mL HOTYR 0.12 ng/mL HVAlc LOQ: 0.36 ng/mL HVAlc 1.39 ng/mL HOTYR Urinary Recovery: NR LOD & LOQ: NR		LLE ×1.33 CF (plasma) ×25CF (urine) I.S.: hydoxyphenyl-propanol (15 ng/mL plasma and 200 ng/2.5 mL urine)	Free and liberated from conjugates HOTYR and HVAlc	**MS** (SIM) MSTFA derivitization Acidic hydrolysis (HCl concentrated) vs. enzymatic hydrolysis	Plasma kinetics of total HOTYR and HVAlc; No free HOTYR and HVAlc in plasma; Urine excretion: Free HOTYR and HVAlc; Rate of conjugated forms Different $AUC_{0\text{-}8h}$ and C_{max}, urinary recoveries values after acidic and enzymatic hydrolysis.	Miro-Casas, 2003
Rats Intestinal perfusion technique OLE 1mmol/L in water	Plasma Flowing perfused samples (1 mL)	Recovery: NR % LOD NR µmol/L LOQ: 0.5 µmol/L	C_{18} **LC**; Isocratic elution	LLE ×5 CF I.S.: p-coumaric acid (1 mmol/L)	Free OLE	**FP**[xiii] with excitation/emission 280/312 nm (OLE) and 319/405 nm (I.S.)	OLE rat intestine permeability under hypotonic and iso-osmotic intestinal lumen. OLE absorption and via intestine wall transport.	Edgecombe, 2000
Humans OA: PhExOO (100 mg): (i) nonpolar, (ii) polar and (iii) OLE-glycoside	24-h Ileostomy effluent, 24-h urine;	Eleostomy effluent Recovery: ≥110% all LOD 10.1 nmol TYR and 7.1 nmol HOTYR UrineRecovery: ≥ 100% LOD: 0.04 µmol/L TYR 0.05 µmol/L HOTYR	**GC**	LLE I.S.: α-naphtol (1 µg/mL)	Free HOTYR and TYR in ileostomy effluent; Free and librated from glucuronides in urine	**MS** (SIM) for BSTFA derivatized HOTYR and TYR β-glucuronidase hydrolysis for conjugates	PhExOO absoption in small intestine and re-extraction in urine as HOTYR and TYR after ingested doses.	Vissers, 2002
		Recovery: 60% nonpolar 90% OLE-glycoside LOD 48 µmol/24h and 45 µmol/24h for OLE- and LGS-glycoside	**LC** Gradient: elution	LLE Semi-quantification:	OLE- and LGS-glycosides, non-polar compounds	**UV** (280 nm)	Urinary Recovery of OLE-glycoside in form of HOTYR (eleostomy subjects).	
Rats,	Plasma	Recovery: NR	**LC**	Protein	Free HOTYR;	**FP** 281 nm for	Plasma concentrations:	Tan, 2003

IVI[xiv] OLE 25 mg/kg (physiological solution)	(50 µL)	LOD NR LOQ: 3 µg/mL OLE and 30 ng/mL HOTYR	Isocratic elution	precipitation ×2 DF; I.S.: dimetho-xyphenyl ethanol, (100 ng/mL)	and OLE	excitation; 316 nm for emission	HOTYR and OLE and their distribution after IV dosing of OLE.	
Rats, OA: OLE (100 mg/kg) in soya oil:destil water (1:1)	Plasma (100 µL) 24-h urine (500 µL)	Plasma Recovery: 100% LOD 1.25 ng/mL LOQ: 2.5 ng/mL Urine Recovery: 60% LOD: 2.5 ng/mL LOQ: 5 ng/mL	RP-**LC** Gradient elution	LLE Plasma ×2 DF Urine ×2.5 CF I.S.: taxifolin (1 ug/mL)	Free and liberated from glucuronides HOTYR and OLE	**MS** ESI(-)-MRM: enzymatic hydrolysis	Plasma kinetics for free + librated from glucuronides OLE. No HOTYR was detected in plasma. Urinary excretion of OLE and HOTYR glucuronides	Del Boccio 2003
Humans	Plasma (NR mL)	Recovery: >85% all; LOD 50 ng/mL OLE and TYR; and 75 ng/mL HOTYR LOQ: 166 ng/ml (all)	RP –**LC** Gradient elution	SPE I.S.: hydroxy-phenylethanol (500 ng/mL)	Free HOTYR, TYR and OLE	**UV** (240 nm (OLE) and 280 nm (HOTYR, TYR and I.S)	NR	Tsarbopoulos, 2003
Humans	Plasma (1 mL)	Recovery: 80% OLE 98% TYR LOD 0.36 µg/mL OLE 0.09 µg/mL TYR	C_{18} **LC** Isocratic: elution	LLE ×4 CF; I.S.: vanillin (1.25 µg/mL)	Free TYR and OLE	**UV** 280 nm (OLE and TYR)	NR	Grizis, 2003
Rats OA: 50 g/kg EVOO and OLE 0.15 g/kg	Urine (200 µL)	Recovery: 76% TYR, 83% HOTYR, 72% EA LOD 300 pg TYR and EA; 2.5 pg HOTYR LOQ: 1 ng/mL (all)	**GC**	SPE: Oasis HLB CF: ×20 I.S.: hydroxy-phenylethanol (500 ng/mL)	Free and liberated from glucuronides HOTYR and TYR, EA	**MS** (MS/MS scan) MSTFA derivitization enzymatic hydrolysis	EA was not detected Free/(free+conjugated) ratio in basal and EVOO related levels of phenols, but elevated for OLE derived HOTYR	Bazoti, 2005
Humans OA: 50 mL EVOO, (648 g/L total phenols, 70.6 g/L HOTYR 27 g/L TYR, OLE+LGS 84% of total phenols)	LDL 60 min post-prandrial blood	Recovery: >70% (both) LOD 0.89 ng/mL HVA. 0.3.2 ng/mL HOTYR LOQ: 3 ng/mL HVA, 1 ng/mL HOTYR	C_{18} - **LC** Gradient: elution	SPE × 6.6 CF; I.S.: taxifolin (100-200 ng/mL of LDL) Metabolites relative semi-quantification	HOTYR, TYR, HVA and their monoglucuronides and monosulfates	**MS** ESI(-)-MRM and double MRM mode	Identification of HOTYR, TYR and HVA metabolites in LDL isolated from postprandrial blood: HOTYR monoglucuronides, monosulfates.	De la Torre-Carbot, 2006, 2007

Humans OA: 30 mL EVOO, (400 mg/mg total phenols)	Plasma 1h and 2h post-prandial (1 mL or 350 µL)	Recovery: 60% (all) LOD 0.1 µM/mL HOTYR, 1.4 µM/mL TYR, 0.09 µM/mL HVA LOQ: 0.5 µM/mL HOTYR, 4.8 µM/mL TYR, 0.3 µM/mL HVA	C_{18} - **LC** Gradient: elution	SPE/µSPE × 10/6 CF; I.S.: p-coumaric acid (2 µg/mL) Metabolites relative semi-quantification	HOTYR, TYR, HVA, mono-glucuronides and sulfates; other olive oil phenols and their metabolites	**MS** ESI(-)-SRM[xv]	Identification of HOTYR, TYR and HVA metabolites in postprandial plasma.	Suárez 2009;
Exploratory analysis of olive oil polyphenols in biological fluids								
Rats OA & IVI: 225 mg in aqueous or oil solutions of H^3-labeled HOTYR and TYR (~23 mg and ~14 mg per 1300 mg of solvent, respectively)	Urine		**LC** Isocratic elution	Normalization: to amount of radioactivity administrated	Free and liberated form conjugated HOTYR and TYR	**UV** (280 nm) and amount of radioactivity; enzymatic hydrolysis	Differences in bioavailabilities of HOTYR and TYR due to the vehicle and via of administration. Identification sulphates and glucuronidates	Tuck, 2001
						MS and **NMR** for isolated metabolites	Structural characterization of HOTYR sulfate-, glucuronide- and possible methyl-conjugate	Tuck, 2002
Rats IVI : C^{14}-HOTYR (~23 mg) Rat and human blood (*in vitro* incubation)	Urine; Tissues GI-content; Feces. Plasma and cell fraction		RP - **LC** Gradient: elution	Blood and plasma: protein precipitation Tissue/feces/liquid samples: solubilization in BTS-450	Free and librated from conjugates HOTYR, and its oxidized and methylated metabolites	**UV** (278 nm) and amount of radioactivity β-glucuronidase and sulfatase hydrolysis of conjugated forms	Organs and tissues uptake. Excretion and tissue distribution. Identified of oxidized and methylated metabolites: DOPAL, DOPAC, MOPET and HVA; and sulfo-conjugates;	D´Angelo, 2001

[i] - LLE – Liquid-Liquid Extraction;
[ii] - CF – concentration factor;
[iii] - BSTFA - bis-trimethylsilyl-trifluoro-acetamide;
[iv] - OA – Oraly administrated;
[v] - OOPhEx – olive oil phenols extract;
[vi] - NR – Not Reported;
[vii] - DF – Dilution Factor;
[viii] - "total" – refers to a fraction of TYR librated from different derivatives of TYR origin (mainly LGS) present in olive oil by the mean of chemical hydrolysis simulating physiological condition of GI digestive fluid;
[ix] - RP – Reverse Phase;
[x] - LC – Liquid Chromatography
[xi] - SPE – Solid Phase Extraction;
[xii] - MSTFA - N-Methyl-N-trifluoroacetamide;
[xiii] - FP - Fluorescence detector;
[xiv] - IVI – Intra Venous Injection;
[xv] - SRM – selective reaction monitoring;

Supplemental Table IV

APPENDICES

APPENDIX A: RNACLIN study protocol

C S B Consorci Sanitari de Barcelona

IMAS
Institut Municipal
d'Investigació Mèdica. IMIM

Título del estudio

Protocolo para la evaluación de las técnicas de obtención de RNA a partir de muestras biológicas en estudios clínicos. **(Estudio RNACLIN)**

Investigadores del estudio

Unidad de Farmacología (R. de la Torre, J. Ortuño, M. Pujadas, I. Sánchez, O. Khymenets)
Unidad de Lípidos y Epidemiología Cardiovascular (M.I Covas, M. Fitó, T. Weinbrenner, M Alcántara)

Antecedentes

El desarrollo de las tecnologías denominadas "-ómicas" (genómica, transcriptómica, proteómica, metabonómica) han tenido un gran crecimiento en los últimos años. En el contexto de estudios clínicos, hasta ahora se han podido hacer exploraciones a nivel fisiológico (p.e. actividad cardiovascular) y bioquímico (p.e. actividades enzimáticos, marcadores de determinadas actividades biológicas...). El desarrollo de las tecnologías del post-genoma abre las puertas para poder conocer cada vez a niveles más básicos las respuestas de los sistemas biológicos ante tratamientos farmacológicos, intervenciones nutricionales o bien condiciones fisiopatológicas. El tener acceso a la expresión génica se considera de interés para que podamos de forma simultánea y global abarcar sistemas biológicos completos (p.e. estrés oxidativo, metabolismo de fármacos, marcadores del proceso aterogénico) cosa muy difícil de abordar con la aproximación metodológica actual. La realización de estudios de expresión génica en humanos todavía padece de problemas metodológicos inherentes a la novedad de la aproximación. De allí el interés de realizar una serie de estudios diseñados para estandardizar las condiciones experimentales de cómo se debe abordar ese tipo de estudios en humanos.

Objetivos

El estudio RNACLIN pretende evaluar diferentes aproximaciones metodológicas para la obtención de RNA a partir de muestras biológicas de voluntarios sanos y pacientes para utilizar la expresión génica (también conocido como transcriptómica) como herramienta en estudios clínicos para evaluar los efectos de tratamientos farmacológicos, intervenciones a nivel nutricional, y/o condiciones fisiopatológicas.
En concreto, se quiere evaluar:

- Puesta a punto de la metodología para realizar análisis cuantitativos de expresión génica
- Evaluar la variabilidad intra-individual en la expresión de genes seleccionados durante un período de tiempo dado
- Evaluar los perfiles de expresión de genes seleccionados en voluntarios sanos.

Selección de los sujetos

En el estudio RNACLIN se incluirán voluntarios sanos no fumadores de ambos sexos. En total se seleccionaran hasta 20 sujetos (10 por género). Los sujetos del sexo femenino, sólo se incorporaran en la tercera condición experimental. La dimensión de la población que participa en los estudios no se ha hecho teniendo en cuenta ningún variable que podría prometer hacer una estimación de la misma. Teniendo en cuenta el objetivo metodológico del estudio, sólo se pretende disponer de un número mínimo de sujetos para poder determinar determinados estadígrafos.

Desarrollo del estudio

Durante la **visita inicial** se realizaran los controles siguientes:

- Revisión médica general de su estado de salud. Incluye un análisis de sangre y de orina y un electrocardiograma.
- Los sujetos participaran en tres experimentos diferentes que es realizaran de forma consecutiva en diferentes períodos de tiempo.
- Las extracciones de sangre se realizaran mediante venopunción por personal de enfermería de la URAF y/o ULEC.
-

Primer experimento

- Extracción de sangre de 35 mL para tal de evaluar la calidad del RNA obtenido aplicando diferentes protocolos de aislamiento (en total 7 técnicas de extracción de RNA). La distribución de la sangre será como sigue: 4,5ml en tubo Vacuntainer por estudios hematológicos, 2,5ml en tubos PAXgene™ (Blood RNA Tube), 8 ml en tubo Vacuntainer® CPT Cell Preparation Tube (aislamiento de monocitos y procedimiento Ultraspec), 8 ml en tubo Vacuntainer® CPT Cell Preparation Tube, (aislamiento monocitos y aplicación del QIAamp RNA Isolation Mini Kit i ABI Prism 6700 Workstation for RNA isolation). 10 ml en tubo BD Vacuntainer™ (ABI Prism 6700 Workstation for RNA Isolation, QIAamp RNA Mini Isolation Kit). 8ml (RNA by Ribo-Pure Kit).
- Todas las muestras de sangre serán procesadas según los procedimientos tal como están definidos en los protocolos propuestos en cada caso.
- Para determinar la calidad y la cantidad de RNA extraído, se utilizara el Agilent 2100 Bioanalyzer. Los resultados se referirán a la cantidad aislada de ácido nucleico por volumen de sangre total o bien por número de leucocitos o monocitos.

Segundo experimento

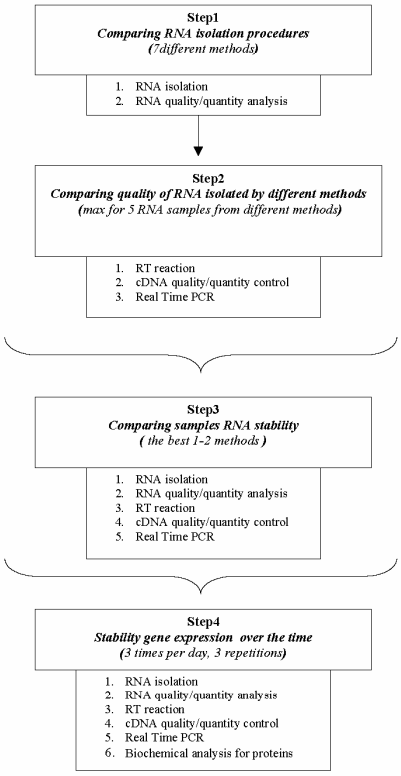
- Extracción de sangre de 35 mL para evaluar la estabilidad del RNA obtenido según dos de las técnicas empleadas en el primer día experimental

Tercer experimento

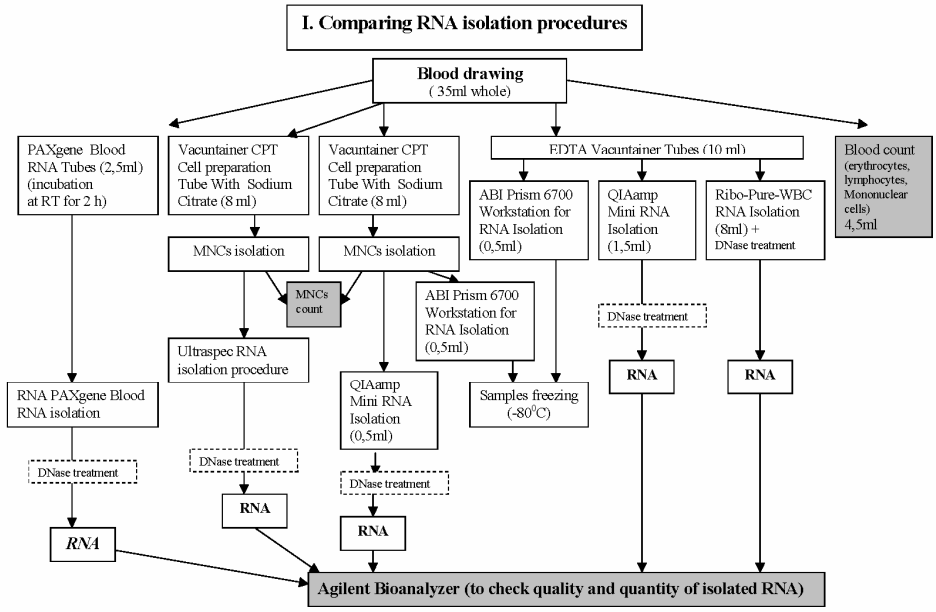
Se trata de evaluar la estabilidad de la expresión génica durante un mismo día y durante tres semanas consecutivas para cada uno de los sujetos participantes del estudio, para evaluar la variabilidad intra-diaria e inter-diaria en la expresión génica en los sujetos que participan en

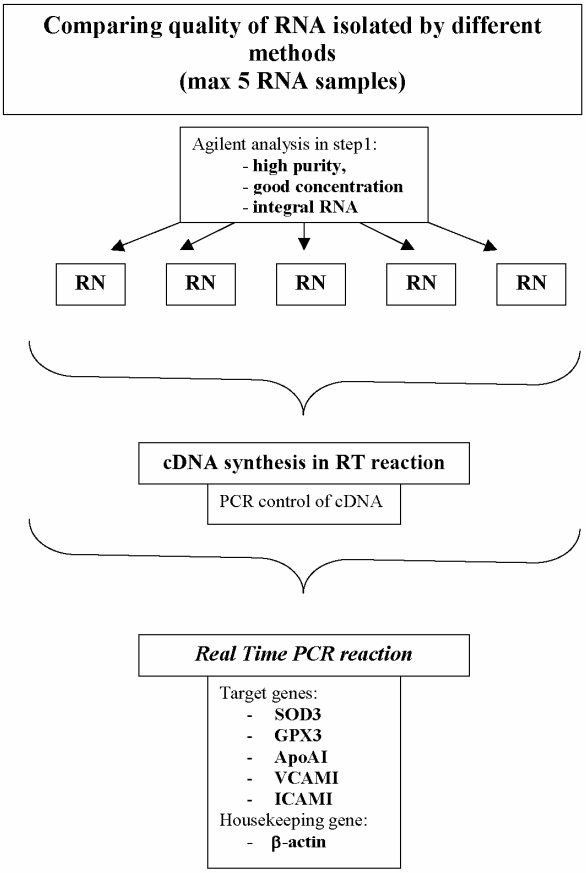
el estudio. En total se extraerán 153 mL de sangre (volumen total) obtenidos en un período de 3 semanas

- 1ª semana 51 mL repartidos en tres extracciones (0h, 3h y 6 h) con una comida ligera a partir de las 3 horas.
- 2ª semana 51 mL repartidos en tres extracciones (0h, 3h y 6 h) con una comida ligera a partir de las 3 horas.
- 3ª semana 51 ml repartidos en tres extracciones (0h, 3h y 6 h) con una comida ligera a partir de les 3 horas.



En el caso de las mujeres estas semanas coincidirán con el período menstrual (fases lúteas y foliculares). Para participar en este estudio no debe cambiar ni sus actividades habituales ni su estilo de vida.




Comparing quality of RNA isolated by different methods
(max 5 RNA samples)
Agilent analysis in step1:
- high purity,
- good concentration
- integral RNA
RN
RN
RN
RN
RN
cDNA synthesis in RT reaction
PCR control of cDNA
Real Time PCR reaction
Target genes:
- SOD3
- GPX3
- ApoAI
- VCAMI
- ICAMI
Housekeeping gene:
- β-actin

Beneficios y riesgos

La salud de los voluntarios, no se beneficiará directamente por su participación en este estudio. Por el otro lado los riesgos que incurren son los propios de que sean objeto de extracciones sanguíneas. Las muestras biológicas una vez recogidas serán analizadas y sus residuos eliminadas. En ningún caso se aprovechará estas muestras para crear un banco con datos genéticos de los individuos participantes.

Voluntariedad

La participación en el estudio es voluntaria y por lo tanto el sujeto se puede retirar en cualquier momento del mismo. También puede solicitar por escrito que sus muestras tanto si han estado analizadas como pendientes de análisis sean eliminadas del estudio. También puede ser retirado del estudio por el investigador en casos de enfermedades que podrían aumentar el riesgo o bien invalidar los resultados.

Confidencialidad

Totdos los datos recogidos sobre la participación en este estudio son considerados como confidenciales y tan solo serán utilizados por los investigadores para finalidades científicas. En los cuadernos de recogida de datos, que pueden ser inspeccionados por el investigador principal (o personas autorizadas por él, por ejemplo auditores), miembros del Comité Ético de Investigación Clínica, la Unidad de Garantía de Calidad del IMIM o bien las autoridades sanitarias, sólo constaran sus iniciales o un código, de esta manera su identidad no será conocida durante las inspecciones. En el informe final del estudio o en caso de comunicar estos resultados a la comunidad científica, su personalidad se mantendrá en el anonimato.

Este protocolo respetará la legislación vigente respecto a la protección de datos personales (Ley Orgánica 15/1999 del 13 de Diciembre de Protección de Datos de Carácter Personal - LOPD). La realización del estudio respetará la Guía Tripartita y armonizada por las buenas prácticas clínicas (ICH Ginebra 1996) y, la Declaración de Helsinki (rev. Edimburg 2000).

Compensación Económica. Seguro.

Por su participación en el estudio los sujetos recibirán una compensación económica. En caso de retirada, percibirán una compensación proporcional a su participación.

Consentimiento

Los voluntarios firmarán un consentimiento informativo (adjunto)

C S B Consorci Sanitari de Barcelona

IMAS
Institut Municipal
d'Investigació Mèdica. IMIM

Título del estudio

Protocolo para la evaluación de las técnicas de obtención de RNA a partir de muestras biológicas en estudios clínicos. **(Estudio RNACLIN)**

Investigadores del estudio

Unidad de Farmacología (R. de la Torre, J. Ortuño, M. Pujadas, I. Sánchez, O. Khymenets)
Unidad de Lípidos y Epidemiología Cardiovascular (M.I Covas, M. Fitó, T. Weinbrenner, M Alcántara)

Antecedentes

El desarrollo de las tecnologías denominadas "-ómicas" (genómica, transcriptómica, proteómica, metabonómica) han tenido un gran crecimiento en los últimos años. En el contexto de estudios clínicos, hasta ahora se han podido hacer exploraciones a nivel fisiológico (p.e. actividad cardiovascular) y bioquímico (p.e. actividades enzimáticos, marcadores de determinadas actividades biológicas...). El desarrollo de las tecnologías del post-genoma abre las puertas para poder conocer cada vez a niveles más básicos las respuestas de los sistemas biológicos ante tratamientos farmacológicos, intervenciones nutricionales o bien condiciones fisiopatológicas. El tener acceso a la expresión génica se considera de interés para que podamos de forma simultánea y global abarcar sistemas biológicos completos (p.e. estrés oxidativo, metabolismo de fármacos, marcadores del proceso aterogénico) cosa muy difícil de abordar con la aproximación metodológica actual. La realización de estudios de expresión génica en humanos todavía padece de problemas metodológicos inherentes a la novedad de la aproximación. De allí el interés de realizar una serie de estudios diseñados para estandardizar las condiciones experimentales de cómo se debe abordar ese tipo de estudios en humanos.

Objetivos

El estudio RNACLIN pretende evaluar diferentes aproximaciones metodológicas para la obtención de RNA a partir de muestras biológicas de voluntarios sanos y pacientes para utilizar la expresión génica (también conocido como transcriptómica) como herramienta en estudios clínicos futuros diseñados para evaluar los efectos de: tratamientos farmacológicos, intervenciones a nivel nutricional y/o condiciones fisiopatológicas.
El objetivo es evaluar cual es el mejor método de manipulación de las mustras biológicas para poder determinar el RNA.
En concreto, se quiere evaluar:

- Puesta a punto de la metodología para realizar análisis cuantitativos de expresión génica
- Evaluar la variabilidad intra-individual en la expresión de genes seleccionados durante un período de tiempo dado
- Evaluar los perfiles de expresión de genes seleccionados en voluntarios sanos.

Selección de los sujetos

En el estudio RNACLIN se incluirán voluntarios sanos de ambos sexos.

Desarrollo del estudio

Durante la **visita inicial** se realizarán los controles siguientes:

- Revisión médica general de su estado de salud. Incluye un análisis de sangre y de orina y un electrocardiograma.

Primer experimento

- Extracción de sangre de 35 mL para evaluar la calidad del RNA obtenido aplicando diferentes protocolos de aislamiento.

Segundo experimento

- Extracción de sangre de 35 mL para evaluar la estabilidad del RNA obtenido según dos de las técnicas empleadas en el primer día experimental

Tercer experimento

Se trata de evaluar la estabilidad de la expresión génica durante un mismo día y durante 3 semanas consecutivas para dada uno de los sujetos participantes del estudio. En total se extraerán 153 mL de sangre (volumen total) obtenidos en un período de 3 semanas

- 1ª semana 51 mL repartidos en tres extracciones (0h, 3h y 6 h) con una comida ligera a partir de las 3 horas.
- 2ª semana 51 mL repartidos en tres extracciones (0h, 3h y 6 h) con una comida ligera a partid de las 3 horas.
- 3ª semana 51 ml repartidos en tres extracciones (0h, 3h y 6 h) con una comida ligera a partir de las 3 horas.

En el caso de que las mujeres durante estas semana coincidieran con el período menstrual (fases lúteas y foliculares). Para participar en este estudio no debe cambiar sus actividades habituales ni su estilo de vida.

Beneficios y riesgos

Su salud, no se beneficiará directamente por su participación en este estudio.

Incomodidades y riesgos

En total usted vendrá a nuestro centro en diversas ocasiones para realizar los controles. En principio, el principal riesgo de su participación es el echo de que hay que realizar extracciones de sangre. Para extraer la sangre se requiere hacer una extracción venosa que requiere una punción con aguja que puede provocar dolor, hematoma y cambios en el color de la piel (morados).
Las extracciones de sangre serán realizadas por personal de enfermería con experiencia con este procedimiento.

Voluntariedad

La participación en el estudio es voluntaria y por lo tanto el sujeto se puede retirar en cualquier momento del mismo. También puede solicitar por escrito que sus muestras tanto si han estado analizadas como pendientes de análisis sean eliminadas del estudio. También puede ser retirado del estudio por el investigador en casos de enfermedades que podrían aumentar el riesgo o bien invalidar los resultados.

Compensación Económica. Seguro.

Por su participación en el estudio recibirá una compensación económica. En caso de retirada, percibirá una compensación proporcional a su participación.

Confidencialidad

Todos los datos recogidos sobre su participación en este estudio serán considerados como confidenciales y tan solo serán utilizados por los investigadores para finalidades científicas. En los cuadernos de recogida de datos, que pueden ser inspeccionados por el investigador principal (o personas autorizadas por él, por ejemplo auditores), miembros del Comité Ético de Investigación Clínica, la Unidad de Garantía de Calidad del IMIM o bien las autoridades sanitarias, sólo constaran sus iniciales o un código, de esta manera su identidad no será conocida durante las inspecciones. En el informe final del estudio o en caso de comunicar estos resultados a la comunidad científica, su personalidad se mantendrá en el anonimato.

Este protocolo respetará la legislación vigente respecto a la protección de datos personales (Ley Orgánica 15/1999 del 13 de Diciembre de Protección de Datos de Carácter Personal - LOPD). La realización del estudio respetará la Guia Tripartita y armonizada por las buenas prácticas clínicas (ICH Ginebra 1996) y, la Declaración de Helsinki (rev. Edimburg 2000).

Investigadores responsables. Como ponerse en contacto.

Si tiene alguna duda sobre aspectos del estudio o le gustaría comentar algún aspecto de esta información, por favor deje de hacerlo saber a los miembros del equipo investigador. Se puede poner en contacto con ellos (laborables de 8:00 a 17:00) de forma personal en la Unidad de Farmacología del IMIM (Doctor Aiguader 80, 08003 Barcelona) o por teléfono (93 221 10 09; 93 225 75 97).

Consentimiento

En el caso de que una vez leído la información y aclarados las dudas que puedan surgir decide participar en el estudio tendrá que firmar al final de este documento en los apartados adecuados.

Este estudio fue aprobado por el Comité Ético de Investigación Clínica del Institut Municipal d'Assistència Sanitària de Barcelona (CEIC-IMAS) y está financiado en parte por el proyecto EUROLIVE (EU V Programa Marc) y por el Plan Nacional sobre Drogas (Ministerio del Interior)

Yo (nombre y apellidos) DNI nº

He leído la hoja de información que se me ha entregado

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con:

Investigador (Nombre y apellidos)

Entiendo que mi participación es voluntaria.

Entiendo que puedo retirar me del estudio:

1º Cuando quiera.

2º Sin que sea preciso dar explicaciones.

Doy libremente mi conformidad de participar en el estudio.

Fecha Firma del participante

Fecha Firma del investigador

APPENDIX B: GEpilot study protocol

C S B Consorci Sanitari de Barcelona

IMAS
Institut Municipal
d'Investigació Mèdica. IMIM

Título del Estudio

Expresión génica tras una intervención nutricional consistente en la administración de dosis repetidas de aceite de oliva virgen: selección de genes candidatos. Estudio Piloto

Investigadores del Estudio:

Unitat de Farmacologia (R. de la Torre, M. Farré, O. Khymenets, J. Ortuño, M. Pujadas, I. Sánchez)
Unitat de Lípids i Epidemiologia Cardiovascular (M.I Covas, M. Fitó, D. Muñoz, M Alcántara)

Antecedentes

Las tecnologías "-òmicas" (genómica, transcriptómica, proteómica, metabonómica) han tenido un gran desarrollo en los últimos años. En el contexto de estudios clínicos, hasta la fecha se han podido realizar exploraciones a nivel fisiológico (p.e. actividad cardiovascular) y bioquímico (p.e. actividades enzimáticas, biomarcadores de estrés oxidativo...). El desarrollo de las tecnologías del post-genoma abre las puertas a poder estudiar a niveles más básicos las respuestas de los sistemas biológicos frente a tratamientos farmacológicos, intervenciones nutricionales o condiciones fisiopatológicas. Se considera de interés tener acceso a la expresión génica por que se puede de forma simultanea y global evaluar sistemas biológicos completos (p.e. estrés oxidativo, metabolismo de fármacos, marcadores del proceso aterogénico) algo muy difícil de abordar con las aproximaciones metodológicas actuales.

El presente protocolo está relacionado con otros dos protocolos de investigación aprobados por el CEIC-IMAS:

(i) Efecto del consumo de aceite de oliva sobre el daño oxidativo en la poblaciones europeas (Proyecto EUROLIVE). El estudio en la actualidad ya ha sido materializado. Fue aprobado por el Comité Ético de Instituto Municipal de Asistencia Sanitaria (CEIC-IMAS : 2002/1326/I).
(ii) Protocol per a l'avaluació de les tècniques d'obtenció de RNA a partir de mostres biològiques en estudis clínics. (Estudi RNACLIN). El estudio en la actualidad ya ha sido materializado.Fue aprobado por el Comité Ético de Instituto Municipal de Asistencia Sanitaria (CEIC-IMAS : 2003/1574/I).

El primer protocolo se interesaba al efecto sobre biomarcadores de daño oxidativo de la administración aleatorizada y cruzada a varias poblaciones europeas de tres aceites de oliva con distintos contenidos en sustancias fenólicas con actividad antioxidante: a) refinado (ausencia de compuestos fenólicos, b) comercial (mezcla de aceite de oliva refinado y extra virgen con un contenido global medio de compuestos fenólicos y c) aceite de oliva extra virgen con el mayor contenido en compuestos fenólicos. En dos de los centros participantes en el estudio, se extrajeron muestras para posteriormente poder aislar el RNA y poder realizar estudios de expresión génica.
El segundo protocolo se interesaba a los distintos procedimientos de aislamiento de RNA a partir de sangre total y distintas subpoblaciones celulares sanguíneas (monocitos y leucocitos) procedentes de muestras clínicas (en este protocolo se obtuvieron de voluntarios sanos). Se evaluaron distintas técnicas de aislamiento de RNA, la estabilidad de las muestras plasmáticas y de la expresión génica, y la influencia del género de los sujetos y del momento del día en que se obtenían las muestras en la

expresión génica. La expresión génica de *Sod1* y *Sod2* aplicando técnicas de RTPCR fue escogida para evaluar los distintos apartados del proyecto.
Es decir que en la actualidad se dispone de los protocolos experimentales para la realización de experimentos de expresión génica así como muestras procedentes de un estudio multicéntrico en las que realizar los mismos. Dados los costes asociados a la realización de los experimentos de expresión génica, se considera oportuno realizar un estudio piloto que permita seleccionar genes candidatos cuya expresión es modificada por la administración de aceite de oliva.

Objetivos:

a) selección de genes candidatos cuya expresión se vea alterada por la administración de aceite de oliva
b) evaluar los cambios temporales en la expresión génica de genes candidatos inducidos por la intervención nutricional durante un período de 3 semanas

Selección de los sujetos

En este estudio piloto participarán voluntarios sanos (6 fumadores y 6 no fumadores de ambos sexos) con edades comprendidas entre 18 y 60 años. En total es seleccionaran 12 sujetos. No se ha realizado una estimación exacta del tamaño de la muestra ya que teniendo en cuenta el carácter de estudio piloto del protocolo, solo se pretende disponer de un mínimo de sujetos para poder determinar ciertos descriptotes estadísticos.

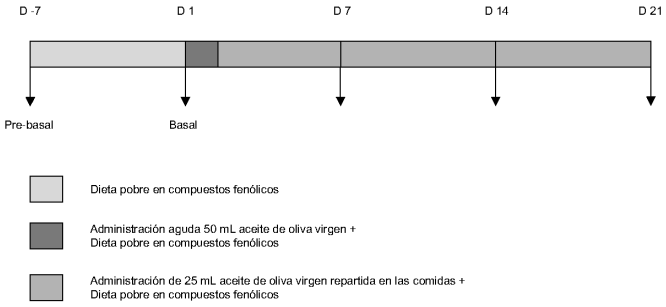
Desarrollo del estudio

En la visita inicial se realizaran los controles siguientes:
- Revisión médica general de su estado de salud. Incluye un análisis de sangre y de orina y un electrocardiograma.
- Las extracciones de sangre se realizaran por venopunción por personal de enfermería de la URAF y/o ULEC.

Intervención nutricional:

a) En el inicio del protocolo, se extraerán 20 ml de sangre para aislamiento de RNA. Durante una semana previa a la intervención dietética, los voluntarios se someterán a una dieta pobre en compuestos fenólicos (los voluntarios recibirán una serie de recomendaciones dietéticas y registrará la dieta diaria)
b) Intervención dietética:
   El 1er día se administraran 50 mL de aceite de oliva virgen, durante tres semanas, los voluntarios ingerirán 25 mL al día de aceite de oliva virgen repartidos durante las comidas.
   1er día:
   i. en las instalaciones de la URF (laboratorio de ensayos clínicos) los voluntarios vendrán en ayunas y antes de la ingesta de aceite de oliva extra virgen se extraerán 30 ml de sangre para aislamiento de sangre
   ii. los voluntarios ingerirán de forma aguda 50 mL de aceite de oliva virgen con 100-200 g de pan
   iii. se extraerán muestras de sangre (30 mL) al cabo de 1, 6 y 24 horas tras la administración del aceite de oliva
   iv. se recogerá orina de 24 horas (0-2h, 2-4h , 4-6 h y 6-24 h)

   días 7, 14 y 21
   v. se extraerán muestras de sangre (30 mL) a primera hora de la mañana en ayunas



Determinaciones

Sangre
Volumen total 30 mL
20 mL se utilizarán para aislar plasma y leucocitos
(i) compuestos fenólicos en plasma
(ii) aislamiento de LDL
(iii) ácidos grasos en plasma
(iv) extracción de RNA de leucocitos

10 mL se utilizarán para aislar monocitos
(v) extracción de RNA de monocitos

Orina
Recogida de 0-6 horas y de 6-24 horas, se utilizará para determinación de compuestos fenólicos

Días 1

Experimentos de expresión génica

Se hará un pool de RNA de los 12 sujetos obtenido a las 0, 1, 6 y 24 horas del día 1 del estudio y se determinará la expresión génica mediante microarrays (GENEchip, Affymetrix)

Se seleccionarán los genes candidatos, y mediante RTPCR sobre Microfluidic cards (Applied Biosystems) se analizarán la muestras del presente ensayo y las del estudio EUROLIVE

Estadística
Al tratarse de un estudio piloto solo se realizará estadística descriptiva de los resultados.

Beneficios y riesgos
La salud de los voluntarios, no es beneficiará directamente por su participación en este estudio. Por otra parte, los riesgos en que incurren son los propios del hecho de ser objeto de extracciones sanguíneas.
Las muestras biológicas un vez recogidas serán analizadas y eliminados sus residuos. En ningún caso se aprovecharán estas muestras para crear un banco con datos genéticos de los individuos participantes.

Voluntariedad
La participación en el estudio es voluntaria y por tanto el sujeto se puede retirar en cualquier momento del mismo. También puede solicitar por escrito que sus muestras tanto si han sido analizadas como pendientes de análisis sean eliminadas del estudio. También puede ser retirado del estudio por el investigador en casos de enfermedades que pudiesen aumentar el riesgo o bien invalidar los resultados.

Confidencialidad
Todos los datos recogidos sobre la participación en este estudio son consideradas como confidenciales y solo serán utilizadas por los investigadores con finalidades científicas. En los cuadernos de recogida de datos, que poden ser inspeccionados por l'investigador principal (o persones autorizadas por el, por ejemplo auditores), miembros del Comitè Ètic d'Investigació Clínica, la Unidad de Garantía de la Calidad del IMIM o bien las autoridades sanitarias, solo constaran sus iniciales o un código, así su identidad no será conocida durante las inspecciones. En el informe final del estudio o en caso de comunicar estos resultados a la comunidad científica, se mantendrá su personalidad en el anonimato.

Este protocolo respetará la legislación vigente respecto a la protección de datos personales (Ley Orgánica 15/1999 de 13 de Diciembre de Protección de Datos de Carácter Personal - LOPD). La realización del estudio respetará la Guía Tripartita y harmonizada para las buenas prácticas clínicas (ICH Ginebra 1996) y, la Declaración de Helsinki (rev. Edimburgo 2000).

Compensación Económica. Seguro.
Por su participación en el estudio, los sujetos recibirán una compensación económica. En caso de retirada, percibirán una compensación proporcional a su participación. No existe una póliza específica de seguro para este estudio al no tratarse de un ensayo clínico con medicamentos. La investigación deberá estar cubierta por la póliza general del centro.

Consentimiento
Los voluntarios recibirán copia de la hoja de información y firmarán un consentimiento informado (adjunto)

C S B Consorci Sanitari de Barcelona

IMAS
Institut Municipal d'Investigació Mèdica. IMIM

HOJA DE INFORMACIÓN A LOS POSIBLES PARTICIPANTES

Título del Estudio

Expresión génica tras una intervención nutricional consistente en la administración de dosis repetidas de aceite de oliva virgen: selección de genes candidatos.

Investigadores del Estudio:

Unitat de Farmacologia (R. de la Torre, M. Farré, O. Khymenets, J. Ortuño, M. Pujadas, E. Menoyo)
Unitat de Lípids i Epidemiologia Cardiovascular (M.I Covas, M. Fitó, D. Muñoz, M Alcántara)

Antecedentes

Las tecnologías "-òmicas" (genómica, transcriptómica, proteómica, metabonómica) han tenido un gran desarrollo en los últimos años. En el contexto de estudios clínicos, hasta la fecha se han podido realizar exploraciones a nivel fisiológico (p.e. actividad cardiovascular) y bioquímico (p.e. actividades enzimáticas, biomarcadores de estrés oxidativo...). El desarrollo de las tecnologías del post-genoma abre las puertas a poder estudiar a niveles más básicos las respuestas de los sistemas biológicos frente a tratamientos farmacológicos, intervenciones nutricionales o condiciones fisiopatológicas. Se considera de interés tener acceso a la expresión génica por que se puede de forma simultanea y global evaluar sistemas biológicos completos (p.e. estrés oxidativo, metabolismo de fármacos, marcadores del proceso aterogénico) algo muy difícil de abordar con las aproximaciones metodológicas actuales.

Objetivos:

a. selección de genes candidatos cuya expresión se vea alterada por la administración de aceite de oliva
b. evaluar los cambios temporales en la expresión génica de genes candidatos inducidos por la intervención nutricional durante un período de 3 semanas

Selección de los sujetos

En el estudio participarán voluntarios sanos (6 fumadores y 6 no fumadores de ambos sexos) con edades comprendidas entre 18 y 60 años. En total es seleccionaran 12 sujetos. El tamaño de la población que participa en el estudio no se ha realizado teniendo en cuenta que permitiera hacer una estimación de la misma. Teniendo en cuenta el carácter de estudio piloto del protocolo, solo se pretende disponer de un mínimo de sujetos para poder determinar ciertos descriptotes estadísticos.

Desarrollo del estudio

En la visita inicial se realizaran los controles siguientes:
- Revisión médica general de su estado de salud. Incluye un análisis de sangre y de orina y un electrocardiograma.

- Las extracciones de sangre se realizaran por venopunción por personal de enfermería de la URAF y/o ULEC.

Intervención nutricional:

a. En el inicio del protocolo, se extraerán 20 ml de sangre para aislamiento de RNA. Durante una semana previa a la intervención dietética, los voluntarios se someterán a una dieta pobre en compuestos fenólicos (los voluntarios recibirán una serie de recomendaciones dietéticas y registrará la dieta diaria)
b. Intervención dietética: El 1er día se administraran 50 mL de aceite de oliva virgen, durante tres semanas, los voluntarios ingerirán 25 mL al día de aceite de oliva virgen repartidos durante las comidas.
    1er día:
        i. en las instalaciones de la URF (laboratorio de ensayos clínicos) los voluntarios vendrán en ayunas y antes de la ingesta de aceite de oliva extra virgen se extraerán 30 ml de sangre para aislamiento de sangre
        ii. los voluntarios ingerirán de forma aguda 50 mL de aceite de oliva virgen con 100-200 g de pan
        iii. se extraerán muestras de sangre (30 mL) al cabo de 1, 6 y 24 horas tras la administración del aceite de oliva
        vi. se recogerá orina de 24 horas (0-2h, 2-4h , 4-6 h y 6-24 h)

    días 7, 14 y 21
        iv. se extraerán muestras de sangre (30 mL)

Beneficios y riesgos

La salud de los voluntarios, no es beneficiará directamente por su participación en este estudio. Por otra parte, los riesgos en que incurren son los propios del hecho de ser objeto de extracciones sanguíneas. Las muestras biológicas un vez recogidas serán analizadas y eliminados sus residuos. En ningún caso se aprovecharán estas muestras para crear un banco con datos genéticos de los individuos participantes.

Voluntariedad

La participación en el estudio es voluntaria y por tanto el sujeto se puede retirar en cualquier momento del mismo. También puede solicitar por escrito que sus muestras tanto si han sido analizadas como pendientes de análisis sean eliminadas del estudio. También puede ser retirado del estudio por el investigador en casos de enfermedades que pudiesen aumentar el riesgo o bien invalidar los resultados.

Confidencialidad

Todos los datos recogidos sobre la participación en este estudio son consideradas como confidenciales y solo serán utilizadas por los investigadores con finalidades científicas. En los cuadernos de recogida de datos, que poden ser inspeccionados por l'investigador principal (o persones autorizadas por el, por ejemplo auditores), miembros del Comitè Ètic d'Investigació Clínica, la Unidad de Garantía de la Calidad del IMIM o bien las autoridades sanitarias, solo constaran sus iniciales o un código, así su identidad no será conocida durante las inspecciones. En el informe final del estudio o en caso de comunicar estos resultados a la comunidad científica, se mantendrá su personalidad en el anonimato.

Este protocolo respetará la legislación vigente respecto a la protección de datos personales (Ley Orgánica 15/1999 de 13 de Diciembre de Protección de Datos de Carácter Personal - LOPD). La realización del estudio respetará la Guía Tripartita y harmonizada para las buenas prácticas clínicas (ICH Ginebra 1996) y, la Declaración de Helsinki (rev. Edimburgo 2000).

Compensación Económica. Seguro.

Por su participación en el estudio, los sujetos recibirán una compensación económica. En caso de retirada, percibirán una compensación proporcional a su participación.

Investigadores responsables. Como ponerse en contacto.

Si tiene alguna duda sobre algún aspecto del estudio o le desearía comentar algún aspecto de esta información, por favor no deje de hacerse lo saber a los miembros del equipo investigador (M. Farré o E. Menoyo). Puede comunicarse con ellos (laborables de 8:00 a 17:00) de forma personal en la Unitat de Farmacologia de l'IMIM (Doctor Aiguader 80, 08003 Barcelona) o por teléfono (93 221 10 09; 93 225 75 97). Fuera de este horario puede llamar al IMIM y le atenderá el personal de seguridad que le pondrá en contacto con los investigadores.

Consentimiento

En el caso de que una vez leída la información y aclaradas las dudas que pudiesen surgir decidiese participar en el estudio, tendrá que firmar al final de este documento en los apartados que corresponda.

Este estudio fue aprobado por el Comitè Ètic de Investigació Clínica del Institut Municipal d'Assistència Sanitària de Barcelona (CEIC-IMAS) y está financiado en parte por el proyecto EUROLIVE (EU V Programa Marc) y por la Red de Excelencia del ISCIII: PREDIMED

_____

Yo (nombre y apellidos) DNI nº

He leído la hoja de información que se me ha dado

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con:

_____

Investigador (nombre y apellidos)

Entiendo que mi participación es voluntaria.

Entiendo que puedo retirarme del estudio:

1º Cuando quiera.

2º Sin que sea preciso dar explicaciones

Doy mi conformidad a participar en el estudio.

_____ _____

Fecha Firma del participante

_____ _____

Fecha Firma del investigador