Tyrosol and its endogenous conversion into hydroxytyrosol in humans

Dietary sources, genetic modulation and clinical effects

Anna Boronat Rigol

TESI DOCTORAL UPF / 2019

Thesis supervisor

Dr. Rafael de la Torre Fornell

Grup de Recerca en Farmacologia Integrada i Neurociència de Sistemes

IMIM – Institut Hospital del Mar d'Investigacions Mèdiques

DEPARTAMENT OF HEALTH AND EXPERIMENTAL SCIENCES

Universitat Pompeu Fabra Barcelona

FINANTIAL SUPPORT

This work has been funded by:

- Fondo de Investigación Sanitaria - Instituto de Salud Carlos III FEDER (FIS PI14/00072)
- PFIS predoctoral fellowship from the Instituto Carlos III (PFIS-FI16/00106)
- Mobility fellowship M-AES from the Instituto Carlos III (M-AES-MV18/00004)
- ERAB (European foundation for alcohol research) Exchange Awards 2018
- CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN) is an initiative of the Instituto de Salud Carlos III, Madrid, Spain.
- DIUE de la Generalitat de Catalunya (2107 SGR 138)
- Centro de Información Cerveza y Salud, Fundación Manuel de Oya

Abstract

Hydroxytyrosol is a health-promoting dietary phenol mainly present in extra virgin olive oil. Wine and beer are sources of tyrosol, a related phenolic compound. We have demonstrated that in humans tyrosol is endogenously converted into hydroxytyrosol following wine and beer consumption. Therefore, tyrosol rich foods could trigger equivalent health effects than those of hydroxytyrosol. The conversion of tyrosol into hydroxytyrosol is mediated by the polymorphic cytochrome P450 isoenzymes *CYP2A6* and *CYP2D6*. In a randomized controlled clinical trial, we have shown that tyrosol and its conversion into hydroxytyrosol improved endothelial function in individuals at high cardiovascular risk following a 4-weeks of a dietary intervention enriched in tyrosol. A polygenic activity score to predict the metabolic fate of tyrosol to hydroxytyrosol based on *CYP2A6* and *CYP2D6* genotypes has also been developed. This score was able of predicting the metabolism of tyrosol and the magnitude of the derived biological effects in a personalized manner.

Resum

L'hidroxitirosol és un compost fenòlic amb propietats beneficioses per la salut present principalment en l'oli d'oliva verge extra. El vi i la cervesa són fonts de tirosol, un compost fenòlic relacionat. Hem demostrat que en humans el tirosol és convertit endògenament a hidroxitirosol després del consum d'aliments rics en tirosol com el vi o la cervesa. Conseqüentment, els aliments rics en tirosol, generarien efectes saludables equivalents als aliments rics en hidroxitirosol. La conversió és mediada per *CYP2A6* i *CYP2D6*, dos isoenzims del citocrom P450 altament polimòrfics. Mitjançant un assaig clínic aleatori controlat s'ha evidenciat que el tirosol i la seva conversió a hidroxitirosol produeixen una millora en la funció endotelial en voluntaris amb risc cardiovascular després de 4 setmanes amb una dieta enriquida en tirosol. S'ha desenvolupat també un paràmetre de valoració numèric en base als genotips de *CYP2A6* i *CYP2D6* per predir el metabolisme del tirosol i la magnitud dels efectes biològics esperables de forma personalitzada.

Resumen

El hidroxitirosol es un compuesto fenólico con propiedades beneficiosas para la salud presente principalmente en el aceite de oliva virgen extra. El vino y la cerveza son fuentes de tirosol, un compuesto fenólico relacionado. Hemos demostrado que en humanos el tirosol es convertido endógenamente en hidroxitirosol tras el consumo de alimentos ricos en tirosol como el vino o la cerveza. Consecuentemente, los alimentos ricos en tirosol, generarían efectos saludables equivalentes al de aquellos ricos en hidroxitirosol. La conversión de tirosol en hidroxitirosol es mediada por *CYP2A6* y *CYP2D6*, dos isoenzimas del citocromo P450 altamente polimórficas. Mediante un ensayo clínico aleatorio controlado se ha evidenciado que el tirosol y su conversión a hidroxitirosol producen una mejora en la función endotelial en individuos con riesgo cardiovascular después de 4 semanas con una dieta enriquecida en tirosol. Se ha desarrollado también una puntuación poligénica en base a los genotipos de *CYP2A6* y *CYP2D6* para predecir el metabolismo del tirosol y la magnitud de los efectos biológicos esperables de forma personalizada.

Preface

Diet and nutritional state are key contributors to health in all stages of life. The Mediterranean diet is the typical dietary pattern followed in the basin of the Mediterranean Sea. It potentiates synergies among foods and is characterized by the consumption of a wide range of bioactive substances at low doses that, all together, trigger an accumulative protective health effect. The exact protective mechanisms of the Mediterranean diet are still unclear, but it is believed that a high intake of bioactive substances, such as phenolic compounds, play an important role. Extra virgin olive oil and red wine are two principal elements of this diet and they are the key contributors to the total daily phenolic intake.

Hydroxytyrosol is the main antioxidant present in extra virgin olive oil. The health effects of this phenolic compound have been extensively studied in the context of the Mediterranean diet and enriched olive oils. Red wine has a minor concentration of hydroxytyrosol, but its higher recoveries following its consumption led us to study the existence of potential precursors. Tyrosol, a structural and metabolically related phenolic compound, was pointed out as the most probable precursor.

In the present thesis we have studied the metabolism of tyrosol and hydroxytyrosol in humans, demonstrating the role of tyrosol as the precursor of hydroxytyrosol in the context of the dietary intake of wine and beer. Furthermore, we have studied the cardiovascular effects of tyrosol per se and those derived from the generation of hydroxytyrosol in a clinical setting. Our findings reinforce the idea that dietary antioxidants have an activity that goes beyond simply oxidant scavengers, showing that they are capable of modulating important signaling and regulatory pathways.

The results obtained in the thesis shed light on the metabolism and health effects of tyrosol and hydroxytyrosol, two important dietary phenols, and will help to understand the mechanisms of action behind the health effects of the Mediterranean diet.

Thesis structure

The present PhD dissertation is structured in several sections. The first section is an **Introduction** to Tyrosol and Hydroxytyrosol with special attention into their dietary sources, metabolism and finally, their potential effects to human health. The second section is **Objectives** stating the aim of the present work. Thirdly, **Methods and Results** sections are divided in four different chapters. Each chapter is preceded by a brief introduction specific for topic discusses, followed by the methods and results obtained and finally a short discussion. **Chapter 1** includes the methodology and results obtained from a clinical trial aimed at demonstrating the endogenous conversion of tyrosol into hydroxytyrosol and to evaluate its effects in cardiovascular biomarkers. **Chapter 2** outline the dietary sources of the studied phenols in the diet, with a special focus in beer. **Chapter 3** describes the methodology and results of a clinical trial assessing tyrosol and hydroxytyrosol metabolism following red wine and beer consumption. **Chapter 4** describes a nutrigenetic approach to evaluate tyrosol to hydroxytyrosol conversion. The next section includes a **General Discussion** of the main findings of the present thesis, providing a global perspective of the results obtained and also including future directions. Finally, the main **Conclusions** drawn from the thesis are outlined. The last sections include all the scientific literature consulted and the Annex section includes the scientific publications derived from the doctoral thesis.

The development of this doctoral had led to the publication of three scientific articles:

- **Boronat A*,** Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, Muñoz D, Barbosa F, Morales JC, Gaedigk A, Langohr K, Covas MI, Pérez-Mañá C, Fitó M, Tyndale RF, de la Torre R. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. Free Radic Biol Med. 2019 Aug 31; 143:471-481. doi: 10.1016/j.freeradbiomed.2019.08.032.

- **Boronat A*,** Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, Muñoz D, Barbosa F, Morales JC, Gaedigk A, Langohr K, Covas MI, Pérez-Mañá C, Fitó M, Tyndale RF, de la Torre R. Data on the endogenous conversion of tyrosol into hydroxytyrosol in humans. Data in brief. 2019 *Submited*

- Soldevila-Domenech N*, **Boronat A*,** Mateus J, Diaz-Pellicer P, Matilla I, Pérez-Otero M, Aldea-Perona A, de la Torre R. Generation of the Antioxidant Hydroxytyrosol from Tyrosol Present in Beer and Red Wine in a Randomized Clinical Trial. Nutrients. 2019 Sep 18; 11(9):2241; doi:10.3390/nu11092241.
  (An * denotes the first author/co-authors of the publication)

The scientific outputs of the current thesis had been presented in different international congresses as oral and poster presentation. The presented works are listed below:

Oral presentation:

- **A Boronat** et al., Wines enriched with tyrosol increase hydroxytyrosol metabolites in high cardiovascular risk individuals; a nutrigenetic approach. Presented in Wine and Health 2017 Meeting. La Rioja, Spain; 02/16/2017 to 02/18/2017.
- **A Boronat** et al., Tyrosol bioconversion into Hydroxytyrosol in humans: regulation by CYP2A6 and CYP2D6 polymorphisms. Selected to be presented as an ePoster discussion in "Nutrition 2018" organized by the American Society for Nutrition; Boston, United States; 09/06/2018 – 12/06/2018

Poster presentation:

- **A Boronat** et al., Determination of free and conjugated hydroxytyrosol in human plasma following olive oil administration. Presented in 10th World Congress on Polyphenols Applications: Porto Polyphenols 2016. Oporto, Portugal; 06/29/2016 to 07/01/2016.
- **A Boronat** et al., Tyrosol bioconversion into Hydroxytyrosol in humans: regulation by CYP2A6 and CYP2D6 polymorphisms. Presented in "Nutrition 2018" organized by the American Society for Nutrition; Boston, United States; 09/06/2018 – 12/06/2018.
- **A Boronat** et al., Bioavailability of Tyrosol present in beer and its biotransformation into the antioxidant hydroxytyrosol in humans; preliminary results. Presented in the International Meeteing on Alcohol and Health; Louvain, Belgium; 24/09/2019 – 25/09/2019.

- **A Boronat** et al., Generation of the antioxidant Hydroxytyrosol from Tyrosol present in beer in a randomized clinical trial. Presented in "Nutrition 2019" organized by the American Society for Nutrition; Baltimore, United States; 09/06/201 9– 12/06/2019

Additionally, during the time period of the presented thesis, the candidate has been involved in other research projects. They have not been included in the thesis since the scope was not strictly related to the topic of the current work or the candidate contributed as a collaborator, and not as the leading investigator. Nevertheless, this work has enriched the PhD learning experience. The work done has been published in the following scientific papers:

- Rodríguez-Morató J, **Boronat A**, … de la Torre R. Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. Drug Metab Rev. 2016 May; 48(2):218-36. doi:10.1080/03602532.2016.1179754.
- Garcia-Marchena N, … **Boronat A**, …Serrano A. Plasma concentrations of oleoylethanolamide and other acylethanolamides are altered in alcohol-dependent patients: effect of length of abstinence. Addict Biol. 2017 Sep;22(5):1366-1377. doi: 10.1111/adb.12408.
- Rodríguez-Morató J, … **Boronat A**, … de la Torre R. CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol. Food Chem. 2017 Feb 15; 217: 716-725. doi: 10.1016/j.foodchem.2016.09.026.
- Martínez-Huélamo M, Rodríguez-Morató J, **Boronat A**, de la Torre R. Modulation of Nrf2 by Olive Oil and Wine Polyphenols and Neuroprotection. Antioxidants (Basel). 2017 Sep 26;6(4). pii: E73. doi: 10.3390/antiox6040073.

- **Boronat A**, Martínez-Huélamo M, Cobos A, de la Torre R. Wine and Olive Oil Phenolic Compounds Interaction in Humans. Diseases. 2018 Sep 1;6(3). pii: E76. doi: 10.3390/diseases6030076.
- Rivera P, … **Boronat A**, … Suárez J. Acetaminophen-Induced Liver Injury Alters the Acyl Ethanolamine-Based Anti-Inflammatory Signaling System in Liver. Front Pharmacol. 2017 Oct 6;8:705. doi: 10.3389/fphar.2017.00705.
- Romero-Sanchiz P, … **Boronat A**, … Rodríguez de Fonseca F. Plasma concentrations of oleoylethanolamide in a primary care sample of depressed patients are increased in those treated with selective serotonin reuptake inhibitor-type antidepressants. Neuropharmacology. 2019 May 1; 149:212-220. doi: 10.1016/j.neuropharm.2019.02.026.
- Soldevila-Domenech N, **Boronat A**, Langohr K, de la Torre R. N-of-1 Clinical Trials in Nutritional Interventions Directed at Improving Cognitive Function. Front Nutr. 2019 Jul 23;6:110. doi: 10.3389/fnut.2019.00110.

Finally, the author also had the opportunity to be involved in the writing of two chapters of the following book:
Role of the Mediterranean Diet in the Brain and Neurodegenerative Diseases (2017) Editors: Tahira Farooqui, Akhlaq A. Farooqui Paperback ISBN: 9780128119594, Academic Press, Date: 24th October 2017, Page Count: 484:

- **Boronat A**, Rodríguez-Morató J, Fitó M and De la Torre R. Cardioprotective properties of wine: Implications for the management of neurodegenerative diseases.
- Rodríguez-Morató J, **Boronat A**, Dierssen M and De la Torre R. Neuroprotective properties of wine: Implications for the prevention of cognitive impairment.

List of abbreviations

AHA	American heart association
AI	Augmentation index
AS	Activity score
ASA	acetylsalicylic acid
AT	O-acetyl transferase
ATIII	Antithrombin III
AU	Arbitrary units
BMI	Body mass index
CD40L	CD40 Ligand
CHD	Coronary heart disease
CHF	Complement factor H
Cmax	Maximum concentration
CNV	Copy number variation
COMT	Catechol-O-methyl transferase
CPIC	Clinical pharmacogenomics implementation consortium
CVD	Cardiovascular disease
CYP2A6	Cytochrome P450 2A6 isoform
CYP2D6	Cytochrome P450 2D6 isoform
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
eNOS	Endothelial nitric oxide synthase
ET1	Endothelin 1
EtG	Ethyl glucuronide
EVOO	Extra virgin olive oil
GC/MS	Gas chromatography coupled to mass spectrometry
GWAS	Genome-wide association studies
Hcy	Homocysteine
HDL	High density lipoprotein
HLM	Human liver microsomes
hsCRP	High sensitivity C reactive protein
HT	Hydroxytyrosol
HVAL	Homovanillyl alcohol
iNOS	Inducible nitric oxide synthase

ISTD	Internal standard
L	Liter
LA	Low activity
LC/MS-MS	Liquid chromatography coupled to mass spectrometry
LDL	Low density lipoprotein
Log	Logarithm
M	Molar
MedDiet	Mediterranean diet
Min	Minute
NA	Normal activity
NF-kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor erythroid 2-related factor
PAI-1	Plasminogen activator inhibitor-1
PAS	Polygenic activity score
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RA	Rapid activity
RELA	Transcription factor p65
RHI	Reactive hyperaemia index
RNA	Ribonucleic acid
RW	Red wine
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SULT	Sulfotransferase
Tyr	Tyrosol
UGT	UDP-glucuronosyl transferase
VEGFA	Vascular growth factor A
wGRS	Weighted genetic risk score
WO	Wash-out
wPAS	Weighted polygenic activity score
WW	White wine
WW+Tyr	White wine plus tyrosol capsules

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Mediterranean diet: from epidemiological studies to randomized clinical trials

Diet and nutrition are key factors on promoting and maintaining health and well-being while reducing the risk of several chronic diseases throughout all stages in life (World Health Organisation, 2003). Over the last years, nutritional research has shifted to a more holistic approach, focusing on the complete dietary pattern instead of studying the effects of single nutrients or single foods. Among the different dietary patterns, the so-called Mediterranean Diet (MedDiet) has received most of the attention for being associated with health and longevity benefits. The term MedDiet was coined after the "Seven Countries Study" by Ancel Keys in 1960. It was the first epidemiological study that compared the incidence of cardiovascular diseases (CVD) and mortality with different dietary patterns. The study observed a lower incidence of CVD and total mortality in the Mediterranean countries, where diet was low in saturated fat and presented a higher proportion of plant-based calories compared to animal calories (Menotti and Puddu, 2015).

A traditional MedDiet is typically a high-fat diet, being extra-virgin olive oil (EVOO) the main source of fat. Furthermore, it is characterized by a high consumption of plant-based products (fruit, vegetables, legumes, nuts and whole-grain cereals), a moderate consumption of fish, seafood, dairy products and poultry, and a very low consumption of red and processed meat. Finally, yet importantly, it includes a moderate alcohol consumption, mostly red wine, consumed during meals.

A number of epidemiological studies had related a high adherence to the MedDiet with a reduction in total mortality (Sofi *et al.*, 2014), and the incidence of CVD (Sofi *et al.*, 2014; Rosato *et al.*, 2019), cognitive decline (Wu and Sun, 2017) and certain cancers (Sofi *et al.*, 2014; Schwingshackl *et*

al., 2017). Meta-analyses of randomized controlled trials also provided strong evidence that interventions with MedDiet decreased CVD incidence, mortality, and diabetes apart from improving anthropometric, metabolic and inflammatory risk parameters compared to control interventions (Dinu *et al.*, 2018). Notwithstanding, it is still unclear whether a shift towards a MedDiet can be effective, feasible and sustainable in non-Mediterranean populations as it is in Mediterranean populations (Martínez-González *et al.*, 2017).

Special mention should be made to the PREDIMED study (Prevención con Dieta Mediterránea, http://www.predimed.es/); a large randomized multicenter study aimed at evaluating the long-term effects of the MedDiet in the prevention of primary cardiovascular events. This study involved 7.447 individuals at high cardiovascular risk who were followed for an average of 4.8 years. Participants were randomly allocated into one of three possible diets: i) a MedDiet supplemented with EVOO, ii) a MedDiet supplemented with nuts or iii) a reduced-fat diet (the usual prescribed diet to individuals with a high risk of CVD). MedDiet supplemented with EVOO or nuts decreased around 30% the incidence of primary cardiovascular events (Estruch *et al.*, 2018). Moreover, MedDiet interventions also prevented type II diabetes and improved classical and emerging CVD risk factors such as blood pressure, insulin sensitivity, inflammation, lipid profiles and oxidative stress (Martínez-González *et al.*, 2015).

1.2 Contribution of phenolic compounds to the health benefits of Mediterranean diet

The exact mechanism by which MedDiet exerts its protective effects remains unclear. A potential mechanism explaining, at least part of MedDiet health effects, is para-hormesis. Based on this, the exposure of low-grade or low-doses of potentially damaging conditions or compounds foster a beneficial effect by promoting the activation and upregulation of the stress-

response pathways (Forman, Davies and Ursini, 2014). The activation of hormetic mechanisms in different animal models is associated with an increase in life-expectancy and the delay on the onset of age-related pathologies (Martucci *et al.*, 2017). It is believed that specific compounds of the MedDiet are likely to act as hormetins, inducing the para-hormetic effect.

One of the presumed main contributors to the para-hormetic effects of MedDiet is the high intake of phenolic compounds. Phenolic compounds are proposed to act as nutritional hormetins triggering antioxidant and anti-inflammatory biologically activities. They are a heterogeneous group of phytochemicals containing phenol rings, which confer them the antioxidant activity. They are especially abundant in fruits, vegetables, whole grain products, legumes, EVOO, coffee and red wine (Guasch-Ferré *et al.*, 2017). Total polyphenol intake was estimated within the participants of PREDIMED study. At baseline, the mean daily intake was 0,8 g. EVOO and olives accounted for an average of 11 % of the total polyphenols intake while red wine contribution was of 6% (Tresserra-Rimbau *et al.*, 2013). An inverse association was observed between total polyphenols intake at baseline and the incidence of major cardiovascular events. Participants placed on the highest quintile presented a 46% risk reduction compared to the lowest quintile of total polyphenol daily intake (Tresserra-Rimbau *et al.*, 2014).

As mentioned before, EVOO is a key and central element of the MedDiet and an important contributor to the total polyphenol intake. Beneficial effects of EVOO were firstly attributed to its fatty acid composition, rich in monounsaturated fatty acids (e.g. oleic acid). Nonetheless, equivalent health effects were not associated to the consumption of other oils with similar fatty acid composition like rapeseed and canola oil. For this reason, the

attention was shifted towards the phenolic compounds present in EVOO, and particularly to the high concentrations of the antioxidant hydroxytyrosol (HT). In 2006, the EUROLIVE study demonstrated a dose-dependent association between EVOO phenolic fraction, HDL-cholesterol levels and protection against low density lipoproteins (LDL) oxidation (Covas *et al.*, 2006). In the EUROLIVE study, 25 mL of three olive oils with similar proportion of monounsaturated fatty acids and micronutrients but differing in the phenolic content (resulting in a low, a medium and a high-phenolic olive oil) were administrated daily. On the basis of the observed results, the European Food Safety Authority (EFSA) released a claim concerning the benefits of a daily ingestion of 5 mg of HT and its derivates on protecting LDL from oxidation (EFSA, 2011).

1.3 Tyrosol and Hydroxytyrosol

1.3.1 Dietary sources of tyrosol and hydroxytyrosol

The phenols tyrosol (Tyr) and hydroxytyrosol (HT) are present in the diet mainly in EVOO and in red wine (Figure 1). The chemical conjugation and the origin of both compounds vary widely in both dietary sources.

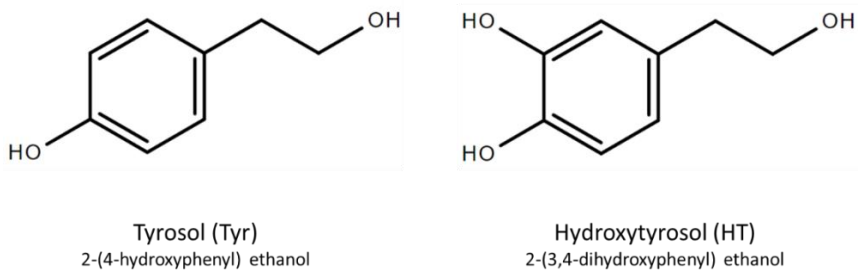


Figure 1. Tyrosol and hydroxytyrosol chemical structures

Tyr and HT are phenolic alcohols mostly found in a conjugated form in the hydrophilic fraction of olive oil. As shown in Figure 2, ligstroside and oleuropein are the most abundant conjugated forms of Tyr and HT, respectively. These compounds are secoiridoids containing an eleanolic acid moiety bound to a glucose and to either Tyr or HT. The second most abundant conjugated forms of Tyr and HT are esters with deacetoxy eleanolic acid corresponding to oleacin and oleocantal, respectively (Figure 2). The Tyr and HT secoiridoid aglycones are present in much lower amounts and their levels increase during olive oil manufacturing process of crushing and malaxation (Figure 2) (Servili *et al.*, 2014; Rodríguez-Morató *et al.*, 2016). Free Tyr and HT are present in very low amounts. Apart from the mentioned phenolic alcohols, olive oil contains phenolic acids (vanillic, caffeic and ferulic acids), flavonoids (apigenin and luteolin) and lignans ((+)-1-pinoresinol) (Rodríguez-Morató *et al.*, 2015; Serreli and Deiana, 2018).

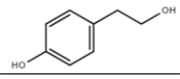
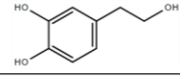
Abundance	Compound	Chemical structure	Tyrosol 	Hydroxytyrosol 
+	Secoiridoids	R + eleanolic acid + glucose	Ligstroside (p-HPEA-EDA)	Oleuropein (3,4 DHPEA-EDA)
	Ester	R + deacetoxy eleanolic acid	Oleacin	Oleocanthal
	Secoiridoids aglycon	R + eleanolic acid	Ligstroside aglycone (p-HPEA-EA)	Oleuropein aglycone (3,4 DHPEA-EA)
-	Free forms	-	Free Tyr (p-HPEA)	Free HT (3,4 DHPEA)

Figure 2. Phenolic alcohol conjugates present in olive oil in order of abundance.

The olive oil phenolic fraction is affected by agricultural factors such as olive fruit variety, ripeness and environmental conditions. Likewise, the

process of obtention of olive oil has an impact on its phenolic composition; being crushing, malaxation and extraction methods the most critical steps (Servili *et al.*, 2014; Rodríguez-Morató *et al.*, 2016). The final concentration of total phenolic compounds can range from 5 mg/ 100 g in the refined oils to 94 mg/ 100g in the purest EVOO (Servili *et al.*, 2014). Table 1 outlines the average content of Tyr and HT on different olives oil and olives.

In contrast, wine is another source of Tyr and HT, although in their free form. They are produced as secondary metabolites derived from the amino acid tyrosine, which is metabolized by yeasts during alcoholic fermentation (Rodríguez-Morató *et al.*, 2016). Tyrosine is converted into Tyr via the Ehrlich pathway and then hydroxylated to form HT. Tyr concentrations in wine are significantly higher than those of HT. Their content is proportional to amount of tyrosine present in the must (Garde-Cerdán and Ancín-Azpilicueta, 2008) and is highly influenced by the yeast strain used in the fermentation process (Álvarez-Fernández *et al.*, 2018). The content present in wine has been described to range depending on the type of wine, being white wine (WW) the lowest and red wine (RW) and sherry the richest in Tyr and HT (Table 1).

Table 1. Total tyrosol and hydroxytyrosol content of food typical from the Mediterranean diet

Food			Tyrosol		Hydroxytyrosol	
Group	Class	Units	Mean	Range	Mean	Range
Olive oil	EVOO	mg/ 100 g	1,1	0,1 – 3,6	0,8	0,0 – 3,5
	Virgin		0,4	0,0 – 6,5	0,7	0,0 – 7,4
	Refined		0,5	0,3 – 0,8	0,4	0,2 – 2,1
Olives	Green	mg/ 100 g	14,4	0,9 – 119,6	65,9	4,3 – 116,
	Black		7,5	0,0 – 21,0	55,6	0,0 – 413,3
Wine	Red	mg/100 mL	3,1	0,6 – 4,6	0,5	0,1 – 1,0
	Rose		0,5	NA (n=1)	0,6	NA (n=1)
	White		0,2	0,1 – 0,3	0,2	0,2 – 0,3
	Sparkling wine		2,4	1,2 – 3,6	ND	ND
	Sherry		5,7	NA (n=1)	ND	ND

NA: Not available: the range is not available since only one sample was analysed. ND: no data on the database.

Data extracted from www.phenol-explorer.eu

1.3.2 Endogenous formation of tyrosol and hydroxytyrosol

Tyr and HT are not only present in the diet but can also be produced endogenously in the human body. HT is formed in normal conditions as a minor metabolite derived from the oxidative metabolism of dopamine (Figure 3). The major metabolite from this pathway is DOPAC, which is further methylated into homovanillic acid. A secondary branch of the pathway involves the conversion of DOPAL into DOPET (equivalent to HT) which can be further methylated to produce homovanillyl alcohol. During moderate ethanol consumption, there is a shift in dopamine oxidative metabolism due to a reductive environment. This results in a higher production of DOPET (HT) and a decreased production of DOPAC. Studies in humans confirmed that the formation of HT directly correlated with the dose of ethanol consumed (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015).

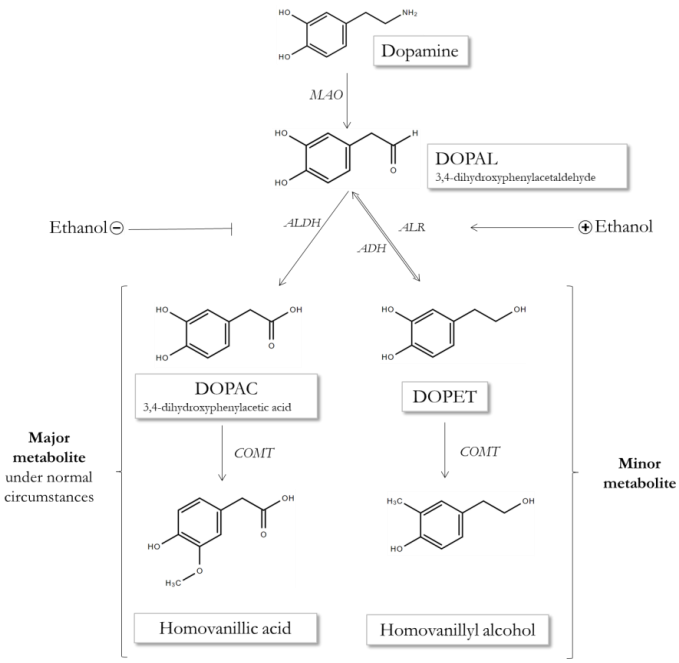


Figure 3. Oxidative pathway of dopamine. MAO: Monoamine oxidase; ALR: aldehyde reductase; ALDH: aldehyde dehydrogenase; ADH: alcohol dehydrogenase, COMT: catechol-O-methyl transferase.

Tyr is also produced endogenously as a byproduct of tyramine metabolism, originated from the decarboxylation of the amino acid tyrosine by the enzyme aromatic L-amino acid decarboxylase (Figure 4). Tyramine is deaminated by the action of MAO to form 4-hydrophenylacetic acid, which alternatively may be converted into Tyr. In a similar way than in dopamine metabolism, ethanol triggers a shift towards a reductive pathway, increasing Tyr production while decreasing the 4-hydrophenylacetic formation (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015). Similarly, studies involving human participants confirmed a dose dependent Tyr formation following ethanol intake (Pérez-Mañá, Farré, Pujadas, *et al.*, 2015).

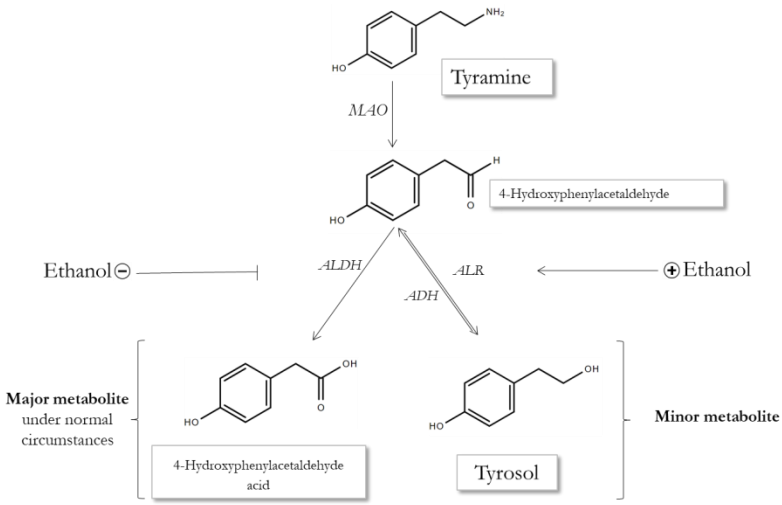


Figure 4. Oxidative pathway of tyramine. MAO: Monoamine oxidase; ALR: aldehyde reductase; ALDH: aldehyde dehydrogenase; ADH: alcohol dehydrogenase, COMT: catechol-O-methyl transferase.

1.3.3 Metabolic disposition of tyrosol and hydroxytyrosol

Tyr and HT bioavailability are highly dependent on the matrix in which they are contained. Olive oil matrix enables a more efficient absorption compared to an equivalent water-based solution (Tuck *et al.*, 2001). In the case of wine, ethanol has been described to promote their absorption. Ethanol has the

capacity to modulate tight junctions, enhancing paracellular permeability within epithelial cells and hence, facilitating intestinal absorption (Yu *et al.*, 2013). The role of ethanol on promoting phenolic absorption was additionally supported by a clinical study in which the phenolic urinary recovery was decreased after a dealcoholized wine compared to a normal wine (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015).

Tyr and HT are absorbed in the small intestine and in the colon by passive diffusion in a dose-dependent manner (Visioli et al., 2000). In the case of EVOO, the phenolic fraction includes the conjugated secoiridoid and aglycone forms. They are de-conjugated in the intestine prior absorption, giving rise to Tyr and HT free forms. Once absorbed, Tyr and HT are distributed systemically, being rapidly uptaken by the organs. Tyr and HT have been detected in muscle, kidney, liver, lungs and are also reported to be capable of crossing the blood-brain barrier (D'Angelo *et al.*, 2001). A dose-response accumulation of HT has been observed not only in blood and urine but also in liver, kidney and brain (López de las Hazas *et al.*, 2015).

Tyr and HT are well absorbed but their systemic bioavailability is low. They go through an extensive first-pass gastric/hepatic metabolism giving rise to phase II metabolites. The predominant metabolites formed are sulfates, glucuronides and methyl-conjugates (Figure 5). The process is of relevance since unconjugated forms are almost undetectable in systemic biological fluids (Pastor *et al.*, 2016). The low concentrations of free HT and free TYR observed *in vivo* cannot explain the biological activities associated with phenolic consumption in clinical and epidemiological studies. Therefore, metabolites have been proposed to exhibit relevant biological properties, either by having activity by themselves or by being deconjugated intracellularly, hence giving rise to free active forms (Serreli and Deiana, 2018), as it has been described for other dietary phenols such as flavonoids (Fernández-Castillejo *et al.*, 2019).

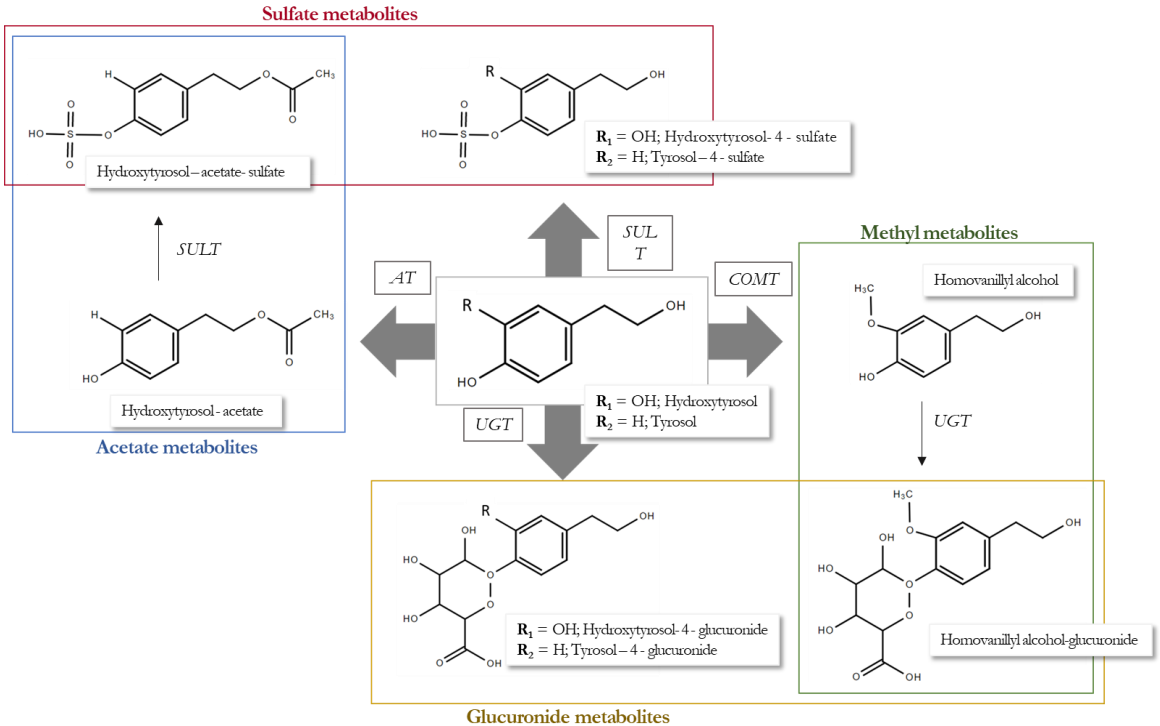


Figure 5. Tyrosol and hydroxytyrosol phase II metabolism. AT: O-acetyl transferase; COMT: catechol-O-methyl transferase; UGT: UDP-glucuronosyl transferase; SULT: sulfotransferase.

1.3.4 Biological activity of tyrosol and hydroxytyrosol

HT is well known for its strong antioxidant properties given by the o-dihydroxyphenyl moiety present in its chemical structure (Rodríguez-Morató *et al.*, 2016). Tyr shares the same structure but lacks a catechol group, resulting in a lower antioxidant activity. The catechol group of HT enhances its capacity to stabilize free radicals. In the case of Tyr, the single hydroxyl group did not provide any direct antioxidant activity (Visioli, Poli and Galli, 2001). The scavenging capacity of dietary antioxidants seems to be more relevant locally in gut than once absorbed when considering its concentrations in these compartments. Nevertheless, based on their *in vivo* biological activity, their capacity to modulate intracellular stress-response system is likely to be mediated by mechanisms beyond scavenging free radicals, as proposed by the para-hormesis paradigm (Forman et al., 2014).

Due to its antioxidant capacity HT and, to a lesser extent, Tyr have traditionally been studied for the prevention of chronic diseases associated with chronic oxidative stress such as CVD, neurodegenerative diseases, diabetes and cancer. Although the exact mechanism behind the healthy effect of Tyr and HT is still unclear, several target pathways seem to be involved. One likely candidate is the nuclear factor E-2 related factor 2 (Nrf2) pathway. Its activation promotes the expression of detoxifying enzymes involved in antioxidant defense. Several *in vitro* and pre-clinical studies have described a Nrf2 upregulation following treatments with HT (Valenzuela *et al.*, 2017), Tyr (Wang *et al.*, 2017) and olive oil phenolic extracts (Bayram *et al.*, 2012). Another proposed pathway downregulated by these phenolic compounds is NF-κB, a critical transcription factor of the inflammatory cascade. Its downregulation by the mentioned phenolic compounds has been reported to decrease the level of several cytokines, chemokines and pro-inflammatory agents in *in vitro* studies (St-Laurent-Thibault *et al.*, 2011; Sangiovanni *et al.*, 2012; Serra *et al.*, 2018).

Most of the *in vitro* studies have focused on the activity of Tyr and HT, but, as stated before, the bioavailability of these compounds in their free form is low. Therefore, the biological activity of the major metabolites of Tyr and HT are being currently studied, as higher concentration are reached in biological systems compared to the free forms, and hence, being more likely involved in the health effects associated to Tyr and HT consumption (Serreli and Deiana, 2018). Tyr and HT glucuronides have been described to lose their direct radical scavenging properties once conjugated (Khymenets *et al.*, 2010). Nevertheless, additional studies had shown the capacity of HT-glucuronide to protect cultured epithelial cells from lipid peroxidation (Deiana *et al.*, 2011). Another study highlighted the capacity of HT-glucuronide to protect endothelial dysfunction induced by oxidative stress. This protective effect was dependent on the activity of β-glucuronidase, suggesting the existence of a prior de-conjugation step, which would release free HT that could enter to the cell (Peyrol *et al.*, 2018). Additional studies demonstrated that Tyr-glucuronide and to a higher extent, Tyr-sulphate prevented NF-κB activation in endothelial cells and hence, the resulting over-expression of adhesion molecules (Muriana *et al.*, 2017). Lastly, HT and Tyr sulphate counteracted the pernicious action of oxidized cholesterol on an intestinal cell model (Atzeri *et al.*, 2016). In contrast to glucuronide metabolites, the existence of sulphate transporters widely across the membranes would made them able to act without being de-conjugated.

In addition, the relevance of HT on health benefits provided by the MedDiet was further supported recently in the frame of the PREDIMED study. High urinary homovanillyl alcohol (HVAL) concentrations, a stable HT metabolite, were independently associated with a lower risk of CVD and lower total mortality in elderly individuals. EVOO and wine consumption were the key dietary factors for HVAL concentrations (De La Torre *et al.*, 2017).

1.4 Potential role of tyrosol and hydroxytyrosol in endothelial function

The vascular endothelium is the functional lining of cells in blood vessels. It has a crucial role in the maintenance of vascular homeostasis keeping the correct balance between vasodilatation and vasoconstriction (Park and Park, 2015). Endothelial cells locally produce nitric oxide that regulates vascular tone, coagulation, inflammatory cell adhesion and smooth cell proliferation. Endothelial dysfunction is characterized by a reduction in the bioavailability of nitric oxide, triggering a deficiency in vasoprotective mediators and followed by an upregulation of pro-inflammatory and pro-thrombotic agents within the endothelium. The presence of endothelial dysfunction highly predicts cardiovascular events in subjects in early stages of disease, beyond any conventional risk factors. Endothelial dysfunction is the earliest detectable stage in the atherosclerotic process. However, it is treatable and reversible. When endothelial dysfunction persists, arteries walls lose their elasticity, becoming hard and thick, and finally lead to arterial stiffness, an independent predictor for CVD. Overall, the process promotes the development and progression of atherosclerosis (Reriani, Lerman and Lerman, 2010) (Figure 6). Traditional and emergent cardiovascular risk factors are involved in the aetiology of endothelial dysfunction, being oxidative stress a common hallmark, and considered the main mechanism involved in its pathogenesis (Konukoglu and Uzun, 2016).

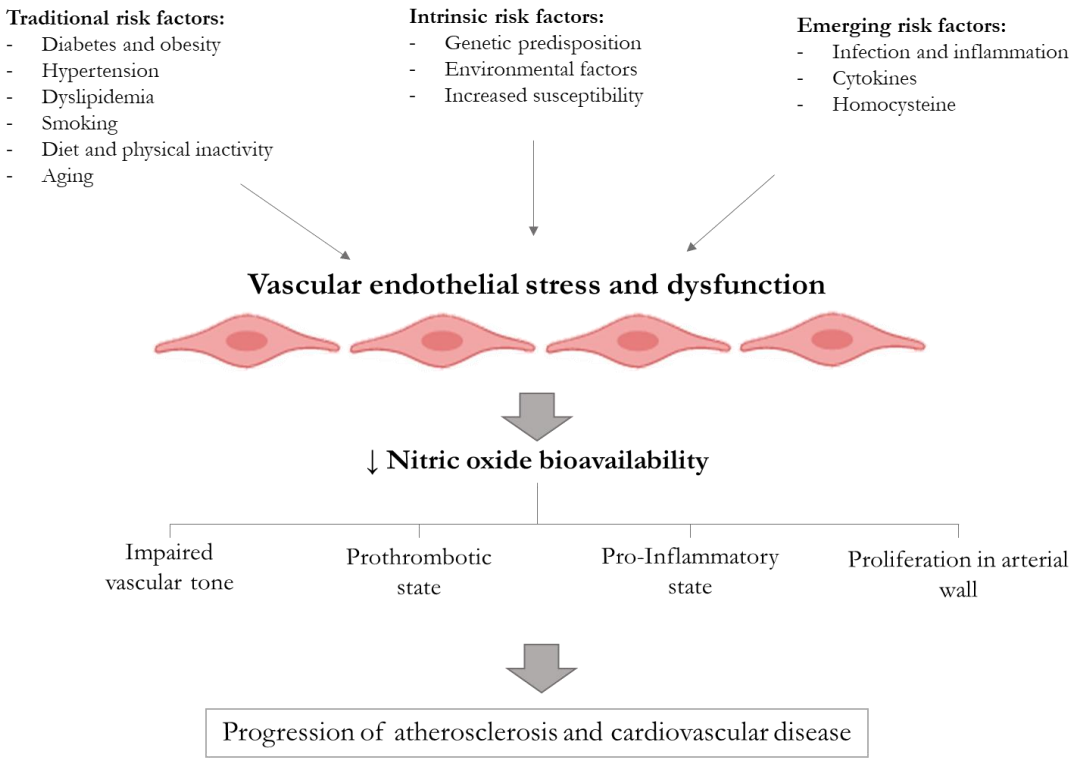


Figure 6. Aetiology and pathology of endothelial dysfunction; progression of atherosclerosis and cardiovascular diseases

To the best of our knowledge, there is limited evidence of the direct effect of Tyr and HT on the endothelial function as a clinical target. Nevertheless, potential mechanisms have been proposed to improve endothelial function. Tyr and HT seem to be able to modulate intermediate biomarkers of endothelial health in *in vitro* and in pre-clinical studies. Nevertheless, limited evidence exists about the translation of the previous mechanisms in humans and their ability to trigger a clinically significant improvement of endothelial function.

Many clinical trials have investigated the effect of food and preparations rich in Tyr and HT on endothelial function and endothelial health biomarkers. A protective effect of EVOO enriched in its own phenolic compounds was observed at a postprandial level, improving endothelial function and reducing oxidized LDL (Valls *et al.*, 2015) as well as in promoting an anti-thrombotic effect (Ruano *et al.*, 2007). A diet supplemented with EVOO rich in polyphenols followed for two months improved endothelial function, as well as inflammatory and oxidative biomarkers (Moreno-Luna *et al.*, 2012). Likewise, moderate red wine consumption has also been associated with an improvement in postprandial endothelial function (Whelan *et al.*, 2004; Boban *et al.*, 2006). Interestingly, it has been reported that this beneficial effect only after red wine intake and not dealcoholized red wine and neither a polyphenol-stripped red wine, suggesting a synergistic effect among phenols and ethanol present in the wine matrix (Boban *et al.*, 2006). On the other hand, the effect of olive leaf extracts has also been studied in endothelial function and associated endothelial risk factors like blood pressure and nitric oxide metabolism. Lockyer at al. (2015), reported a postprandial improvement in the endothelial stiffness index following an acute ingestion of an olive leaf extract equivalent to 51 mg of oleuropein and 10 mg of HT (Lockyer *et al.*, 2015). A second study providing a chronic administration of olive leaf extracts observed an

improvement in 24 h ambulatory blood pressure (Lockyer *et al.*, 2015). Finally, the administration of a nutraceutical preparation of HT (15 mg/day) for 3 weeks was associated with a reduction in nitrites and nitrates levels, metabolites of nitric oxide, and modulated the expression of inflammation and oxidative stress-related genes (Colica *et al.*, 2017). On the contrary, two clinical trials administrating pure HT failed to observe any significant health effect (Crespo *et al.*, 2015; López-Huertas and Fonolla, 2017). Nevertheless, it is important to emphasize that the bioavailability of Tyr and HT compounds from the olive leaf extracts or nutraceutical preparations is probably low, fact that could explain the mild biological effects observed. Overall, limited evidence exists about the health effects on endothelial function of Tyr and HT singled out from their food matrix in humans.

1.5 Nutrigenetic regulation of tyrosol to hydroxytyrosol conversion

The contribution of a single dose of EVOO and a single dose of red wine to the metabolism of Tyr and HT were studied in a clinical setting. Total HT dose administered in the study in the form of EVOO was five-fold higher than the one in red wine. Nonetheless, urinary recoveries of HT were higher after red wine administration (De la Torre *et al.*, 2006). The increase in HT recovery was not totally attributable to ethanol consumption and its effect on dopamine metabolism, suggesting other sources and precursors of HT presents in the wine matrix. Further studies in animal models observed that Tyr was endogenously converted into HT and confirmed, as previously mentioned, that ethanol improves Tyr bioavailability. Finally, the simultaneous administration of Tyr and ethanol to animal models triggered an increased urinary excretion of HT metabolites in a dose-dependent manner (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015). Further *in vitro* experiments with human liver microsomes using specific cytochrome P450 (CYP) inhibitors and human recombinant proteins identified two

isoenzymes mediating the hydroxylation of Tyr into HT: *CYP2A6* and *CYP2D6* (Rodríguez-Morató *et al.*, 2017) (Figure 7). Despite these *in vitro* and pre-clinical studies, the conversion of Tyr into HT has yet to be confirmed in humans.

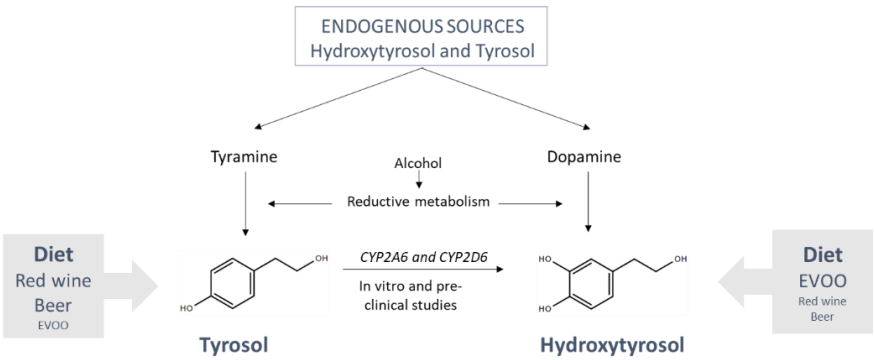


Figure 7. Direct and indirect dietary and endogenous sources of hydroxytyrosol.

The activities of the enzymes involved in the conversion, *CYP2A6* and *CYP2D6*, present an extensive inter-individual variation in the population, primarily due to genetic variation. These genes are known to be highly polymorphic. Thus, *CYP2A6* has more than 40 allelic characterized variants, and over 100 alleles and sub-alleles are described in the case of *CYP2D6* (Hicks, Swen and Gaedigk, 2014; Tanner and Tyndale, 2017). Moreover, certain drugs, dietary compounds, and hormones had been described to affect their activity *in vivo*.

The field of pharmacogenetics has emerged to study variations in DNA sequence related to drug response and is currently being used to personalize drug therapy (Gaedigk *et al.*, 2008). An activity score system has been developed to standardize phenotype assignment from *CYP2D6* genotype (Gaedigk *et al.*, 2008), which has been adopted by the Clinical Pharmacogenetics Implementation Consortium (CPIC) to develop drug

therapy guidelines (Gaedigk *et al.*, 2018). The area of pharmacogenetics and the relevance of genetic variation are increasingly being acknowledged for its clinical importance and are starting to be implemented in the clinical setting. Nevertheless, little attention has been paid to genetic variation in the metabolic disposition of dietary phenolic compounds and how this variability may affect their health effects in humans. For this reason, further studies should be performed to understand how genetic background can affect phenolic compounds response *in vivo*.

2. OBJECTIVES

2. OBJECTIVES

Tyr has been proposed as an endogenous precursor of HT in pre-clinical and *in vitro* studies. Therefore, the fact that dietary Tyr could be metabolized into HT *in vivo* has highlighted the potential role of Tyr-rich food as alternative sources of HT and hence, to trigger equivalent health effects in the organism. However, the fact that the endogenous bioconversion of Tyr into HT is catalyzed by the highly polymorphic cytochrome P450 isoenzymes *CYP2A6* and *CYP2D6* has led to propose the efficiency of the conversion of dietary Tyr into HT could highly depend on the individual genetic background. Based on these hypotheses, the following objectives were planned:

- **Objective 1:** Demonstrate the *in vivo* endogenous transformation of dietary Tyr into HT in the context of a randomized controlled clinical trial.
- **Objective 2:** Assess the clinical effects of Tyr to HT conversion on endothelial function and cardiovascular biomarkers in individuals at high cardiovascular risk.
- **Objective 3:** Characterize the different dietary sources of Tyr and HT in the diet, including their most well-known sources (EVOO and wine) and explore beer as an alternative source of these phenolic compounds.

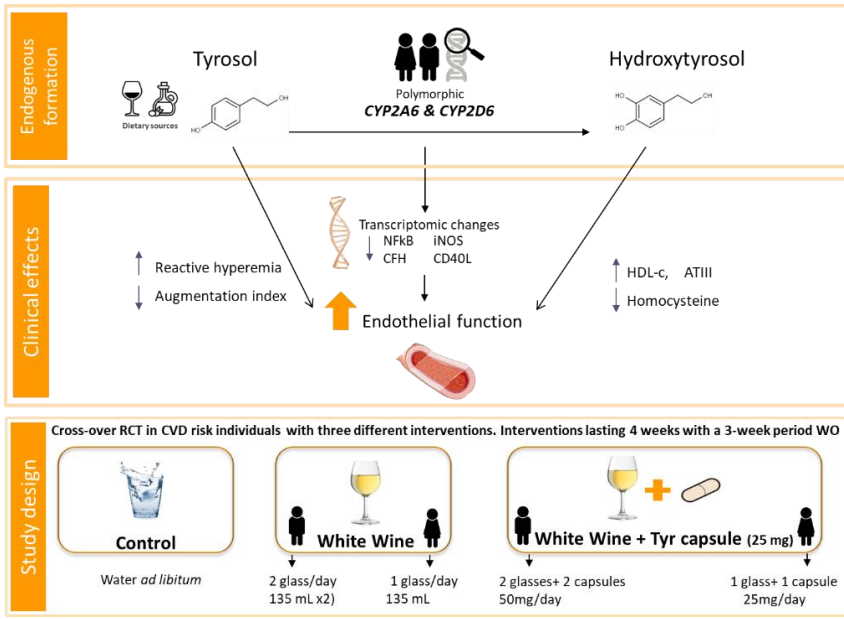
- **Objective 4:** Evaluate Tyr to HT conversion in the context of moderate beer consumption to determine Tyr bioavailability and factors interacting with it.
- **Objective 5:** Measure the *in vivo* nutrigenetic impact of polymorphic *CYP2A6* and *CYP2D6* in Tyr to HT bioconversion and its influence on derived health effects.

2. METHODS AND RESULTS

3.1

CHAPTER 1

Tyrosol and its endogenous conversion into hydroxytyrosol, effects on endothelial function and cardiovascular biomarkers



Adapted from:

Boronat A*, Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, Muñoz D, Barbosa F, Morales JC, Gaedigk A, Langohr K, Covas MI, Pérez-Mañá C, Fitó M, Tyndale RF, de la Torre R. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. Free Radic Biol Med. 2019 Aug 31; 143:471-481.

Boronat A*, Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, Muñoz D, Barbosa F, Morales JC, Gaedigk A, Langohr K, Covas MI, Pérez-Mañá C, Fitó M, Tyndale RF, de la Torre R. Data on the endogenous conversion of tyrosol into hydroxytyrosol in humans. Data in brief. 2019

Boronat A*, Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, Muñoz D, Barbosa F, Morales JC, Gaedigk A, Langohr K, Covas MI, Pérez-Mañá C, Fitó M, Tyndale RF, de la Torre R. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. Free Radic Biol Med 2019. doi:10.1016/j.freeradbiomed.2019.08.032.

3.1.1 Abstract

The simple phenol hydroxytyrosol (HT) has been associated with the beneficial health effects of extra virgin olive oil (EVOO). Wine contains HT at lower amounts but notably more tyrosol (Tyr). Pre-clinical studies identified Tyr hydroxylation as an additional source of HT, generated by the activity of cytochrome P450 isoforms *CYP2A6* and *CYP2D6*. In this work we aimed to (i) confirm Tyr to HT bioconversion *in vivo* in humans, (ii) ~~to~~ assess the cardiovascular benefits of this bioconversion, and (iii) determine the interaction between the benefits observed and a polygenic activity score (PAS) from *CYP2A6* and *CYP2D6* genotypes. To do so, a randomized crossover controlled clinical trial was performed. Individuals at cardiovascular risk (n=33) were randomly assigned to three 4-week interventions: 1) white wine (WW), 2) WW supplemented with Tyr capsules (WW+Tyr) and 3) water *ad libitum* (control). Participants were classified by a PAS as low versus normal activity metabolizers. HT urinary recovery following WW+Tyr was higher than after the other interventions ($p<0.05$). Low PAS individuals had lower urinary HT/Tyr ratios compared to individuals with normal PAS. WW+Tyr improved endothelial function, increased plasma HDL-cholesterol and antithrombin III, and decreased plasma homocysteine, endothelin 1, and CD40L, P65/RELA, and CFH gene expression in peripheral blood mononuclear cells ($p<0.05$). WW + Tyr intervention also abolished the increase in the expression of iNOS, eNOS, VEGFA, and CHF promoted by WW ($p<0.05$). In conclusion, Tyr, and its partial biotransformation into HT, promoted cardiovascular health-related benefits in humans after dietary doses of Tyr. The study design allowed the health effects of individual phenols to be singled out from the dietary matrix in which they are naturally found.

3.1.2 Introduction

A cross-sectional study in the frame of the PREDIMED trial described an association between urinary excretion of HT and consumption of alcohol and wine (Schröder *et al.*, 2009). Despite EVOO having larger concentrations of HT, a higher urinary recovery of HT was observed after red wine consumption when both were consumed at real-life moderate/dietary doses (De la Torre *et al.*, 2006). Small amounts of HT are endogenously produced as a secondary byproduct of dopamine oxidative metabolism (De la Torre *et al.*, 2006; Rodríguez-Morató *et al.*, 2016). This minor pathway in dopamine oxidative metabolism becomes more relevant after ethanol ingestion, in a dose-dependent manner (Pérez-Mañá, Farré, Pujadas, *et al.*, 2015). However, the increase in HT recovery after red wine intake could not totally be attributable to ethanol consumption, suggesting other sources of HT in wine. Wine represents a source of HT, and particularly of Tyr (Piñeiro *et al.*, 2011). Studies in animal models have shown that (i) Tyr is endogenously converted into HT, (ii) ethanol improves Tyr bioavailability, and (iii) Tyr + ethanol co-administration increases urinary excretion of HT metabolites in a dose-dependent manner (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015). However, this has never been demonstrated in humans. Additionally, *In vitro* experiments with human liver microsomes using specific CYP inhibitors and human recombinant proteins identified two isoenzymes of cytochrome P450, *CYP2A6* and *CYP2D6*, as mediators of the hydroxylation of Tyr into HT (Rodríguez-Morató *et al.*, 2017).

As mentioned before, HT shows a strong antioxidant activity *in vitro*. Apart, several relevant biological activities have been attributed to HT such as anti-inflammatory, anti-proliferative, pro-apoptotic, anti-microbial, and neuroprotective properties (Robles-Almazan *et al.*, 2018). Although HT has been extensively studied; most of these evidences come from *in vitro* and

pre-clinical studies and the mechanisms underlying many of the observed biological activities are still unknown (Marković *et al.*, 2019).

In contrast, less is known about the biological effects of Tyr, specially as it refers to *in vivo* studies. Tyr possess a weaker *in vitro* antioxidant activity compared to HT (Visioli, Poli and Galli, 2001). Both molecules share the same structure, but Tyr lacks the catechol group. The catechol group of HT is known to enhance its capacity to stabilize free radicals (Visioli, Poli, & Galli, 2001). Overall, the current evidence on the direct effect of HT and Tyr in humans is limited. Clinical trials had always relied on the effect of high HT or high Tyr products such as EVOO. Nevertheless, the global effects of EVOO cannot be directly extrapolated to the specific effects of HT and Tyr. Two clinical trials administering pure HT failed to observe any significant health effect (Lopez-Huertas et al., 2017, Crespo et al., 2015). Nonetheless, positive effects were reported by Colica et al (2017) in a clinical trial administering a nutraceutical preparation of HT on biomarkers associated with endothelial health, inflammation and antioxidant profile. The bioavailability of Tyr and HT is known to be highly dependent on the matrix in which they are contained. In the case of nutraceutical preparations, their bioavailability is low, a fact that could explain the scarce biological effects observed when administering pure compounds.

In this context, we performed a randomized, crossover, controlled clinical trial "Effects on cardiovascular risk factors of the endogenous hydroxytyrosol (3,4-dihydroxyphenylethanol, DOPET) generation after the combined intake of wine and tyrosol in humans" (NCT02783989). The trial followed a cross-over design and included three interventions: white wine, white wine plus Tyr capsules, and water acting as control. Volunteers included were at high cardiovascular risk.

The main aim of the study was to assess the Tyr biotransformation into HT in humans and the beneficial effects at a cardiovascular level of both Tyr supplementation and its biotransformation into HT. A secondary aim of this work was to evaluate the relevance of the different genotypic profiles of *CYP2A6* and *CYP2D6* on Tyr to HT biotransformation and the modulating effect of specific / particular genetic polymorphisms in the magnitude of the observed effects.

3.1.3 Material and methods

Methodology is separated in three sections: i) description of the clinical trial ii) metabolic disposition of tyrosol and hydroxytyrosol and its genetic regulation and, iii) clinical effects of the interventions.

3.1.3.1 Description of the clinical trial

a) Study design

The study consists in a randomized, crossover, controlled clinical trial performed at the Clinical Research Unit of the Hospital del Mar Medical Research Institute. The study included three interventions: water (control), white wine (WW), and WW plus Tyr capsules (WW+Tyr). Participants were randomly assigned to one of six possible orders to receive the intervention (Figure 8) through a computerized block-randomization method for sequence generation, performed by an independent statistician.

Order 1	WO	Water	WO	WW+Tyr	WO	WW
Order 2	WO	Water	WO	WW	WO	WW+Tyr
Order 3	WO	WW	WO	Water	WO	WW+Tyr
Order 4	WO	WW	WO	WW+Tyr	WO	Water
Order 5	WO	WW+Tyr	WO	Water	WO	WW
Order 6	WO	WW+Tyr	WO	WW	WO	Water

Figure 8. Latin square for 6 treatments in the randomized, crossover, controlled trial

Intervention periods lasted 4 weeks and were preceded by 3-weeks wash-out periods. Participants followed a low-phenol diet throughout all the study and were requested to avoid additional sources of alcohol. Dietary compliance was assessed by performing a 24 h food recall before and after each intervention period. Physical activity was recorded at the beginning and at end of the study, assessed by the Minnesota Leisure Time Physical Activity Questionnaire, validated for the Spanish population (Elosua *et al.*, 2000). A general physical examination, routine urine, blood and hematological analyses, were performed at the beginning and end of the study (Figure 9). The study was conducted in accordance with the Helsinki Declaration and approved by the local Ethical Committee (CEIm-Parc de Salut Mar), the clinical trial was registered in ClinicalTrials.gov: NCT02783989.

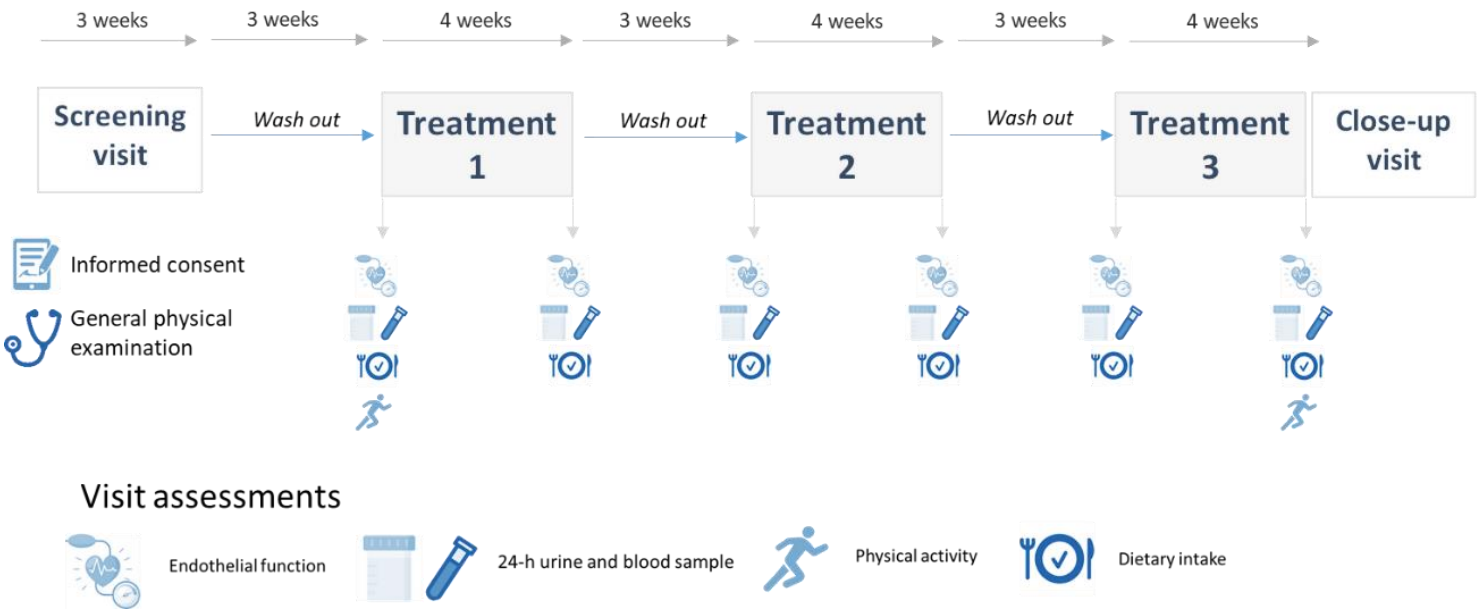


Figure 9. Study design and timeline: Cross-over randomized controlled clinical trial with three 4-weeks treatments separated by a 3-weeks wash out periods. Endothelial function, 24-hour urine, blood test and dietary intake were assessed at the beginning and at the end of each treatment. Physical activity was assessed at the beginning and at the end of the study

b) Interventions

Each study intervention lasted 4 weeks. Doses were different for women and men. The administered doses of alcohol where chosen following the American Heart Association guidelines, which limit alcohol consumption to one standard drink in the case of women (14 g of alcohol) and to two standard drinks for men (28 g of alcohol).

- Control intervention: participants were only allowed to drink water (no alcohol, wine, or supplemented Tyr or HT).

- White wine (WW) intervention: consisted in a glass of WW (135 mL) in the case of women and two glasses (270 mL) in the case of men. WW was consumed during meals. The WW used in the experiments was Bach Viña Extrísimo Blanco Seco 2016 (12,5 % Alc. Vol.) and was provided by the Codorniu Raventós Group (Barcelona, Spain). Tyr content (10,4 ± 0,6 mg/L) and that of HT (1,3 ± 0,1 mg/L) was quantified by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS).

- White wine plus tyrosol (WW+Tyr) intervention: WW was consumed as previously described and additionally a capsule containing 25 mg of Tyr was consumed with each glass of WW. Therefore, women were supplemented with 25 mg of Tyr whereas men were supplemented with 50 mg. Supplemented doses of Tyr were within the range of its maximal content per liter in red and fortified wines (e.g. sherry) (http://phenol-explorer.eu/contents/polyphenol/673). Tyr was administered in gelatin white opaque capsules containing 25mg Tyr/unit, produced by the manufacturing department of the Jordi Cabezas Pharmacy (Barcelona, Spain).

Table 2: Alcohol, tyrosol and hydroxytyrosol composition of each intervention.

	Control: Water	White wine				White wine + tyrosol			
		mL	mg alc.	Tyr	HT	mL	mg alc.	Tyr	HT
Woman	*Ad libitum*	135	13,5	1,4	0,2	135	13,5	26,4	0,2
Men		270	27	2,8	0,4	270	27	52,8	0,4

c) Participants

Recruitment was carried out through a volunteer center database, primary healthcare centers, and word of mouth, starting on January 2016 and finishing on December 2017. Inclusion criteria were: to be at high risk for coronary heart disease (CHD) meeting 3 or more risk factors, [current smoking (>1 cig/day during the last month), hypertension (≥140/90 mmHg or antihypertensive medication), high LDL cholesterol (>130 mg/dl or lipid-lowering therapy), low HDL-cholesterol (≤40 mg/dl in men and ≤50 mg/dl in women), overweight/obesity (body mass index ≥25 kg/m^2), a family history of premature CHD, and type II diabetes treated with oral hypoglycemic agents]; and to have a social or recreational use of ethanol/wine consumption at least once during lifetime. Exclusion criteria are detailed in table 3.

Before inclusion, candidates were interviewed to exclude any concomitant medical conditions, and underwent a general physical examination, laboratory tests, and 12-lead ECG. Written informed consent to participate was obtained prior to any study-related procedure.

Table 3. Summary of inclusion and exclusion criteria

INCLUSION CRITERIA	EXCLUSION CRITERIA
60- 80 years	History of cardiovascular disease
≥ 3 cardiovascular risk factors	Severe chronic illness or inflammatory disease
• Smokers	Excessive alcohol consumption (>80g/day)
• Hypertension	Alcohol hypersensitivity or intolerance
• High LDL	Illicit drug consumption
• Low HDL	Intake of antioxidant supplement
• Overweight or obesity	Sedative drugs
• Family history of premature coronary heart disease	Multiple allergies or intestinal diseases
• Type II diabetes	Vegetarian or special diets
Social or recreational use of ethanol/wine	Condition limiting mobility and limiting the assistance to study visits
	Illiteracy

d) Sample collection

At the beginning and at the end of each intervention, blood was collected into EDTA tubes (Vacutainer Tubes, Becton-Dickinson, Franklin Lakes, NJ, USA). Samples were centrifuged at 1700 g for 15 minutes at 4ºC. Plasma aliquots were kept at -80ºC and buffy coat was kept for DNA isolation performed with QIAamp DNA Blood Midi Kit (Qiagen, Dusseldorf, Germany). Each participant collected a 24-hour urine sample the day prior to the beginning, and at the end of each intervention. The total amount of urine collected was registered. Aliquots were acidified to avoid phenolic compounds degradation and frozen at -80ºC.

3.1.3.2 Metabolic disposition of tyrosol and hydroxytyrosol and its genetic regulation

a) Hydroxytyrosol and tyrosol metabolites urinary determination

The excretion of the main Tyr and HT metabolites was measured in 24h-urinary samples before and after each intervention. Quantified Tyr metabolites included Tyr-4-sulfate and Tyr-4-glucuronide. HT metabolites quantified were HT-3-sulfate, HT-4-sulfate, HT-acetate-3-sulfate, HT-3-glucuronide, HT-4-glucuronide, and homovanillyl alcohol (HVAL)-4-glucuronide. Total Tyr and HT corresponded to the molar sum of their respective quantified metabolites. Briefly, 0.5 mL of urine were diluted with 0.5 mL of purified water, spiked with 10 μL of internal standard mixture (containing 10 μg/mL of 3-(4-hydroxyphenyl)-1-propanol glucuronide and 10 μg/mL HT-1'-O-sulfate) and stabilized with 1 mL of phosphoric acid 4% (v/v). Thereafter, samples went under a solid-phase extraction using Oasis HLB columns 3 mL, 60-mg cartridges from Waters Corporation (Milford, MA, USA). First, samples were loaded into cartridges then washed with 2 mL of purified water. Finally, the compounds of interest were eluted from the cartridge with 2 mL of pure methanol. The methanol extracts were then evaporated until dryness under a stream of nitrogen (29ºC, 10-15 psi). Dried extracts were reconstituted with a mixture of mobile phases (95 % A and 5% B (v/v)), transferred into HPLC inserts, and injected in the LC-MS/MS. To prepare blank samples and calibration curves, urine from volunteers after consuming a diet poor in Tyr and HT containing foods was used in which Tyr and HT metabolite concentrations were below quantification limits. Blank urine was spiked with increasing concentrations of the metabolites of interest, and then processed in the same manner as samples.

Identification and quantification of the metabolites was performed using an Agilent 1200 series HPLC system coupled to a triple quadrupole (6410 Triple Quad LC-MS) mass spectrometer with an electrospray interface from Agilent Technologies (Santa Clara, CA, USA). For the chromatographic separation, an Acquity UPLC®BEH C18 column (100 mm × 3.0 mm i.d., 1.7 μm particle size) from Waters Corporation (Milford, MA USA) was used at 40ºC. The composition of mobile phase A was 0.01% (v/v) formic acid in water, and mobile phase B was acetonitrile with 0.01% (v/v) of formic acid. The injection volume was 10 μL and the flow rate was set at 0.25 mL/min. The ion source operated in negative ionization for 27 minutes.

b) Ethyl glucuronide

Ethyl glucuronide is a well-known biomarker of alcohol consumption. We measured its 24 h-urinary concentrations to assess the compliance during the wash-out periods and at the end of each intervention to evaluate volunteers' adherence. The method used a dilute-and-shoot approach for sample preparation, and analysis was performed LC-MS/MS (17). Briefly, 30μL of urinary samples were spiked with 10 μL of internal standard containing 10 g/mL ethyl-glucuronide-d5 and diluted into a final volume of 150 μL with mobile phase. Identification and quantification were performed using a LC-MS/MS Agilent 1200 series HPLC (Agilent technologies, Santa Clara, California USA) coupled to a triple quadrupole with an electrospray (6410 Triple Quad LC-MS). Mobile phase compositions were: A; 0.1% (v/v) of formic acid in water and mobile phase B with 0.1% (v/v) of formic acid in acetonitrile. To perform the chromatographic separation, we used an Acquity UPLC® BEH C18 column with a 1.7 μm particle size, 3 mm x 100 mm (Waters, Milford, Massachusetts, USA).

c) Pharmacokinetics nested sub-study

Within the parent study, a pharmacokinetic sub-study involving nine participants from the whole study population was performed. On the first day of each intervention period, following the first treatment administration, participants collected their urine for 24 hours in two different time period fractions: 0-8 hours and 8-24 hours. The day that the pharmacokinetic study was conducted, men and women took the same dosage: one glass of WW or one glass of WW supplemented with 1 capsule of Tyr (25 mg) or water. This sub-study allowed: (i) the assessment of HT and Tyr metabolism following each intervention in two urine collection time periods, and (ii) the identification of potential sex differences, as in this sub-study subjects received the same dose of Tyr.

d) Genotyping

Volunteers were genotyped for *CYP2A6* and *CYP2D6* allelic using TaqMan genotyping assays (Applied Biosystems, Foster City, CA, USA). Allelic variants tested included mostly single nucleotide polymorphisms (SNP) but also copy number variations (CNV), which were analyzed differently. When these allelic variants were not detected, the allele *1 was assumed.

d.1) SNP genotyping

Table 4 shows the characteristics of *CYP2A6* and *CYP2D6* tested allelic variants. The following SNPs were analyzed: for *CYP2A6* *2 and *9, and for *CYP2D6* *2, *4, *9, *10, *35, and *41. To determine their presence TaqMan SNP genotyping assay was performed, using FAM™ and VIC™ dye-labeled TaqMan pre-designed proves. Real-time polymerase chain reaction (PCR) was performed in a QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were prepared with 15 ng of DNA, 0.25 µL of TaqMan SNP Genotyping Assay,

and 2.5 µL of TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA). SNP determination was made using allelic discrimination plots with TaqMan Genotyper Software (Applied Biosystems, Foster City, CA, USA).

Table 4 Characteristics of *CYP2A6* and *CYP2D6* tested SNPs

Tested Allelic Variants	Reference Number	Nucleotide Substitution	Amino acid substitution	TaqMan Assay ID
CYP2A6	rs1801272	479T>A	Leu160His	C_27861808_60
	rs28399433	- 48T>G	Upstream	C_30634332_10
CYP2D6	rs1135840	4181G>C	Ser486Thr	C_27102414_10
	rs16947	2851C>T	Arg296Cys	C_27102425_10
	rs3892097	1847G>A	Intron Variant	C_27102431_DO
	rs5030656	2616_2618delAAG	Lys281del	C_32407229_60
	rs1065852	100C>T	Pro34Ser	C_11484460_40
	rs769258	31G>A	Val11Met	C_27102444_80
	rs28371725	2989G>A	Intron Variant	C_34816116_20

d.2) Copy number variation (CNV) detection analysis

TaqMan CNV assays were used to analyze *CYP2A6* allelic variants *4, *12 (Hs07545274_cn; Hs07545275_cn), and *CYP2D6* allelic variants *5 (deletion), *1xN, *2xN, and *35xN (Hs00010001_cn). Real time qPCR was performed using specific TaqMan assays. Quantitative PCR was performed in QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, USA). Reaction was carried in 384-well plates with a mixture of TaqMan Master Mix (Applied Biosystems, Foster City, USA), CNV assays, 10 ng of DNA and RNase P as reference (Applied Biosystems, Foster City, USA). Reactions were performed in duplicates. Copy number calls were

made using the Expression Suite Software v1.0.3 (Applied Biosystems, Foster City, USA).

e) Polygenic activity score

CYP2A6 and *CYP2D6* activities were estimated from the obtained genotypes. A scoring system was established for each enzyme based on the method described by Gaedigk et al. (2018) for *CYP2D6* and we adapted it for *CYP2A6*. Tested allelic variants were categorized according to functionality into non-function, reduced-function, normal function, and increased function. A score of 0, 0,5 or 1 was assigned for the presence of each allele (Table 5), obtaining a final activity score ranging from 0 to 2 for each gene. The allelic score could be later multiplied if presenting duplications of a specific allele. Then, a pooled polygenic activity score (PAS) was obtained by adding both enzymes activity scores and a maximal potential activity score may go up to 8 (in this study the maximal score reached was 5). According to their PAS, volunteers were then placed into three different broad phenotypic categories: low-activity group (LA, range 1-2.5), normal activity group (NA, range 3-4) and rapid activity group (RA range 5).

Table 5. Tested allelic variants features

Tested Variants	Allelic	Functional consequence	Activity score	Defining SNP
CYP2A6	*2	No function	0	479 T>A
	*4	No expression	0	Gene deletion
	*9	Decreased	+0.5	- 48 T>G
	*12	Decreased	+0.5	Hybrid allele with CYP2A7
	*1xN	Increased	+2	Multiple copies
CYP2D6	*2	Normal	+1	2851 C>T 4181 G>C
	*4	No function	0	1847 G>A *
	*5	No expression	0	Gene deletion
	*9	Decreased	+0.5	2616 del AGG
	*10	Decreased	+0.5	100 C>T 4181 G>C
	*35	Normal	+1	31G>A 2851 C>T 4181 G>C
	*41	Decreased	+0.5	2989 G>A 2851 C>T 4181 G>C
	*1xN *2xN *35xN	Increased	xN	Multiple copies

*4 sub-alleles can commonly present other SNPS such as 100 C>T, 4181 G>C and/or 2851 C>T.

3.1.3.3 Clinical effects of Tyrosol supplementation

a) Primary outcome: endothelial function

Endothelial function was assessed before and after interventions by monitoring endothelium-mediated changes (reactive hyperemia index, RHI) in the digital pulse waveform, known as the Peripheral Arterial Tone (PAT) signal (EndoPAT 2000; Itamar Medical Inc., Caesarea, Israel). Specially designed finger probes were placed on the middle finger of each subject's

dominant hand. The probes comprised a system of inflatable latex air cuffs connected by pneumatic tubes to an inflating device controlled through a computer algorithm. A constant counter pressure (pre-determined by baseline DBP) was applied through the air cushions. Pulsatile volume changes of the distal digit induced pressure alterations in the finger cuff, which were sensed by pressure transducers and transmitted to and recorded by the EndoPAT 2000 device. EndoPAT 2000 also provides the augmentation index (AI), a measurement of arterial stiffness via pulse-wave analysis, which was normalized to 75 bpm heart rate. Measurements were performed by a trained professional with the participants in resting supine conditions, in a quiet room, at a constant temperature and after 10 minutes of stabilization.

b) Secondary outcome

b.1) Lipid and inflammatory biomarkers

Plasma glucose, triglycerides, total and HDL cholesterol (HDL-c) were measured by automated enzymatic methods. LDL cholesterol was calculated by the Friedewald formula whenever triglycerides were lower than 300 mg/dL. Serum high-sensitivity C reactive protein (hs-CRP) was determined by immunoturbidimetry (Horiba, Montpellier, France). All analyses were carried out before and after interventions, and samples from the same individual were analyzed in the same analytical run.

b.2) Vasodilatation related markers

Homocysteine (Hcy), nitrates, nitrites and endothelin-1 were measured as vasodilatation related biomarkers. Plasmatic levels of Hcy were measured before and after each intervention by GC/MS. Shortly, plasma aliquots of 40 µL were spiked with 40 µL Hcy-d4 and subjected to a phase reduction by adding 10 µL of THP tris(hydroxypropyl)phosphine) (23nM). Subsequently,

the mixture was subjected to a process of deproteinization using 40 µL TCA (Trichloroacetic) at 0.6M. Then, samples were derivatize with 40 µL of 1-propanol/pyridine (3:1), 130 µL TMP/BAC/PCF (Isooctane, Butyl acetate, Propyl chloroformate) (10:3:1) and 100 µL of chloroform containing 1% of PCF. Finally, samples were subjected to a liquid-liquid extraction with chloroform. The organic extracts were dried under a steam of nitrogen (29º, 10-15 psi) and reconstituted with chloroform containing 1% of PCF. The total Hcy concentration was determined by gas chromatography–mass spectrometry (GC/MS). Plasma concentrations of nitrates and nitrites were determined at the first visit and at the end of each intervention using a colorimetric kit (Cayman Chemical, Michigan, USA). Finally, plasma endothelin 1 (ET1) concentrations were measured by ELISA (Invitrogen, California, USA) at the end of each intervention. All samples were analyzed by duplicate.

b,3) Coagulation and fibrinolysis biomarkers

Antithrombin III (ATIII) and dimer D plasmatic levels were measured by automated enzymatic methods. The plasminogen activator inhibitor-1 (PAI-1) plasmatic concentration was measured by ELISA (Affymetrix, California, USA). All samples were analyzed in duplicate.

b.4) Gene expression

Based on their relationship with endothelial health and atherosclerosis, and the available data of gene expression response after VOO ingestion, several candidate genes were selected (Castañer *et al.*, 2012; Farràs *et al.*, 2013) (Figure 10). They were: AKT serine/threonine kinase 2 (*AKT2*); arachidonate 5-lipoxygenase (*ALOX5*); CD40 ligand (*CD40L)*; complement factor H (*CFH*); endothelial nitric oxide synthase 3 (e*NOS*); endothelial plasminogen activator inhibitor (*SERPINE1*); inducible nitric oxide synthase (i*NOS*); interferon gamma (*IFNG*); interleukins (IL)1B (*IL1B*) and

6 (*IL6*); matrix metalloproteinases (*MMP*) 2 (*MMP2*) and 9 (*MMP9*); mitogen-activated protein kinase 14 (*MAPK14*); monocyte chemoattractant protein 1 (*MCP1*); nuclear factor (NF) (erythroid-derived 2)-like 2 (*NEF2L2*); NF-kappa B inhibitor alpha (*NFKBIA*); platelet-derived growth factor subunit B (*PDGFB*); peroxisome proliferator-activated receptor alpha (*PPARα*); sirtuins *(SIRT)* 1 (*SIRT1*), 2 (*SIRT2*), and 6 (*SIRT6*); transcription factor p65 (*RELA*); tumor necrosis factor alpha (*TNF*-α); and vascular endothelial growth factor (*VEGFA*). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and B-actin were used as endogenous controls to correct changes in gene expression. Isolation of RNA from PBMC was performed with the RNeasy Mini Kit (Qiagen, Duesseldorf, Germany). DNA complementary conversion was then carried out with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

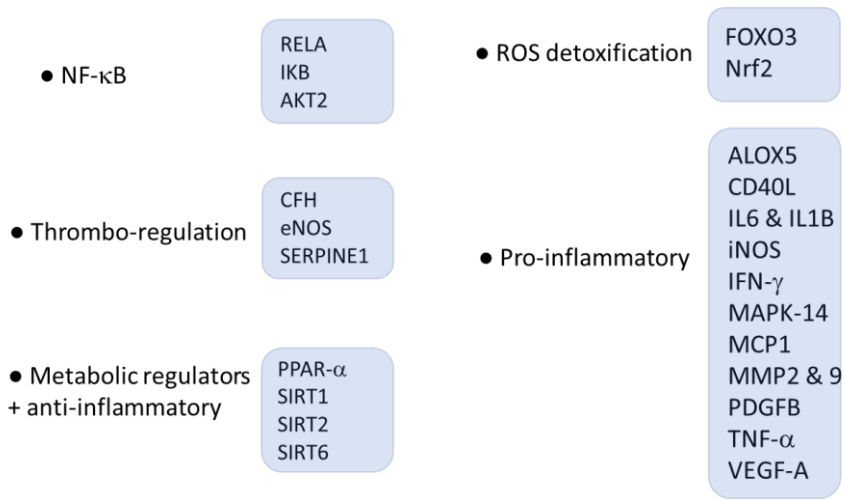


Figure 10. List of screened genes grouped by its main role in the cardiovascular system

The expression of selected genes was measured before and after interventions, by a PCR with a QuantStudio™ 12K Flex real-time PCR

System (Applied Biosystems, Foster City, USA) and SYBR Green dye-based analysis. Samples were analyzed in duplicate. Results were obtained with the Expression Suite Software v1.0.3 (Applied Biosystems, Foster City, USA). Changes in gene expression were assessed firstly by calculating the relative quantification, applying the $2^{-\Delta\Delta CT}$ of each sample. Thereafter, the fold change of each intervention was extracted by calculating the ratio between values at the end and at the beginning of each intervention.

3.1.3.4 Statistical analysis

a) Sample size and power analyses

A total sample of 32 participants would allow at least 80% power to detect a statistically significant difference among groups of 0.205 units in the RHI measurement, assuming a dropout rate of 5% and type I error of 0.005 (2-sided). The standard deviation of the measurement assumed was 0.4 (Rubinshtein *et al.*, 2010).

b) Statistical Analyses

Normality of continuous variables was assessed by normal probability plots and data was transformed when required. Intra-treatment comparisons were assessed by Student's t-test for paired samples. Comparisons among treatments were made by an ANOVA for repeated measures and adjusted by age, sex, smoking, acetylsalicylic acid (ASA) medication, and baseline concentrations. In the case of lipids an additional adjustment for LDL cholesterol values at the beginning of the study was performed. A general lineal model was used to assess linear and quadratic trends. For the post-hoc pairwise comparison, the Tuckey test was used. Statistical analyses were performed with R (R Foundation for Statistical Computing, Vienna, Austria), version 3.0.2. and R packages lme and multcomp. Significance was defined as $P < 0,05$.

3.1.4 Results

3.1.4.1 Study design

a) Participants

From the 192 subjects assessed for eligibility, 157 were excluded. The remaining 33 participants (21 men and 12 women) were randomly allocated to the interventions, and finally 32 participants completed the study (Figure 11). One subjected did not complete the last intervention period, corresponding to the WW intervention. Baseline characteristics of participants are shown in additional information (Table 16, Additional information). Participants' mean age was 65,3 ± 6,2 years with a mean BMI of 32,6 ± 4,2 kg/m². Main clinical features were overweight/obesity (97,0%), hypertension (84,8%), and dyslipidemia (75,6%). No differences in baseline characteristics were observed among the sequence of administration groups with the exception of LDL cholesterol which was higher (157 ± 24 mg/dL) in sequence 3 than in sequence 5 (98 ± 32 mg/dL). Due to this, lipid variables were adjusted by LDL cholesterol in the statistical analyses. No changes in the level of physical activity were observed from the beginning to the end of the study in any group. No dietary differences were observed among interventions (Table 17, Additional information). Basal values of ethyl-glucuronide and those after water treatment were undetectable (i.e. below 4.5 nmol/mL based on assay detection limits). Ethyl-glucuronide total urinary excretion after WW intervention was 1,6 ± 1,1 μmol, and 2,2 ± 1,6 μmol following WW+Tyr.

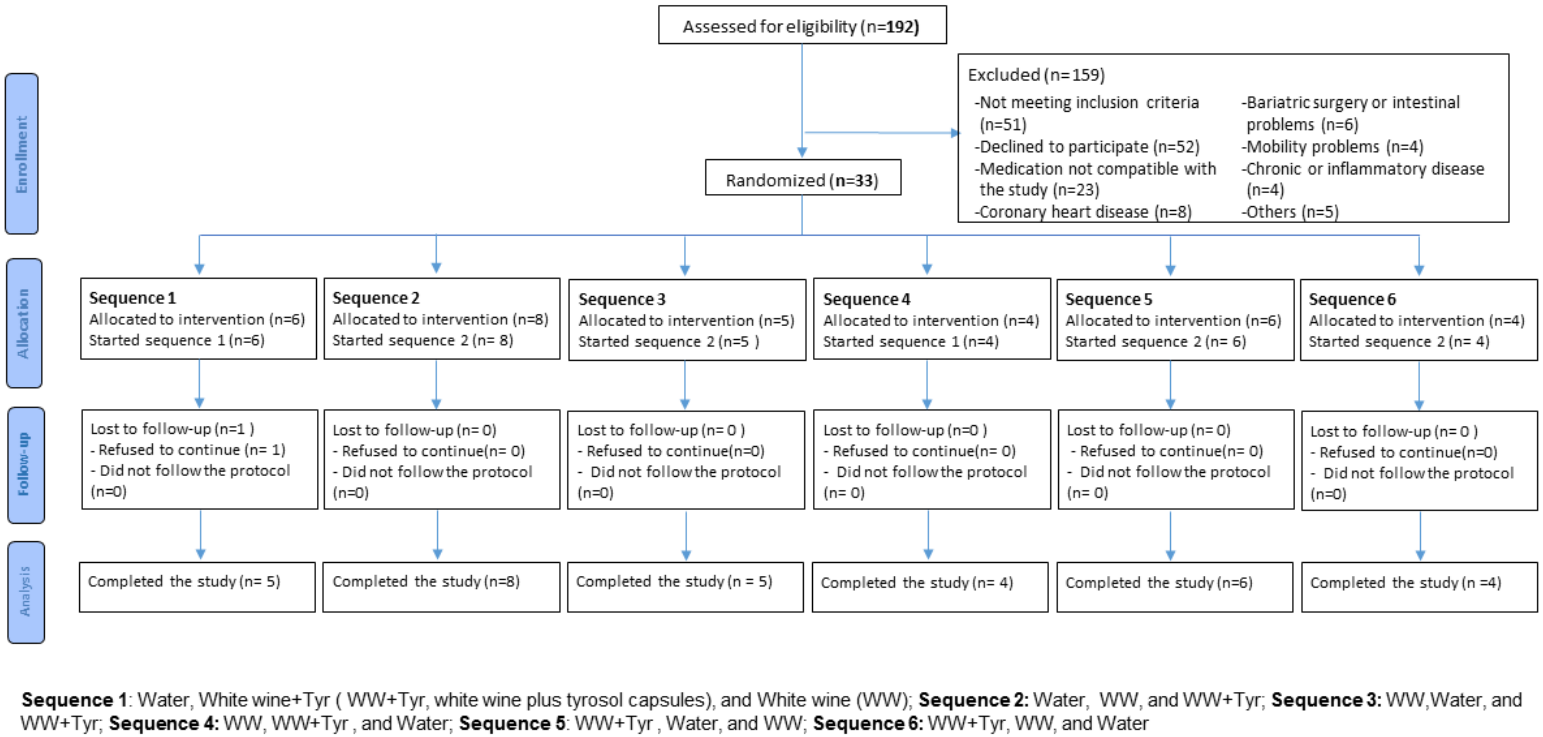


Figure 11. Consort Flow Diagram of the study

3.1.4.2 Metabolic disposition of Tyrosol and Hydroxytyrosol and its genetic regulation

a) Genotyping

All volunteers were genotyped for *CYP2A6* and *CYP2D6* allelic variants and gene duplications. Their frequencies are summarized in the supporting information. From their genotype of both genes, a polygenic activity score (PAS) was calculated. According to PAS, individuals were categorized into three different groups: 11 in the low activity one, 19 as normal activity, and 2 individuals as rapid activity group. Age and sex were equally distributed among the three groups (Table 6).

Table 6. Distribution of volunteers according to the polygenic activity scores for *CYP2A6* and *CYP2D6* variants.

	Low activity	Normal activity	Rapid activity
[n(%)]	11 (34,4%)	19 (59,4%)	2 (6,3%)
Age (y) [1]	65,1 ± 5,7	64,8 ± 6,1	72.0 ± 5,5
Sex [n(%)]			
Women	4 (36,4%)	7 (36,9%)	1 (50,0%)
Men	7 (63,6%)	12 (63,1%)	1 (50,0%)
Range of polygenic activity score	1-2,5	3-4	5

[1] Mean ± SD

b) Hydroxytyrosol and tyrosol metabolites urinary determination

Table 7 shows Tyr and HT urinary recoveries after each intervention. Baseline values (urines collected 24h prior intervention) did not differ among treatments. Total Tyr urinary recovery compared to its baseline increased only after the WW+Tyr intervention ($p<0,01$). The Tyr recovery observed after the WW+Tyr intervention was significantly different

compared to the observed after control (p<0,001), and after WW (p=0,030). Tyr recovery after WW did not differ from that of the control group. Total HT urinary recovery increased after the WW+Tyr intervention (p<0,01) compared to its baseline. This change in HT recovery (baseline vs. end of intervention) was also superior to those observed after the control (p=0,001), and WW (p=0,002) interventions (Figure 12).

Table 7. Total Tyr and HT metabolites urinary recovery (0-24 h) in study volunteers (N=32)

	Control		WW		WW+Tyr	
	Baseline	Final	Baseline	Final	Baseline	Final
Total Tyr metabolites (µmols)	1,9 ± 1,6	1,8 ± 1,3	1,9 ± 2,3	2,4 ± 2,2	1,6 ± 1,5	8,8 ± 9,2 bb cc ww
Total HT metabolites (µmols)	4,0 ± 5,6	3,4 ± 3,5	4,0 ± 4,8	4,7 ± 5,6	3,6 ± 3,5	10,2 ± 12,8 bb cc w

Urinary excretion (µmols) of Total Tyr and Total HT at baseline and at the end of each intervention. [b] p<0,05, [bb] p<0,01 vs baseline, [c] p<0,05, [cc] p<0,01 vs change in control, [w] p<0,05, [ww] p<0,01 vs change in WW interventions. Data shown as mean ± SD.

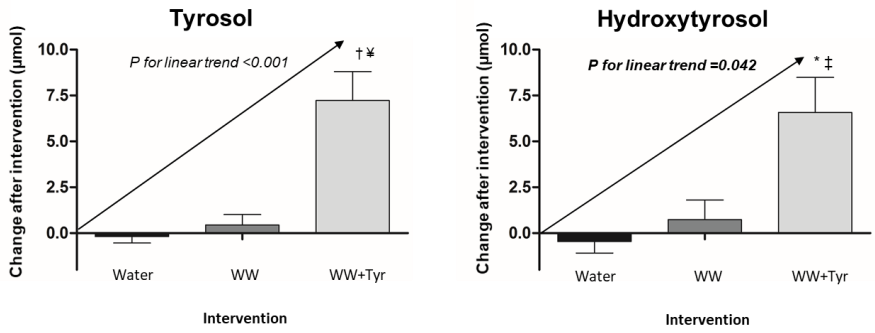


Figure 12. Changes in 24h-urinary recovery of tyrosol and hydroxytyrosol (Ends vs baseline of the intervention). Results are expressed as mean± SEM. WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; * p<0,05, † p<0,001 versus water; ‡ p<0,05, ¥ p<0,001 versus WW

c) Tyr and HT phase II metabolites

Tyr-4-sulfate and, most notably, Tyr-4-glucuronide urinary recovery increased significantly following the WW+Tyr intervention compared to the control and WW intervention (only Tyr-4-glucuronide). Likewise, an increased HT-3-sulfate urinary recovery was observed following WW+Tyr compared to both interventions ($p<0,01$). In contrast, HT-acetate-3-sulfate, HT-3- and 4-glucuronide and HVAL-4-glucuronide remained unchanged among the interventions (Table 8).

Table 8. Urinary recovery of Tyr and HT metabolites among treatments.

	Control	*WW*	*WW+Tyr*
Tyr-4-sulfate	0,4 ± 0,8	0,5 ± 1,5	1,5 ± 2,3 b, c
HT-3-sulfate	2,1 ± 2,71	3,3 ± 4,3	8,4 ± 1,1 bb, cc, ww
HT-acetate-3-sulfate	0,3 ± 0,4	0,4 ± 0,5	0,4 ± 0,5
Tyr-4-glucuronide	1,3 ± 1,1	1,8 ± 1,8	7,3 ± 7,9 bb, cc, ww
HT-3/4-glucuronide	0,7 ± 1,1	0,8 ± 1,1	0,7 ± 1,2
Hvalc-4-glucuronide	0,5 ± 0,5	0,6 ± 0,7	0,7 ± 0,7

Data expressed as mean ± SD. b $p<0,05$, bb $p<0,01$ versus baseline; c, $p<0,05$ cc $p<0,01$ versus control, ww $p<0,01$ versus WW.

d) Pharmacokinetics nested sub-study

The pharmacokinetics sub-study confirmed the endogenous generation of HT following the WW+Tyr intervention. It included 9 participants (3 women and 6 men). Increased recoveries of HT metabolites were observed in the first 8 hours post administration ($p=0.004$ for linear trend) and maintained elevated in the 8-24-hour fraction ($p=0.045$ for linear trend). On average, following WW+Tyr administration, 57 % of Tyr metabolites and 44 % of HT ones were excreted in the first 8 hours post administration (Figure 13).

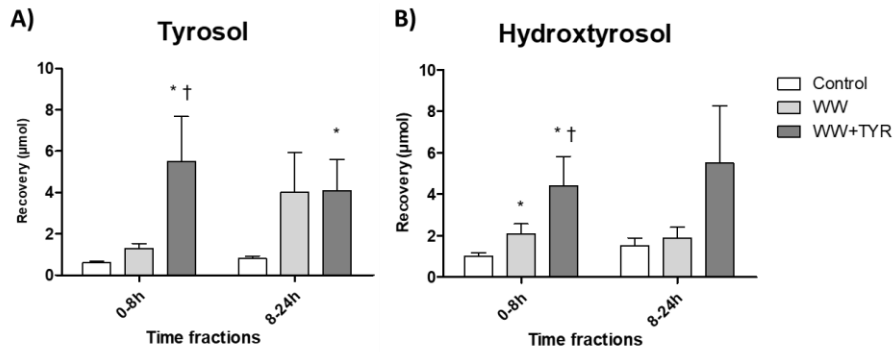


Figure 13. Pharmacokinetics study. Total Tyr (A) and Total HT (B) metabolites recovery, the first day of each intervention. Urine was collected in two time fractions: 0-8 hours and 8-24 hours, (n=9, 3 women and 6 men) Data is expressed as mean ± SEM. * p<0,050, ‡ p<0,001 versus water; † p<0,050, ¥ p<0,001 versus white wine compared to equivalent time fraction in women.

e) Interaction between genotype and Tyr and HT metabolism

The impact of *CYP2A6* and *CYP2D6* polymorphisms on the recoveries of total Tyr and total HT metabolites were evaluated. Figure 14 shows recoveries following WW+Tyr according to the PAS categories assigned to participants. Comparisons were adjusted to the administered dose of Tyr (recoveries were divided by the dose administered). The LA group presented higher recoveries of urinary total Tyr compared to NA one (0,38 ± 0,32 vs 0,12 ± 0,05 µmol of Total Tyr/administered Tyr; p=0,021). Conversely, HT metabolite recovery was higher in NA compared to LA (0,17 ± 0,16 vs 0,32 ± 0,34 µmol of Total HT/ Tyr administered p=0,115). As expected, HT/Tyr ratio were higher in NA compared to LA (p =0,025). RM were excluded from the statistical analysis due to its low number, however, their adjusted recovery for Tyr metabolites were 0,12 ± 0,11 µmol and their HT recovery 0,09 ± 0,34 µmol.

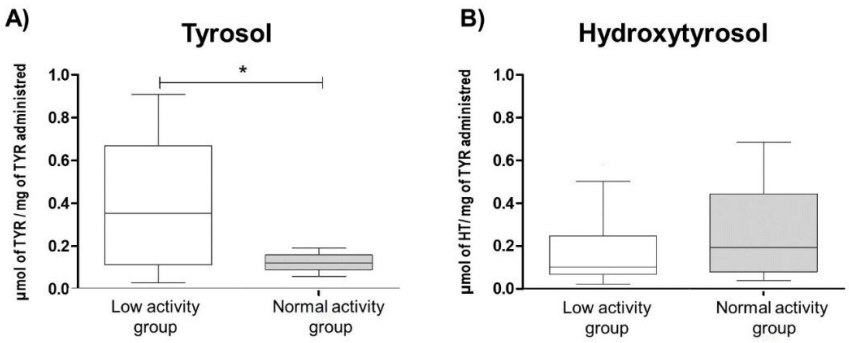


Figure 14. Phenotype interaction with Tyr and HT metabolites following WW+Tyr: recovery of Tyr (A), recovery of HT (B) standardized by Tyr dose administered. Data expressed as median and percentile 10-90th.

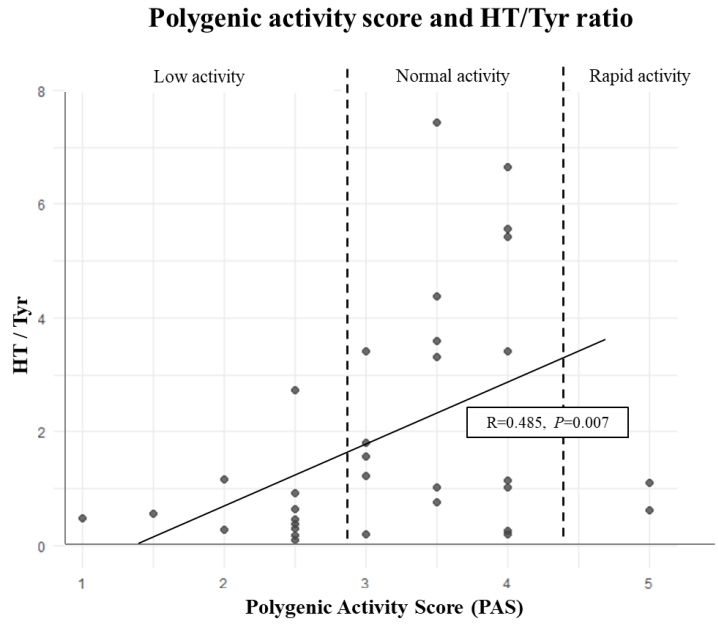


Figure 15. Correlation between the polygenic activity score and the HT/Tyr ratio. Broken lines indicate separation of low, normal and rapid activity groups. The determination coefficient has been assessed excluding individuals from the rapid activity group.

PAS correlated negatively with the adjusted Tyr recovery (r=-0,452; *p*=0,011). Figure 15 shows the positive correlation between PAS and HT/Tyr ratio (r= 0,448; *p*=0,011), excluding subjects with the rapid activity score. These associations were only found including both enzymes, and not when performing the same analyses separately for *CYP2A6* and *CYP2D6*.

f) Sex differences

The pharmacokinetic sub-study assessed the potential sex dimorphisms on the metabolic disposition of the studied phenol to a single and equal dose of WW+Tyr: 1 glass of WW and 25 mg of Tyr. Only individuals with *normal activity* were considered in order to avoid any interference of individuals of the *LA* or *RA activity* group (n=6, 3 women and 3 men). Figure 16 shows sex differences in the recoveries of total Tyr and HT. At both time fractions, women presented higher Tyr recovery (Figure 16.A). On the contrary, higher HT recovery (Figure 16.B) was observed in men. Figure 16.C represents the difference in the metabolic conversion rate, where men exhibited higher HT/Tyr ratios than women.

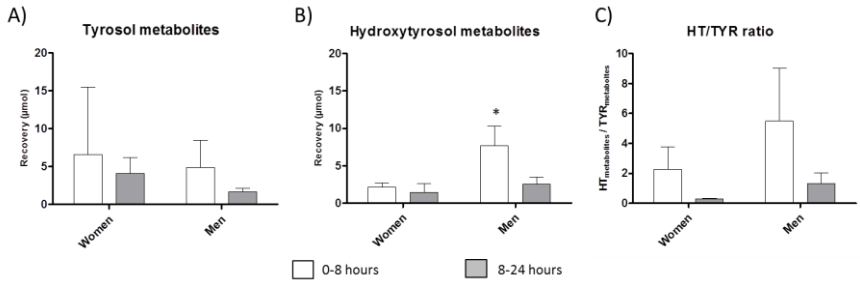


Figure 16. Sex differences in the pharmacokinetics sub-study. Tyr (A) and HT (B) metabolite recovery, and HT/Tyr ratio (C) the first day of WW+Tyr intervention. Urine was collected from 0-8 hours and 8-24 hours. Comparison only includes individuals with a normal PAS; 3 women and 3 men, using log transformed data and a t-test. Data are expressed as mean ± SEM. * $p < 0{,}050$ compared to the equivalent time fraction in women.

The parent study enabled the assessment of sex dimorphism following repeated doses of WW+Tyr. As expected, given the difference in the administered dose, Tyr recovery following WW+Tyr was higher in men than women (11,2 ± 11,2 vs 4,1 ± 3,5 μmols, p=0,013). The same accounted for the recovery of HT metabolites (10,7 ± 15,1 vs 7,9 ± 6,0 μmols, p=0,557) although significance was not reached. In terms of metabolites, and concurring with the previous observation, Tyr-glucuronide urinary recovery was higher in men than in women (9,2 ± 9,0 vs 2,7 ± 2,3 μmols, p=0,047) and the same trend was observed for Tyr-sulfate (1,91 ± 2,7 vs 0,6 ± 0,9 μmols, p=0,146). HT-3-sulfate recovery, although higher in men, was not statistically different (9,2 ± 13,4 vs 6,9 ± 5,7 μmols; p=0,598). The same analyses were performed considering only individuals of the *normal activity* group (n=19; 7 women and 12 men) to avoid the potential interference of extreme activity of *low* and *rapid* groups. Conclusions were the same than those reached with the whole population.

3.1.4.3 Clinical effects of Tyrosol supplementation

a) Primary outcome: endothelial function

Table 9 shows the changes in the reactive hyperemia index (RHI) and augmentation index (AI) after each intervention. One volunteer had skin petechiae the first time the ENDOPAT test was performed and endothelial function was not measured in this subject, thus only 31 participants were assessed. RHI increased after the WW+Tyr intervention compared to its baseline (p=0,048), remaining significant only in men (p=0,041) when analyses were made by sex. When assessing the PAS genotype interaction, RHI increased after the WW+Tyr intervention compared to its baseline (p=0,020) only in the case of the LA group, the increase reaching significance *versus* changes in the WW intervention (p=0,040). AI decreased significantly after WW+Tyr compared to changes in control intervention (p=0,047), but significance was not reached when analyses were made by

sex. When assessing the PAS genotype interaction, the decrease in AI reached significance *versus* changes in the control group in the case of the LA group (p=0,007).

Table 9. Changes in endothelial function after interventions

	Intervention				
	Control	WW	WW+Tyr	*p* value for WW+Tyr	
				vs Control	vs WW
Reactive hyperemia index (AU)					
All participants	0,0 ± 0,4	0,0 ± 0,5	**0,2 ± 0,5***	0,521	0,309
Women	0,0 ± 0,5	- 0,1 ± 0,8	0,1 ± 0,4	0,990	0,725
Men	0,1 ± 0,3	0,1 ± 0,2	**0,2 ± 0,5***	0,147	0,196
Genotype Interaction					
LA	- 0,0 ± 0,4	- 0,1 ± 0,6	**0,3 ± 0,4***	0,252	**0,040**
NA	0,1 ± 0,4	0,3 ± 0,5	0,1 ± 0,5	0,955	0,987
Augmentation Index (AU)					
All participants	2,5 ± 10,3	-2,2 ± 11,1	-3,8 ± 17,2	**0,047**	0,821
Women	5,0 ± 11,5	-4,3 ± 8,6	-2,4 ± 18,6	0,282	0,972
Men	1,1 ± 9,6	-1,2 ± 12,8	-5,2 ± 17,2	0,231	0,639
Genotype Interaction					
LA	1,2 ± 7,0	-0,8 ± 8,2	-5,0 ± 15,5	**0,007**	0,265
NA	2,3 ± 11,6	-1,1 ± 9,2	-3,9 ± 10,7	0,436	0,990

Results are expressed as mean ± SD (n=31). AU, arbitrary units; WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex, smoking habits, acetylsalicylic acid consumption, and baseline levels. $^{*}p < 0.05$ versus its baseline; p value, significance for inter-intervention comparisons.

b) Secondary outcome

b.1) Lipid and inflammatory biomarkers

Table 10 shows the changes in HDL-c after each intervention. A significant increase from their respective baselines was observed after the WW (p=0,027) and WW+Tyr (p<0,001) interventions, the latter remaining in men when analyses were performed by sex (p<0,001). The increase in HDL-c after WW+Tyr reached significance *versus* changes in the control group (p=0,025), and this significance remained in males (p< 0,001 *vs* control and p= 0,027 *vs* WW) and in the NA group (p=0,014 *vs* control) when analyses were performed by sex and by PAS genotype respectively. HDL-c increased in a dose-dependent manner with the content of alcohol plus Tyr administered in all participants (p=0,027 for linear trend), in men (p=0,001 for linear trend), and with a borderline significance in the NA group (p=0,082) (Figure 7). No differences by intervention group, sex, or PAS genotype were observed in total and LDL cholesterol, triglycerides, hsCRP, and glucose (Table 11).

Table 10. Changes in HDL cholesterol (mg/dL) after interventions

	Interventions				
	Control	WW	WW+Tyr Tyr	*P* value for WW+Tyr	
				vs Control	vs WW
Total	-0,1 ± 6,0	**1,6 ± 5,1***	**3,1 ± 4,0†**	**0,025**	0,417
Women	2,6 ± 5,1	3,1 ± 6,7	1,3 ± 3,9	0,794	0,697
Men	-1,7 ± 6,0	0,8 ± 3,8	**4,2 ± 3,7†**	**< 0,001**	**0,027**
Genotype interaction					
LA	0,7 ± 9,9	3,4 ± 7,7	**3,4 ± 3,2***	0,802	0,995
NA	-0,1 ± 2,4	**1,2 ± 2,3***	**2,9 ± 4,7***	**0,027**	0,304

Results are expressed as mean ± SD (n=32). WW, white wine; WW+Tyr, white wine plus Tyr capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex and smoking habits, acetylsalicylic acid consumption, LDL cholesterol at the beginning of the study, and baseline levels. *p< 0,05, †p<0,001 versus its baseline. p value, significance for inter-intervention comparisons.

Table 11. Changes in lipid and inflammatory biomarkers (mg/dL)

	Interventions				
	Control	WW	WW+Tyr	*P* value for WW+Tyr	
				vs Control	vs WW
Total Cholesterol	1,8 ± 12,5	2,9 ± 21,8	7,7 ± 24,4	0,665	0,753
LDL cholesterol	-1,0 ± 14,1	0,1 ± 17,8	4,7 ± 22,3	0,511	0,761
Triglycerides	14,7 ± 62,0	6,0 ± 25,9	-0,9 ± 25,7	0,290	0,788
Glucose	0,9 ± 13,4	2,7 ± 10,3	2,6 ± 12,0	0,849	0,999
hsPCR	-0,2 ± 1,3	0,02 ± 0,3	-0,01 ± 0,2	0,443	0,516

Results expressed as mean ± SD (N=32). LDL, low density lipoprotein; hsCRP, high sensitivity C reactive protein. ANOVA adjusted by age, sex and smoking habits, acetylsalicylic acid consumption, LDL cholesterol at the beginning of the study, and baseline levels.

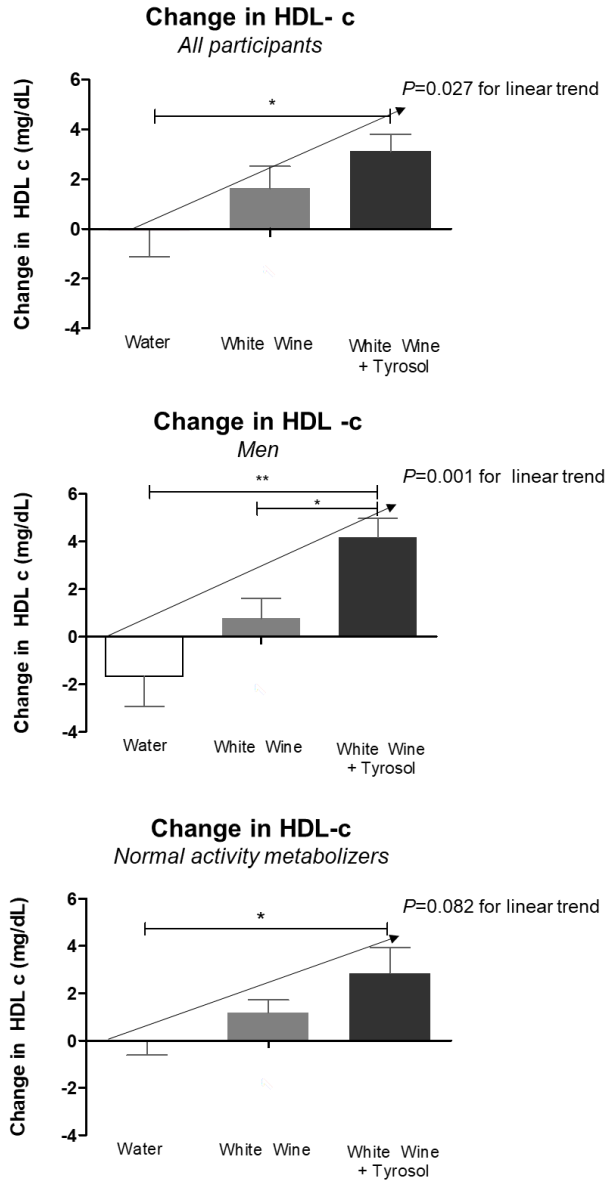


Figure 17: Changes in HDL cholesterol (HDL-c) after interventions. Change in HDL-c compared to the baseline of the intervention expressed as mean and SD in all participants (A), only men (B) and only normal activity metabolizers (C). ANOVA adjusted by age, sex and smoking habits, LDL cholesterol at the beginning of the study, and baseline levels * p <0,050; ** p < 0,001

b.3) Vasodilatation related markers

Table 12 show the changes in Hcy concentrations. Hcy increased after WW (p=0,049) and decreased after the WW+Tyr intervention (p= 0,041) in all participants. WW+Tyr decreased Hcy in both sex although without significance. When changes among interventions were compared, the decrease after WW+Tyr reached significance versus changes after WW (p=0,028). The latter trend remained in the NA group when stratified by PAS genotype (p=0,095). ET1 concentrations after interventions were: 2,2 ± 0,9; 2,3 ± 1,1 ng/mL, and 2,0 ± 0,8 ng/mL, after water, WW, and WW+Tyr interventions, respectively (Table 13). Concentrations after WW+Tyr were significantly lower versus WW intervention (p=0.031). No differences were observed when analyses were performed by sex. No differences by intervention group, sex, or PAS groups were observed in nitrate and nitrite values (Table 13).

Table 12. Homocysteine changes (µmol/L) after interventions

	Interventions				
	Control	WW	WW+Tyr	p value for WW+Tyr	
				vs Control	vs WW
All participants	-0,1 ± 1,1	**0,4 ± 1,1***	**-0,4 ± 1,0***	0,975	**0,028**
Women	-0,2 ± 1,2	0,7 ± 1,4	-0,6 ± 1,2	0,675	0,315
Men	0,0 ± 1,1	0,2 ± 0,9	-0,2 ± 0,8	0,826	0,358
Genotype interaction					
LA	0,1 ± 0,6	0,5 ± 1,0	-0,3 ± 0,9	0,973	0,533
NA	-0,2 ± 1,5	0,4 ± 1,2	-0,5 ± 1,1	0,963	0,095

Results are expressed as mean ± SD (n=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex smoking, acetylsalicylic acid consumption, and baseline levels. *p<0,050 versus its baseline; P value, significance for inter-intervention comparison

Table 13. Endothelin levels (ng/dL) after interventions and nitrites and nitrates (μM) change versus baseline.

	Interventions				
	Control	WW	WW+Tyr	P value for WW+Tyr	
				vs Control	vs WW
Entodhelin-1					
All participants	2,2 ± 0,9	2,3 ± 1,2	2,0 ± 0,8	0,572	**0,031**
Women	2,4 ± 1,2	2,6 ± 1,1	2,1 ± 0,8	0,479	0,108
Men	2,0 ± 0,8	2,2 ± 1,2	2,0 ± 0,9	0,990	0,463
Genotype interaction					
LA	1,93 ± 0,7	2,3 ± 1,0	1,9 ± 0,7	0,981	0,203
NA	2,24 ± 0.95	2,48 ± 1,32	2,13 ± 1,07	0,747	0,068
Nitrates and Nitrites					
All participants	1,7 (-12,5; 7,3)	2,8 (-15,3; 21,0)	0,4 (-12,9; 16,3)	0,987	0,835
Female	7,6 (-16,4; 22,4)	2,9 (-15,5; 27,0)	-0,10 (-25,4; 6,6)	0,673	0,740
Male	1,2 (-11,7; 4,7)	2,7 (-10,2; 14,1)	1,2 (-5,9; 16,4)	0,570	0,972
Genotype interaction					
LA	5,5 (-2,7; 16,1)	6,7 (-6,1; 18,0)	2,4 (-3,5; 14,9)	0,515	0,917
NA	-0,6 (-15,9; 4,7)	0,9 (-11,8; 21,1)	-0,1 (-8,2; 12,8)	0,688	0,532

Results are expressed as mean ± SD for ET-1 and as median (25th-75th percentiles) for Nitrates and nitrites (N=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex, smoking, acetylsalicylic acid consumption, and baseline levels. Prior to statistical analysis, nitrates and nitrites data was transformed using the following formula ln(x+1)).

b.5) Coagulation and fibrinolysis biomarkers

Table 14 shows the changes in ATIII after each intervention. ATIII increased after WW+Tyr compared to its baseline (p=0,005), the increase being significant versus changes after control (p=0,044) and WW (p=0,005) interventions. When data were analyzed by sex, a borderline increase (p=0,060) after WW+Tyr vs baseline remained in males, the increase reaching significance versus changes after WW (p=0,002). Concerning PAS groups, after the WW+Tyr intervention, ATIII plasma concentrations increased versus its baseline (p=0,010) in the NA group. No differences by intervention group, sex, or PAS groups were observed in PAI and dimer D plasma concentrations.

Table 14. Changes in antithrombin III (mg/dL) after interventions

	Interventions				
	Control	WW	WW+Tyr	*P* value for WW+Tyr	
				vs Control	vs WW
All participants	0,0 (-3,0; 0,0)	0,0 (-0,4; 0,2)	**0,0* (0,0; 7,4)**	**0,044**	**0,005**
Women	0,0 (-2,6; 0,0)	0,0 (0,0; 0,1)	0,0 (0,0; 6,6)	0,422	0,491
Men	0,0 (-3,2; 0,7)	0,00 (-5,6; 0,4)	0,7 (0,7; 9,0)	0,205	**0,002**
Genotype interaction					
LA	1,9 (0,5; 11,4)	0,0 (-9,1; 4,5)	0,7 (-1,6; 2,7)	0,670	0,054
NA	0,0 (-3,0; 0,0)	0,0 (-0,2; 0,2)	**0,0* (0,0; 7,4)**	0,245	0,408

Results are expressed as median (25th-75thpercentiles) (n=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex and smoking habits, acetylsalicylic acid, and baseline levels. Prior to statistical analysis, data were transformed using the following formula ln(x+1)). *P < 0,05; †p<0,01 versus its baseline; P value, significance for inter-intervention comparisons.

b.6) Gene expression

Figure 18 shows comparisons among the expression of genes related to cardiovascular risk with significant changes in all participants. Subgroup changes are depicted in table 15. *CD40L* expression decreased following WW+Tyr (p<0.001), the decrease was significant versus changes after control (p=0,041) and WW (p=0,003) interventions. When analyses were stratified by gender, *CD40L* decreased versus its baseline after WW+Tyr in both genders (p<0,050), the decrease was significant versus control (p=0,016) and WW (p=0,024) for males. Concerning PAS groups, in the NA group the *CD40L* expression decreased after WW+Tyr versus control (p=0,011) and WW(p=0,020) groups. The expression of *P65/RELA* decreased versus changes after WW intervention (p=0,048). *CFH* expression increased *versus* its baseline in all participants and among women (p<0,050) after WW intervention. After the WW+Tyr intervention, there was a decrease versus changes after WW intervention in all subjects (p=0.025). When analyses were stratified by gender, there was a decrease CFH expression in males versus baseline after the WW+Tyr intervention (p=0,010) which was significant versus control (p= 0,013) and WW (p=0,048). *iNOS* increased after WW intervention (p=0,032), the increase was significant versus WW+Tyr intervention in all subjects (p=0,007) and among males (p=0,019). The expression of *eNOS* and *VEGFA* increased after the WW intervention (p=0,034 and p=0,045, respectively), and decreased after the WW+Tyr intervention versus WW intervention in all subjects (p=0,045). No changes were observed in expression of other genes which were evaluated.

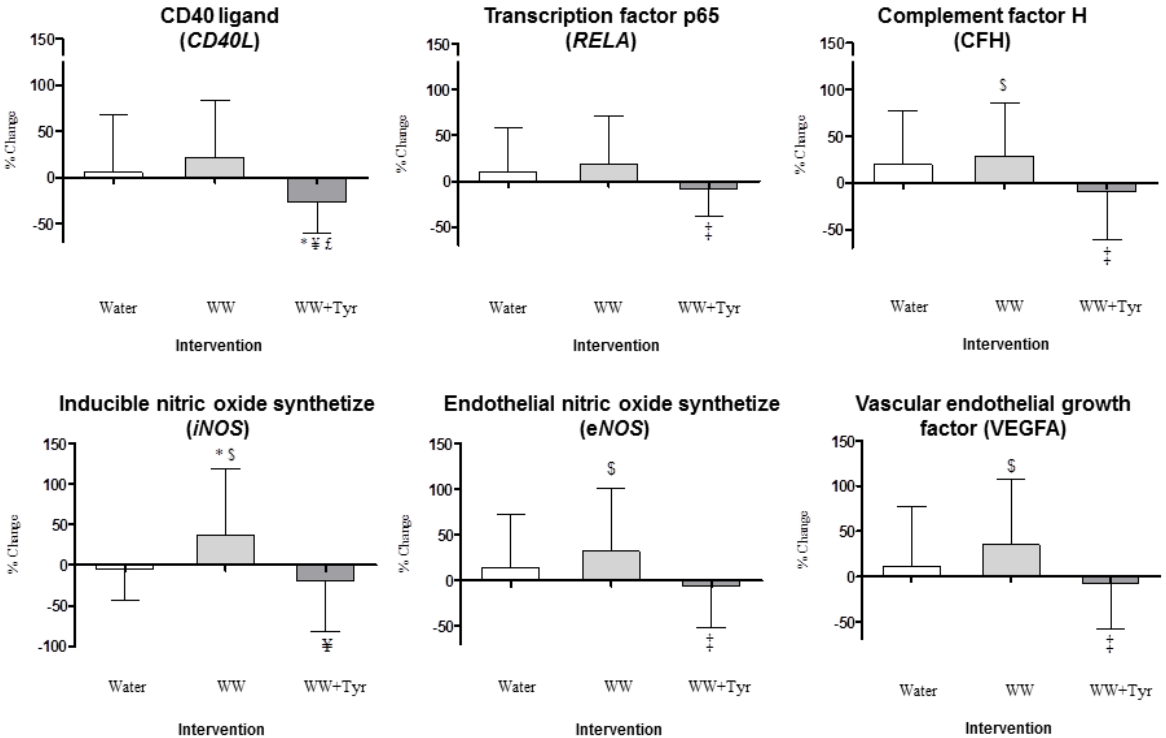


Figure 18. Changes in the expression of cardiovascular related risk genes. WW, white wine; WW+Tyr, white wine plus tyrosol capsules. * p<0,05 *versus* water; ‡ p<0,05, ¥ p<0,001 *versus* white wine; $ p<0,05, £ p<0,001 versus its baseline.

Table 15 Transcriptomic changes (% change versus baseline) after interventions

	Intervention				
	Control	WW	WW+Tyr	*P* value for WW+Tyr	
				vs Control	vs WW
CD40L					
All participants	8,9 ± 60,6	21,7 ± 62,3	**-26,8±34,2†**	**0,042**	**0,003**
Women	-10,7±75,4	28,8 ± 59,5	**-29,9±33,7***	0,743	0,063
Men	24,6±47,2	20,4 ± 66,2	**-25,5±35,8***	**0,016**	**0,024**
Genotype interaction					
LA	-4,8± 66,7	11,1± 59,8	-17,1 ± 35,6	0,874	0,514
NA	20,1± 60,0	16,7 ± 56,5	**-28,2± 33,9†**	**0,011**	**0,020**
P65/RElA					
All participants	1,1 ± 0,5	1,18 ± 0,5	0,9 ± 0,3	0,229	**0,048**
Women	-7,6± 54,0	26,9 ± 63,3	-16,9 ± 29,8	0,896	0,089
Men	22,9± 41,9	13,4 ± 48,2	-2,2 ± 28,6	0,157	0,484
Genotype interaction					
LA	16,7 ± 63,5	6,3 ± 45,3	-3,0 ± 43,4	0,584	0,886
NA	6,7 ± 41,1	22,5± 58,3	-12,5 ± 20,9	0,414	0,054
CFH					
All participants	19,8 ± 57,6	**28,8 ± 56,5***	-9,1 ± 51,5	0,115	**0,025**
Women	9,2 ± 57,5	**48,4 ± 51,9***	11,9 ± 63,2	0,994	0,334
Men	27,9 ± 59,5	16,6 ± 58,9	**-18,1 ± 40,9***	**0,013**	**0,048**
Genotype interaction					
LA	21,4± 77,7	35,2 ± 74,8	-16,7 ± 29,5	0,359	0,150
NA	22,4± 46,9	22,6 ± 45,1	0,0 ± 62,8	0,438	0,433

Table 15 (continuation)

iNOS					
All participants	-5,0±38,3	**36,7 ± 82,6***	-19,7 ± 62,4	0,734	**0,007**
Women	-16,9±28,8	56,1 ± 109,4	2,3 ± 24,1	0,897	0,303
Men	-2,7± 41,2	29,9 ± 71,3	-27,5 ± 70,4	0,470	**0,019**
Genotype interaction					
LA	6,3 ± 36,2	17,0 ± 66,9	-33,0 ± 52,4	0,299	0,091
NA	-4,7±38,1	46,2 ± 26,7	-10,4± 69,6	0,996	0,080
eNOS					
All participants	11,7±65,6	**34,9 ± 72,2***	-8,2 ± 50,2	0,509	**0,035**
Women	13,2±71,7	**42,2 ± 42,1***	14,7 ± 48,8	0,997	0,565
Men	12,9±65,5	26,4 ± 83,7	-20,5 ± 49,4	0,351	0,115
Genotype interaction					
LA	-4,8±54,9	28,9 ± 77,8	-16,2 ± 62,6	0,910	0,344
NA	28,3±72,9	34,8 ± 74,6	-0,2 ± 44,8	0,536	0,334
VEGFA					
All participants	14,3±58,1	**32,2 ± 69,7***	-6,2 ±45,2	0,398	**0,045**
Women	12,6±59,0	30,4 ± 68,9	-3,0 ± 61,6	0,533	0,112
Men	9,4 ± 55,4	26,2 ± 67,9	**-10,0± 36,3***	0,870	0,500
Genotype interaction					
LA	26,3±65,9	19,4 ± 75,6	-3,0 ± 40,6	0,497	0,665
NA	7,7 ± 57,6	27,7 ± 60,1	-1,1 ± 46,5	0,896	0,256

Results are expressed as mean ± SD (N=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. *CD40L,* CD40 ligand; *CFH*, complement factor H; e*NOS,* endothelial nitric oxide synthase 3; i*NOS*, inducible nitric oxide synthase; *p65/RELA,* transcription factor p65 (RELA); *VEGFA,* vascular endothelial growth factor. ANOVA adjusted by age and sex. *$P < 0,05$, † $P < 0,001$ versus its baseline; *P* value, significance for inter-intervention comparisons.

3.1.5 Discussion

The present study provides scientific evidence of the endogenous bioconversion of dietary Tyr into HT and confirms the modulation of this reaction by *CYP2A6* and *CYP2D6* polymorphisms in humans. Furthermore, our findings enhance our understanding of the biological activity of Tyr and HT, which goes beyond simple ROS scavenging to play a relevant role in modulating crucial signalling pathways. The presented data also indicates that both Tyr *per se* and its endogenous conversion into HT promote cardiovascular health benefits in humans. Thus, our results have shown that ingestion of an enriched WW+Tyr diet improved endothelial function, plasma levels of HDL-c, Hcy, ET1, and ATIIII, and the expression of CD40L, p65/RELA, and CFH in PBMC. The addition of Tyr to WW abolished the increase in iNOS, eNOS, VEGFA, and CHF observed after WW. Tyr seems the preferential phenol involved in benefits on RHI and AI, whereas HT appears to be the leading phenol for explaining HDLc, Hcy, ATII systemic levels, and CD40L expression improvements.

3.1.5.1 Metabolic disposition of tyrosol and hydroxytyrosol and its genetic regulation

In the present report we have shown a novel biochemical mechanism by which foods rich in Tyr can act as an endogenous source of HT. The co-administration of WW and Tyr lead to a significant increase in HT urinary metabolites, not observable when WW was consumed alone. In this study, WW was chosen as the matrix for performing the clinical studies for two reasons. First, wine alcohol content improves Tyr bioavailability (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015). Secondly, the content of phenolic compounds of WW is the lowest from among grape products (such as red or sparkling wine), reducing confounders for the interpretation. In this context, the simultaneous administration of WW and Tyr in the form

of capsules easily allowed the detection of HT bioconversion over the background of other phenolic compounds present in the WW. The WW intervention favoured the determination of the HT formation due to the effect of ethanol on the endogenous dopamine oxidative metabolism. HT urinary metabolites increased an average of 19% over the baseline of the WW intervention. In contrast, WW+Tyr intervention resulted in a 283% increase in HT urinary metabolites compared to its baseline. Therefore, this remarkable difference between WW and WW+Tyr interventions can, essentially, be attributed to the endogenous synthesis of HT from the Tyr provided during the WW+Tyr intervention.

The doses of wine administered in the present study are within those recommended in the frame of its moderate consumption and the dose of Tyr selected (25 to 50 mg/day) are in the range of its maximal content per litter in red wine and fortified wines (sherry) ((http://phenol-explorer.eu/contents/polyphenol/673). The objective of the present study was not to evaluate the health effects derived from moderate and regular wine consumption, as in that case a red wine would have been the right choice. Actually, we wanted to unveil a novel *in vivo* biochemical reaction leading to the formation of HT from dietary Tyr that may contribute to its recognized health benefits, particularly the cardioprotective effects attributed to moderate red wine consumption (Costanzo *et al.*, 2011). This beneficial effect is thought to be attributed to the combination of low doses of alcohol and the phenolic compounds present in wine (Cooper, Chopra and Thurnham, 2004; Arranz *et al.*, 2012). Among phenolic compounds, resveratrol and catechins had been widely studied. Nevertheless, no relationship has been found between cardiovascular protection and resveratrol urinary levels (Semba *et al.*, 2014) or plasmatic catechin levels (Boban *et al.*, 2006).

Dietary Tyr and HT are well absorbed but their systemic bioavailability is low. They go through an extensive first-pass hepatic metabolism, resulting in almost undetectable free forms in systemic biological fluids (Pastor *et al.*, 2016). Moreover, the low concentrations of the free forms observed *in vivo* cannot explain the biological activities associated with phenolic consumption in clinical and epidemiological studies. Therefore, derived metabolites have been proposed to exhibit relevant biological properties, either by having activity by themselves or by being deconjugated intracellularly giving rise to free active forms, as it has been described for other dietary phenols (Serreli and Deiana, 2018; Fernández-Castillejo *et al.*, 2019). Our study shows an increase in the following Tyr and HT metabolites recovery following the WW+Tyr intervention: Tyr-4-sulfate, Tyr-4-glucuronide, and HT-3-sulphate. Interestingly, Tyr and HT sulphate metabolites had been reported to counteract the effects of oxidized cholesterol in an *in vitro* intestinal cell membrane model (Atzeri *et al.*, 2016). Protective effects of HT-sulphate in front of neuronal oxidative stress had also been observed *in vitro* (López de las Hazas *et al.*, 2018) (40). Another study reported that Tyr-sulphate and, to a lesser extent, Tyr-glucuronide prevented the activation of NF-κB, and hence down-regulated the expression of adhesion molecules in endothelial cells (Muriana *et al.*, 2017). Finally, a mixture of HT metabolites, including sulphates and glucuronides, promoted a decrease in endothelial dysfunction biomarkers in *in vitro* human aortic endothelial cells (Catalán *et al.*, 2015). Overall, the increased Tyr and HT metabolite recovery in our WW+Tyr intervention may be able to trigger biological properties relevant for the cardiovascular system.

Our findings have also confirmed the involvement of *CYP2A6* and *CYP2D6* enzymes in the *in vivo* endogenous formation of HT from Tyr. The fact that individuals with low PAS have higher Tyr recoveries and those with normal PAS showed a higher HT/Tyr ratio at the end of the WW+Tyr

intervention confirms a role for genetic variation in Tyr metabolism. Nonetheless, results from the rapid activity group were surprising. Although urinary Tyr recovery followed the expected trend, HT recovery was lower compared to individuals with low activity. We hypothesize that, in those individuals, Tyr is converted into HT which is further rapidly transformed into another unidentified compound. However, caution should be taken when interpreting the results of the rapid polygenic activity group, as only 2 individuals (with *CYP2D6* duplications, one *2xN and one *35XN) were included in the group. Future studies should assess the metabolic disposition of Tyr and HT in this specific group of individuals

3.1.5.2 Clinical effects of Tyrosol supplementation

Endothelial dysfunction is a predictor for CVD (Rubinshtein *et al.*, 2010; Pushpakumar, Kundu and Sen, 2014). Diet is an important contributor to endothelial health and a healthy dietary pattern has been associated with improved endothelial function (Fung *et al.*, 2005). In our study RHI, a marker for the endothelium-dependent vasodilator function (Matsuzawa *et al.*, 2015), increased by a mean of 0.176 AU after the WW+Tyr intervention. This value corresponds to a 0.103 AU when data are on a logarithmic scale (Ln-RHI). A 0.1 increase in Ln-RHI has been associated with a 21% decrease in CVD according to a meta-analysis that included 1602 individuals (Matsuzawa *et al.*, 2015). AI is a marker for arterial stiffness, an independent predictor of cardiovascular and cerebrovascular diseases, and a consequence of endothelial function impairment (Li *et al.*, 2017; Tomiyama *et al.*, 2018). In our study, AI decreased after the WW+Tyr intervention. Thus, our data agree with previous reports concerning the benefits of polyphenol-rich VOO and red wine on endothelial function in humans (Covas, De La Torre and Fitó, 2015; van Bussel *et al.*, 2018).

The increase in HDL-c is a well-known effect of moderate alcohol consumption. As expected, HDL-c increased after both treatments which involved alcohol (Huang *et al.*, 2017). Nevertheless, a larger increase was observed following WW+Tyr intervention, suggesting an additional contribution of the phenolic content on the observed effect. HDL protects endothelial function by preventing LDL oxidation (Riwanto *et al.*, 2015). Within the frame of the EUROLIVE Study, the following observations were reported: 1) a dose-dependent increase of plasma HDL-c, 2) a decrease in LDL oxidation (Covas *et al.*, 2006), 3) an improvement of HDL functionality associated with the phenolic content (mainly HT and Tyr) of the olive oil administered (Hernáez *et al.*, 2014) and 4) the capacity of the HT derived metabolites to bind to the HDL lipoprotein in a dose-dependent manner with the HT content of the olive oil administered (Hernáez *et al.*, 2014). Together, these aspects have an impact on HDL fluidity (Hernáez *et al.*, 2014) and protect HDL from oxidation, thus rendering the particle fully functional (Riwanto *et al.*, 2015). In agreement with EUROLIVE previous data, in the present study HDL-c increased in a dose-dependent manner with the Tyr and HT content of the beverage administered.

Endothelial dysfunction is characterized by an imbalance between vasodilation and vasoconstriction factors. It is a multifactorial process that involves different signalling pathways. Hcy is considered a risk factor for CVD and deep vein thrombosis, as it promotes endothelial dysfunction through several mechanisms including NF-κB activation (Pushpakumar, Kundu and Sen, 2014). Here we showed an increase in Hcy levels after the WW treatment, consistent with the previously described increases in Hcy levels after moderate alcohol and wine consumption (Gibson *et al.*, 2008). The addition of Tyr to WW abolished the increase in Hcy associated to alcohol consumption while also decreasing its levels compared to baseline.

Olive oil phenolic compounds, including Tyr and HT, had been described to protect from the Hcy-induced pro-inflammatory effects through a downregulation of adhesion molecules (Manna *et al.*, 2009). Prothrombotic effects had also been associated with higher Hcy levels (Di Minno *et al.*, 2010). Cerebrovascular disease patients with high urinary Hcy/creatinine ratio have been reported to have lower levels of ATIII (Choi *et al.*, 2014). Accordingly, we observed a concomitant increase of ATIII parallelly to the decrease in Hcy. ATIII is a pleiotropic molecule with anti-coagulation and anti-inflammatory effects reported to contribute to an adequate blood flow (Allingstrup *et al.*, 2016). The anti-inflammatory properties of ATIII include the inhibition of neutrophil recruitment and NF-κB pathways (Yin *et al.*, 2017). NF-κB is the main mediator of the inflammatory response. Its activation is known to promote that of the inflammatory factor iNOS (Chen *et al.*, 1995), the nitric oxide synthetase which expression is induced by an oxidative environment and pro-inflammatory cytokines. Likewise, iNOS inhibition improves endothelium-dependent vasodilatation in aged rats (Tian *et al.*, 2010). iNOS expression is upregulated by Hcy, disrupting the angiogenesis of the microvascular endothelium (Mayo *et al.*, 2014). On the contrary, the anti-inflammatory capacity of ATIII also includes inhibition of iNOS expression (Pata *et al.*, 2002). In agreement with the changes observed in Hcy and ATIII in our study, we observed an increase in iNOS after WW that was abolished in the WW+Tyr intervention. In experimental models, Tyr, HT and their biological metabolites were able to inhibit iNOS expression (Asakura *et al.*, 2005; Serreli *et al.*, 2019). iNOS inhibition is known to attenuate ET1 levels (Asakura *et al.*, 2005). In agreement with this, in our study, post-treatment ET1 concentrations after WW+Tyr intervention decreased versus WW intervention.

As iNOS, soluble CD40L also induces endothelial dysfunction (Chen *et al.*, 2008) and it is considered to trigger atherogenesis and thrombosis, by

promoting inflammation (Castañer *et al.*, 2012). A decrease in CD40L expression *in vivo* in healthy volunteers was associated with the high phenolic content (mainly Tyr and HT) of VOO (Castañer *et al.*, 2012). Consistent with this, in the present study, WW+Tyr intervention reduced CD40L expression. Similar decreases occurred after wine and cocoa flavonoid intake (Pignatelli *et al.*, 2005; Monagas *et al.*, 2009). In parallel, a decrease in p65 (RELA) and CFH was observed after WW+Tyr. p65 (RELA) is a transcription factor belonging to the NFκB family and its activation, like that of CFH, exacerbates the inflammatory status (Calippe *et al.*, 2017). CFH competes with C1q for binding to target surfaces (Tan *et al.*, 2011), and C1q exerts inhibitory effects on pro-inflammatory gene expression by reducing NF-κB mediated transcription through a p65 decreased activation (Fraser *et al.*, 2007). Thus, in our study we observed concomitant reductions in p65 and CFH expressions after WW+Tyr intervention. Red wine polyphenol extracts decrease both NF-κB (p65) and iNOS expressions (Janega *et al.*, 2014), and inhibition of p65 translocation by olive tree (Olea Europea L) methanolic extracts has been recently reported (Song *et al.*, 2019). Tyr-4-sulfate prevented TNF-α-induced NF-κB signalling in endothelial cells by reducing p65 phosphorylation (Muriana *et al.*, 2017). VEGF induces proangiogenic changes such as vessel dilatation and increased vascular permeability predominantly via eNOS (Fukumura *et al.*, 2001), and VEGF inhibition decreases CFH in the eye and kidney (Keir *et al.*, 2017). The WW+Tyr treatment prevented the increase observed in the expression of p65/RELA, VEGFA, eNOS, and CFH after WW. A schematic representation of the possible interrelationships among all these variables is depicted in figure 19, and comparisons among the observed effects after WW and WW+Tyr interventions are represented in figure 20.

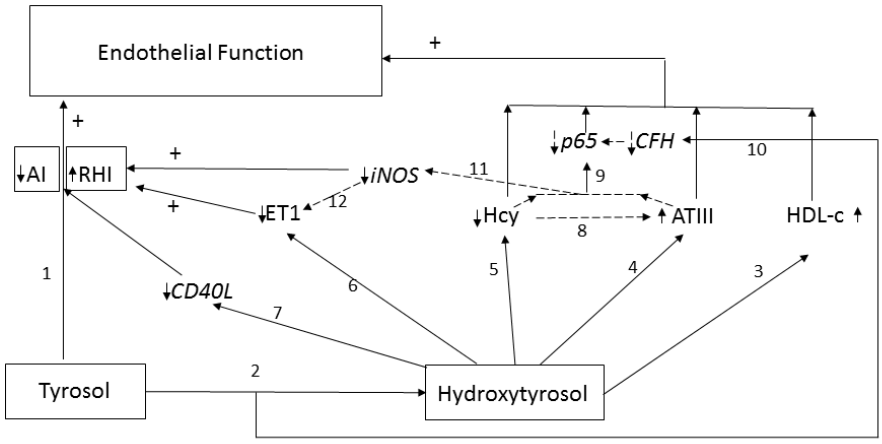


Figure 19. Interrelationship among tyrosol and its biotransformation into hydroxytyrosol with endothelial function and its risk factors. (1), tyrosol seems to be the main phenol involved in the direct improvement of reactive hyperaemia (RHI) and arterial stiffness (AI). Its conversion to hydroxytyrosol (2), however, also improves endothelial function through an increase in HDL-c (3) and antithrombin III (ATIII) (4), and a decrease in homocysteine (Hcy) (5), endothelin 1 (ET1) (6), CD40L (7), and p65 (9) expressions. The increase in ATIII could be mediated by the decrease in Hcy (8) and both changes could improve endothelial function through a reduction in NF-κB (p65-RELA) (9), potentiated by the CFH decrease (10), and in iNOS (11) that in turn contributes to ET1 reduction (12). Full lines depict the study findings, and dashed lines indicate related mechanisms.

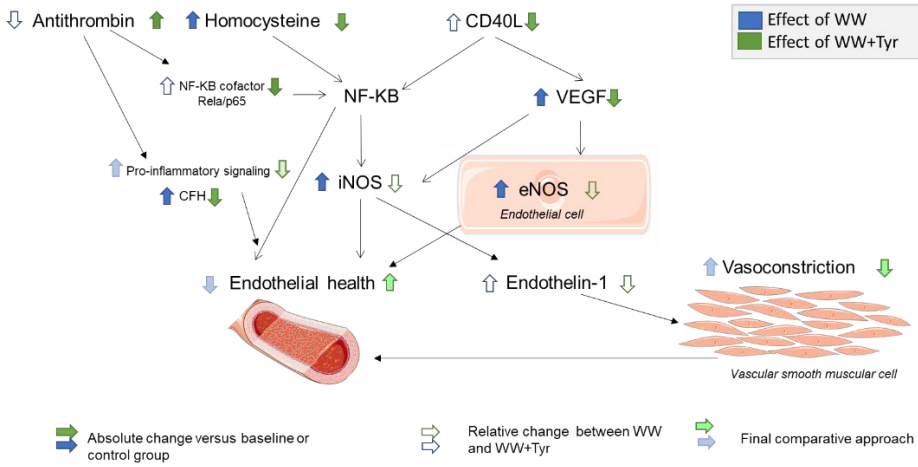


Figure 20. Comparison of the effects of white wine (WW) (blue) versus those of white wine plus tyrosol (WW+Tyr) (Green) interventions. CD40L, CD40 ligand; NF-κB, nuclear factor kappa B; CFH, complement factor H; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase

Our findings have confirmed the involvement of *CYP2A6* and *CYP2D6* enzymes in the endogenous formation of HT from Tyr *in vivo* in humans and show an impact on the magnitude of the biological effects observed. Differences in the observed health effects following interventions between LA and NA metabolizers allowed the identification of which phenolic compound, Tyr or HT, was preferentially involved in the changes observed. The fact that the increase in RHI and the decrease in AI after the WW+Tyr intervention occurred preferentially in the LA group, suggests Tyr as the likely main responsible phenol for RHI and AI improvements. Conversely, the fact that the decrease in Hcy and the increases in ATIII and HDL-c after the WW+Tyr treatment occurred particularly in the NA group, points HT as the more likely phenol involved in these improvements. Similarly, decreases in CD40L occurred particularly in the NA group, suggesting HT as the key phenol involved.

Our study has strengths and limitations. One strength is the crossover design which minimizes the role of possible confounding variables and between-subject variation. The similarity of matrices between WW and WW+Tyr treatments allowed the assessment of the role of the exogenous Tyr administered and its bioconversion into HT. One limitation is the sample size, particularly for women. The length of the study (6 months) and the restricted inclusion criteria may have decreased study recruitment for both genders. Tyr and alcohol doses were higher in men than in women, fact that may contribute to the higher responsiveness to interventions in men. Although Tyr and HT effects are influenced by the designed PAS, not all of the variability in HT and Tyr recoveries could be explained by variants assessed for *CYP2A6* and *CYP2D6* and included in the PAS. Besides, further untested variants, and potential alternative enzymes could contribute to the observed variation. Additionally, enzyme expression can be affected by further factors such as drugs, diet or hormones. We were unable to assess treatment interference due to dietary components or medication. The fact that participants were individuals at high CVD risk limits the extrapolation of the results to the general population. Whether additional or different effects would have been observed over longer treatment periods is unknown, nevertheless, greater intervention periods could have compromised the compliance of participants.

3.1.6 Conclusions

This is the first study that demonstrates that the endogenous conversion of Tyr into HT occurs in humans *in vivo*. This describes a new mechanism that could explain, in part, the potential benefits associated with moderate wine consumption and of other Tyr-rich components of our diet. Moreover, we have shown that both Tyr and its partial biotransformation into HT promoted cardiovascular health-related benefits in humans after the intake of Tyr and WW. The study design allowed the health effects of individual phenols to be singled out from the dietary matrix in which they were built-in. Tyr bioconversion to HT is modified by *CYP2A6*/*CYP2D6* genetic polymorphisms, and therefore some individuals may benefit more than others from the biological activities of these antioxidants. The results obtained in the present work encourage further study of Tyr-rich foods and/or Tyr supplements with other foods or beverages for added enhancement of the healthy bioactivities *in vivo*.

3.1.7 Additional information

Table 16. Baseline characteristics of the participants

Variable	Values
Age, y	65.3 ± 6.2
Gender, n (%)	
Women	12 (36.4%)
Men	21 (63.6%)
BMI, kg/m²	32.6 ± 4.2
LDL cholesterol, mg/dL	118 ± 34.4
HDL cholesterol, mg/dL	50.2 ± 12.9
Total cholesterol, mg/dL	192 ± 39.3
Triglycerides, mg/dL	120 ± 72.2
Cardiovascular Risk factors, n (%)	
Current smokers	6 (18.2%)
Family history of premature CHD	6 (19.4%)
Obesity (BMI ≥ 25kg/m²)	32 (97.0%)
Type 2 Diabetes	13 (39.4%)
Hypertension	28 (84.8%)
High LDL cholesterol (> 130 mg/dL)	25 (75.6%)
Low HDL cholesterol (<40 mg/dL for men or <50 mg/dL for women)	8 (24.2%)
Medications, n (%)	
Alfa blockers	2 (6.1%)
Beta blockers	6 (18.2%)
ACE inhibitors	14 (42.4%)
Angiotensin II receptor antagonists	11 (33.3%)
Diuretics	13 (39.4%)
Statins	16 (48.5%)
Oral hypoglycemic drugs	12 (36.4%)
Acetylsalicylic acid	10 (30.3%)

Data presented as mean ± SD or n (%) (n=33). BMI, body mass index; LDL, low density lipoproteins; HDL, high density lipoproteins; CHD, coronary heart disease.

Table 17: Energy, nutrients, and fiber at the beginning and at the end of the study.

Variable	Treatment						P*
	Control	P	WW	P	WW+Tyr	P	
Energy, kcal/day Baseline	1695 ± 446		1663 ± 421		1624 ± 370		NS
12-week	1643 ± 361	0,616	1650±354	0,868	1737±450	0,082	
HC, % energy Baseline	38,2 ± 8,6		40,2 ± 6,4		38,8 ± 6,3		NS
12-week	38,3± 7,3	0,906	37,6 ± 7,7	0,095	37,8±7,5	0,360	
HC, grams Baseline	159 ± 48		165± 43		157 ± 38		NS
12-week	156 ± 41	0,811	153 ± 40	0,209	163 ± 45	0,532	
Protein, % energy Baseline	20,9 ± 4,0		19,2 ± 3,0		21,5 ± 4,5		
12-week	21,2 ± 4,1	0,578	19,5 ± 4,8	0,028	19,0± 3,9	,142	NS
Protein, grams Baseline	88 ± 25		79 ± 19		86 ± 24		
12-week	87 ± 25	0,939	81 ± 30	0,651	82 ± 23	0,184	NS
Total Fat, % energy Baseline	40,7 ± 7,5		40,3 ± 6,2		39,2 ± 6,6		
12-week	40,2 ± 7,2	0,625	36,7 ± 9,0	0,496	38,6 ± 6,3	0,230	NS
Total Fat, grams Baseline	78 ± 29		76 ± 28		72 ± 24		
12-week	74 ± 23	0,489	68 ± 24	0,015	76± 28	0,809	NS
SFA, % energy Baseline	11,4± 3,8		10,1 ± 3,0		11,4 ± 4,2		
12-week	10,7 ± 3,7	0,526	10,1 ± 3,9	0,953	10,4 ± 3,7	0,272	NS
SFA, grams Baseline	22 ± 12		19 ± 9		21 ± 12		
12-week	20 ± 10	0,428	19 ± 10	0,873	21 ± 11	0,855	NS

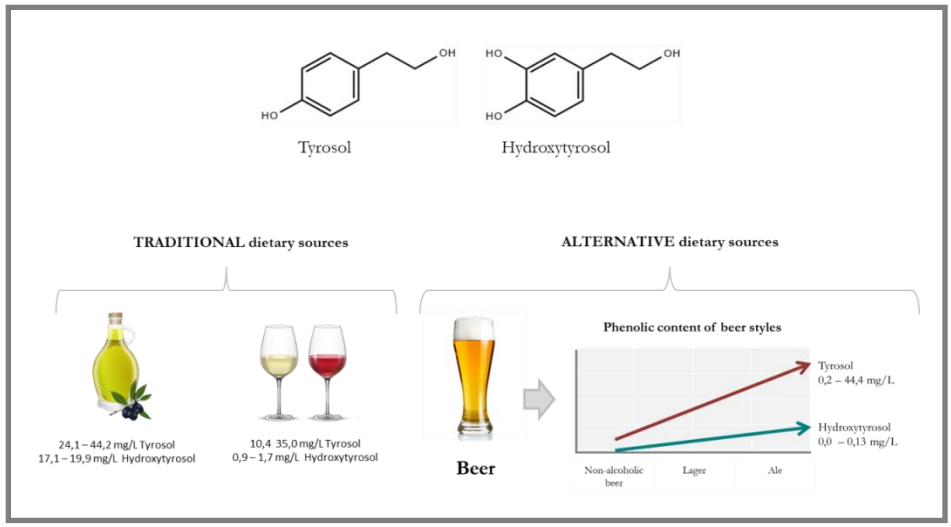
Table 2 (cont)										
MUFA, % energy										
Baseline	19,8 ± 5,0			19,7 ± 4,2			18,6 ± 5,0			
12-week	20,1 ± 4,2			18,4 ± 5,1			19,6 ± 3,5			NS
	0,893			0,177			0,172			
MUFA, grams										
Baseline	53,3 ± 21,0			53,8 ± 15,5			53,0 ± 16,6			
12-week	51,9 ± 21,2			46,5 ± 13,3			50,0 ± 14,3			NS
	0,475			0,274			0,054			
PUFA, % energy										
Baseline	6,1 ± 2,6			6,8 ± 2,5			6,0 ± 2,1			
12-week	5,9 ± 2,3			5,2 ± 2,1			5,4 ± 1,9			NS
	0,697			0,222			0,179			
PUFA, grams										
Baseline	12 ± 6			13 ± 8			11 ± 4			
12-week	11 ± 7			10 ± 5			11 ± 6			NS
	0,901			0,025			0,960			
Fiber, g/day										
Baseline	20 ± 7			23 ± 11			20 ± 8			
12-week	20 ± 8			23 ± 11			21 ± 9			NS
	0,894			0,848			0,408			

Results expressed as mean ± SD (N=32). HC, carbohydrates; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. P Intra-treatment comparisons by Student's t test. * P value for ANOVA repeated measures adjusted by age and sex.

3.2

CHAPTER 2

Tyrosol and hydroxytyrosol content in the Mediterranean diet: special focus on beer as a dietary source



Abstract

Tyrosol (Tyr) and hydroxytyrosol (HT) are health-promoting dietary phenols present at relatively high levels in extra virgin olive oil (EVOO) and red wine. However, the content of these phenolic compounds in other common dietary products such as beer has been poorly described. Here we report the analysis of Tyr and HT content of 45 beers available in the Spanish market. The Tyr and HT content in EVOO and red wine samples using the same methodology has also been determined for comparative purposes. The different sources of these phenolic compounds on a typical Mediterranean diet have contextualized to assess the overall contribution of beer to this diet. As expected, EVOO has the highest levels of HT (11,8 mg/mL) and represents its main source in the diet. The concentration of HT in fermented beverages is much lower, with mean values of 1,5 mg/L in red wine and 0,03 mg/L in beer. In contrast, fermented beverages represent a good source of Tyr. On average, red wine has a higher Tyr concentration than beer (31,1 mg/L vs 11,5 mg/L). Interestingly, some beer styles presented similar concentrations of Tyr than red wine. Ale beers showed higher concentration of Tyr and HT compared to lager beers. Non-alcoholic beers exhibited significantly lower levels of Tyr and almost undetectable levels of HT.

3.2.2 Introduction and aim

In 2011, EFSA released a health claim concerning the role of hydroxytyrosol (HT) and its derivates to prevent LDL oxidation. The panel considered that a daily ingestion of 5 mg of HT or its derivates, including free HT and oleuropein (a conjugated form of HT), would trigger this beneficial effect (EFSA, 2011). Extra virgin olive oil (EVOO) is the most well-known dietary source of HT and Tyr, mainly in the form of their respective secoiridoid derivatives: oleuropein and ligstroside (Rodríguez-Morató *et al.*, 2016). Phenolic concentrations in EVOO are known to be affected by several factors such as the variety of the olive fruit, harvest time, techniques for oil processing and exposed temperature at cooking (Gómez-Rico *et al.*, 2006; Perez-Herrera *et al.*, 2013).

In the case of red wine (RW) and beer, Tyr and HT are not naturally present in any of the row materials (grapes and cereals) but are generated during the manufacturing process. In wine, Tyr is produced by *Saccharomyces cerevisiae* during fermentation, as a byproduct of the metabolism of the amino acid tyrosine following the Ehrlich pathway (Figure 21) (Piñeiro *et al.*, 2011). In winemaking, Tyr levels had been described to be directly correlated to the amount of amino acids present in the must (Bordiga *et al.*, 2016). Less is known about Tyr and HT content in beer (Cerrato-Alvarez *et al.*, 2019). It is likely that tyrosine content of the cereals used for the brewing process would be also relevant for its generation.

The amounts of olive oil consumed in a balanced Mediterranean diet range from 25 to 50 mL per day. In the case of wine and beer, its daily consumption is limited by its alcohol content. A recent meta-analysis described a J-shaped inverse association between wine and beer consumption with cardiovascular risk (Costanzo *et al.*, 2011), being a daily moderate consumption protective compared to abstainers or heavy drinkers.

The American Heart Association (AHA) considers moderate drinking as one standard drink per day in women, equivalent to 14 g of alcohol, and two standard drinks per day in men, equivalent to 28 g of alcohol (Lichtenstein *et al.*, 2006).

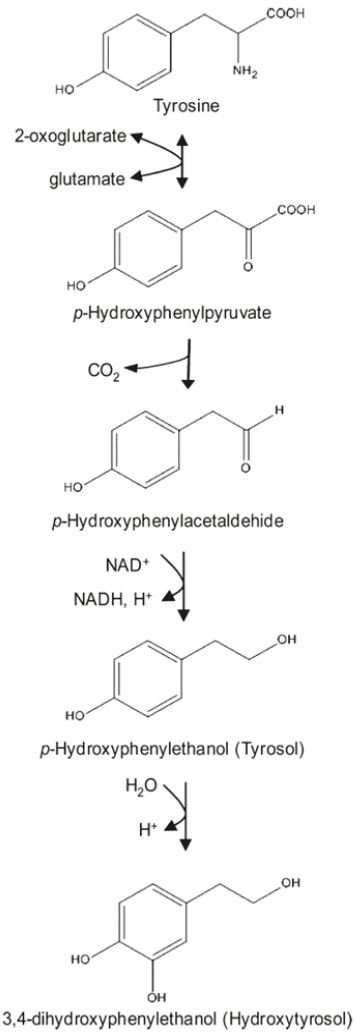


Figure 21. Ehrlich pathway; Tyr and HT formation from Tyrosine in wine and beer (Image from Piñeiro et al., 2011).

The main aim of this section was to evaluate the potential of beer as a source of Tyr and HT and its eventual contribution to the total phenolic intake in the diet. A secondary goal of this study was to characterize the factors that could determine the final beer phenolic composition.

3.2.3 Material and methods

3.2.3.1 Samples

The analysis included two samples of EVOO, two samples of white wine (WW), three samples of red wine (RW) and 45 samples of different beers. All products were purchased in the Spanish market.

3.2.3.2 Tyrosol and hydroxytyrosol determination in extra virgin olive oil, wine and beer

a) Determination in extra virgin olive oil

Tyr and HT content in EVOO were quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after a triple liquid-liquid extraction and a hydrolysis as previously described (Miró-Casas *et al.*, 2003). Shortly, 1 mL aliquots of olive oil were spiked with 10 μL of an internal standard containing 100 μg/mL of HT-D3 and 3-(4-hydroxyphenyl)-1-propanol). An initial liquid-liquid extraction was performed by adding 10 mL of a methanol/water solution (80:20, v/v) containing 1 mM of ascorbic acid in order to protect samples from phenol degradation during extraction process. Tubes were shaken for 60 min and then centrifuged (2000xg, 5 min). The organic phase was transferred into a new tube and evaporated under nitrogen until a final remaining volume of 2 mL of an aqueous extract of olive oil. Thereafter, metabisulfite was added to the samples to prevent oxidation. In order to hydrolyse all the conjugated forms of TYR and HT, samples were incubated at 37ºC for 30 min with HCl (1.5 mmol/tube), to mimic gastrointestinal conditions during digestion. Consecutively, a second liquid-liquid extraction was performed by adding 4 mL of a mixture of ethyl acetate and acetonitrile (4:1 v/v), shaking for 30 min and centrifuging (2000xg, 5 min). The organic phase was transferred into a new tube and the liquid-liquid extraction was repeated. Finally, both organic phases were combined into the same tube and the mixture evaporated until complete

dryness. The composition of mobile phase A was 0.01% of ammonium acetate (pH 5) in water. Mobile phase B was pure methanol. Extracts were reconstituted with 100 μL of a mobile phase containing 80% of mobile phase A and 20% of mobile phase B and injected into the LC-MS/MS. Calibration curves were prepared by adding standards of TYR and HT to 1 mL of refined oil. All the samples were analysed in triplicate.

b) Determination in wine and beer

TYR and HT content were determined by liquid chromatography coupled to a tandem mass spectrometry (LC-MS/MS) following a dilute-and-shoot approach. In the case of beer, foam was removed by sonication. Wine and beers were diluted 40 times with mobile phase (65% A: 35%B) and spiked with 10μL of an internal standard containing 1μg/mL of 3-(4-hydroxyphenyl)-1-propanol and 1μg/mL of HT-D3. Mobile phase A contained 0,5 mM of ammonium fluoride in water. Mobile phase B contained 0,5 mM of ammonium fluoride in methanol. Calibration curves were prepared adding standards of Tyr and HT to pure water.

c) Instrumentation

For both extraction procedures, sample extracts were analysed using an Agilent Technologies 6410 Triple Quad (Santa Clara, CA, USA). The separation was carried out with an Acquity UPLC® BEH C18 column (Waters, Milford, MA, USA) with a 1.7 μm particle size, 3 mm x 100 mm (Waters, Milford, MA, USA). The injection volume was 10 μL and the ion source operated in negative ionization mode.

3.3.3 Statistical analysis

Statistical analysis was performed using R, version 3.0.2. R packages used were "multcomp", "nlme" and "ggplot2". Square root transformation was used to achieve a normal distribution of Tyr and HT concentrations.

ANOVA was used to compare concentrations of different beer groups. Tukey's HSD (honestly significant difference) test was used to perform post-hoc pairwise comparisons. Pearson correlation coefficient was calculated to evaluate the existence of linear associations between TYR, HT and percentage of alcohol. Significance was set as $p < 0,05$.

3.2.4 Results

3.2.4.1 Analysis of tyrosol and hydroxytyrosol in oil and wine samples

Table 18 summarizes the content of Tyr and HT of EVOO, WW and RW analyzed. EVOO samples were subjected to a hydrolysis before Tyr and HT determination. Therefore, the presented results are the total Tyr and HT content after deconjugation.

Table 18: Tyrosol and hydroxytyrosol content of the analyzed EVOO, WW, and RW

Sample	N	Tyr (mg/L)			HT (mg/L)		
		Range	Mean	SD	Range	Mean	SD
EVOO	2	24,1 – 44,2	34,2	14,2	17,1 – 19,9	18,5	2,0
WW	2	10,4 – 12,0	11,2	1,1	0,9 – 1,3	1,1	0,3
RW	3	25,3 – 35,0	31,1	5,1	1,5-1,7	1,5	0,1

EVOO: Extra virgin olive oil, WW: white wine, RW: red wine.

3.2.4.2 Beer classification

The 45 beers were classified in terms of their style and type following Beer Judge Certification Program (BJCP) (Strong and England, 2015). Beer were classified according to their type into ale, lager or non-alcoholic beer, and within each group, a further sub-classification was made in terms of their style as described in figure 22.

Figure 22: Beer classification according to the beer type and style.

3.2.4.3 Analysis of tyrosol and hydroxytyrosol in beer samples

a) Beer type

Figure 23 summarized the Tyr and HT content across the three beer types. The mean Tyr content of non-alcoholic beer was significantly lower (3,4 ± 2,6 mg/L) compared to ale beer (13,8 ± 12,8 mg/L; p=0,027) and lager beer (11,4 ± 8,9 mg/L; p=0,05). Likewise, HT content in non-alcoholic beer was the lowest (0,007 ± 0,007 mg/L). Ale HT content was higher (0,05 ± 0,03 mg/L) compared to lager (0,02 ± 0,02 mg/L) and non-alcoholic beer (p=0,018 and p=0,008 respectively). Statistical analysis was performed in transformed data using squared root to achieve normality.

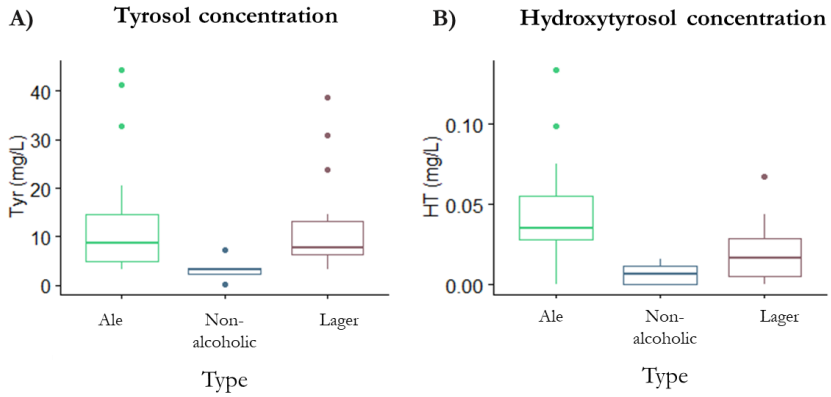


Figure 23: Tyrosol and hydroxytyrosol beer concentration according to the beer type

b) Beer style

Each beer type was subdivided in several groups according to their style. Table 19 describes Tyr and HT content across the different beer styles. Statistical comparisons were only performed within beer style groups containing at least 3 samples (Figure 24). Belgian strong ale presented the highest concentration of Tyr (p<0,050 vs IPA and pale lagers and p=0,067 vs wheat beers). In terms of HT, IPA tended to represent the highest source (p=0,063 vs pale lager).

Table 19. Tyrosol and hydroxytyrosol content according to beer styles

Type	*Style*	*N*	*Tyr (mg/L)*		*HT (mg/L)*	
			Mean	SD	Mean	SD
Ale	Belgian strong ale	3	29,2	14,3	0,04	0,01
	Blonde ale	2	24,3	28,4	0,05	0,01
	IPA	6	9,7	6,2	0,06	0,05
	Pale ale	1	7,4	-	0,03	-
	Stout	2	5,7	2,4	0,01	0,02
	Wheat	3	8,7	5,8	0,05	0,02
	Winter	1	8,6	-	0,03	-
Lager	Amber lager	2	8,8	3,5	0,01	0,01
	Bock	1	13,9	-	0,04	-
	Dark lager	1	8,7	-	0,02	-
	Lite lager	1	7,8	-	0,02	-
	Pale lager	15	10,5	9,3	0,02	0,02
	Special	2	22,6	11,5	0,04	0,03

Values are expressed as mean and SD when more than one sample per group was analyzed.

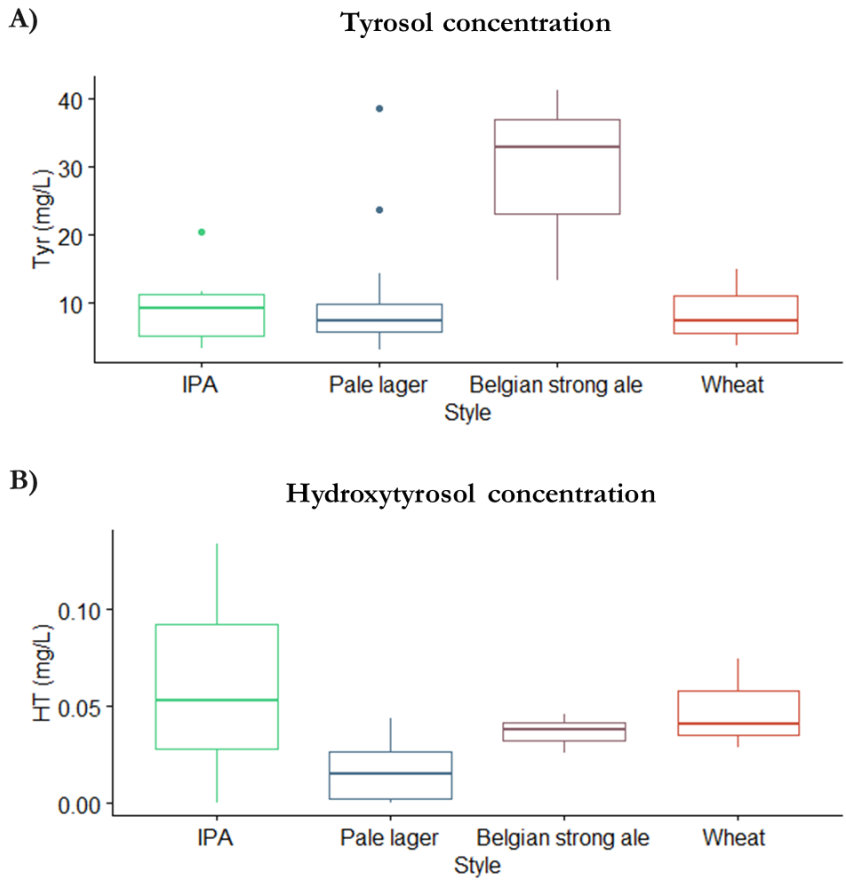


Figure 24. Tyrosol (A) and hydroxytyrosol (B) content of beers classified into the main styles: IPA, Pale lager, Belgian strong ale and wheat beers.

c) Other factors:

No significant differences were observed when comparing crafted (N=10) and industrial beers (N=35). The effect of filtration was only assessed on samples in which this information was available. No significant effect of filtration was observed in Tyr and HT content.

3.2.4.4 Tyrosol and hydroxytyrosol correlation with beer alcoholic content

The content of Tyr was positively correlated with beer alcoholic content (r=0,569, p<0,001, figure 25.A). In the case of HT, there was a mild positive correlation (r = 0,364, p=0,021, figure 25.B). As expected, Tyr and HT beer content were positively correlated among them (r=0,568, p<0,001, figure 25.C).

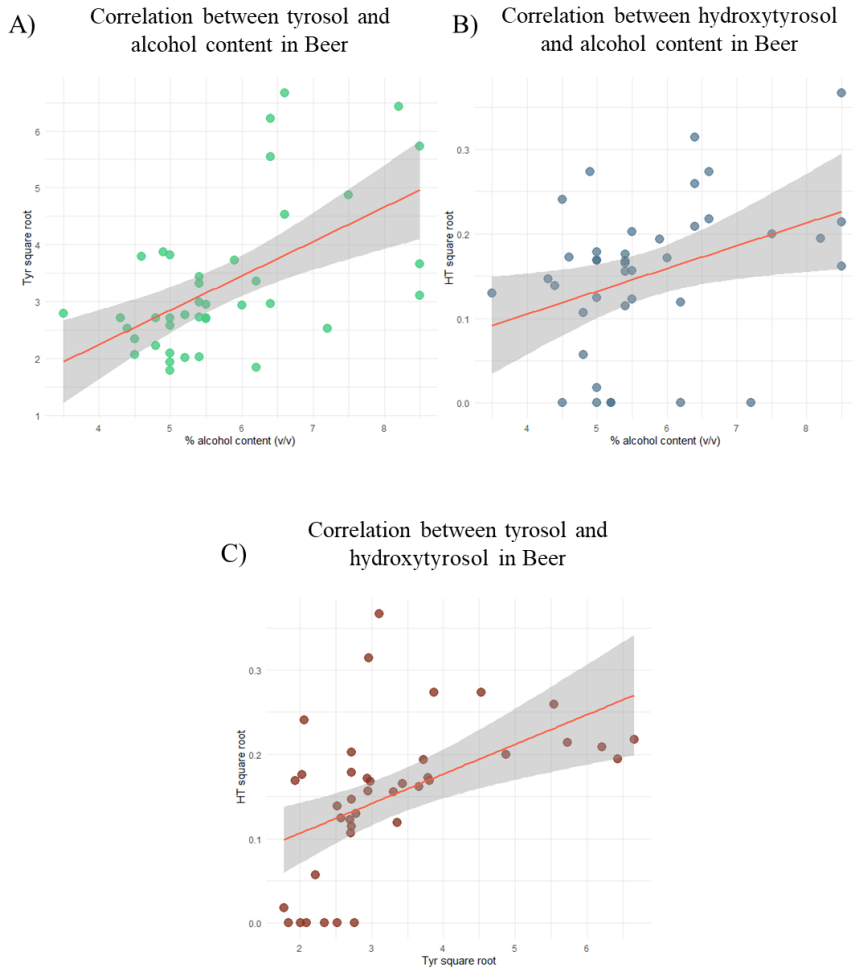


Figure 25. Correlation between tyrosol, hydroxytyrosol and alcohol content (% v/v) among analyzed beers. Non-alcoholic beers were excluded of the graph.

3.2.4.5 Dietary consumption of Tyr and HT

Dietary contribution of Tyr and HT were calculated based on the most frequent consumed doses of EVOO, wine and beer in the Spanish population (Table 20). Beers represented were grouped as non-alcoholic beer, ale, lager. Additionally, Belgian strong ale and IPA were also represented as beers with the highest content in Tyr and HT respectively. Tyr, HT and alcohol total contribution were calculated from the obtained mean of each group.

EVOO represented the highest source of HT, with a daily consumption of 25-50 mL, providing a dose of 0,46-0,93 mg. In the case of Tyr, RW, Ale and Belgian strong ale beers represented the most relevant sources, all over 4,7 mg per dose. Figure 26 and 27 outline the Tyr and HT contribution in the diet in their normal consumed doses and the alcohol content of each doses. Moderate alcohol recommendations are also outline in the figures.

Table 20. Total contribution of tyrosol and hydroxytyrosol in the frequent consumed doses

Source	*Dose*	*Tyrosol (mg)*	*Hydroxytyrosol (mg)*	*Alcohol (g)*
EVOO	25 mL	0,9	0,463	0,0
	50 mL	1,7	0,925	0,0
WW	150 mL	1,7	0,165	15,0
RW	150 mL	4,7	0,225	15,0
Non-alcoholic	200 mL	0,7	0,001	0,0
	330 mL	1,1	0,002	0,0
	400 mL	1,4	0,003	0,0
Ale	200 mL	2,7	0,009	9,8
	330 mL	4,6	0,015	16,2
	400 mL	5,5	0,018	19,7
Lager	200 mL	2,3	0,004	8,6
	330 mL	3,8	0,006	14,2
	400 mL	4,6	0,008	17,3
Belgian strong ale	200 mL	5,9	0,007	13,4
	330 mL	9,7	0,012	22,2
	400 mL	11,7	0,015	26,9
IPA	200 mL	1,9	0,012	10,1
	330 mL	3,2	0,020	16,7
	400 mL	3,9	0,024	20,3

Results are expressed as total mg/g. Calculations were done based on the average content of Tyr, HT and alcohol of the analyzed samples.

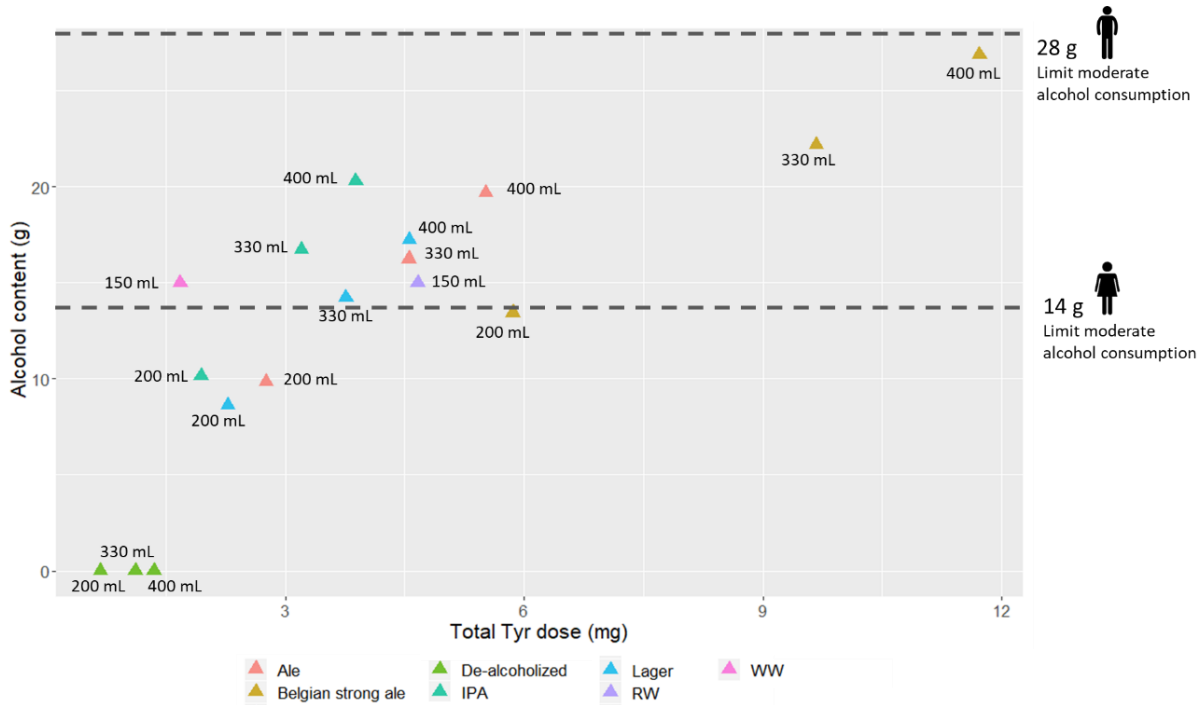


Figure 26. Tyrosol contribution and alcohol content of wines and beer based on frequent consumed doses. Dashed lines represent the limit of moderate alcohol consumed according to the AHA (14 g/day for women and 28 g/day for men). Doses of beer represented are 200 mL, 330 mL and 400 ml. IPA: Indian pale ale; RW: red wine; WW: white wine.

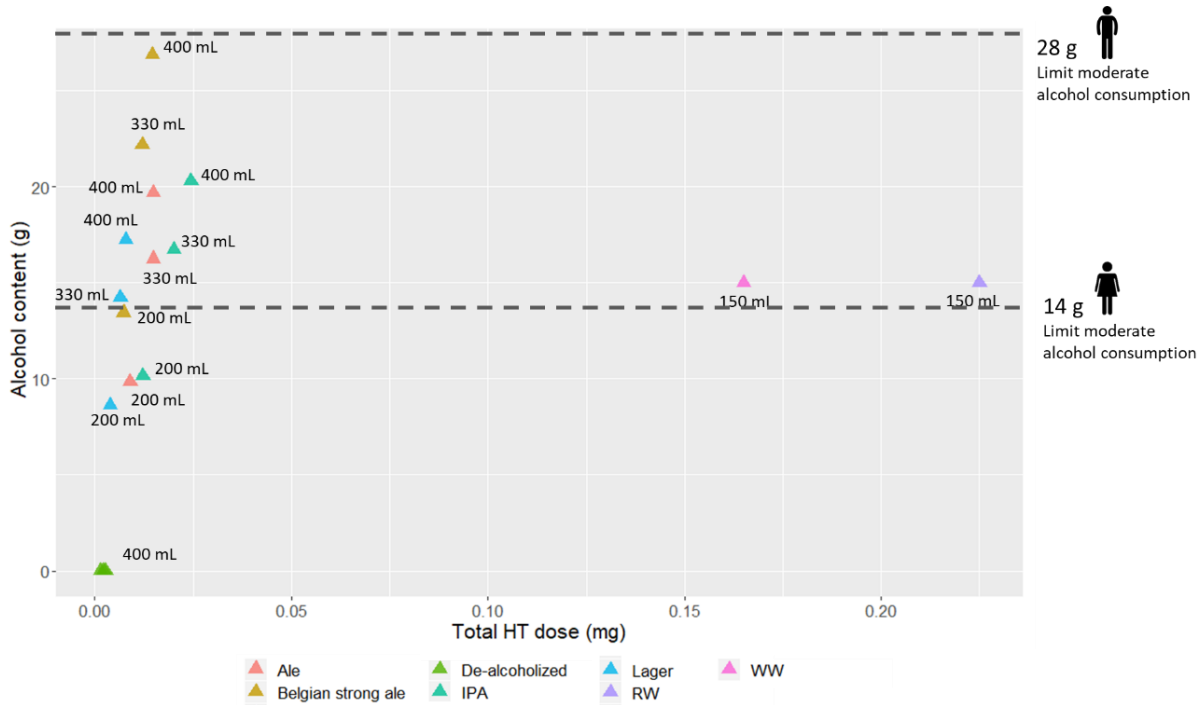


Figure 27. Hydroxytyrosol contribution and alcohol content of wines and beer based on frequent consumed doses. Dashed lines represent the limit of moderate alcohol consumed according to the AHA (14 g/day for women and 28 g/day for men). Doses of beer represented are 200 mL, 330 mL and 400 ml. IPA: Indian pale ale; RW: red wine; WW: white wine.

3.2.5 Discussion

The present work demonstrates that beer is a dietary source of Tyr. Tyr average content in beer is comparable to WW content, and certain types of beer have Tyr levels in the same range as RW. However, HT content in beer is particularly low and even undetectable in some cases. Beer basic ingredients are water, malted barley, hops and yeast. Changes in the type and proportion of each ingredient gives rise to different types and styles and can have an impact on the phenolic content of the final product.

As a fermented beverage, beer composition and characteristics relies to a great extent on the fermentation process. Based on the type of fermentation, beer can be divided into two broad types: ale and lager. According to our findings, ale beers have higher Tyr and HT than lager beers as it has been described previously (Cerrato-Alvarez *et al.*, 2019). The main difference between the two beer types is the yeast strains used. Lager beer uses *Saccharomyces pastorianus* while ale beers are fermented using *Saccharomyces cerevisiae* (Preedy, 2008). Hence, our data suggest that *S. cerevisiae* would be more efficient in producing Tyr and HT than *S. pastorianus*. A factor worth mentioning is that *S. cerevisiae* has a greater capacity to overcome the stress caused by the ethanol produced during the brewing process than the *S. pastorianus*. Hence, in ale beers, the fermentation activity is maintained for a longer period of time. This fact, may increase the exposure time of the yeast to metabolize tyrosine to produce Tyr and HT. Another point worth mentioning is the positive correlation found between Tyr and HT content and the alcohol content. This reinforces the fact that Tyr and HT are actually by-products of the beer fermentation process. The positive correlation found between the concentration of both molecules in beer suggests that HT is formed from Tyr in a dose-dependent manner.

Beer types can be sub-classified in different styles, based on common organoleptic characteristics, specific production methods, recipes and origin. In the case of the samples analysed in the present study, the most common style present within the lager beers was pilsner, whereas in the case of ale beers was IPA. Nonetheless, some beer styles were only represented by one sample. These cases were excluded from the statistical analysis. In agreement with our observations and in the context of beer consumption, Belgian strong ales would represent the best source of Tyr whereas IPA would be the best source of HT. In the case of Belgian strong ale beers, a relevant distinctive feature is the use of specific yeasts. In the case of IPA beers, they are characterized by their high content in hops. Our stud represents the first attempt to examine beer phenolic composition according to beer styles.

Non-alcoholic beer popularity has risen together with the concern about health and alcohol abuse consequences. The production of beer with a limited alcohol content can be achieved by two approaches that modify the brewing process: limiting the fermentation process, and hence the alcohol production, or by using physical methods to remove the alcohol at the end of the brewing process (Basso, Alcarde and Portugal, 2016). According to our data, and in agreement with a previous study (Cerrato-Alvarez *et al.*, 2019), non-alcoholic beers present lower Tyr and lower HT content. Based on the fact that Tyr and HT are produced as by-products during fermentation, the limitation of this process is likely to negatively affect their accumulation levels. On the other hand, the use of physical methods, often including thermal processes to induce alcohol evaporation, could trigger the degradation of Tyr and HT, explaining in this way the low concentrations of these phenolic compounds observed in the non-alcoholic beers. Our results suggest that the non-alcoholic brewing process has a detrimental impact on Tyr and HT content on beer.

Nowadays, the consumption of craft beer is becoming more popular. Nevertheless, it is worth to mention that there is not a clear or fixed definition of what is considered craft beer. In our set of samples, 22 % of them were considered craft beer. Our results have not revealed any significant difference in the Tyr and HT content between craft and commercial beers. Crafted beers normally tend to be unfiltered. However, our results do not suggest any negative effect of beer filtration on Tyr and HT concentration.

Beer composition and characteristics are strongly determined by yeast metabolism. New technologies and innovations in beer fermentation are based on the use of novel yeast strains. Variation in the yeast used for beer brewing changed the antioxidant activity and total phenolic composition of the beer produced (Capece A, 2019). Furthermore, the presence of polyphenols with antioxidant activity within the wort has been described to protect yeast viability in front of the stress generated by higher levels of ethanol (Gharwalova *et al.*, 2017). In the case of beer, the presence of flavonoids with antioxidant activity could contribute to yeast stability and enhance Tyr and HT formation. Hopes are the main contributor to beer phenolic composition. IPA beer, with a high content in hops, presented a high HT content. This could be a result of the stabilization activity of hop-flavonoids present in the wort and hence, promoting Tyr and HT formation. Overall, the study of new yeast strains that would selectively produce more Tyr and HT and the addition of other antioxidant compounds in the matrix could be an approach to potentiate phenolic composition of beer. Another crucial factor for Tyr and HT content in beer are tyrosine levels in the cereal used. The selection of tyrosine-rich cereals would be an additional approach to maximize the Tyr and HT beer's content.

The overall contribution of a specific food to the total phenolic daily intake is dependent on the amounts of the specific food consumed and the potential interaction with other compounds and food matrices. A balanced MedDiet includes consumption of EVOO and moderate alcohol consumption, both products having an important contribution on the total polyphenol intake per day. Our results confirm EVOO as the most important source of HT in the diet. Thus, a diet rich in EVOO could represent an intake of almost 1 mg of HT per day. HT daily intake could increase a 10% by including one glass of RW in the case of women or up to 20% by including two glasses in the case of men. Based on our results, beer would have a small contribution on HT total intake.

The results presented in this study indicate that RW and beer are good dietary sources of Tyr. Nonetheless, their total contribution on the diet is limited by its alcoholic content. RW has a higher content of Tyr but, since its alcoholic content is also higher, its total consumption is limited to one glass for women and two glasses for men. On the other way, an advantage of beer is its lower alcohol content, that enables the consumption of higher doses. In the case of women, 200 mL of beer would be the maximum to be taken to be considered within moderate drinking. Choosing the beer with the highest content of Tyr, approximately 5,9 mg of Tyr could be obtained from beer in the case of women. In the case of men, by drinking 400 mL of Tyr- rich beer, a daily dose of 11,7 mg of Tyr/day could be achieved.

The presented data represents a wide picture of the available beer in the Spanish market. However, some beer styles were under-represented due to its low popularity and hence low availability on the market. The lack of specific information about beer ingredients makes more difficult drawing definitive conclusions of the specific factors contributing to Tyr and HT

phenolic content. This work represents a starting point to understand Tyr and HT formation in beer brewing and suggests several approaches to increase their phenolic composition. Certainly, beer is a complex matrix including alcohol and a wide range of other phenolic compounds that could interact among them. Future studies should assess the bioavailability of Tyr and HT present in beer in the context of moderate consumption and its potential health effects.

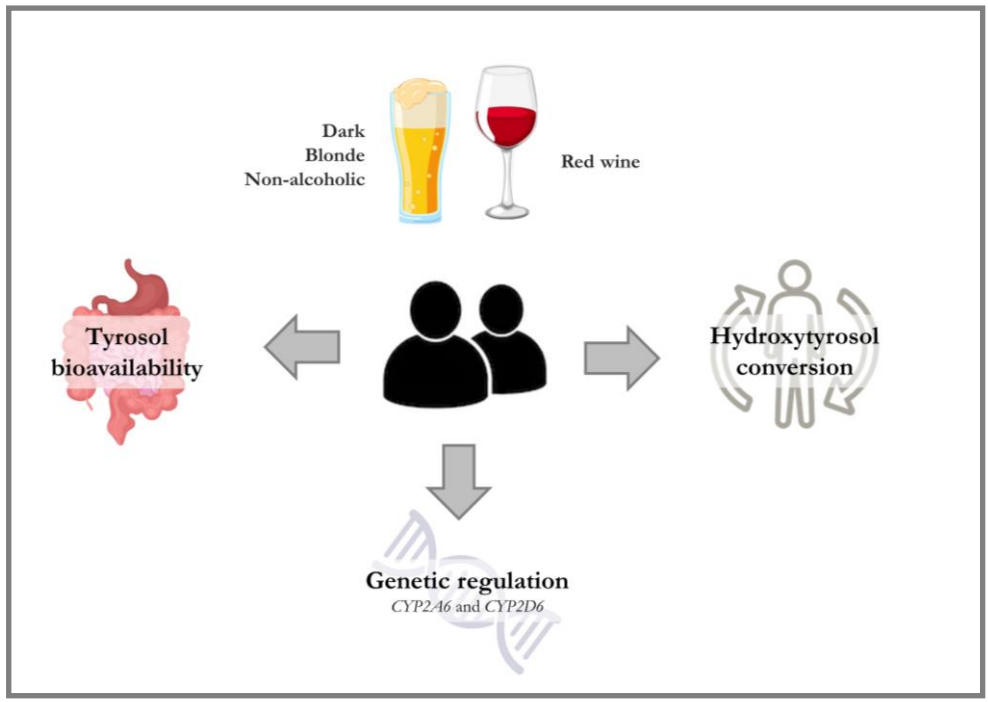
3.2.6 Conclusions

Extra virgin olive oil, red wine and beer are important dietary sources of Tyr and HT that can be consumed in a balanced MedDiet. EVOO and RW stand out for their content on HT. The HT content of beer is barely meaningful on the overall diet. RW and beer represent a relevant source of Tyr in the diet. However, their consumption is limited by its alcohol content. Fermentation is a crucial step on the formation of Tyr and HT during beer brewing and their final amount directly correlates with the alcoholic content of the beer. Non-alcoholic beer presents a low Tyr and negligible HT concentrations.

3.3

CHAPTER 3

Generation of hydroxytyrosol from tyrosol following beer and red wine administration



Adapted from: Soldevila-Domenech N*, **Boronat A*,** Mateus J, Diaz-Pellicer P, Matilla I, Pérez-Otero M, Aldea-Perona A, de la Torre R. Generation of the Antioxidant Hydroxytyrosol from Tyrosol Present in Beer and Red Wine in a Randomized Clinical Trial. Nutrients. 2019 Sep 18; 11(9):2241; doi:10.3390/nu11092241.

Soldevila-Domenech N*, **Boronat A*,** Mateus J, Diaz-Pellicer P, Matilla I, Pérez-Otero M, Aldea-Perona A, de la Torre R. Generation of the Antioxidant Hydroxytyrosol from Tyrosol Present in Beer and Red Wine in a Randomized Clinical Trial. Nutrients. 2019 Sep 18; 11(9):2241; doi:10.3390/nu11092241.

3.3.1 Abstract

Tyrosol (Tyr) is a natural phenolic compound produced within the beer fermentation brewing process. Evidence from wine studies suggests that Tyr can be endogenously transformed into the antioxidant hydroxytyrosol (HT) in vivo. Our study evaluates Tyr bioavailability and HT biotransformation following beer consumption in relation to red wine (RW) consumption. To do so, we performed a cross-over randomized controlled clinical trial (N=20) administering one single dose of RW (150 mL) and three different beers (250 mL): Indian pale ale (IPA), blonde and non-alcoholic beer, differing in their Tyr and alcohol content while being HT content almost undetectable. Additionally, individuals were genotyped for *CYP2A6* and *CYP2D6*. RW triggered the highest increase in Tyr and HT metabolites, followed by IPA and blonde beer and with a minor effect of non-alcoholic beer. HT formation following RW and beer consumption was correlated with Tyr administered doses. *CYP2A6* and *CYP2D6* genotype and sex had a significant impact on the efficiency of Tyr to HT biotransformation following RW consumption. In conclusion, Tyr is bioavailable in beer and it gets biotransformed into HT *in vivo*, as it has been previously described for RW. The results point out to a common mechanism between RW and beer that could explain health effects attributed to their moderate consumption.

3.3.2 Introduction

Beer has become the most prevalent form of alcohol consumption, accounting for 40,0% of the total alcohol consumed in Europe (WHO, 2014). The relationship between alcohol and CVD morbidity and mortality follows a J-shaped curve, indicating that moderate drinkers are at lower risk than abstainers and heavy drinkers (Poli *et al.*, 2013). Some studies indicate that fermented alcoholic beverages containing phenolic compounds such as wine or beer, may provide additional benefits than spirits, with low phenolic content (Costanzo *et al.*, 2011; de Gaetano *et al.*, 2016). Evidence suggest that beers and wine beneficial health effects would be result of a synergistic effect between alcohol content and RW/beer's phenolic compounds.

Beer is a fermented drink rich in natural phenolic compounds. The total phenolic content of beer is slightly higher than in white wine and lower than red wine. At the same time, its alcohol content is lower compared to other popular alcoholic drinks. Therefore, beer's low alcohol content together with its high phenolic composition made it as a potential trigger of health effects while minimizing detrimental effects associated to alcohol consumption.

Beer has been described recently as an alternative dietary source of Tyr. Our previous data confirmed that Tyr present in wine was well absorbed and then was biotransformed into HT in vivo. However, the bioavailability of Tyr and its further metabolism into HT has never been studied in the context of beer consumption. Beer and wine intrinsic matrix differences arise several potentially factors that could have an impact on the bioavailability and conversion of Tyr: alcohol present in the matrix, the carbon dioxide dissolved in beer, the potential interaction/competence with other polyphenols present in beer, apart from the previous described sex and genotypic differences.

The aim of the present chapter is to confirm Tyr bioavailability and its biotransformation into HT in the context of moderate beer consumption, determining the effect of alcohol and other compounds present in the matrix on Tyr bioavailability. Additionally, the impact of sex and *CYP2A6* and *CYP2D6* genetics on HT formation is evaluated.

3.3.3 Material and methods

3.3.3.1 Study design

The study consisted in a double-blinded randomized controlled clinical trial with four different interventions: RW, and three different classes of beer: Indian pale ale (IPA), blonde and non-alcoholic beer. Participants were randomly allocated in different orders for the treatment's administration. Each treatment was given on separate experimental sessions. On the day of the study, participants attended the clinical research unit in, at least, 10-hour fasting conditions. Urine was collected during the first 2 hours to obtain a baseline sample (-2 – 0 h). Treatment administration was given in opaque glasses within less than 10 minutes. Thereafter, post-administration urine was collected for the following 24 hours at different time collection periods: 0-2 hours, 2-4 hours, 4-6 hours, 6-12 hours and 12-24 hours. Volunteers were kept in fasting conditions until the 2nd hour post administration, when they were given a light meal. Volunteers remained at the research unit until the 4th hour post-administration. Each session day was preceded by at least 72 hours wash-out periods, in which participants were asked to follow a low-phenol diet (available in 3.3.7 Additional information) and abstain from any alcoholic drink. Timeline of the experimental session is detailed in figure 28.

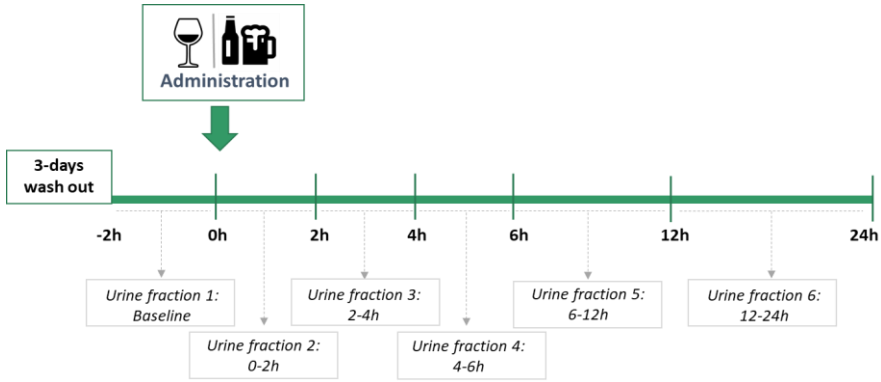


Figure 28. Timeline of the experimental sessions

3.3.3.2 Interventions

The RW used in the present study was Jardins Negre 2017 (14% Alc. Vol) from the Castillo de Perelada S.A. winery (Girona, Spain). Blonde ale (Blonde; 4.5 % Alc. Vol) and India Pale Ale (IPA; 8.5 % Alc. Vol) were provided by Cervesa Espiga (Sant Llorenç d'Hortons, Spain). The non-alcoholic beer was Aigua de Moritz (0.0 % Alc. Vol) manufactured by Cervezas Moritz S.A (Barcelona, Spain). All four products were obtained from the Spanish market. Tyr and HT content of administered treatments was determined using LC/MS-MS and are detailed in table 21.

Table 21. Tyrosol, hydroxytyrosol and alcohol content of wine and beers administered in the study

	Treatment			
	Red wine	**IPA**	**Blonde ale**	**Non-alcoholic**
Tyr (mg/L)	25,30	9,68	4,23	3,35
HT (mg/L)	1,79	0,13	0,06	0,01
Alcohol (% vol/vol)	14,00	8,50	4,50	0,00
Dose (mL)	150	250	250	250
Tyr administered (mg)	3,80	2,42	1,06	0,84
HT administered (mg)	0,27	0,03	0,01	0,00
Alcohol (g)	16,57	16,77	8,88	0,00

The doses of RW and beers administered were based on normally consumed amounts within the Spanish population and followed moderate alcohol consumption recommendations. In the case of beer, 250 mL were given while a lower volume of RW was given; 150 mL which was supplemented with 100 mL of water to match total volumes. The choice of beers was made based on alcohol and Tyr content in comparison to RW (Figure 29). IPA belongs to a group of beers with a relatively high content

of Tyr and high content of HT while alcohol dose matched the one of RW. Hence, both treatments resulted in the same amount of alcohol and both were high in Tyr. Blonde ale was selected for having a lower content in Tyr and alcohol. Finally, non-alcoholic beer was chosen matching its Tyr content to blonde ale to assess the contribution of alcohol to Tyr absorption.

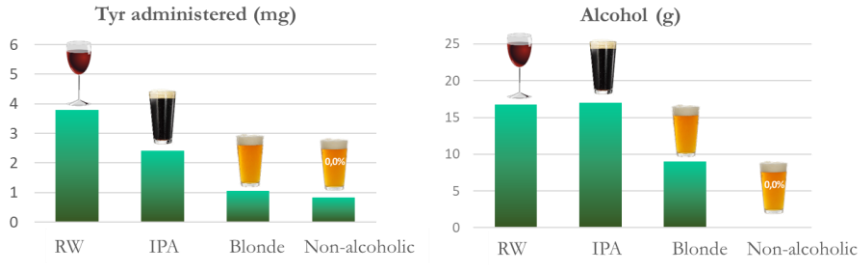


Figure 29. Tyrosol and alcohol content of the administered treatments

3.3.3.3 Participants

The study was carried out with a total of twenty healthy subjects (50% women). They were recruited using an internal volunteer database and by worth of mouth from June 2018 to February 2019 in the Clinical Research Unit of Hospital del Mar Medical Research Institute. Volunteers were healthy individuals aged from 18 to 45 years with a recreational consumption of alcohol. Exclusion criteria were: smokers, suffering a severe chronic diseases, body mass index < 18.5 or >30.0 kg/m^2, multiple allergies, intestinal, hepatic or renal conditions that may affect normal phenol metabolism, participants following restrictive diets or taking antioxidant supplements, participants with previous history of alcohol hypersensitivity or intolerance, participants consuming > 50 g of alcohol per day, pregnant or breastfeeding women and illiteracy. (Table 22)

Table 22. Inclusion and exclusion criteria

INCLUSION CRITERIA	EXCLUSION CRITERIA
Healthy individuals	Smokers
Aged from 18 to 45 years	Severe chronic disease
Recreational consumption of alcohol	BMI < 18.5 or > 30.0 kg/m^2
	Multiples allergies
	Intestinal, hepatic or renal conditions
	Restrictive diets
	Taking antioxidant supplements
	Alcohol hypersensitivity or intolerance
	Alcohol consumption <50g/day
	Pregnant or breastfeeding women
	Illiteracy

The present study was conducted following good clinical practices and in accordance with the Helsinki declaration. The clinical trial was approved by our local ethical committee (CEIm-Parc de Salut Mar) and registered in ClinicalTrials.gov: NCT03614520. Written informed consent was obtained from all screened participants prior to any clinical procedure. Before inclusion, all potential volunteers underwent a general physical examination, routine laboratory tests, urinalysis, and a 12-lead electrocardiogram presenting results within normal values. At the end of the study, another general physical examination and routine blood and urine laboratory test were performed.

3.3.3.4 Sample collection

For all urinary samples collected, the total volume and pH were registered. Thereafter, samples were acidified to avoid phenolic degradation and kept at -20º until analysis. At the beginning of the study, an EDTA blood tube was collected for DNA sampling. Tubes were centrifuge (1700g, 15 min, 4ºC) to isolate buffy coat. Two volunteers refused to consent for DNA examination.

3.3.3.5 Volunteer genotyping

CYP2A6 and *CYP2D6* genotypes were determined for the eighteen volunteers who gave consent to the DNA sample extraction. Genomic DNA was isolated from the buffy coat using the QIAamp DNA blood Mini kit (Qiagen, Dusseldorf, Germany).

Volunteers were genotyped as described in section 3.1.3.2 subsection D for the most common allelic variants in the Spanish population of *CYP2A6* (*2, *4, *9, *12, *1Xn (xN indicates more than one copy) and *CYP2D6* (*2, *4, *5, *9, *10, *35, *41, 1xN, 2xN, 35xN) (Menoyo, Del Rio and Baiget, 2006) using the TaqMan allelic discrimination method (Applied Biosystems, Foster City, CA, USA). *1 allele was assumed when none of the tested allelic variants were detected. Then, a global *CYP2A6* and *CYP2D6* polygenic activity score was calculated as previously described in section 3.1.3.2. subsection E.

3.3.3.5 Tyrosol and hydroxytyrosol urinary recovery

Urinary recovery of Tyr and HT metabolites was quantified by means of a solid-phase extraction followed by LC-MS/MS analysis as previously described in section 3.1.3.2 subsection A. In order to calculate phenolic compounds recovery, urinary concentrations were standardized by the total volume of urine collected in each time fraction. Total Tyr was equivalent to the molar sum of free Tyr, Tyr-4-sulphate and Tyr-4-glucuronide. Total HT was the molar sum of free HT, HT-4-sulphate, HT-glucuronide (HT-3-glucuronide plus HT-4-glucuronide), HT-acetate-3-sulphate, HT-acetate, free Hval and Hval-4-glucuronide.

3.3.3.6 Ethyl glucuronide determination

The alcoholic biomarker ethyl glucuronide was measured in urine using a dilute-and-shoot approach as described in section 3.1.3.2 subsection B. It was used to assess volunteers' compliance with alcohol abstinence throughout the study.

3.3.3.7 Sample size and power analysis

A total sample size of 20 participants (10 of each sex) would allow 85% power to detect a statistically significant difference in total HT recovery of 1.2 μmols, assuming a normal standard deviation of 5.4 μmols, an adjusted type I error of 0.05, 2-sided. The sample size was calculated with the GLIMMPSE software (https://glimmpse.samplesizeshop.org/#/) considering the multiple comparisons of the study.

3.3.3.8 Statistical analysis

Analyses were performed with R, version 3.0.2. R packages used were "multcomp", "nlme" and "ggplot2". Linear mixed effects models were devised to perform comparisons among treatments. Tukey's HSD (honestly significant difference) test was used to perform post-hoc pairwise comparisons. Pearson correlation coefficient was calculated to evaluate the existence of linear associations between the dose of Tyr administered and the total Tyr and HT recoveries. Significance was set as $p < 0,05$.

3.3.4 Results

3.3.4.1 Characteristics of study participants

A total of 20 individuals successfully completed the study. Mean age was 24,3 ± 3,9 years with a BMI of 22.2 ± 2.0 kg/m^2. Male volunteers presented significantly greater weight than female volunteers (73,0 ± 8,3 *vs* 60,3 ± 8,4 kg respectively, p=0,003) and higher BMI (23,2 ± 2,0 *vs* 21,1 ± 1,4 kg/m^2 respectively, p=0,019).

Volunteers' compliance with the low phenolic diet and alcohol abstinence was assessed in baseline urines. Baseline EtG levels revealed that one men volunteer did not follow the alcohol abstinence requested in the wash-out periods in two of the four study visits corresponding to blonde and non-alcoholic beers (baseline EtG levels were 2,8 and 12,9 μmol, respectively). Consequently, these two visits from this volunteer were removed from the statistical analysis.

3.3.4.2 Genotyping

Eighteen volunteers (two declined genetic testing) were genotyped for *CYP2A6* and *CYP2D6*; from this information, a PAS was calculated, ranging from 2 to 4. Sex was not equally distributed among PAS groups; male volunteers presented higher polygenic activity than women (p=0,018).

3.3.4.3 Tyrosol and hydroxytyrosol metabolites recovery in urine

a) Total tyrosol and hydroxytyrosol

Urinary recoveries of total Tyr and total HT in different urinary fractions are represented in figure 30. Differences of the Tyr and HT recovery among the

different interventions were evaluated for each time fraction Significant differences were observed in Tyr recovery among treatments in all urinary fractions (0-2, 2-4, 4-6, and 6-12 hours, Figure 30.A) except 12-24 hours. In the case of HT, significant differences were only found in the urine fractions included in the first 6 hours post-administration (0-2, 2-4 and 4-6 hours, Figure 30.B).

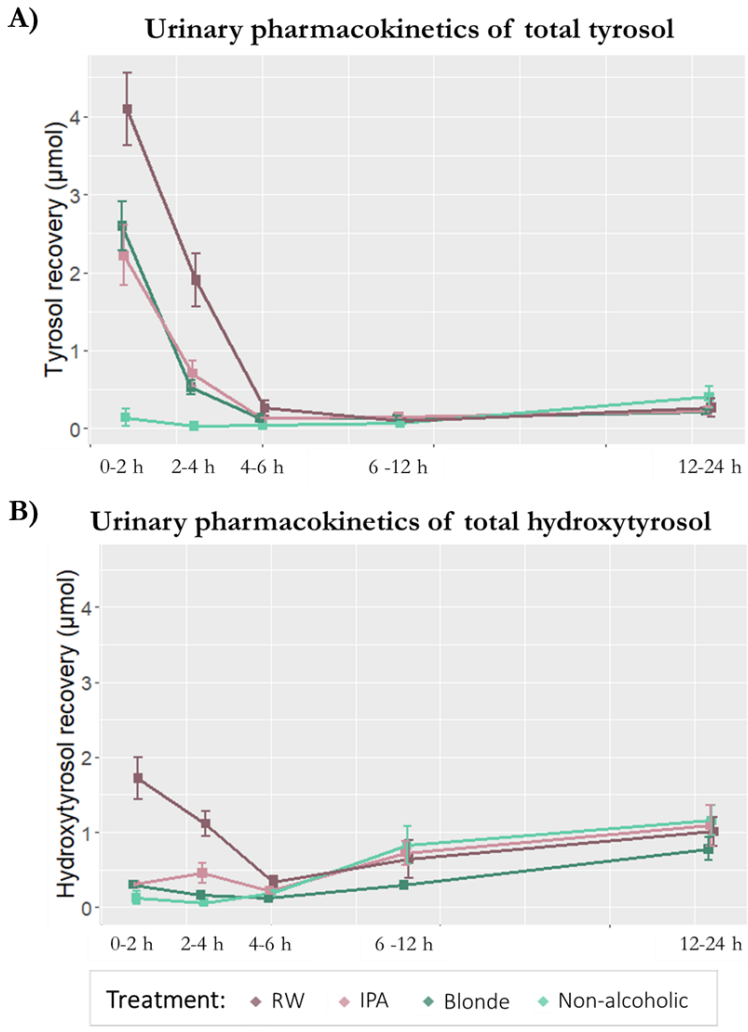


Figure 30. Urinary pharmacokinetics of total tyrosol (A) and total hydroxytyrosol (B) following each treatment in urinary fractions from 0 to 24 h post administration.

Accumulated Tyr and HT after 6 and 24 hours following each treatment are presented in Table 22. After 6 hours post-administration, RW triggered the highest increase in total Tyr (6,2 ± 2,9 µmol) followed by blonde (3,3 ± 1,8 µmol) and IPA (3,1 ± 2,4 µmol). Non-alcoholic beer presented the lowest recovery of Tyr: 0,3 ± 1,0 µmol (p<0,001 compared to the other treatments).

In terms of total HT excretion within the first 6 hours, the highest recoveries were observed following RW, with of 3,1 ± 1,3 µmol (p<0,001 *vs* all treatments) (Table 23). The highest HT recovery after beer intake was observed in the case of IPA (1,0 ± 0,6 µmol), followed by blonde and finally non-alcoholic beer. Similar trends were observed in Tyr and HT recovery when including urine collection of 24 hours post-administration. Baseline levels of total Tyr and HT were low (median and interquartile rank were <0,1 µmol), compatible with a low-phenolic diet and did not differ significantly among interventions.

Table 23. Total tyrosol and Total hydroxytyrosol pharmacokinetic urinary data

Metabolite	Fraction	RW	IPA	Blonde	Non-alc beer	*p*
Total Tyr	0-6h	6,2 ± 2,9	3,1 ± 2,4^{w}	3,3 ± 1,8^{w}	0,2 ± 0,5 ^{w,i,b}	<0,001
	0-24h	6,5 ± 2,9	3,4 ± 2,5^{w}	3,7 ± 1,8^{w}	0,6 ± 0,8 ^{w,i,b}	<0,001
Total HT	0-6h	3,1 ± 1,3	1,0 ± 0,6^{w}	0,6 ± 0,3^{w}	0,4 ± 0,5^{w}	<0,001
	0-24h	4,8 ± 2,4	2,8 ± 1,4^{w}	1,5 ± 0,7^{w}	2,3 ± 1,6^{w}	<0,001

Results are expressed in µmol as mean ± SD. P for ANOVA repeated measures. w, i, b= Tukey's HSD post-hoc comparisons indicated as w: p<0,05 compared to RW; i: p<0,05 compared to IPA; b: p<0,05 compared to blonde.

Based on our results, Tyr was rapidly excreted in urine within the first 6 hours post-administration: representing approximately 90% of the total 24-h Tyr excretion in the case of RW, IPA and blonde beers. On the contrary, non-alcoholic beer, Tyr within the first 6 hours represented the 30% of the total Tyr excreted in 24 hours. In relation to HT excreted during the first 6 hours, it represented 65% for RW, and approximately 35% for IPA and blonde beers, and 16% for non-alcoholic beer in relation to total 24-h HT .

Figure 31 shows the mean dose recoveries observed in the study in the form of Tyr and in the form of HT following each treatment during the first 6 hours post-administration. Tyr dose recovery ranged from 3.5% to 43%, being non-alcoholic beer the lowest and blonde beer the highest. The percentages of the doses recovered in the form of HT ranged from 5.6% to 11.3%; RW presenting the highest recovery and IPA beer the lowest.

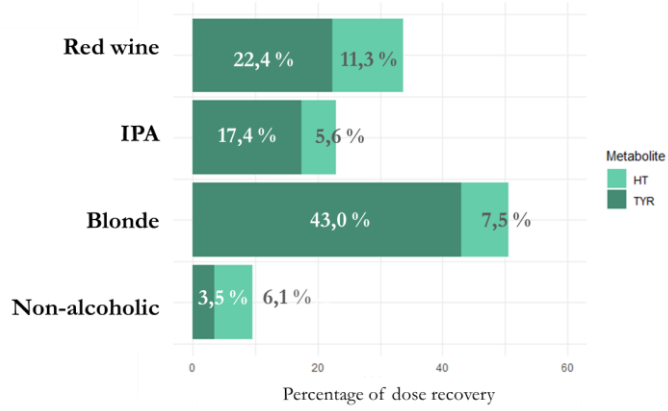


Figure 31. Dose recovery in the form of total tyrosol (dark green) and total hydroxytyrosol (pale green) (6 hours post-administration).

Figure 32 shows the correlation between total Tyr and HT recovery (0 - 6h) and the dose of Tyr administered in each intervention adjusted by volunteer's body weight. There was a linear relationship between the dose of Tyr and total Tyr (r=0,691, p<0,001) and total HT (r=0,737, p<0,001) urinary recoveries.

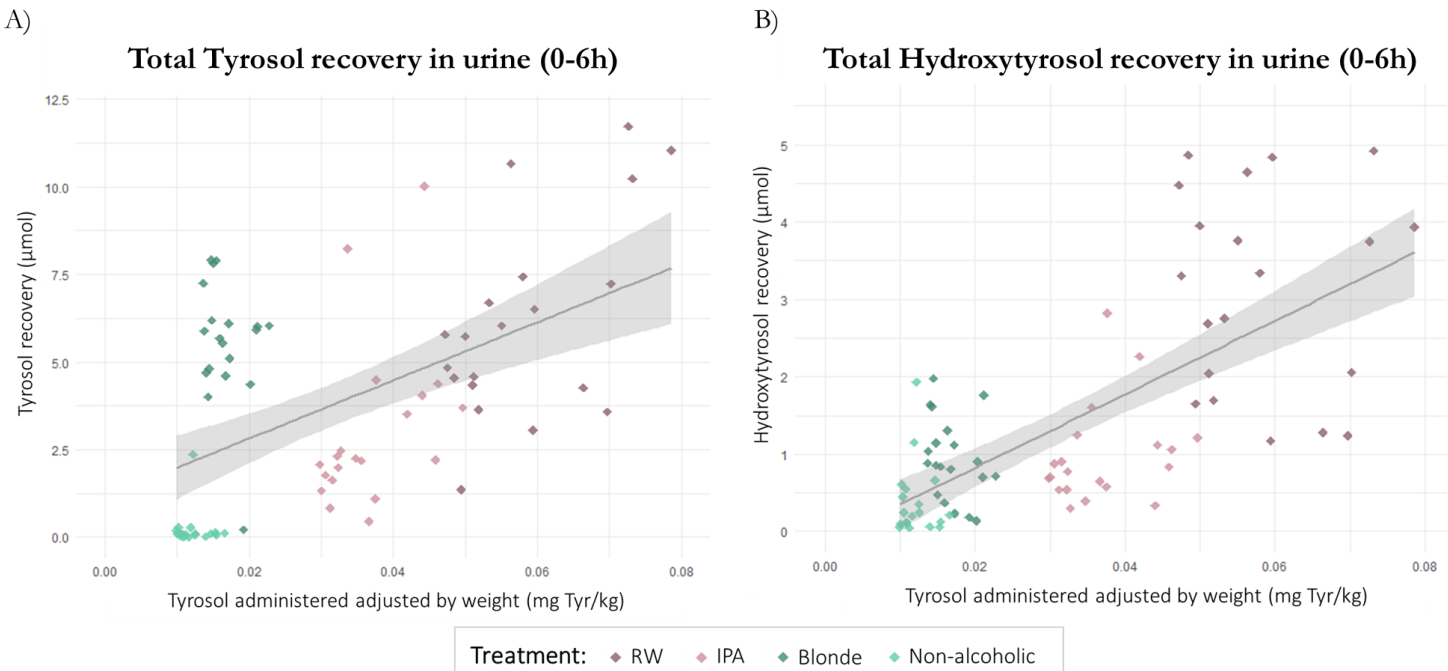


Figure 32. Correlation between tyrosol administered adjusted by body weight and total tyrosol 6h recovery (A) and total hydroxytyrosol 6h recovery (B) following each treatment.

b) Tyrosol and hydroxytyrosol metabolites

Figure 33 summarized the metabolic disposition of Tyr and HT metabolites following RW and beer treatments. A global significant increase in Tyr-metabolites was observed at the first 6 hours; being Tyr-4-glucuronide the most prevalent metabolite, followed by Tyr-4-sulfate, remaining free Tyr minor (approximately 6%) (p<0,001). Tyr accumulated levels from 6-24 hours were low and not statistically significant among treatments. An increase in HT-metabolites was observed from 0-6 and 6-24 hours in HT-4-sulphate (p<0,001) Hval-4-glucuronide (p<0,001 at 0-6 hours and p=0,047 at 6-24 hours) and a mild increase in free HT (approximately 8% of total HT, p=0,004). HT-acetate-3-sulphate, HT-acetate, HT-3-glucuronide/HT-4-glucuronide and free Hval were not statistically different among treatments (p>0,100).

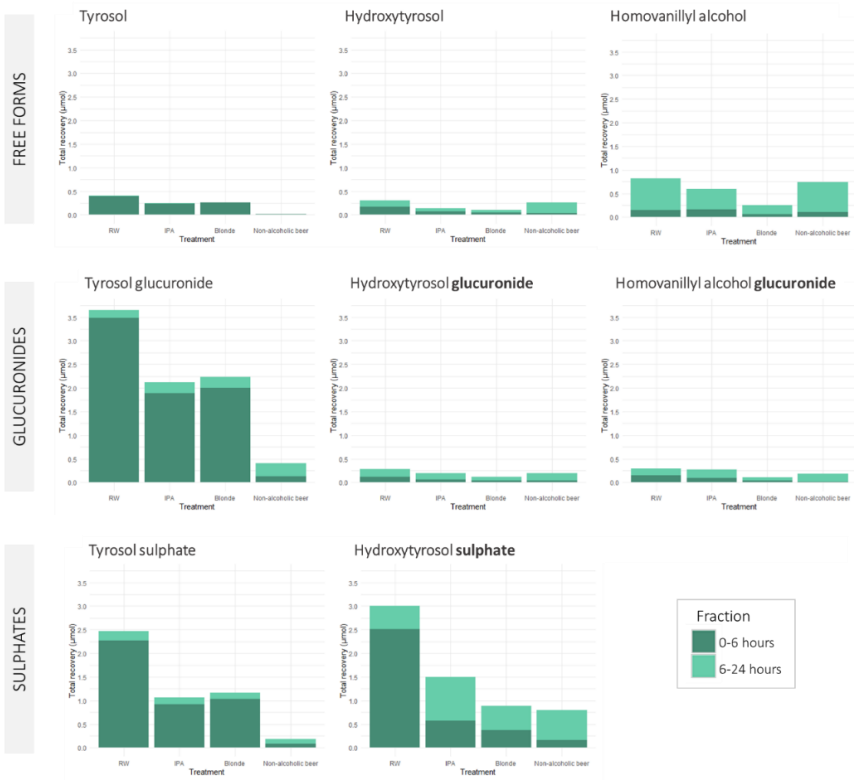


Figure 33. Metabolic disposition of tyrosol and hydroxytyrosol metabolites (µmols) for each treatment in time fractions 0-6 and 6-24 hours.

c) Sex dimorphism in tyrosol and hydroxytyrosol metabolism

Total Tyr and total HT recoveries (0-6 hours) in urine were stratified by sex to study potential differences between men and women. No significant differences were observed with respect to total metabolites, although distinct marginal trends were found following RW and IPA beer. Tyr recovery was marginally greater in women compared to men following RW (p=0,102) while HT recovery was higher in women compared to men following IPA beer (p=0,067). Figure 34 shows HT/Tyr ratios comparison between men and women across the different treatments. Significant differences were observed in the case of RW within the first 6 hours; in which men presented greater ratios than women (p=0,004). Statistical analyses were adjusted by PAS.

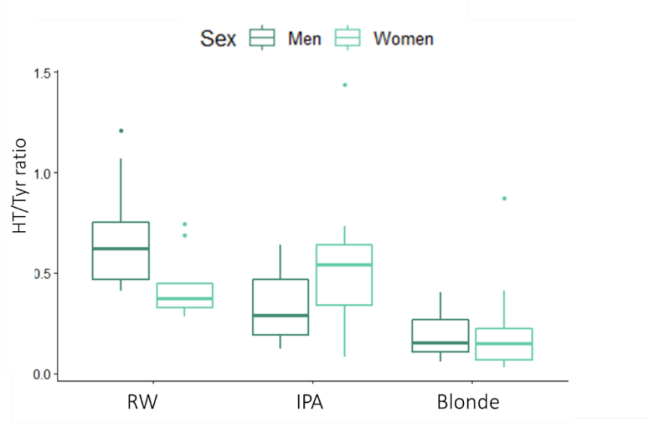


Figure 34. Ratio between total hydroxytyrosol/total tyrosol (HT/Tyr ratio) recoveries collected from 0 to 6 hours for different RW, IPA and blonde interventions stratified by sex.

Figure 35 shows the matrix of correlation of HT/ Tyr ratios across the different interventions stratified by sex. Men exhibited good correlation between HT/Tyr ratios across the different treatments. However, women HT/Tyr ratios only were correlated in the case of both beer treatment, but RW response was not correlated with any of the treatments.

	RW	IPA	Blonde
RW	-	R=0,723 *p* =0,017	R=0,566 *p*=0,089
IPA	R=0,464 *p*=0,177	-	R=0,808 *p*=0,005
Blonde	R=0,020 *p* =0,956	R=0,641 *p*=0,046	-

MEN

WOMEN

Figure 35. Matrix of correlation of total hydroxytyrosol / total tyrosol (HT/Tyr) ratios across the different interventions: Differences between men and women. Pearson correlation between log transformed HT/Tyr ratios. Cells colored with darker green corresponded to men while cells colored with paler green corresponded to women.

d) Interaction between PAS and tyrosol and hydroxytyrosol metabolism

There was a positive correlation between PAS and the HT/Tyr ratio at 0-6 hours following RW consumption (r=0,534 spearman correlation test, *p*=0,022). This linear association was not seen in the case of IPA, blonde, and non-alcoholic beers.

e) Effect of urinary pH on tyrosol and hydroxytyrosol recovery

No influence of urinary pH on phenols excretion was observed in any of the urinary fractions after the consumption of the different treatments.

3.3.4.4. EtG urinary recovery

Baseline EtG levels were undetectable in most of the samples and did not differ significantly among interventions (*p*=0,416). As shown in Table 24, 24-hour EtG recovery was similar between RW and IPA beer and both significantly higher than blonde and non-alcoholic beers. Urinary recovery of EtG followed a similar kinetic pattern, reaching its maximum in the 2-4 hours fraction and then declined. Finally, women presented higher EtG

levels than men, reaching borderline significance after IPA intervention (p=0,058).

Table 24. EtG urinary recovery (0-24 hours). Results are expressed in µmol

	RW	IPA	Blonde	Non-alc beer
All sample	22,1 ± 7,9	19,7 ± 7,0	9,1 ± 3,7 ^{w,i}	0,2 ± 1,1 ^{w,i,b}
Men	21,2 ± 8,7	16,8 ± 7,0	9,5 ± 4,0 ^{w,i}	0,5 ± 1,5 ^{w,i,b}
Women	23,0 ± 7,4	22,7 ± 6,1	8,7 ± 3,5 ^{w,i}	0,0 ± 0,0 ^{w,i,b}

Results are expressed in µmol as mean ± SD. *ANOVA repeated measures. w, i, b= Tukey's HSD post-hoc comparisons indicated as w: p<0,05 compared to RW; i: p<0,05 compared to IPA; b: p<0,05 compared to blonde.

3.3.5 Discussion

The results of this study show the *in* vivo bioavailability of Tyr and its biotransformation into HT following beer consumption. Alcoholic content and beer matrix composition seem to be key determinants of Tyr bioavailability. On the other hand, sex and genetics seem to affect Tyr to HT bioconversion capacity. Thus, helping to understand the variability observed in the metabolism of these simple phenols.

In the present study we had been able to compare Tyr absorption in different treatments: RW and three beers, differing in its Tyr and alcohol content. RW and IPA presented equivalent alcohol total content. When assessing the dose of Tyr recovered (adjusted by the given dose), we observed a low recovery following IPA intake compared to RW (17,4 % vs 22,4% of total Tyr given dose respectively). Tyr absorption following blonde beer was the highest observed: 43,0%. In contrast, non-alcoholic beer Tyr absorption was the lowest: 3,5%, although Tyr content was similar to blonde beer. Taken together, these results confirm the role of alcohol to enhance phenolic bioavailability as it has been previously described (Pérez-Mañá, Farré, Rodríguez-Morató, *et al.*, 2015). Beers are rich in other polyphenols and their content is known to vary widely among styles (Intelmann, Haseleu and Hofmann, 2009; Quifer-Rada *et al.*, 2015; Ragusa *et al.*, 2017). These other phenolic compounds could compete for its absorption with Tyr and hence affecting to its final bioavailability, explaining the differences in Tyr dose recovery between IPA and blonde beers.

Another important finding of our study is the confirmation of the endogenous HT formation following beer consumption. Beer's HT content was low; however, we observed an increase in HT metabolites following its consumption, which was positively correlated to Tyr ingested doses. Our results point out beer as an indirect source of HT, supporting the hypothesis

of Tyr being an endogenous precursor of HT in vivo (Rodríguez-Morató *et al.*, 2017). Nonetheless, a higher HT recovery (adjusted by the given dose) was observed following RW (11,3%) than the one observed following beer intake (5,6 to 7,5%). As it has been previously described, there is a shift in dopamine metabolism following alcohol consumption, resulting in endogenous HT formation as a byproduct of dopamine oxidative metabolism in a dose response manner to the alcohol consumed (Pérez-Mañá, Farré, Pujadas, *et al.*, 2015). This could explain part of the different HT relative recoveries following the intake of beverages with varying alcohol content. However, the low HT recovery following beer consumption suggests that Tyr to HT conversion could be affected by matrix factors as it was more efficient in RW than in IPA and blonde beers. A unique ingredient of beer are hops, which highlight for their content in prenylated phenols. They have been reported to be potent and selective inhibitors of multiple members of the CYP family (Yuan *et al.*, 2014). A study worth mentioning evaluated the inhibitory effect of different beer extracts on *CYP2D6*. Its activity was inhibited by all beer extracts, with the greatest inhibition obtained with porter beer followed by ales, whilst lager beers were the least inhibitors (Foster *et al.*, 2011). To the best of our knowledge, no effect of hops constituents has been described on *CYP2A6* activity. According to these findings, hops-phenolic compounds present in beer are likely to interfere in the Tyr to HT conversion. Thus, this inhibitory effect could be behind the low production of HT observed after beer consumption and also explain the lower impact of PAS on Tyr to HT conversion after beer consumption.

Furthermore, our results describe the *in vivo* urinary kinetics of Tyr and HT formation following real life doses. Our study suggests a distinct urinary kinetics for Tyr than from HT. Tyr was rapidly excreted within the first 4

hours while HT excretion was sustained until the 6th hour post-administration of our treatments, confirming its metabolic origin.

Our research confirms the extensive phase II metabolism of Tyr and HT following their absorption, giving rise to phase II metabolites and being the free forms minority as it has been previously described (Pastor *et al.*, 2016; Rodríguez-Morató *et al.*, 2016). In the present study, we show an increase in the same Tyr and HT phase II metabolite recovery following RW and beer treatments. The main metabolites recovered were Tyr-4-sulfate, Tyr-4-glucuronide, and HT-3-sulfate following all the intervention. Theses metabolites have been shown to possess *in vitro* biological activity (Atzeri *et al.*, 2016; Muriana *et al.*, 2017; López de las Hazas *et al.*, 2018). The fact that beer produced a rise in the same metabolites as RW leads us to think that similar health effects could be expected from moderate beer consumption, although at a lower level. Therefore, Tyr, and HT metabolites could also explain, in part, the health effects associated to moderate beer consumption.

The current work provides further support to the role of *CYP2A6* and *CYP2D6* in the mediation of Tyr to HT biotransformation (Rodríguez-Morató *et al.*, 2017). Following RW consumption, PAS was positively correlated with HT/Tyr, understood as a measurement of the reaction efficiency. Nonetheless, the effect was only observable in the RW treatment, probably due to its higher Tyr dose. An additional fact worth considering when evaluating these results is the potential interaction of CYPs with beer polyphenols, which may hamper the observation of potential differences among different genotypic profiles.

This research extended our knowledge of sex differences in the metabolism of Tyr and HT. In particular, differences in HT / Tyr ratios pointed out to a difference on the efficiency of Tyr to HT conversion: men presented higher

ratios than women following RW. The present findings seem to be consistent to the ones described in the PREDIMED study, in which men presented higher values of HT adjusted by alcohol consumption than women (Schröder *et al.*, 2009; Rodríguez-Morató *et al.*, 2016)[32]. Our findings are also in agreement with de Bock et al., (2013), describing a sex dimorphism following the administration of olive leaf extracts in a clinical setting. Likewise, men exhibited greater concentrations of HT metabolites compared to women in response to the same treatment (de Bock *et al.*, 2013). Interestingly, in our study, HT/Tyr ratios among treatments were well correlated in men but not in women. In the case of men, Tyr to HT conversion capacity would be equivalent across the different treatments. Nonetheless, the fact that women HT/Tyr ratios fluctuate among the treatments suggest that other factors could be affecting Tyr to HT biotransformation capacity. It is well known that *CYP2A6* activity is upregulated by estrogen levels (Tanner and Tyndale, 2017), indicating a higher activity in women compared to men, however, this was not observed in our study. It seems possible that the influence of estrogen status and the fluctuation in estrogen levels intrinsic to the menstrual cycle could affect *CYP2A6* activity distinctly among the experimental sessions, which may explain the absence of correlation between rations among treatments. Caution should be taken when interpreting these sex differences as women presented lower PAS than men.

EtG is a secondary alcohol metabolite used as a biomarker of alcohol consumption (Andresen-Streichert *et al.*, 2018). It was used to assess the compliance with alcohol abstention prior to the experimental sessions. Its recovery following our treatments matched their alcohol content. Noteworthy, another sex dimorphism was observed in EtG recovery, particularly following IPA beer. In this case, women exhibited greater EtG

than men. This observation would be in line with reports showing a distinct alcohol metabolism between men and women (Baraona *et al.*, 2001).

Currently, beer is the most popular drink in the western world (WHO, 2014) whilst RW consumption is decreasing. Nevertheless, at similar given doses of Tyr and equal doses of alcohol, RW exhibited a higher recovery on Tyr and HT metabolites, suggesting a better bioavailability of Tyr and a more efficient conversion to HT. Therefore, it is likely that the beneficial effects attributed to RW moderate consumption are superior to the benefits observed following beer consumption. A large body of evidence exists about the health effects attributed to moderate RW consumption whilst less is known about moderate beer consumption, and data are mostly derived from epidemiological studies (de Gaetano *et al.*, 2016). Further randomized controlled clinical trials should be performed in order to identify the biological effects of moderate beer consumption and also assess the contribution of Tyr and HT on the observed effects.

Our findings improve our understanding of the antioxidant composition of beer and its potential health effects. The study of traditional beer and non-alcoholic beer can help to understand the role of ethanol and its interaction with beer phenolic compounds and their biological effects. Beer alcoholic content is lower than RW and, as shown in the present study, beer consumption triggered an increase in similar phenols. Non-alcoholic beer led to a mild increment in Tyr and HT metabolites due to its lower bioavailability. Nevertheless, it could represent a source of these compounds in cases where alcohol consumption must be limited.

Our study presents certain strengths and limitations. One strength is the cross-over design that minimized the interferences with possible confounders and controlled for between-subject variation. Our study design

enabled the evaluation of potential sex differences in the metabolism of dietary phenolic compounds. Another strength of this study is that the administered doses of RW and beers are achievable in a normal diet and agree with moderate alcohol consumption recommendations. The current investigation was, however, limited by the low sample size which hampered the simultaneous grouping and comparison of volunteers according to their sex and PAS. This would have been relevant since PAS was not equally distributed between the both sexes. In addition, treatments were given at a single dose; it is possible that longer administration periods would have resulted in different results attributed to sex or PAS. Finally, further studies should be performed to assess the biological relevance of the clinical effects of Tyr and endogenous HT formation associated with moderate beer consumption, at a postprandial level, and after chronic consumption.

3.3.6 Conclusions

Overall, ours is the first study that has evaluated the metabolism of Tyr and HT following beer consumption. Beer's HT content was almost undetectable; however, there was an increase in HT urinary metabolites correlated with Tyr administered doses. Therefore, Tyr present in beer would be an indirect source of HT as previously observed following RW. Hence, a common mechanism between beer and RW is pointed out here. Nevertheless, Tyr bioavailability and HT biotransformation seem to be affected by matrix components, sex and genetics.

3.3.7 Additional information

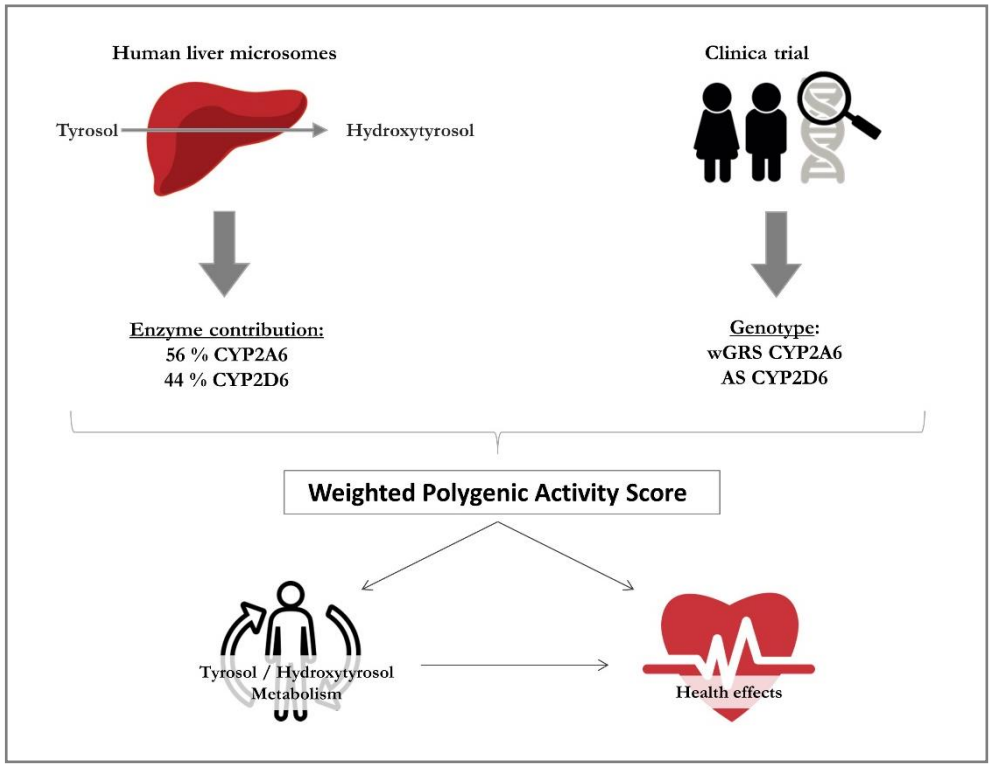
a) Low-phenol content diet during the study

Study participants were asked to follow a controlled diet with a low antioxidant content, and to abstain from the following foods and beverages thorough the study: alcoholic drinks (except in the framework of treatment allocations), vinegar and foods derived from olives or containing olives (e.g. olives, olives pate, olive oil, canned products in olive oil, pre-cooked products with olive oil). The consumption of coffee and energy drinks was limited to one serving/day. The use of cosmetics that contained alcohol (e.g. deodorant, colognes) was forbidden on the day of the session until the end of urine collection. Other prohibitions included the use of mouthwashes with alcohol, cosmetics with olive oil, performing strenuous physical exercise and taking any type of drug.

3.4

CHAPTER 4

Genetic regulation of tyrosol to hydroxytyrosol conversion



The work presented in this chapter was conducted in the Rachel Tyndale's laboratory, in the department of toxicology of the Faculty of Medicine in the University of Toronto from June 2019 to August 2019.

3.4.1. Abstract

Dietary Tyrosol (Tyr) is endogenously biotransformed into the antioxidant Hydroxytyrosol (HT) in vivo. This reaction is catalysed by the isoforms of cytochrome P450 including *CYP2A6* and *CYP2D6*. These enzymes are highly genetically polymorphic, leading to a high variability of the enzyme functionality within the population. In the present study, we have evaluated the involvement of these two CYP isoforms in this reaction using a large number of hepatic tissue samples from a human liver bank which were previously characterized for CYP2A6 and CYP2D6 activity. To do so, human liver microsomes were incubated with Tyr at two different concentrations 5 and 50 μM. Our results point out to CYP2A6 as a major contributor of the reaction and CYP2D6 as the secondary one. According to the obtained results, we developed a weighted polygenic activity score (wPAS) based on *CYP2A6* and *CYP2D6* individual's genotype. The resulting wPAS was applied to volunteers from two clinical studies administering Tyr-rich products: white wine enriched with Tyr and RW. The wPAS could predict 20,3% of the variance in HT/Tyr metabolite ratio. Our studied population was divided into three broad groups: low, normal and rapid wPAS. Individuals at normal group presented significant improvements on cardiovascular biomarkers following a Tyr-rich treatment. Our results suggest that the relevance of the biological activity of Tyr-rich food (wine, beer, olive oil…) is partially dependent on individual's genetic background.

Introduction

In the recent years, the term "Precision Nutrition" has arisen as a new popular concept based on the idea that individuals respond differently to dietary components as a result of the interplay between environmental, social, metabolic, and genetic factors. Included within this broad concept, the area of "nutrigenetics" is the discipline focused on the study of different phenotypic responses to specific diets depending on the genotype of an individual (Corella *et al.*, 2017; Ordovas *et al.*, 2018). The nutrigenetics approach has evolved from the study of the interaction of single nucleotide polymorphisms (SNPs) to whole-genome strategies interacting with the dietary patterns (Ordovas *et al.*, 2018). Although a large effort has been made to increase the evidence on this field, the heterogeneity on the study designs and populations analysed, as well as the lack of replication studies are major concerns. Moreover, the complexity of measuring accurately dietary intake is another challenge that has to be overcome (Corella *et al.*, 2018).

At the same time, the field of pharmacogenetics has emerged to study genetic variation and its effect on drug response (Daly, 2017). Several guidelines are currently available in order to adapt a specific drug's dosage using an individual's genotype. A lot of attention has been paid on the genetic variation of CYP-family enzymes and their effects on drug metabolism. CYP-family catalyses the oxidative metabolism of a wide range of endogenous substrates and xenobiotics, including drugs but also dietary compounds. Nevertheless, little attention has been paid to genetic variation and the metabolic disposition of dietary phenolic compounds and how this variability could impact their health effects in humans.

As shown before, Tyr to HT conversion is catalysed by CYP isoenzymes *CYP2A6* and *CYP2D6*. In the previous chapters we have shown an

association between metabolic disposition of Tyr and HT metabolites and *CYP2A6* and *CYP2D6* genotypic profiles. To do so, a polygenic activity score (PAS) was developed based on the premise that both enzymes contributed equally to the reaction, however, the exact contribution of each enzyme is unknown. Additionally, the activity score that we used was initially developed only for *CYP2D6* (Gaedigk *et al.*, 2018). Due to the lack of a scoring system for standardizing *CYP2A6* genotype, we extrapolated the same scoring system to *CYP2A6*. In the actuality, a weighted genetic score specific for *CYP2A6* has been recently developed based on novel genome-wide association studies (GWAS) and nicotine clearance (El-Boraie *et al.*, 2019).

The **aim of the present chapter** is to develop a weighted polygenic activity score (wPAS) to predict Tyr to HT formation. To do so, a number of *in vitro* drug metabolism experiments were performed using a human liver bank which has been previously characterized for its *CYP2A6* and *CYP2D6* activities.

3.4.2 Material and methods

3.4.1.1Study population

The present study is a secondary analysis of the two former clinical trials, including all participants and representing a total of 51 subjects. The Tyr and HT metabolic data used were in the case of the first study, the Tyr and HT recovery following WW supplemented with Tyr, in the case of the second study, the Tyr and HT recovery following RW (0-6h urinary collection). Comparisons were performed based on HT/Tyr ratios. To overcome potential interferences from the different treatments, log transformed HT/Tyr ratios were standardized using a Z-score separately for each study.

3.4.1.2 *CYP2A6* and *CYP2D6* scores

a) CYP2A6 activity score

CYP2A6 activity score was calculated based on the weighted genetic risk score (wGRS) previously described (El-Boraie *et al.*, 2019). To do so, the volunteers were genotyped for three additional *CYP2A6* SNPs: rs51223850, rs113288603 and rs2316304. The score was based on a total of seven variants: including the aforementioned novel SNPs, and the well-known variants *2, *4, *9 and *12. The exact weight of each risk allele is summarized in table 25.

Table 25. Variants and weights included in *CYP2A6* wGRS

Variant	Reference allele	Risk allele	Location of the variance in reference of CYP2A6 GENE	Weight for Risk allele
rs51223850	T	C	Intron 4	+ 0,135
rs2316304	C	T	5 kb 3'	+ 0,080
rs113288603	C	T	6 kb 5'	- 0,025
*2	A	T	Exon 3 (L160H)	- 0,250
*4	-	Deletion	Deletion of exon 1 – 9	- 0,350
*9	A	C	Promoter (TATA box)	- 0,160
*12	-	Hybrid	Translocation exons 1-2	- 0,272

b) CYP2D6 activity score

CYP2D6 activity score (AS) was based on the Clinical Pharmacogenetics Implementation Consortium (CPIC) latest consensus document published last March 2019. The specific activity score for each tested allele are detailed in table 26 (Gaedigk *et al.*, 2018; Clinical Pharmacogenetics Implementation Consortium (CPIC), 2019). In case of presenting duplications, the obtained score was later multiplied.

Table 26. Punctuation of CYP2D6 activity score according to CPIC

Functional Status	Activity Score (AS)	Tested Alleles
Functional / normal activity	1	*1, *2, *34, *35
Reduced function/ decrease activity	0,5	*9, *41
	0,25	*10
Non-functional / no activity	0	*3, *4, *5

The AS of the genotype is the sum of the values assigned to each allele.

3.4.1.3 Human Liver bank

Human liver tissues used proceeded from two different liver banks: Saint Jude Liver Resources and the University of Washington Human Liver Bank. Human liver microsomes (HLM) were obtained from liver samples as previously described (Shirasaka *et al.*, 2016). Thereafter, protein concentration of the microsomal extracts was measured using Bio-Rad protein assay kit based on Bradford dye-binding technique (Bio-Rad Laboratories, California, USA). *CYP2A6* enzyme activity was measured as the rate of hydroxylation of coumarin to form 7-hydroxycoumarin. *CYP2D6* activity was measured as the rate of hydroxylation of metoprolol to form α-hydroxymetoprolol. The human liver bank was previously characterized for *CYP2A6* to determine the predictors for its enzymatic activity (Tanner *et al.*, 2017).

3.4.1.4 Tyrosol to hydroxytyrosol incubation experiments

HLM were incubated with Tyr to assess their HT formation velocity. Methodology was adapted from a previously published paper (Rodríguez-Morató *et al.*, 2017). Incubation experiments were performed at two different Tyr concentrations: 5 and 50 μM. Linearity for time and protein concentration were tested beforehand at both concentrations 5 and 50 μM in pooled HLM. Incubations were carried in a shaking water bath at 37º for 20 minutes. The final mixture contained Tyr, HLM (1 mg protein / mL), and NADPH (1 mM) in 100 mM of sodium phosphate buffer (pH 7,4; stabilized with magnesium chloride hexahydrate (3 mM)) in a final volume of 50 μL. Mixtures were pre-incubated for 3 minutes, using NADPH to start the reaction. After the 20 minutes period, the reaction was stopped adding 50 μL of ice-cold methanol and 50 μL of ISTD (50 ng/mL of HT-

D_4). Samples were ultra-centrifuge to precipitate protein (15.000 rpm, 5 min, RT). The supernatant was filtered (0,22 μm) and transferred to LC-MS/MS vials. The resulting mixture was stabilized by adding ammonium acetate buffer (1mg/mL) in a proportion 1:4.

HT detection and quantification were performed in an Agilent 1260 LC system coupled to a Agilent 6430 triple quadrupole mass spectrometer with an electrospray interface. Chromatographic separation was performed using an Acquity UPLC® BEH C18 column with a 1.7 μm particle size, 3 mm x 100 mm (Waters, Milford, Massachusetts, USA). Mobile phases used were A: acetic acid 0,1% (v/v) in water and B: pure acetonitrile.

3.4.1.5 Clinical significance of wPAS

The developed wPAS was used to evaluate the magnitude of the clinical effects of a WW enriched with Tyr intervention as a proof-of-concept. As previously done, participants were divided into three broad categories in terms of their wPAS: low, normal and rapid wPAS groups. The effect of the treatment was evaluated across the different groups in the main biomarkers studied in the clinical trial.

3.4.1.6 Statistical analysis

Statistical analysis was completed using SPSS version 18 (IBM Corporation) and R-studio, version 3.0.2. R packages used were "multcomp", "nlme" and "ggplot2". Normality was tested, and data was log transformed when needed to achieve a normal distribution. Pearson correlation coefficient was calculated to evaluate the existence of linear associations between enzyme activities and HT formation. Significance was set as $p < 0,05$. Linear regression analyses were used to calculate weighted final model.

3.4.2 Results

3.4.2.1 *CYP2A6* and *CYP2D6* scores

a) CYP2A6 score

The original wGRS obtained ranked from -0,33 to 0,43. Our second step was to standardize the score to a 0 – 2 scale similar to the CPIC score for *CYP2D6*. Based on the previously described wGRS (El-Boraie *et al.*, 2019), we ranked as the lowest possible score -0,41, equivalent to 0 in the new scale. At the same time, the highest score, 0,43 was equivalent to a value of 2 in the new scale. Figure 36 describes the HT/Tyr ratio (Z-score standardized values) across 4 groups with increasing wGRS. No correlation was observed between *CYP2A6* wGRS and HT/Tyr ratio (r=0,126 spearman correlation test, p= 0,379).

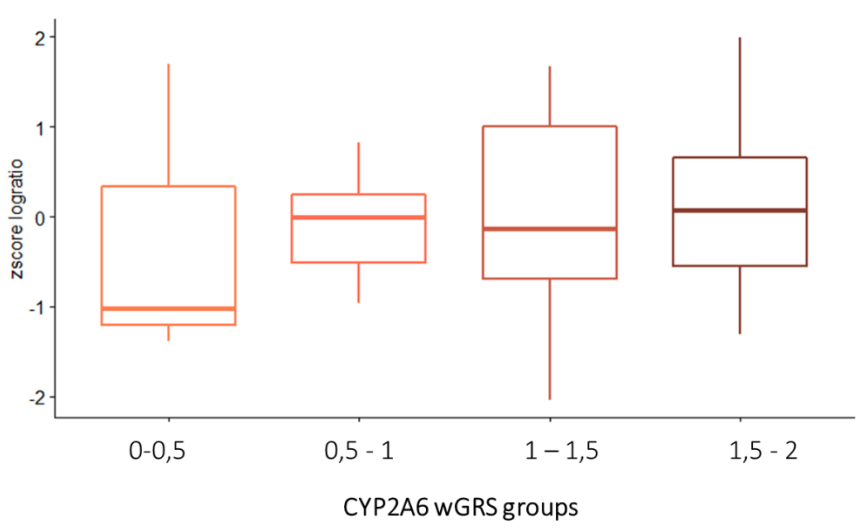


Figure 36. HT/Tyr ratios across different *CYP2A6* wGRS groups

b) CYP2D6 activity score

CYP2D6 activity score (AS) obtained ranked from 0 to 3 (due to duplications) in the studied population. Figure 37 describes the HT/Tyr ratio (Z-score standardized values) across 5 groups with increasing *CYP2D6* AS. A positive correlation was observed between *CYP2D6* AS and HT/Tyr ratio (r=0,379 spearman correlation test, *p*=0,004).

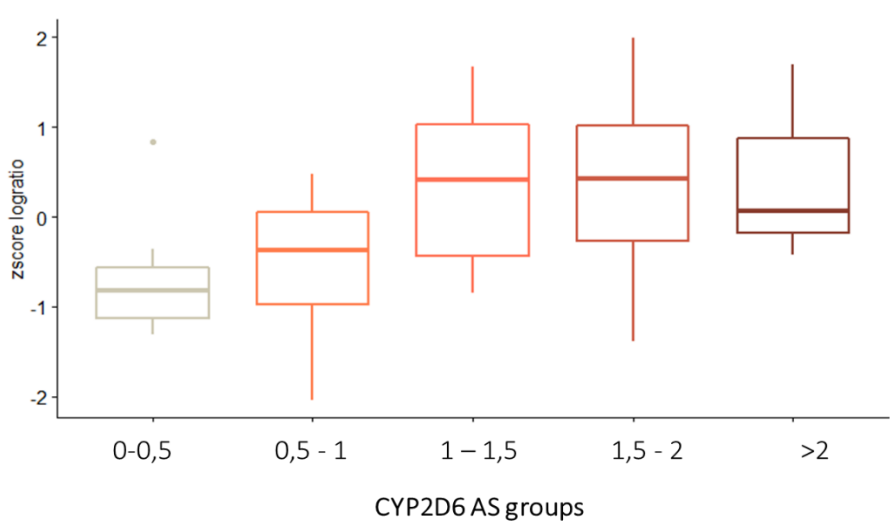


Figure 37. HT/Tyr ratios across different CYP2D6 AS groups

3.4.2.2 Tyrosol hydroxylation in HLM

HT formation followed a linear trend from 0 to 60 minutes, at 5 and 50 µM in pooled HLM (R^2 > 0,95). HT formation also exhibited linearity with total HLM protein concentration, from 0,5 to 1,5 mg of protein/mL (R^2 > 0,90).

3.4.2.3 Tyrosol hydroxylation in the human liver bank

HT formation was evaluated at a concentration of 50 μM in a total of 169 HLM samples and at a concentration of 5 μM in 147 HLM samples. HT formation velocity (pmol/mg total protein and minute) was calculated for all samples. In the 50 μM dataset, HT formation ranged from 0,47 to 175,44 pmol/mg total protein / minute. In the 5 μM dataset, HT formation ranged from 0,96 to 15,37 pmol/mg total protein / minute.

3.4.2.4 Correlation of hydroxytyrosol formation with *CYP2A6* and *CYP2D6* activity

The correlation between enzyme activity and HT formation was studied separately at the two concentrations of Tyr tested. Coumarin to 7-hydroxycoumarin formation was positively correlated with HT formation in the two datasets (Figure 38.A and 38.B), exhibiting a stronger correlation in 5 μM. On the contrary, metoprolol to α-hydroxymetoprolol formation was only correlated in 5 μM but not in in 50 μM (Figure 39.A and 39.B).

Figure 38. Correlation between hydroxytyrosol formation and CYP2A6 activity measured as coumarin to 7-hydroxycoumarin formation at 50 µM (A) and 5 µM (B) of tyrosol

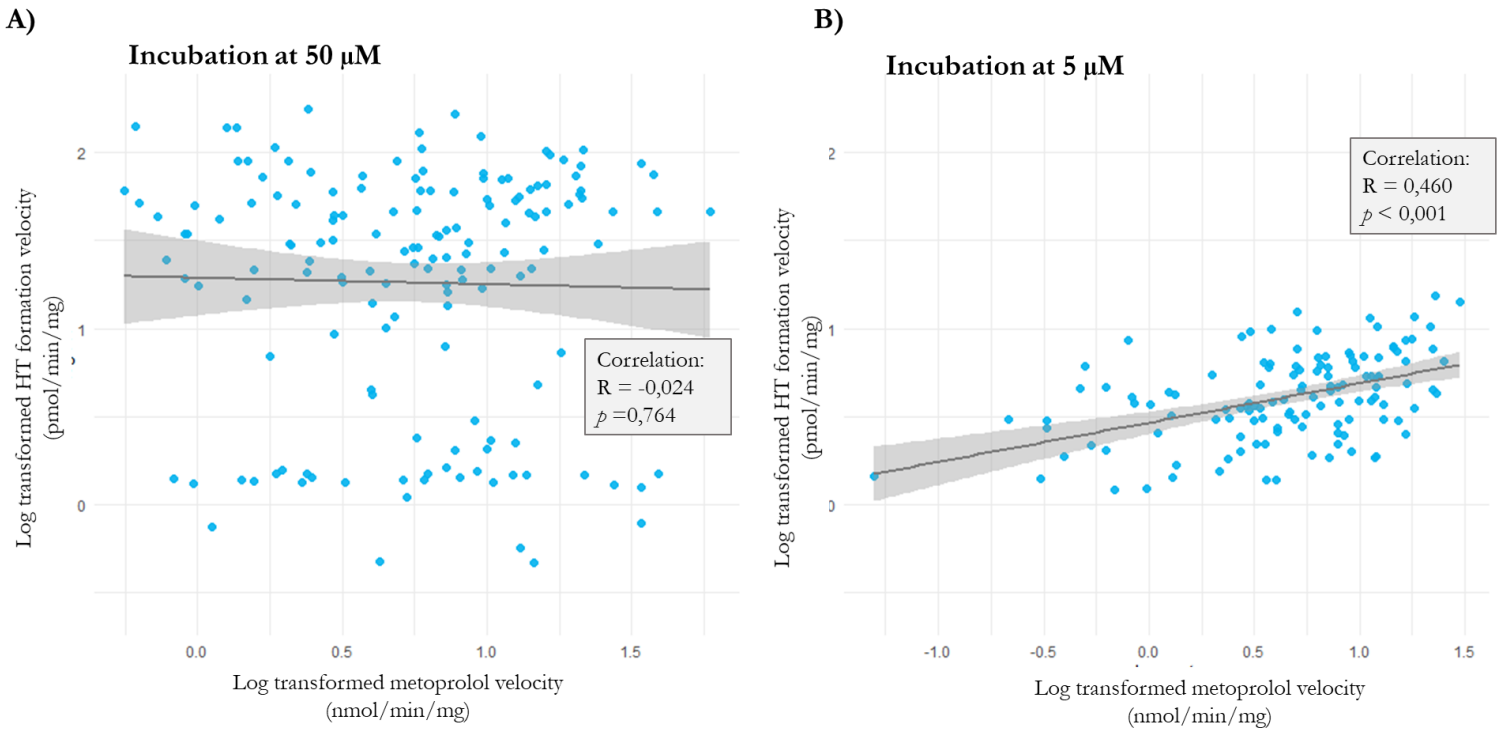


Figure 39. Correlation between hydroxytyrosol formation and CYP2A6 activity measured as coumarin to 7-hydroxycoumarin formation at 50 µM (A) and 5 µM (B) of tyrosol

3.4.4.5 Linear regression model for hydroxytyrosol formation

Using a linear regression analysis, we could assess the contribution of each enzyme to HT formation *in vitro* using the data from 5 μM experiments. The model could explain 32.1 % of the variation obtained. The standardized betas obtained were 0,362 for coumarin activity and 0,286 for metoprolol activity, highlighting the weighting of each enzyme in the model. Details of the resulted model are summarized in table 27.

Table 27. Linear regression analysis of HT formation

Predictor variable	B	Beta (standardized)	% variation accounted	*p* Value
Coumarin activity	0,144	0,362	11,7 %	<0,001
Metoprolol activity	0,012	0,286	7,7 %	0,001

Adjusted R^2 = 0,321; P < 0,001

3.4.4.6 Development of the wPAS and proof-of-concept

In order to calculate the weighting of the contribution of each enzyme to Tyr to HT formation, we used the adjusted betas obtained in the model, multiplying the *CYP2A6* wGRS per 0,362 and *CYP2D6* AS per 0,286. The resulting wPAS ranged from 0,36 to 1,58 in our sample.

Figure 40 represents the linear regression of wPAS with HT/Tyr ratios (Z-score standardized values). It predicted 20,3 % of the variability obtained (p<0,001). *CYP2D6* ultra-rapid metabolizers were excluded from the analysis since they presented unexplainable low recovery values for Tyr and

HT as mentioned before. Separating the two clinical trials, the wPAS explained 11.0 % of the variability observed in HT/Tyr ratio following WW supplemented with Tyr treatment (p=0,039). The second clinical trial, wPAS explained 37,3% of the variability following RW consumption (p=0,004).

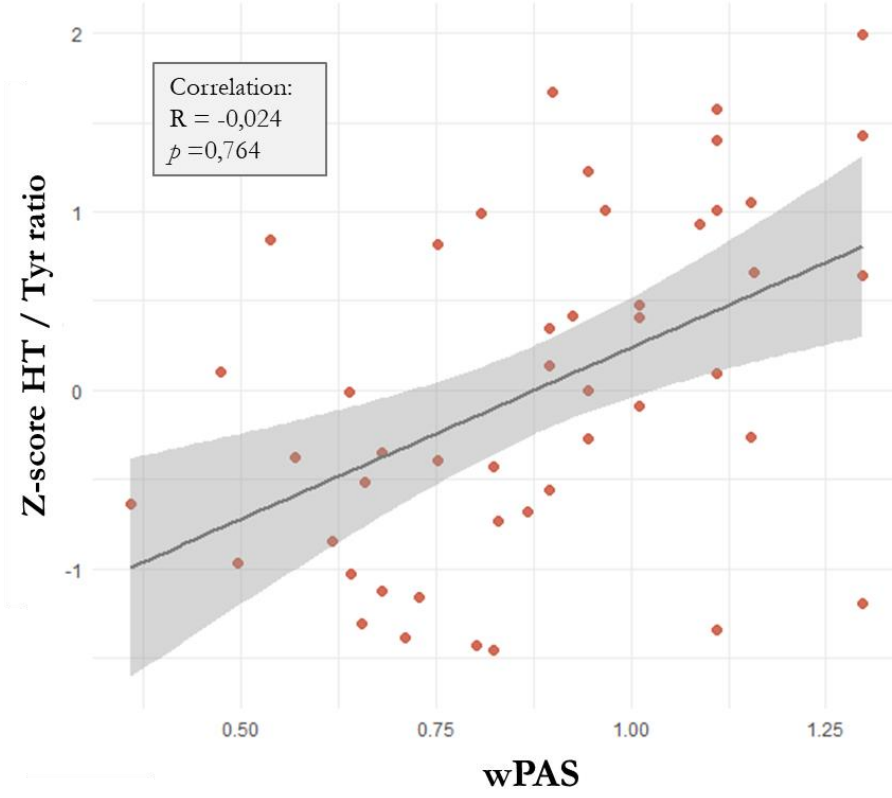


Figure 40: Linear regression of wPAS with the HT/Tyr ratio.

3.4.4.7 Clinical significance of wPAS

Population from the clinical trial administering WW supplemented Tyr was divided into three broad categories according to their wPAS: low wPAS (L-wPAS, n= 11), normal wPAS (N-wPAS, n=19), rapid-wPAS (R-wPAS, n=2). Characteristics of each group are summarized in table 28.

Table 28. Distribution of volunteers in three groups based on their wPAS

	Low wPAS	Normal wPAS	Rapid wPAS
[n(%)]	11 (34,4%)	19 (59,4%)	2 (6,3%)
Age (y) [1]	65,5 ± 5,6	64,6 ± 6,4	72.0 ± 5,5
Sex [n(%)]			
Women	3 (27,3%)	8 (42,1%)	1 (50,0%)
Men	8 (72,7%)	11 (57,9%)	1 (50,0%)
Range of wPAS	0,36 – 0,81	0,83 – 1,3	1,58

[1] Mean ± SD

Table 29 summarizes the differences in the main clinical features and biomarkers measured in the intervention stratified by wPAS groups. Endothelial function did not exhibit any significant difference in any of the groups. Conversely, a decrease in augmentation index was observed in N-wPAS after WW+Tyr compared to its baseline (p=0,025). Only N-wPAS group exhibited significant changes in HDL-c, homocysteine and antithrombin III (ATIII) following WW+Tyr compared to their baseline and compared to WW intervention. In the case of HDL-c, the change was also significant compared to control intervention. Finally, a trend towards significance was observed when comparing final ET-1 levels following WW with WW+Tyr (p=0,080) only in N-wPAS group. Rapid-wPAS had been excluded from the statistical analysis.

Table 29. Changes in clinical and endothelial biomarkers according to wPAS groups.

	Group	Control	WW	WW+Tyr	*p* value WW+Tyr vs Control	*p* value WW+Tyr vs WW
Endothelial function (RHI)	L-wPAS	0,1 ± 0,6	0,1 ± 0,2	0,2 ± 0,5	0,696	0,799
	N-wPAS	0,0 ± 0,3	0,0 ± 0,6	0,2 ± 0,5	0,312	0,533
Augmentation index	L-wPAS	2,1 ± 9,2	-0,3 ± 6,8	-0,6 ± 14,7	0,939	0,973
	N-wPAS	2,2 ± 10,8	-1,7 ± 10,0	**-7,1 ± 10,6 ***	0,181	0,915
HDL-C	L-wPAS	0,2 ± 10,0	4,1 ± 7,4	2,1 ± 4,4	0,856	0,746
	N-wPAS	-0,1 ± 2,4	0,7 ± 2,3	**3,7 ± 4,0 ****	**< 0,001**	**0,004**
Homocysteine	L-wPAS	0,2 ± 0,6	0,5 ± 0,8	0,1 ± 0,6	0,658	0,894
	N-wPAS	-0,2 ± 1,4	0,5 ± 1,3	**-0,7 ± 1,1 ***	0,840	**0,048**
ATIII	L-wPAS	0,3 ± 0,8	0,0 ± 1,9	0,6 ± 1,3	0,731	0,585
	N-wPAS	-0,2 ± 1,1	-0,2 ± 0,8	**0,6 ± 1,1 ***	0,075	**0,038**
ET1	L-wPAS	2,0 ± 0,5	2,3 ± 0,7	1,9 ± 0,5	0,938	0,181
	N-wPAS	2,2 ± 1,0	2,5 ± 1,4	2,1 ± 1,1	0,852	0,080

Results are expressed as mean ± SD as change vs baseline of the intervention (n=30). Endothelial function (RHI) and Augmentation index are represented in arbitrary units; HDL-c and ATIII are represented as mg/dL; homocysteine is represented as μM and ET1 as ng/dL. RHI: reactive hyperaemia index; HDL-c: high density lipoprotein cholesterol; ATIII: antithrombin III; ET1: endothelin-1; WW, white wine; WW+Tyr, white wine plus Tyr capsules; L-wPAS, low weighted polygenic activity score group; N-wPAS: normal weighted polygenic activity score group. ANOVA adjusted by age, sex, smoking habits, acetylsalicylic acid consumption, and baseline levels. *$p < 0.05$, ** $p <$ 0,001 versus its baseline; p value, significance for inter-intervention comparisons.

3.4.5 Discussion

The present work confirms the involvement of *CYP2A6* and *CYP2D6* enzymes in the bioconversion of Tyr into HT using a large number of samples from a human liver bank. The experiments using HLM suggested that the contribution of each enzyme would be dependent on the Tyr concentration. Based on the obtained data, a weighted polygenic activity score (wPAS) was developed. It was able to explain a 20,3% of the variability on the HT/Tyr ratio following a Tyr-rich treatment in humans.

The main objective of this chapter was to assess the contribution of *CYP2A6* and *CYP2D6* on the Tyr to HT reaction. Based on genetics, the inclusion of both enzymes gave a greater prediction of HT/Tyr ratio variation than the enzymes separately. In the case of *CYP2A6*, the calculated wGRS only predicted the 3,7% of the variation without reaching significance. On the other hand, *CYP2D6* AS only predicted 13,9% of the variation of HT/Tyr ratio. Likewise, our wPAS model was superior to the previous model used, strengthening the hypothesis that both enzymes had different contributions to the reaction. Based on the betas obtained in the linear regression performed with the liver bank, *CYP2A6* was the main contributor (55,9% of the final wPAS) while the *CYP2D6* contribution was lower (44,1% of the final wPAS). Following WW supplemented with Tyr, the former model could predict 10% of the variation while the wPAS could predict 11%. On the case of volunteers taking RW, the unweighted PAS predicted 18,5 % whereas the weighted model reached 37,3% of the variability explained. According to our pharmacokinetic study, we know that the rise in Tyr and HT metabolites attributed to an oral intake can be mostly observed in urine for 6 hours after administration. Therefore, urinary samples from 0-24 hours obtained after WW supplemented with Tyr could be contaminated by other sources of Tyr. Furthermore, volunteers from the second study followed a strict diet on the day of the study, while volunteers

from the first study followed a low-phenolic diet, but allowed a small consumption of olive oil. Taking these facts together, it could explain the discrepancy between the variability predicted by wPAS in both studies. It is likely that our data obtained after RW consumption is less biased by other non-genetics factors than the data obtained after WW supplemented with Tyr.

The experiments performed using the liver bank showed that *CYP2A6* and *CYP2D6* together explained 32,1% of the variation in HT formation velocity. Therefore, there was a remaining 67,9% of the variation unexplained. Based on previous published data using HLM and selective inhibitors, *CYP2A6* was the major contributor to the conversion, followed by *CYP2D6*. Nevertheless, three other CYP-isoenzymes specific inhibitors produced a mild decrement of the reactions: *CYP3A4*, *CYP2B6* and *CYP2C9* (Rodríguez-Morató *et al.*, 2017). The role of *CYP3A4* in Tyr to HT reaction was further confirmed in experiments using baculosomes (Rodríguez-Morató *et al.*, 2017). The three mentioned isoenzymes are also polymorphic within the global population. Besides, some of the polymorphisms described for these enzymes have an impact on the final functionality of the enzyme. Therefore, although *CYP2A6* and *CYP2D6* are the main enzymes involved, different genotypic profiles in the previous mentioned enzymes could also have an impact on the Tyr to HT bioconversion rate. The assessment of the overall role of *CYP3A4*, *CYP2B6* and *CYP2C9* on the conversion should be studied in future research and if confirmed, the inclusion of their genotype in the wPAS could increase the variability explained by our score.

Genetics is the main contributor to explain the variability observed in *CYP2A6* and *CYP2D6* enzyme functionality within general population (Tanner and Tyndale, 2017; Gaedigk *et al.*, 2018). However, there are further

factors affecting final activities. Previous work has shown the limits of genetics to predict *CYP2A6* and *CYP2D6* final enzyme functionality. In the case of the previous published data using the same human liver bank, *CYP2A6* genetics (*2, *9 and *12) together with age, sex and AKR1d1 mRNA levels (a regulator of P450 family) were able to predict 35,0 % of the variation of the activity (Tanner *et al.*, 2017). In the case of wGRS for *CYP2A6*, it was capable of predicting 33,8% of the variation on nicotine metabolism (El-Boraie *et al.*, 2019). Lastly, in the liver bank used, *CYP2D6* activity score could predict the 48,8 % of the variability for *CYP2D6* activity measured as metoprolol hydroxylation velocity (non-published data). Therefore, based on these previous studies and our results, it is clear that part of the functionality of both enzymes is regulated independently of genetics. A broad group of substances had been described to interact with their activity either by inducing or inhibiting the activity, including certain drugs, endogenous substances like hormones and dietary compounds.

A fact worth mentioning is the distinct enzymatic contribution to the reaction at different Tyr concentrations. At lower concentrations of Tyr (5 μM), both enzymes played a role in the Tyr to HT conversion. Nevertheless, at higher concentrations (50 μM), the main contributor was *CYP2A6*, leaving the contribution of *CYP2D6* irrelevant and non-significant. This observation raised two major concerns: in one hand, the inapplicability of the developed wPAS at higher Tyr doses. This may be relevant following the intake of Tyr based nutraceutics preparations. On the other hand, since the contribution of each enzyme was not maintained across the different concentrations used, we cannot guarantee that at lower concentrations of Tyr, the contribution of the enzymes is equivalent to the predicted at 5 μM. It is worth to mention that the concentrations that Tyr reached inside the liver remain unknown. Nevertheless, a study assessing HT plasma levels following EVOO intake described a Cmax of 29 nM. This suggests that real

concentrations of Tyr reached within the liver at dietary doses are more likely to be around the nM range rather than μM range. Although 5 μM is closer to the physiological conditions than 50 μM, it may still not represent real life concentrations. Further studies should study Tyr to HT conversion at lower doses.

The development of the wPAS is intended to predict the efficiency of Tyr to HT bioconversion, based on the fact that HT is likely to produce higher biological effects than Tyr. The *in vivo* predictive capacity of wPAS was proved in the clinical trial administering WW+Tyr. Individuals from the trial were divided into three categories according to their wPAS. Having a normal wPAS was significantly associated to a greater improvement in the augmentation index, HDL-cholesterol, homocysteine and antithrombin III compared to participants having lower wPAS. This observation indicates that a higher efficiency on Tyr to HT conversion is associated with the greater biological activity observed, therefore, pointing out to HT as the phenol involved in the biological effects observed. Although the current study is based on a small sample of participants, the findings suggest that our model could identify the participants that would benefit the most from our intervention. Therefore, wPAS could predict the magnitude of the health effects triggered by Tyr-rich food consumption based on individual's genetic background. Nevertheless, our findings should be replicated on a larger sample size. To the best of our knowledge, this is the first approach of nutrigenetics to predict the metabolic fate of a dietary phenolic compound and to predict the biological effects derived from its intake.

Our study presents some strengths and limitations. The key strength of the study is the combination of *in vitro* and *in vivo* human studies. The large number of samples in the liver bank provides sufficient statistical power to extract clear conclusions of the involvement of *CYP2D6* and *CYP2A6* in

HT formation. The combination of several SNPs into a final score gives a broader picture of the effect of genetics into the studied reaction than assessing single SNPs or single genes. However, when assessing individual's genotype, we tested the most prevalent allelic variants described in the Spanish population. There is always the possibility that individuals may carry other non-tested allelic variants that could have an impact on enzyme functionality. Finally, the use of metabolic data of free-living individuals, may be prone to bias when assessing the intake of dietary compounds, especially with a small sample size.

3.4.6 Conclusions

In conclusion, our study confirms the involvement of *CYP2A6* and *CYP2D6* on Tyr to HT conversion. Based on our results, *CYP2A6* would be the major contributor, followed by *CYP2D6*. We have calculated a weighted PAS to predict the efficiency of Tyr to HT conversion based on genetics. The resulted weighted PAS could also predict the size of the health effects associated to Tyr-rich food consumption at an individual level.

4. DISCUSSION

4.1 GENERAL DISCUSSION

In times of evidence-based medicine, the MedDiet is one of the dietary patterns of reference in nutritional science for its attributes in disease prevention and longevity (Sofi et al., 2014; Di Daniele et al., 2017; Estruch et al., 2018). As a food pattern, MedDiet boosts synergies between foods and potentiates the additive effect of small quantities of different bioactive substances, which combined, show a positive impact in human health. Among bioactive substances, dietary phenolic compounds can modulate signalling and regulatory pathways (e.g. inflammation, oxidation), rendering a protective effect in the development of non-communicable diseases. Clinical trials have demonstrated that a diet rich in phenolic compounds protected from cardiometabolic diseases (Tresserra-Rimbau et al., 2013, 2014; Amiot, Riva and Vinet, 2016). MedDiet is characterized by a high intake of phenolic compounds, being EVOO and RW characteristic contributors on total phenolic intake. HT is the key antioxidant present in EVOO and, together with its content in monounsaturated fatty acids, is considered as the relevant contributor of its health positive effects. The PREDIMED study has shown that a MedDiet enriched in EVOO protected from developing cardiovascular events (Estruch et al., 2018). In the case of RW, its moderate consumption has been associated with a cardioprotective effect (De Gaetano et al., 2002; Chiva-Blanch et al., 2013). RW is rich in multiple phenolic compounds, including resveratrol. However, the compound(s) responsible of its beneficial health effects are still being investigated. It has been suggested that it may be the result of a synergistic effect between moderate doses of alcohol together with a high intake of diverse phenolic compounds.

The initial objective of this PhD project was to demonstrate the endogenous conversion of the dietary phenol Tyr into the antioxidant HT in humans following previous clinical indirect evidences and preclinical studies. The

present work intended to explain the unexpected high recovery of HT following wine consumption observed in previous studies (de la Torre et al., 2006; Pérez-Mañá, Farré, Rodríguez-Morató, et al., 2015). Hereby we have shown that the systemic levels of HT and its metabolites may be the result of a complex combination of different sources. Based on previous studies and on the findings described in the present work, HT levels after wine consumption would come from: i) the HT present in the wine, ii) HT formed from dopamine oxidative metabolism as a result of the ethanol induced reductive environment, and iii) the HT formed from the endogenous hydroxylation of Tyr. The later mechanism was tested in the study based in the administration of WW supplemented with Tyr. In one hand, WW intervention provided an alcoholic matrix low in phenolic compounds. As shown in chapter 2, the content of HT in WW is low. Thus, its administration enabled the assessment of the contribution of points i) and ii) to total HT recovery. The effect of alcohol on dopamine oxidative metabolism to produce HT has been previously described to occur in a dose-dependent manner (Pérez-Mañá, Farré, Pujadas, et al., 2015). However, it does not explain the high amounts of HT recovered following RW intake. On the other hand, the supplementation of WW with Tyr enabled the identification of a new mechanism for HT formation based on the endogenous hydroxylation of Tyr. This third source of HT confirmed previous findings described in in vitro and in pre-clinical studies (Pérez-Mañá, Farré, Rodríguez-Morató, et al., 2015; Rodríguez-Morató et al., 2017). This represented the first-time demonstration that the reaction happens in vivo in humans. The endogenous Tyr to HT bioconversion was further confirmed in the study administering different beers and RW. Beer HT content was almost undetectable, however, a rise in HT metabolites was observable after beer administration and followed a dose-response relationship with the Tyr administered. Therefore, the current work describes Tyr-rich foods (i.e. wine, beer and olive oil) as an indirect source

of HT. Tyr-rich foods could potentially foster equivalent biological effects as the ones attributed to a HT-rich food like EVOO. The results obtained in the present work lead us to propose that the endogenous HT formation could explain, at least in part, the health effects attributed to moderate wine and beer consumption.

Another important finding of the present work is the characterization of beer as a source of Tyr. Beer Tyr content widely varies depending on the type and style of beer. Based on our results, certain types of beer have Tyr concentrations equivalent to RW. During the beer brewing process, fermentation has shown to be a critical step for Tyr and HT formation. The yeast strain used seems to be a determinant factor, since ale beers presented greater concentrations of HT and Tyr than lager beers. Furthermore, it is likely that the amino acid composition of the cereals used has also a significant impact on the final phenolic composition. Finally, yet importantly, the alcoholic content of beer is lower than that of wine, enabling higher daily doses which still fall within moderate alcohol consumption.

The present work enabled the determination of factors affecting Tyr bioavailability. Tyr was well absorbed following the intake of enriched WW, RW and beer. The results of the clinical trial administering beers indicated that its alcoholic content was critical for Tyr absorption. According to our results, a poor Tyr bioavailability was exhibited after non-alcoholic beer consumption, confirming the role of alcohol on enhancing phenolic bioavailability (Pérez-Mañá, Farré, Rodríguez-Morató, et al., 2015). Nevertheless, the effect of alcohol to Tyr bioavailability did not seem to be dose-dependent, as higher dose-relative Tyr recovery was observed following blonde beer, with lower alcohol content, than the recovery following IPA beer. When comparing Tyr bioavailability between beer and

RW, that of wine was apparently greater than that of beer, although similar doses of Tyr were given. Potential matrix effects may be hampering Tyr absorption in beer, like carbon dioxide content generated in the brewing process or potential interaction with other phenols present in beer but not in wine. Further studies assessing these potential interactions in Tyr absorption in the frame of beer consumption should be performed. A good understanding of Tyr bioavailability is of interest when developing Tyr-based nutraceutical preparations.

The metabolic disposition of Tyr and HT observed following the intake of WW enriched with Tyr, was similar to the ones observed after RW and beer. Low levels of free phenolic compounds were observed in both cases. This was expected owing that Tyr and HT undergo through an extensive phase II metabolism. The most prevalent urinary metabolites were Tyr and HT conjugated with sulphate and glucuronide groups. After all the mentioned treatments, there was a significant increase in specific Tyr and HT metabolites. The most abundant Tyr related metabolite was Tyr-4-glucuronide, followed by smaller amounts of Tyr-4-sulphate. In the case of HT, the increase was only observed in HT-3-sulphate. The in vivo effect of phenolic metabolites is currently being studied in front of their parent compounds (Serreli and Deiana, 2018). A large number of studies had been performed assessing the effect of free Tyr and free HT. However, the in vivo concentrations of free compounds reached systemically are lower than the ones tested in the in vitro studies. Additionally, and as mentioned before, the bioavailability of the free forms is low, since they are rapidly metabolized into phase II conjugates. Therefore, authors had claimed the unlikelihood of free phenolic compounds to be the only contributors to the in vivo observed effects (Vissers, Zock and Katan, 2004) and had proposed the potential involvement of Tyr and HT metabolites, since greater concentrations are reached in vivo. Tyr and HT metabolites had shown

biological activity in in vitro studies, having the capacity to modulate important intracellular signalling pathways like NF-□□ and improve cellular defence in front of oxidative and pro-inflammatory stimuli (Serreli and Deiana, 2018). There are two different hypotheses behind the potential activity of Tyr and HT metabolites in vivo: i) they can be active by themselves, or ii) they can act as a reservoir of free forms which are released intracellularly after a de-conjugation process. Among the conjugated forms, sulphate derivatives of Tyr and HT have shown a greater activity than glucuronide derivatives (Muriana et al., 2017). Based on this observation, the functionality of sulfotransferases could be a determinant factor of the benefits observed after Tyr and HT supplementation. Nevertheless, limited evidence exists about the identity of the phase II enzymes involved in the Tyr and HT metabolism.

CVD are the leading cause of morbidity and mortality in the western world. Diet has a central role on the risk of CVD events and CVD-associated risk factors. High adherence to the MedDiet is associated with a primary CVD prevention as shown in the PREDIMED study (Estruch et al., 2018) and contributes to CVD secondary prevention as reported in the Lyon Diet Heart Study (de Lorgeril et al., 1999). Endothelial dysfunction is the earliest detectable stage of CVD, highly predicting a cardiovascular event more than any other risk factor. Nonetheless, it is treatable and, in contrast to the atherosclerotic plaque formation, reversible (El Assar et al., 2012; Daiber et al., 2017; Godo and Shimokawa, 2017). Our treatment with WW supplemented with Tyr improved endothelial function by means of a rise in reactive hyperaemia index and a decrease in arterial stiffness index. As earlier explained, endothelial dysfunction is characterized by an imbalance between vasodilation and vasoconstrictor factors, with an increased expression of pro-inflammatory mediators. WW supplemented with Tyr was able to downregulate homocysteine and iNOS, and hence to downregulate

their associated inflammatory cascade as well. On the same line, Tyr treatment fostered lower levels of ET-1, a potent vasoconstrictor molecule, and the downregulation of the expression of intermediary pro-inflammatory mediators (CD40L, VEGFA and CFH). Finally, ATII, a vasodilation and anti-inflammatory molecule, increased in response to our treatment. The potential common mechanism behind these effects could be the inhibition of NF-□□ nuclear factor, considered the master regulator of the inflammatory response. Along these lines, we observed a downregulation of the expression of p65/RELA, a sub-unit of the NF-□□ complex. Overall, there was a shift in the levels of pro-inflammatory and levels of anti-inflammatory factors towards a cardioprotective phenotype. These findings enhance our understanding of the in vivo biological activity of Tyr and HT and clearly show that their activity goes beyond ROS scavenging. Taken as a whole, our study encourages the recommendation of a HT and Tyr-rich diets to provide cardioprotection to individuals at a high cardiovascular risk.

On the other hand, we observed an increase in HDL-cholesterol (HDL-c) dependent on the total amount of Tyr administered. In agreement with our results, moderate alcohol consumption is associated with a raise in HDL-c levels. Nevertheless, the raise observed following the intake of WW enriched with Tyr was higher than that observed after the intake WW, suggesting a combined contribution of Tyr and HT to the effect of alcohol. The role of these phenolic compounds on HDL-c has been previously described in the EUROLIVE study (Covas et al., 2006) where HDL-c increased in a dose-dependently manner with the amount of phenolic compounds of EVOO. HDL-c is also involved in CVD, mainly due to the cholesterol efflux properties, reducing fat deposition to the atherosclerotic plaque. Additionally, anti-inflammatory and antioxidant properties had also been attributed to HDL particles, linking lipid metabolism with vascular and endothelial health (Campbell and Genest, 2013).

It is worth to mention the distinct effect of WW alone and that of WW enriched with Tyr. In some of the measured biomarkers, WW triggered a detrimental effect, which was later compensated by the enrichment of WW with Tyr. This observation is in line with the meta-analysis showing a protective effect of moderate consumption of fermented beverages, which are rich in phenolic compounds, that was not observed after the intake of equivalent moderated doses of spirits (Costanzo et al., 2011). This suggests that alcohol itself is less capable of triggering beneficial health effects. Nonetheless, when alcohol is combined with a phenolic-rich matrix, a protective effect is observed. Based on our metabolic results, moderate doses of alcohol are required to ensure the absorption of the phenolic compounds, and hence their likelihood to trigger positive health effects. Taken together, our findings support the idea of developing and implementing low-alcohol wines and phenolic-enriched beers (maintaining acceptable stability and organoleptic properties of beverages), in which alcohol content would be reduced to limit the negative effects associated with its consumption, but still be present to enhance phenolic compounds absorption. In this way we would be maximizing the beneficial effects of their phenolic compounds while minimizing the potential harmful effects associated to alcohol.

Until lately, medical studies had been mostly performed in men and results had been simply extended to women. The same is applicable to nutrition research, where potential sex-differences in the metabolism and effects of bioactive dietary compounds are usually ignored. With the raise of precision nutrition, a sex-perspective has been also adopted to tailor nutritional advice. Nevertheless, it is surprising that most of the studies aimed to develop evidence to support personalized nutrition have focused on factors such as individuals genotype and little attention has been paid to other

critical determinants like sex (Corella et al., 2018). In terms of alcohol consumption, it is widely acknowledged that women exhibit a distinct pharmacokinetic profile than men. Following ethanol consumption, women tend to display higher ethanol bioavailability, higher blood alcohol levels and to produce larger amounts of ethanol-oxidative metabolism by-products such us acetaldehyde (Baraona et al., 2001). Overall, alcohol toxic effects are higher in women than in men in front of equivalent alcohol doses. These differences in alcohol metabolism are observable in our sample, by being the EtG levels higher in women than in men in front of the same dose of wine or beer. Based on these metabolic differences, the dose of alcohol recommended in moderate drinking is lower in women than in men. The American Heart Association (AHA) recommends a daily maximum ingestion of a 1 standard drink for women and 2 standard drinks for men (Lichtenstein et al., 2006). In the case of the studied fermented beverages, their contribution to total Tyr and HT intake to the diet is limited by their alcohol content. Women maximum ingestion would be the half of men, and hence, their total Tyr and HT ingestion. For this reason, Tyr-rich products and lower alcohol contents should be chosen especially in the case of women. In the case of the metabolic disposition of the studied phenols, our results suggest that women present a less efficient conversion of Tyr to HT than men in front of a single dose. Nevertheless, this sex dimorphism could not be determined in the chronic study (4 weeks WW supplemented with Tyr) since administered doses of Tyr and wine were different. At the end of the treatment, lesser changes in the measured biomarkers were observed in women compared to men. This distinct effect was clearly observed in the case of HDL-c, in which a very significant effect was observed in men but not even a trend was observed in the case of women (although women presented higher baseline HDL-c levels). Nevertheless, we cannot conclude whether this distinct effect in women is attributed to sex-differences, to the different dosage or to a smaller sample size in the case of women. However,

dosing was based on AHA recommendations, and higher doses for women would be likely harmful. Following all these observations, the question about whether women can extract the same benefits of moderate RW/beer consumption arises. The intake of bioactive compounds from fermented beverages will always be lower in women, therefore raising the concern about reaching sufficient concentration to trigger the described biological effects while counteracting detrimental alcohol-associated effects.

An important question that this PhD thesis sought to determine was the in vivo modulation of Tyr to HT bioconversion by cytochrome P450 (CYP) isoenzymes CYP2A6 and CYP2D6. Our results confirmed their involvement following Tyr-rich food administration as previously described in human liver microsomes (Rodríguez-Morató et al., 2017). We replicated the experiments in human liver microsomes in a large liver bank with two different Tyr concentrations. This gave us the opportunity to observe the contribution of each enzyme to Tyr to HT conversion, highlighting that this contribution was dependent on the Tyr concentration. CYP2A6 was the major contributor, followed by CYP2D6. However, our human liver microsome experiments suggest that other CYP450 isoenzymes could be contributing to HT formation.

The potential relevance of the involvement of CYP2A6 and CYP2D6 into Tyr to HT bioconversion is due to the fact that these two enzymes are highly polymorphic within the general population. These genetic variation is a main determinant for the activity of CYP2A6 and CYP2D6 within the population. Therefore, a wide variability in Tyr to HT conversion capacity could also be expected. Based on our results, we developed a weighted polygenic activity score (wPAS) derived from individual genotypes for CYP2A6 and CYP2D6 in order to predict the metabolic and biological effects of a Tyr-rich intervention. The wPAS was capable of predicting up

to 37% of the variability observed in Tyr and HT metabolic disposition following RW consumption. Thus, significant differences were observed in vivo in the levels of Tyr and HT among people with different wPAS. Taken together, our results suggest that alterations in these enzymes associated to particular gene polymorphisms could have an impact on the efficiency of Tyr to HT reaction.

The clinical significance of wPAS was demonstrated by stratifying volunteers from the clinical trial administering WW supplemented with Tyr in two broad groups: Low wPAS and Normal wPAS. Our results indicated that individuals with a normal wPAS presented greater benefit from a Tyr-rich intervention since significant changes towards a cardioprotective phenotype were observed in normal wPAS group but not in the low wPAS group. Despite its exploratory nature, this observation opens the possibility to speculate that the biological effects of Tyr-rich food could be dependent at least in part on the genetic background of each individual. Additionally, future research should examine the role of phase II enzymes genetics, based on the observation that sulphate metabolites are likely more active than glucuronide metabolites. A natural progression of this work would add phase II genetics to a final score, which would predict phase I and phase II metabolism of a Tyr-rich intervention and, more accurately, the biological effects expected from it.

4.2 Future directions

The present thesis has shown in humans that a simple phenol deemed devoid of relevant biological activity is endogenously biotransformed into hydroxytyrosol one of the most potent dietary bioactive compounds. The regulatory aspects of this bioconversion, including nutrigenomic ones, and their impact on human health have been investigated. Likewise, based on the new findings, several new questions had arisen and could be the starting point for future research:

1. Development of a phenol-rich beer by modifying key steps in the brewing process. Based on our findings we could develop a tyrosol rich beer by choosing the yeast strains that potentiate the tyrosine conversion into tyrosol during the fermentation. The addition of other antioxidant substances could stabilize the fermentation process. Finally, the choice of tyrosine rich cereals would increase the substrate to generate larger amounts of tyrosol and hydroxytyrosol. The resulting beer should contain moderates amounts of alcohol to ensure phenolic absorption. Finally, the bioavailability of Tyr should be studied as well as the organoleptic characteristics and the acceptability of the beer.

2. Study the potential sex differences related to metabolic disposition of tyrosol and hydroxytyrosol *in vivo* highlighted in the present work. To do so, a clinical trial with a larger population and a balanced gender representation should be performed including a pharmacokinetic study to understand the differences in the metabolism, considering the effect of genetics and hormonal status. A study administering same doses for both sexes chronically could be capable of assessing potential gender differences in the biological effects.

3. Evaluation of the role of other cytochrome P450 enzymes in Tyr to HT conversion. The percentage of variation unexplained in the model suggest the involvement of other enzymes in the oxidation of Tyr. Samples from the liver bank could be assessed for the activity or genotype of the potential candidates. In the case they contribute significantly to the reaction, their inclusion in the wPAS should be considered.

4. The interaction of beer hops prenylflavonoids with Tyr to HT reaction. Literature suggests that prenylflavonoids inhibit the activity of *CYP2D6*. The concomitant administration of tyrosol and prenylflavonoids in the context of beer intake could inhibit HT formation, and hence limit the potential health benefits obtained from beer consumption based on this reaction.

5. The application of the wPAS to a larger study population. In the present thesis we have developed a useful tool to assess the magnitude of the expected benefits associated to a Tyr-rich treatment. The replication of the present findings in an independent study would confirm the *in vivo* relevance of wPAS and contribute to the evidence based personalized nutrition.

6. Pharmacogenetics of phase II enzymes. The role of phase II metabolites in the biological activity of Tyr and HT is becoming acknowledged. Among phase II metabolites, sulphates exhibit greater *in vitro* activity than glucuronides. Enzymes involved in the conjugation are also polymorphic, implying a high variability in the capacity to form sulphates and glucuronides. Therefore, variation on the activity of these enzymes could modulate the metabolic

disposition of Tyr and HT and hence, the global biological effects observed, especially if the biological activity of conjugate metabolites is confirmed. If its relevance is confirmed, the addition of the genotype of phase II enzymes in the wPAS should be considered.

5. CONCLUSIONS

CONCLUSIONS

Based on the results presented, the main conclusions that can be drawn from the current work are the following:

1. Tyrosol present in the diet is the biosynthetic precursor of the antioxidant hydroxytyrosol in humans.

2. Beer is an alternative dietary source of tyrosol, which is well absorbed and biotransformed into hydroxytyrosol in the context of moderate beer consumption. Tyrosol content ranges depending on the type and style of beer.

3. The cytochrome P450 isoenzymes *CYP2A6* and *CYP2D6* are involved in the bioconversion of tyrosol into hydroxytyrosol *in vivo*. This bioconversion presents a wide variability in the population due to the high number of polymorphisms in the genes encoding these enzymes

4. A tyrosol-enriched intervention modulated pro-inflammatory and anti-inflammatory mediators, and triggered an improvement in endothelial function and endothelial biomarkers. The observed effects were dependent on sex and genetic factors.

5. The effect of tyrosol and hydroxytyrosol goes beyond their function as antioxidant and oxidant scavengers after unveiling their ability to modulate key regulatory signalling pathways *in vivo*.

6. Sex differences exist in the metabolism of tyrosol and hydroxytyrosol. Women present lower bioconversion capacity than men. Its physiological and clinical relevance is currently unknown.

7. A polygenic activity score based on individual *CYP2A6*/*CYP2D6* genotypes allows the prediction of the metabolic fate of tyrosol and hydroxytyrosol metabolites, and thus the health benefits expected from a tyrosol-enriched intervention in a personalized manner.

6. BIBLIOGRAPHY

Bibliography

Allingstrup, M. *et al.* (2016) 'Antithrombin III for critically ill patients', *Cochrane Database of Systematic Reviews*, p. CD005370. doi: 10.1002/14651858.CD005370.pub3.

Álvarez-Fernández, M. A. *et al.* (2018) 'Determination of hydroxytyrosol produced by winemaking yeasts during alcoholic fermentation using a validated UHPLC–HRMS method', *Food Chemistry*, 242, pp. 345–351. doi: 10.1016/j.foodchem.2017.09.072.

Andresen-Streichert, H. *et al.* (2018) 'übersichtsarbeit: Alkoholmarker bei klinischen und forensischen Fragestellungen', *Deutsches Arzteblatt International*, pp. 309–315. doi: 10.3238/arztebl.2018.0309.

Arranz, S. *et al.* (2012) 'Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer', *Nutrients*. Multidisciplinary Digital Publishing Institute (MDPI), 4(7), pp. 759–781. doi: 10.3390/nu4070759.

Asakura, H. *et al.* (2005) 'Selective inducible nitric oxide synthase inhibition attenuates organ dysfunction and elevated endothelin levels in LPS-induced DIC model rats', *Journal of Thrombosis and Haemostasis*, 3(5), pp. 1050–1055. doi: 10.1111/j.1538-7836.2005.01248.x.

Atzeri, A. *et al.* (2016) 'Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells', *Food and Function*, 7(1), pp. 337–346. doi: 10.1039/c5fo00074b.

Baraona, E. *et al.* (2001) 'Gender differences in pharmacokinetics of alcohol', *Alcoholism: Clinical and Experimental Research*, 25(4), pp. 502–507. doi: 10.1111/j.1530-0277.2001.tb02242.x.

Basso, R. F., Alcarde, A. R. and Portugal, C. B. (2016) 'Could non-Saccharomyces yeasts contribute on innovative brewing fermentations?', *Food Research International*. Elsevier, pp. 112–120. doi:

10.1016/j.foodres.2016.06.002.

Bayram, B. *et al.* (2012) 'A diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression', *Rejuvenation Research*, 15(1), pp. 71–81. doi: 10.1089/rej.2011.1245.

Boban, M. *et al.* (2006) 'Red wine induced modulation of vascular function: Separating the role of polyphenols, ethanol, and urates', *Journal of Cardiovascular Pharmacology*, 47(5), pp. 695–701. doi: 10.1097/01.fjc.0000211762.06271.ce.

de Bock, M. *et al.* (2013) 'Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (Olea europaea L.) leaf extract', *Molecular Nutrition and Food Research*. John Wiley & Sons, Ltd, 57(11), pp. 2079–2085. doi: 10.1002/mnfr.201200795.

Bordiga, M. *et al.* (2016) 'Factors influencing the formation of histaminol, hydroxytyrosol, tyrosol, and tryptophol in wine: Temperature, alcoholic degree, and amino acids concentration.', *Food chemistry*, 197 Pt B, pp. 1038–45. doi: 10.1016/j.foodchem.2015.11.112.

van Bussel, B. C. T. *et al.* (2018) 'Alcohol and red wine consumption, but not fruit, vegetables, fish or dairy products, are associated with less endothelial dysfunction and less low-grade inflammation: the Hoorn Study', *European Journal of Nutrition*, 57(4), pp. 1409–1419. doi: 10.1007/s00394-017-1420-4.

Calippe, B. *et al.* (2017) 'Complement Factor H Inhibits CD47-Mediated Resolution of Inflammation', *Immunity*, 46(2), pp. 261–272. doi: 10.1016/j.immuni.2017.01.006.

Castañer, O. *et al.* (2012) 'Protection of LDL from oxidation by olive oil polyphenols is associated with a downregulation of CD40-ligand expression and its downstream products in vivo in humans', *American Journal of Clinical*

Nutrition, 95(5), pp. 1238–1244. doi: 10.3945/ajcn.111.029207.

Catalán, Ú. *et al.* (2015) 'Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells', *Molecular Nutrition and Food Research*. John Wiley & Sons, Ltd, 59(12), pp. 2523–2536. doi: 10.1002/mnfr.201500361.

Cerrato-Alvarez, M. *et al.* (2019) 'Fast and direct amperometric analysis of polyphenols in beers using tyrosinase-modified screen-printed gold nanoparticles biosensors', *Talanta*, 193, pp. 93–99. doi: 10.1016/j.talanta.2018.09.093.

Chen, C. *et al.* (2008) 'Soluble CD40 ligand induces endothelial dysfunction in human and porcine coronary artery endothelial cells', *Blood*, 112(8), pp. 3205–3216. doi: 10.1182/blood-2008-03-143479.

Chen, F. *et al.* (1995) 'Dependence and Reversal of Nitric Oxide Production on NF-κB in Silica and Lipopolysaccharide Induced Macrophages', *Biochemical and Biophysical Research Communications*, 214(3), pp. 839–846. doi: 10.1006/bbrc.1995.2363.

Choi, J. W. *et al.* (2014) 'Association of the urine homocysteine/creatinine ratio to proinflammatory cytokine, natural anticoagulant, and nitric oxide levels in cerebrovascular disease.', *Annals of clinical and laboratory science*. Association of Clinical Scientists, 44(4), pp. 461–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25361933 (Accessed: 24 May 2019).

Clinical Pharmacogenetics Implementation Consortium (CPIC) (2019) *Final Consensus CYP2D6 genotype to phenotype table - March 2019*. Available at: https://cpicpgx.org/wp-content/uploads/2019/03/Final-Consensus-CYP2D6-genotype-to-phenotype-table_-final_Mar2019.pdf (Accessed: 11 September 2019).

Colica, C. *et al.* (2017) 'Antioxidant Effects of a Hydroxytyrosol-Based Pharmaceutical Formulation on Body Composition, Metabolic State, and

Gene Expression: A Randomized Double-Blinded, Placebo-Controlled Crossover Trial', *Oxidative Medicine and Cellular Longevity*, 2017, pp. 1–14. doi: 10.1155/2017/2473495.

Cooper, K. A., Chopra, M. and Thurnham, D. I. (2004) 'Wine polyphenols and promotion of cardiac health', *Nutrition Research Reviews*, 17(01), p. 111. doi: 10.1079/NRR200482.

Corella, D. *et al.* (2017) 'Utilizing nutritional genomics to tailor diets for the prevention of cardiovascular disease: a guide for upcoming studies and implementations', *Expert Review of Molecular Diagnostics*, pp. 495–513. doi: 10.1080/14737159.2017.1311208.

Corella, D. *et al.* (2018) 'Nutrigenética, nutrigenómica y dieta mediterránea: una nueva visión para la gastronomía', *Nutricion hospitalaria*, pp. 19–27. doi: 10.20960/nh.2120.

Costanzo, S. *et al.* (2011) 'Wine, beer or spirit drinking in relation to fatal and non-fatal cardiovascular events: a meta-analysis', *European Journal of Epidemiology*. Springer Netherlands, 26(11), pp. 833–850. doi: 10.1007/s10654-011-9631-0.

Covas, M.-I. *et al.* (2006) 'The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial.', *Annals of internal medicine*, 145(5), pp. 333–41.

Covas, M. I., De La Torre, R. and Fitó, M. (2015) 'Virgin olive oil: A key food for cardiovascular risk protection', *British Journal of Nutrition*, 113(S2), pp. S19–S28. doi: 10.1017/S0007114515000136.

Crespo, M. C. *et al.* (2015) 'One-week administration of hydroxytyrosol to humans does not activate Phase II enzymes', *Pharmacological Research*, 95–96, pp. 132–137. doi: 10.1016/j.phrs.2015.03.018.

D'Angelo, S. *et al.* (2001) 'Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil', *Drug Metabolism and*

Disposition, 29(11), pp. 1492–1498. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11602527 (Accessed: 7 September 2019).

Daly, A. K. (2017) 'Pharmacogenetics: A general review on progress to date', *British Medical Bulletin*, pp. 65–79. doi: 10.1093/bmb/ldx035.

Deiana, M. *et al.* (2011) 'Hydroxytyrosol glucuronides protect renal tubular epithelial cells against H 2O 2 induced oxidative damage', *Chemico-Biological Interactions*, 193(3), pp. 232–239. doi: 10.1016/j.cbi.2011.07.002.

Dinu, M. *et al.* (2018) 'Mediterranean diet and multiple health outcomes: An umbrella review of meta-analyses of observational studies and randomised trials', *European Journal of Clinical Nutrition*, pp. 30–43. doi: 10.1038/ejcn.2017.58.

EFSA (2011) 'Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), mainte', *EFSA Journal*, 9(4), p. 2033. doi: 10.2903/j.efsa.2011.2033.

El-Boraie, A. *et al.* (2019) 'Evaluation of a weighted genetic risk score for the prediction of biomarkers of CYP2A6 activity', *Addiction Biology*. doi: 10.1111/adb.12741.

Elosua, R. *et al.* (2000) 'Validation of the Minnesota leisure time physical activity questionnaire in Spanish women', *Medicine and Science in Sports and Exercise*, 32(8), pp. 1431–1437. doi: 10.1097/00005768-200008000-00011.

Estruch, R. *et al.* (2018) 'Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts', *New England Journal of Medicine*, 378(25), p. e34. doi: 10.1056/NEJMoa1800389.

Farràs, M. *et al.* (2013) 'Olive oil polyphenols enhance the expression of

cholesterol efflux related genes in vivo in humans. A randomized controlled trial', *The Journal of Nutritional Biochemistry*, 24(7), pp. 1334–1339. doi: 10.1016/j.jnutbio.2012.10.008.

Fernández-Castillejo, S. *et al.* (2019) 'Endothelial Cells Deconjugate Resveratrol Metabolites to Free Resveratrol: A Possible Role in Tissue Factor Modulation', *Molecular Nutrition & Food Research*, p. 1800715. doi: 10.1002/mnfr.201800715.

Forman, H. J., Davies, K. J. A. and Ursini, F. (2014) *How do nutritional antioxidants really work: Nucleophilic tone and para-hormesis versus free radical scavenging in vivo*, *Free Radical Biology and Medicine*. Pergamon. doi: 10.1016/J.FREERADBIOMED.2013.05.045.

Foster, B. C. *et al.* (2011) 'Comparative study of hops-containing products on human cytochrome P450-mediated metabolism', *Journal of Agricultural and Food Chemistry*, 59(9), pp. 5159–5163. doi: 10.1021/jf200090d.

Fraser, D. A. *et al.* (2007) 'Generation of inhibitory NFκB complexes and phosphorylated cAMP response element-binding protein correlates with the anti-inflammatory activity of complement protein C1q in human monocytes', *Journal of Biological Chemistry*, 282(10), pp. 7360–7367. doi: 10.1074/jbc.M605741200.

Fukumura, D. *et al.* (2001) 'Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability', *Proceedings of the National Academy of Sciences of the United States of America*, 98(5), pp. 2604–2609. doi: 10.1073/pnas.041359198.

Fung, T. T. *et al.* (2005) 'Diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction', *American Journal of Clinical Nutrition*. Narnia, 82(1), pp. 163–173. doi: 10.1093/ajcn/82.1.163.

Gaedigk, A. *et al.* (2008) 'The CYP2D6 activity score: Translating genotype information into a qualitative measure of phenotype', *Clinical Pharmacology*

and Therapeutics, 83(2), pp. 234–242. doi: 10.1038/sj.clpt.6100406.

Gaedigk, A. *et al.* (2018) 'Ten years' experience with the CYP2D6 activity score: A perspective on future investigations to improve clinical predictions for precision therapeutics', *Journal of Personalized Medicine*, p. 15. doi: 10.3390/jpm8020015.

de Gaetano, G. *et al.* (2016) 'Effects of moderate beer consumption on health and disease: A consensus document', *Nutrition, Metabolism and Cardiovascular Diseases*. Elsevier, 26(6), pp. 443–467. doi: 10.1016/j.numecd.2016.03.007.

Garde-Cerdán, T. and Ancín-Azpilicueta, C. (2008) 'Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation', *LWT - Food Science and Technology*. Academic Press, 41(3), pp. 501–510. doi: 10.1016/j.lwt.2007.03.018.

Gharwalova, L. *et al.* (2017) 'Resveratrol suppresses ethanol stress in winery and bottom brewery yeast by affecting superoxide dismutase, lipid peroxidation and fatty acid profile', *World Journal of Microbiology and Biotechnology*, 33(11), p. 205. doi: 10.1007/s11274-017-2371-x.

Gibson, A. *et al.* (2008) 'Alcohol increases homocysteine and reduces B vitamin concentration in healthy male volunteers--a randomized, crossover intervention study', *QJM*, 101(11), pp. 881–887. doi: 10.1093/qjmed/hcn112.

Gómez-Rico, A. *et al.* (2006) 'Phenolic and volatile compounds of extra virgin olive oil (Olea europaea L. Cv. Cornicabra) with regard to fruit ripening and irrigation management', *Journal of Agricultural and Food Chemistry*, 54(19), pp. 7130–7136. doi: 10.1021/jf060798r.

Guasch-Ferré, M. *et al.* (2017) 'Dietary Polyphenols, Mediterranean Diet, Prediabetes, and Type 2 Diabetes: A Narrative Review of the Evidence',

Oxidative Medicine and Cellular Longevity. Hindawi, pp. 1–16. doi: 10.1155/2017/6723931.

Hernáez, Á. *et al.* (2014) 'Olive oil polyphenols enhance high-density lipoprotein function in humans: A randomized controlled trial', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(9), pp. 2115–2119. doi: 10.1161/ATVBAHA.114.303374.

Hicks, J., Swen, J. and Gaedigk, A. (2014) 'Challenges in CYP2D6 Phenotype Assignment from Genotype Data: A Critical Assessment and Call for Standardization', *Current Drug Metabolism*, 15(2), pp. 218–232. doi: 10.2174/1389200215666140202215316.

Huang, S. *et al.* (2017) 'Longitudinal study of alcohol consumption and HDL concentrations: A community-based study', *American Journal of Clinical Nutrition*, 105(4), pp. 905–912. doi: 10.3945/ajcn.116.144832.

Intelmann, D., Haseleu, G. and Hofmann, T. (2009) 'LC-MS/MS Quantitation of Hop-Derived Bitter Compounds in Beer Using the ECHO Technique', *Journal of Agricultural and Food Chemistry*, 57(4), pp. 1172–1182. doi: 10.1021/jf803040g.

Janega, P. *et al.* (2014) 'Red wine extract decreases pro-inflammatory markers, nuclear factor-κB and inducible NOS, in experimental metabolic syndrome', *Food and Function*, 5(9), pp. 2202–2207. doi: 10.1039/c4fo00097h.

Keir, L. S. *et al.* (2017) 'VEGF regulates local inhibitory complement proteins in the eye and kidney', *Journal of Clinical Investigation*, 127(1), pp. 199–214. doi: 10.1172/JCI86418.

Khymenets, O. *et al.* (2010) 'Antioxidant Activities of Hydroxytyrosol Main Metabolites Do Not Contribute to Beneficial Health Effects after Olive Oil Ingestion', *Drug Metabolism and Disposition*, 38(9), pp. 1417–1421. doi: 10.1124/dmd.110.032821.

Konukoglu, D. and Uzun, H. (2016) 'Endothelial dysfunction and

hypertension', in *Advances in Experimental Medicine and Biology*. Springer, Cham, pp. 511–540. doi: 10.1007/5584_2016_90.

De la Torre, R. *et al.* (2006) 'Is dopamine behind the health benefits of red wine?', *European journal of nutrition*, 45(5), pp. 307–10. doi: 10.1007/s00394-006-0596-9.

De La Torre, R. *et al.* (2017) 'Protective effect of homovanillyl alcohol on cardiovascular disease and total mortality: Virgin olive oil, wine, and catechol-methylathion', *American Journal of Clinical Nutrition*, 105(6), pp. 1297–1304. doi: 10.3945/ajcn.116.145813.

Li, X. *et al.* (2017) 'Arterial stiffness and cognitive impairment', *Journal of the Neurological Sciences*, pp. 1–10. doi: 10.1016/j.jns.2017.06.018.

Lichtenstein, A. H. *et al.* (2006) 'Summary of American Heart Association Diet and Lifestyle Recommendations Revision 2006', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 26(10), pp. 2186–2191. doi: 10.1161/01.ATV.0000238352.25222.5e.

Lockyer, S. *et al.* (2015) 'Secoiridoids delivered as olive leaf extract induce acute improvements in human vascular function and reduction of an inflammatory cytokine: A randomised, double-blind, placebo-controlled, cross-over trial', *British Journal of Nutrition*, 114(1), pp. 75–83. doi: 10.1017/S0007114515001269.

López-Huertas, E. and Fonolla, J. (2017) 'Hydroxytyrosol supplementation increases vitamin C levels in vivo. A human volunteer trial', *Redox Biology*, 11, pp. 384–389. doi: 10.1016/j.redox.2016.12.014.

López de las Hazas, M.-C. C. *et al.* (2015) 'Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats', *Molecular Nutrition and Food Research*, 59(7), pp. 1395–1399. doi: 10.1002/mnfr.201500048.

López de las Hazas, M. C. *et al.* (2018) 'Brain uptake of hydroxytyrosol and

its main circulating metabolites: Protective potential in neuronal cells', *Journal of Functional Foods*. Elsevier, 46, pp. 110–117. doi: 10.1016/j.jff.2018.04.028.

Manna, C. *et al.* (2009) 'Olive oil phenolic compounds inhibit homocysteine-induced endothelial cell adhesion regardless of their different antioxidant activity', *Journal of Agricultural and Food Chemistry*, 57(9), pp. 3478–3482. doi: 10.1021/jf8037659.

Marković, A. K. *et al.* (2019) 'Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health', *Molecules*. Multidisciplinary Digital Publishing Institute, p. 2001. doi: 10.3390/molecules24102001.

Martínez-González, M. A. *et al.* (2015) 'Benefits of the Mediterranean Diet: Insights From the PREDIMED Study', *Progress in Cardiovascular Diseases*, 58(1), pp. 50–60. doi: 10.1016/j.pcad.2015.04.003.

Martínez-González, M. Á. *et al.* (2017) 'Transferability of the Mediterranean diet to non-Mediterranean countries. What is and what is not the Mediterranean diet', *Nutrients*. Multidisciplinary Digital Publishing Institute, 9(11), p. 1226. doi: 10.3390/nu9111226.

Martucci, M. *et al.* (2017) 'Mediterranean diet and inflammaging within the hormesis paradigm', *Nutrition Reviews*, 75(6), pp. 442–455. doi: 10.1093/nutrit/nux013.

Matsuzawa, Y. *et al.* (2015) 'Prognostic value of flow-mediated vasodilation in brachial artery and fingertip artery for cardiovascular events: A systematic review and meta-analysis', *Journal of the American Heart Association*, 4(11). doi: 10.1161/JAHA.115.002270.

Mayo, J. N. *et al.* (2014) 'Homocysteine Disrupts Outgrowth of Microvascular Endothelium by an iNOS-Dependent Mechanism', *Microcirculation*, 21(6), pp. 541–550. doi: 10.1111/micc.12133.

Menotti, A. and Puddu, P. E. (2015) 'How the Seven Countries Study

contributed to the definition and development of the Mediterranean diet concept: A 50-year journey', *Nutrition, Metabolism and Cardiovascular Diseases*, 25(3), pp. 245–252. doi: 10.1016/j.numecd.2014.12.001.

Menoyo, A., Del Rio, E. and Baiget, M. (2006) 'Characterization of variant alelles of cytochrome CYP2D6 in a Spanish population', *Cell Biochemistry and Function*, 24(5), pp. 381–385. doi: 10.1002/cbf.1258.

Di Minno, M. *et al.* (2010) 'Homocysteine and arterial thrombosis: Challenge and opportunity', *Thrombosis and Haemostasis*, 103(05), pp. 942–961. doi: 10.1160/TH09-06-0393.

Miró-Casas, E. *et al.* (2003) 'Hydroxytyrosol disposition in humans', *Clinical Chemistry*. Clinical Chemistry, 49(6), pp. 945–952. doi: 10.1373/49.6.945.

Monagas, M. *et al.* (2009) 'Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease', *American Journal of Clinical Nutrition*, 90(5), pp. 1144–1150. doi: 10.3945/ajcn.2009.27716.

Moreno-Luna, R. *et al.* (2012) 'Olive oil polyphenols decrease blood pressure and improve endothelial function in young women with mild hypertension', *American Journal of Hypertension*, 25(12), pp. 1299–1304. doi: 10.1038/ajh.2012.128.

Muriana, F. J. G. *et al.* (2017) 'Tyrosol and its metabolites as antioxidative and anti-inflammatory molecules in human endothelial cells', *Food and Function*, 8(8), pp. 2905–2914. doi: 10.1039/c7fo00641a.

Ordovas, J. M. *et al.* (2018) 'Personalised nutrition and health', *BMJ*. British Medical Journal Publishing Group, 361, p. bmj.k2173. doi: 10.1136/bmj.k2173.

Park, K. H. and Park, W. J. (2015) 'Endothelial dysfunction: Clinical implications in cardiovascular disease and therapeutic approaches', *Journal of Korean Medical Science*, pp. 1213–1225. doi: 10.3346/jkms.2015.30.9.1213.

Pastor, A. *et al.* (2016) 'Analysis of free hydroxytyrosol in human plasma following the administration of olive oil', *Journal of Chromatography A*, 1437, pp. 183–190. doi: 10.1016/j.chroma.2016.02.016.

Pata, C. *et al.* (2002) 'The effects of antithrombin-III on inducible nitric oxide synthesis in experimental obstructive jaundice. An immunohistochemical study.', *Pharmacological research*, 46(4), pp. 325–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12361694 (Accessed: 24 May 2019).

Perez-Herrera, A. *et al.* (2013) 'The antioxidants in oils heated at frying temperature, whether natural or added, could protect against postprandial oxidative stress in obese people.', *Food chemistry*, 138(4), pp. 2250–9. doi: 10.1016/j.foodchem.2012.12.023.

Pérez-Mañá, C., Farré, M., Pujadas, M., *et al.* (2015) 'Ethanol induces hydroxytyrosol formation in humans', *Pharmacological Research*, 95–96, pp. 27–33. doi: 10.1016/j.phrs.2015.02.008.

Pérez-Mañá, C., Farré, M., Rodríguez-Morató, J., *et al.* (2015) 'Moderate consumption of wine, through both its phenolic compounds and alcohol content, promotes hydroxytyrosol endogenous generation in humans. A randomized controlled trial', *Molecular Nutrition and Food Research*, 59(6), pp. 1213–1216. doi: 10.1002/mnfr.201400842.

Peyrol, J. *et al.* (2018) 'Involvement of bilitranslocase and beta-glucuronidase in the vascular protection by hydroxytyrosol and its glucuronide metabolites in oxidative stress conditions', *Journal of Nutritional Biochemistry*, 51, pp. 8–15. doi: 10.1016/j.jnutbio.2017.09.009.

Pignatelli, P. *et al.* (2005) 'The polyphenols quercetin and catechin synergize in inhibiting platelet CD40L expression [3]', *Thrombosis and Haemostasis*, pp. 888–889. doi: 10.1160/TH05-04-0888.

Piñeiro, Z. *et al.* (2011) 'Direct liquid chromatography method for the

simultaneous quantification of hydroxytyrosol and tyrosol in red wines.', 59(21). doi: 10.1021/jf202254t.

Poli, A. *et al.* (2013) 'Moderate alcohol use and health: A consensus document', *Nutrition, Metabolism and Cardiovascular Diseases*. Elsevier, pp. 487–504. doi: 10.1016/j.numecd.2013.02.007.

Preedy, V. R. (2008) *Beer in health and disease prevention*, *Beer in Health and Disease Prevention*. Elsevier. doi: 10.1016/B978-0-12-373891-2.X0001-6.

Pushpakumar, S., Kundu, S. and Sen, U. (2014) 'Endothelial Dysfunction: The Link Between Homocysteine and Hydrogen Sulfide', *Current Medicinal Chemistry*, 21(32), pp. 3662–3672. doi: 10.2174/09298673216661407061423 35.

Quifer-Rada, P. *et al.* (2015) 'A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS)', *Food Chemistry*, 169, pp. 336–343. doi: 10.1016/j.foodchem.2014.07.154.

Ragusa, A. *et al.* (2017) 'Composition and Statistical Analysis of Biophenols in Apulian Italian EVOOs', *Foods*. Multidisciplinary Digital Publishing Institute, 6(10), p. 90. doi: 10.3390/foods6100090.

Reriani, M. K., Lerman, L. O. and Lerman, A. (2010) 'Endothelial function as a functional expression of cardiovascular risk factors', *Biomarkers in Medicine*, 4(3), pp. 351–360.

Riwanto, M. *et al.* (2015) 'Dysfunctional HDL: From structure-function-relationships to biomarkers', *Handbook of Experimental Pharmacology*, 224, pp. 337–366. doi: 10.1007/978-3-319-09665-0_10.

Robles-Almazan, M. *et al.* (2018) 'Hydroxytyrosol: Bioavailability, toxicity, and clinical applications', *Food Research International*, 105, pp. 654–667. doi: 10.1016/j.foodres.2017.11.053.

Rodríguez-Morató, J. *et al.* (2015) 'Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases', *Molecules*. Multidisciplinary Digital Publishing Institute, pp. 4655–4680. doi: 10.3390/molecules20034655.

Rodríguez-Morató, J. *et al.* (2016) 'Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol', *Drug Metabolism Reviews*. Taylor & Francis, 48(2), pp. 218–236. doi: 10.1080/03602532.2016.1179754.

Rodríguez-Morató, J. *et al.* (2017) 'CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol', *Food Chemistry*, 217, pp. 716–725. doi: 10.1016/j.foodchem.2016.09.026.

Rosato, V. *et al.* (2019) 'Mediterranean diet and cardiovascular disease: a systematic review and meta-analysis of observational studies', *European Journal of Nutrition*, 58(1), pp. 173–191. doi: 10.1007/s00394-017-1582-0.

Ruano, J. *et al.* (2007) 'Intake of phenol-rich virgin olive oil improves the postprandial prothrombotic profile in hypercholesterolemic patients.', 86(2). doi: 10.1093/ajcn/86.2.341.

Rubinshtein, R. *et al.* (2010) 'Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events', *European Heart Journal*, 31(9), pp. 1142–1148. doi: 10.1093/eurheartj/ehq010.

Sangiovanni, E. *et al.* (2012) 'Inhibition of NF- κ B activity by minor polar components of extra-virgin olive oil at gastric level', *Phytotherapy Research*, 26(10), pp. 1569–1571. doi: 10.1002/ptr.4600.

Schröder, H. *et al.* (2009) 'Alcohol consumption is associated with high concentrations of urinary hydroxytyrosol', *American Journal of Clinical Nutrition*, 90(5), pp. 1329–1335. doi: 10.3945/ajcn.2009.27718.

Schwingshackl, L. *et al.* (2017) 'Adherence to Mediterranean Diet and Risk

of Cancer: An Updated Systematic Review and Meta-Analysis', *Nutrients*, 9(10), p. 1063. doi: 10.3390/nu9101063.

Semba, R. D. *et al.* (2014) 'Resveratrol Levels and All-Cause Mortality in Older Community-Dwelling Adults', *JAMA Internal Medicine*, 174(7), p. 1077.

Serra, G. *et al.* (2018) 'Olive oil polyphenols reduce oxysterols -induced redox imbalance and pro-inflammatory response in intestinal cells', *Redox Biology*, 17, pp. 348–354. doi: 10.1016/j.redox.2018.05.006.

Serreli, G. *et al.* (2019) 'Modulation of LPS-induced nitric oxide production in intestinal cells by hydroxytyrosol and tyrosol metabolites: Insight into the mechanism of action', *Food and Chemical Toxicology*, 125, pp. 520–527. doi: 10.1016/j.fct.2019.01.039.

Serreli, G. and Deiana, M. (2018) 'Biological Relevance of Extra Virgin Olive Oil Polyphenols Metabolites', *Antioxidants*, 7(12), p. 170. doi: 10.3390/antiox7120170.

Servili, M. *et al.* (2014) 'Biological activities of phenolic compounds of extra virgin olive oil', *Antioxidants*, pp. 1–23. doi: 10.3390/antiox3010001.

Shirasaka, Y. *et al.* (2016) 'Interindividual variability of CYP2C19-catalyzed drug metabolism due to differences in gene diplotypes and cytochrome P450 oxidoreductase content', *Pharmacogenomics Journal*, 16(4), pp. 375–387. doi: 10.1038/tpj.2015.58.

Sofi, F. *et al.* (2014) 'Mediterranean diet and health status: an updated meta-analysis and a proposal for a literature-based adherence score.', *Public health nutrition*, 17(12), pp. 2769–82.

Song, C. *et al.* (2019) 'Suppression of Src and Syk in the NF-κB signaling pathway by Olea europaea methanol extract is leading to its anti-inflammatory effects', *Journal of Ethnopharmacology*, 235, pp. 38–46. doi: 10.1016/j.jep.2019.01.024.

St-Laurent-Thibault, C. *et al.* (2011) 'Tyrosol and Hydroxytyrosol Two Main Components of Olive Oil, Protect N2a Cells Against Amyloid-β-Induced Toxicity. Involvement of the NF-κB Signaling', *Current Alzheimer Research*, 8(5), pp. 543–551. doi: 10.2174/156720511796391845.

Strong, G. and England, K. (2015) *Guide of styles 2015 Beer Judge certification program*.

Tan, L. A. *et al.* (2011) 'Interactions of complement proteins C1q and factor H with lipid A and Escherichia coli: Further evidence that factor H regulates the classical complement pathway', *Protein and Cell*, 2(4), pp. 320–332. doi: 10.1007/s13238-011-1029-y.

Tanner, J. A. *et al.* (2017) 'Predictors of variation in CYP2A6 mRNA, protein, and enzyme activity in a human liver bank: Influence of genetic and nongenetic factors', *Journal of Pharmacology and Experimental Therapeutics*, 360(1), pp. 129–139. doi: 10.1124/jpet.116.237594.

Tanner, J. A. and Tyndale, R. F. (2017) 'Variation in CYP2A6 activity and personalized medicine', *Journal of Personalized Medicine*, pp. 1–29. doi: 10.3390/jpm7040018.

Tian, J. *et al.* (2010) 'Inhibition of iNOS protects endothelial-dependent vasodilation in aged rats', *Acta Pharmacologica Sinica*, 31(10), pp. 1324–1328. doi: 10.1038/aps.2010.111.

Tomiyama, H. *et al.* (2018) 'Longitudinal association among endothelial function, arterial stiffness and subclinical organ damage in hypertension', *International Journal of Cardiology*, 253, pp. 161–166. doi: 10.1016/j.ijcard.2017.11.022.

Tresserra-Rimbau, A. *et al.* (2013) 'Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk: The PREDIMED study', *Nutrition, Metabolism and Cardiovascular Diseases*. Elsevier, 23(10), pp. 953–959. doi: 10.1016/j.numecd.2012.10.008.

Tresserra-Rimbau, A. *et al.* (2014) 'Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study', *Nutrition, Metabolism and Cardiovascular Diseases*, 24(6), pp. 639–647. doi: 10.1016/j.numecd.2013.12.014.

Tuck, K. L. *et al.* (2001) 'The In Vivo Fate of Hydroxytyrosol and Tyrosol, Antioxidant Phenolic Constituents of Olive Oil, after Intravenous and Oral Dosing of Labeled Compounds to Rats', *The Journal of Nutrition*, 131(7), pp. 1993–1996. doi: 10.1093/jn/131.7.1993.

Valenzuela, R. *et al.* (2017) 'Molecular adaptations underlying the beneficial effects of hydroxytyrosol in the pathogenic alterations induced by a high-fat diet in mouse liver: PPAR-α and Nrf2 activation, and NF-ϰB down-regulation', *Food and Function*, 8(4), pp. 1526–1537. doi: 10.1039/c7fo00090a.

Valls, R. M. *et al.* (2015) 'Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomised controlled trial', *Food Chemistry*, 167, pp. 30–35. doi: 10.1016/j.foodchem.2014.06.107.

Visioli, F., Poli, A. and Galli, C. (2001) 'Antioxidant and Other Biological Activities of Phenols from Olives and Olive Oil', *Med Res Rev*. John Wiley & Sons, Inc, 22(1), pp. 65–75. doi: 10.1002/med.1028.

Wang, W. *et al.* (2017) 'Protective Effects of Tyrosol against LPS-Induced Acute Lung Injury via Inhibiting NF-ϰB and AP-1 Activation and Activating the HO-1/Nrf2 Pathways', *Biological & Pharmaceutical BulletinBiological and Pharmaceutical Bulletin*, 40(5), pp. 583–593. doi: 10.1248/bpb.b16-00756.

Whelan, A. P. *et al.* (2004) 'Effects of white and red wine on endothelial function in subjects with coronary artery disease', *Internal Medicine Journal*, 34(5), pp. 224–228. doi: 10.1111/j.1444-0903.2004.00507.x.

WHO (2014) *Global status report on alcohol and health. Geneva, Switzerland*, *WHO*. Geneva.

World Health Organisation (2003) *Diet nutrition and the prevention of chronic diseases. Report of the WHO/ FAO Joint Expert Consultation*, *WHO*. World Health Organization. Available at: https://www.who.int/dietphysicalactivity/publications/trs916/summary/en/ (Accessed: 9 September 2019).

Wu, L. and Sun, D. (2017) 'Adherence to Mediterranean diet and risk of developing cognitive disorders: An updated systematic review and meta-analysis of prospective cohort studies', *Scientific Reports*, 7, p. 41317. doi: 10.1038/srep41317.

Yin, J. *et al.* (2017) 'Antithrombin III prevents progression of chronic kidney disease following experimental ischaemic-reperfusion injury', *Journal of Cellular and Molecular Medicine*, 21(12), pp. 3506–3514. doi: 10.1111/jcmm.13261.

Yu, Q. *et al.* (2013) 'The effect of various absorption enhancers on tight junction in the human intestinal Caco-2 cell line', *Drug Development and Industrial Pharmacy*, 39(4), pp. 587–592. doi: 10.3109/03639045.2012.692376.

Yuan, Y. *et al.* (2014) 'Inhibition of human cytochrome P450 enzymes by hops (Humulus lupulus) and hop prenylphenols', *European Journal of Pharmaceutical Sciences*, 53(1), pp. 55–61. doi: 10.1016/j.ejps.2013.12.003.

7. ANNEX

Boronat A, Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, et al. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. Free Radic Biol Med. 2019 Nov 1;143:471–81. DOI: 10.1016/j.freeradbiomed.2019.08.032

Boronat A, Mateus J, Soldevila-Domenech N, Guerra M, Rodríguez-Morató J, Varon C, et al. Data on the endogenous conversion of tyrosol into hydroxytyrosol in humans. Data Br. 2019 Dec;27:104787. DOI: 10.1016/j.dib.2019.104787

Soldevila-Domenech N, Boronat A, Mateus J, Diaz-Pellicer P, Matilla I, Pérez-Otero M, et al. Generation of the Antioxidant Hydroxytyrosol from Tyrosol Present in Beer and Red Wine in a Randomized Clinical Trial. Nutrients. 2019 Sep 18;11(9). DOI: 10.3390/nu11092241