# Lowering effects of hydroxytyrosol on homocysteine plasma concentrations after wine intake in humans

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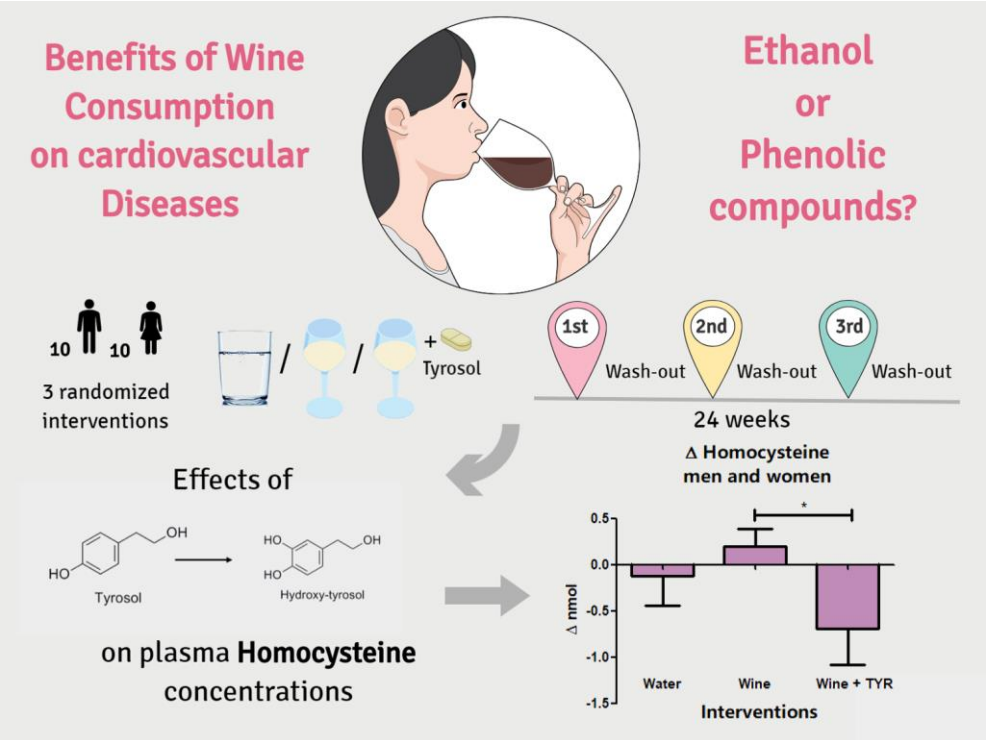
## 0. ABSTRACT

Homocysteine is considered to be a risk factor for atherosclerosis. Moderate alcohol consumption has been associated with beneficial effects on cardiovascular diseases. However, whether these effects are due to ethanol or to non-alcoholic components of alcoholic beverages including wine is still controversial. We designed a study that tries to demonstrate that one of the most potent dietary antioxidant compounds, hydroxytyrosol is responsible for cardiovascular health benefits provided by moderate wine consumption. Instead of administering hydroxytyrosol, we will benefit from the endogenous bioconversion described by us of tyrosol, a simple phenol quite abundant in wine, to hydroxytyrosol. The aim of the present study is to evaluate the effect of hydroxytyrosol after wine + tyrosol intake on homocysteine plasma concentrations. 20 subjects at high cardiovascular risk were randomized in a crossover trial. All received: (i) water ad libitum, (ii) 1 or 2 glasses (men) of white wine (14g ethanol/glass; 1-1,25 mg tyrosol/glass) and (iii) 1 or 2 glasses (men) of white wine each supplemented with a capsule of tyrosol (25mg) in a randomized crossover design. Tyrosol and hydroxytyrosol were measured in urine two times in each intervention, homocysteine concentrations were measured in plasma and endothelial function was measured using the Peripheral Arterial Tone (PAT signal). An increase on hydroxytyrosol urine metabolites was observed after wine + tyrosol intake together with a reduction on homocysteine concentrations. No differences were observed on the endothelial function. These results support a beneficial effect of the phenolic compounds of wine on cardiovascular risk factors/cardiovascular diseases.



## 1. INTRODUCTION

### Background

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality worldwide causing 17.7 million deaths people every year, a 31% of all global deaths (1). In Spain, CVD caused 257 deaths for every 100.000 habitants and it was the first cause of death in women and the second in men (2). Research indicates that CVD is a life course disease that begins with the evolution of risk factors that, in turn contribute to the development of subclinical atherosclerosis that could culminate in overt CVD (3). Atherosclerosis is defined as a continuous inflammatory damage to the arterial intima with increased permeability to plasma, deposition of plasma lipids in plaques and fibrosis and calcification of plaques (4). However, an effective prevention is possible by lifestyle changes reducing the exposure to related CVD risk factors like tobacco use, physical inactivity, harmful use of alcohol and unhealthy diets (3)(5).

### Homocysteine and the methylation cycle

Homocysteine (Hcy) is a sulfhydryl-containing amino acid and an intermediate product in the normal biosynthesis of the amino acids methionine and cysteine (4). It is present in plasma in four different forms: around 1% circulates as a free thiol; 70–80% remains bound to plasma proteins and 20–30% combines with itself to form an homocysteine dimer or with other thiols (4)(6). The term “total plasma homocysteine” (tHcy) refers to the combined pool of all four forms of homocysteine (4,6). Hcy is a key determinant of the methylation cycle: it is first activated to form S-adenosylmethionine (SAM) which serves as a methyl donor in a high number of reactions. The loss of the methyl group generates the S-adenosylhomocysteine (SAH), and the hydrolysis of SAH results in the formation of homocysteine and adenosine (4,7). Later on, the Hcy generated can be metabolized through two pathways: it can be re-methylated into methionine to begin another methyl transfer cycle or it can be combined with serine and trans-sulfurized by the enzyme cystathionine beta-synthase (CBS) to form cystathionine. Regarding the re-methylation pathway, homocysteine is remethylated back to methionine through methionine synthase (MS) which is dependant of vitamin B12 cofactor and it also needs methyltetrahydrofolate, which is generated by the enzyme, methylenetetrahydrofolate reductase (MTHFR) (7)(8). In the trans-sulfuration pathway, the enzyme CBS requires Vitamin B6 as a cofactor and the cystathionine generated is later metabolized to

cysteine, a precursor to the antioxidant glutathione (GSH) (7). The activation of the re-methylation pathway or the trans-sulfuration pathway is regulated by the dietary methionine intake (7).

It must be taken into account that both myocardial and vascular cells are inherently deficient in the enzyme cystathionine beta-synthase and therefore are unable to stabilize the excess of homocysteine through the trans-sulfuration pathway, and they have higher susceptibility to homocysteine toxicity (9).

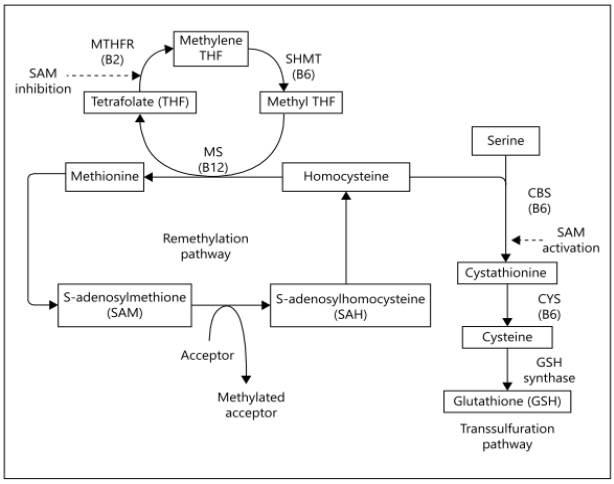



Figure 1: The metabolism pathway of homocysteine. CBS = Cystathione-beta-synthase; CYS = cystathione-gamma-lyase; MS = methionine synthase; MTHFR = methylenetrahydrofolate reductase; SHMT = serine hydroxy- methyltransferase. (7)

**Homocysteine as a risk factor for atherosclerosis**

Hyperhomocysteinemia (HHcy) is defined as a medical condition characterized by an abnormally high level (above 15 µmol/L) of homocysteine in the blood and a value above 100 µmol/L is classified as severe HHcy (4). It is believed that HHcy leads to endothelial cell damage, reduction in the flexibility of vessels, and alters the process of haemostasis (4). In addition, HHcy may lead to an enhancement of the adverse effects related with CVD risk factors like hypertension, smoking, lipids and lipoproteins metabolism. HHcy also induces chronic inflammation which could lead to an impairment in endothelial disruption (4)(7). Researchers have long debated the extent to which Hcy should be considered a risk factor for cardiovascular diseases, since according to some, only 50% of CVD can be explained by “classical” risk factors, and they say that “new” risk factors like HHcy could significantly boost the CVD predictive power. Currently, Hcy is known as an independent risk factor for atherosclerosis and research points towards a relationship between moderately elevated homocysteine levels and higher risk of CVD (4) (3).

Hcy can mediate the formation of CVD by several different mechanisms which include: (i) an increase in proliferation of vascular smooth muscle cells (VSMCs), (ii) endothelial dysfunction, (iii) oxidative damage, (iv) an increase is synthesis of collagen and deterioration of arterial wall elastic material (4). In relation to Hcy-induced oxidative stress, Hcy is known to increase reactive oxygen species (ROS) levels by autoxidation and by interfering with the activity and expression of antioxidant enzymes like glutathione peroxidase (10)(11). Regarding inflammation in VSMCs, a study demonstrated that Hcy

is capable of initiating an inflammatory response in VSMCs by stimulating CRP production, which is mediated through NMDAr-ROS-MAPK-NF-κB signal pathway (12).

**Homocysteine and endothelial function**

Homocysteine-induced endothelial dysfunction is produced by an imbalance between the vasodilators and vasoconstrictors molecules produced by the endothelium, and it has been regarded as the core systemic pathological status in the process of atherosclerosis and CVD. The role of Hcy in endothelial dysfunction is thought to be mediated by oxidative stress, nuclear factor-kb (NF-kb) activation, inflammation and inhibition of endothelial nitric oxide synthase (eNOS) (4,7). There are different mechanisms mediated by the Hcy and that could lead to an impairment of the endothelial function: NADPH Oxidase up-regulation, L-Arginine cellular transport reduction, e-NOS uncoupling and peroxynitrite production, loss of DDAH function and accumulation of ADMA, homocysteine-induced endoplasmic reticulum stress. These different mechanisms will reduce de NO bioavailability and impair endothelial vasodilatation in response to a dilatator stimulus, they will increase ROS species, and also reduce the endothelium integrity (7).

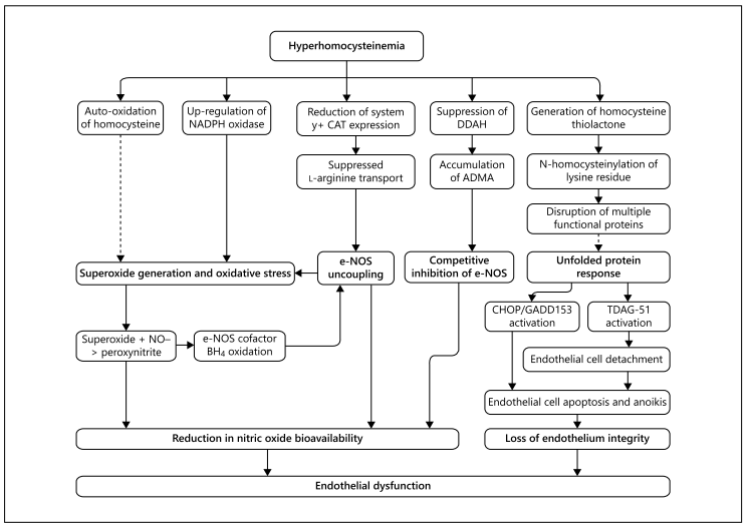


Figure 2: Potential mechanism of Hcy-induced endothelial dysfunction (7)

Local leukocyte recruitment into the vessel wall is the earliest basic step in atherosclerosis and is largely explained by the induction of endothelial activation, featuring the increased expression of endothelial leukocyte adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin. These endothelial adhesion molecules mediate the adhesion and transmigration of leukocytes to the vascular endothelial wall and may also promote plaque growth and instability (10). Recent studies have shown that, moderate Hcy levels induced VCAM-1 expression in human umbilical vein endothelial cells, at the protein and mRNA levels through a prooxidant mechanism involving NF-kappa-B (10). The VCAM-1 induction by Hcy is mediated by an increase of ROS, mainly through activation of NAD(P)H oxidase (10). It was also reported that HHcy induced the expression of other endothelial adhesion molecules like E-selectin and ICAM-1, the later one only if it was previously induced by TNF-alpha (10).

**Mediterranean diet and cardioprotection**

Mediterranean diet (MD) is a dietary pattern found in some of the olive-growing regions in the Mediterranean countries which is characterized by its high proportion in plant foods and the use of olive oil as the main source of fats, resulting in a higher proportion of mono unsaturated fatty acids relative to saturated ones (13). The healthy benefits of the MD regarding cardiovascular disease are widely known and are supported by numerous studies, as the well-known “The Seven Countries Study” or the PREDIMED trial. Both studies showed a reduced CVD related mortality in the Mediterranean countries and a reduction in the risk of major cardiovascular events among high-risk persons respectively (14)(13)(15).

**Phenolic compounds and health benefits of the Mediterranean diet**

Red wine and olive oil, particularly extra-virgin olive oil are thought to be partly responsible of the MD protective health effects. They are the main dietary sources of phenolic compounds like Hydroxytyrosol (HT) and Tyrosol (Tyr) (16). Phenols are organic compounds that contain a hydroxyl group (–OH) bound directly to a carbon atom in a benzene ring. Regarding olive oil, a number of factors including variety, olive fruit maturity and processing determine the final amount of phenolic compounds, typically ranging between 100 and 600 mg/kg (17), among which 140-200 mg/Kg are from HT and 80-140 for Tyr (18) . In the case of wine, the concentrations described for red wine are 1 mg/L (0–5 mg/L) for HT and 31 mg/L (20–40 mg/L) for Tyr (16) and for white wine (WW) 0-2 mg/L of HT and 8-9mg/L of Tyr (19). Epidemiological studies support that light to moderate alcohol drinking (10–20 g per day), may reduce the risk of cardiovascular disease (CVD), stroke, dementia, depression, and all-cause mortality (20)(16) . Phenolic compounds, in vitro, ex-vivo and in animal models, show several antiatherogenic activities, such as the inhibition of LDL oxidation; increasing high-density lipoprotein (HDL)-cholesterol; platelet aggregation; inflammation; improving the endothelial function and also strong antioxidant activities due to the potent redox properties of the phenolic hydroxyl groups (16)(21). In 2006, a health claim was released by the European Food Safety Authority (EFSA) for the consumption of 5 mg per day of HT and its derivatives in olive oil for the prevention of LDL oxidation (22).

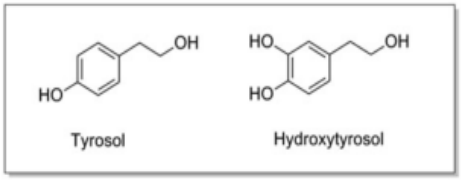


Figure 3: Chemical structures of tyrosol and hydroxytyrosol (16)

As mentioned before, HT is the main phenol present in olive oil and also in minor quantities in wine and it is one of the most potent antioxidants present in the MD (23). Tyr is also a well-known phenolic compound that is mainly present in extra-virgin olive oil and wine. However, in comparison with HT, Tyr has lower antioxidant

activity because it lacks of the hydroxyl group in position 3 of the phenolic ring (23). HT in addition of being a dietary microconstituent, is a metabolite of dopamine metabolism. Dopamine is initially catalysed by monoaminoxidase (MAO) giving rise to the 3,4-dihydroxyphenyl acetaldehyde (DOPAL). DOPAL is further oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC), which is the major metabolite of dopamine in biological matrices (oxidative pathway). A minor metabolic pathway is the reduction of DOPAL to 3,4-dihydroxyphenylethanol (DOPET) also known as HT (Figure)((16,24).

Data from bioavailability studies after red wine administration in healthy volunteers showed a recovery of substantial amounts of HT that could not be explained by the small quantities contained in wine. Moreover, the excretion of HT was higher than olive oil despite the fact that HT content was much lower in red wine (23,25). This suggested an endogenous HT formation after ethanol intake. It has been described that ethanol up-regulates the dopamine reductive pathway and increases DOPET generation over DOPAC in a dose-dependent manner (23)(26). Nevertheless, this ethanol-induced mechanism does not fully explain the amount of HT recovered in urine after wine intake (24). It was reported that Tyr was converted in vivo to HT and the enzymes responsible for this biotransformation are isozymes of cytochrome P450, specifically CYP2D6 and CYP2A6 (24).

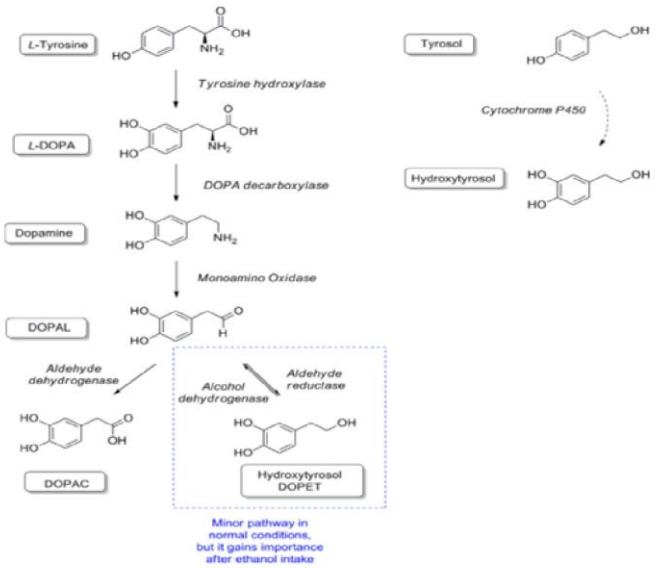



Figure: A) Dopamine biosynthesis and oxidative metabolism (B) Representation conversion of Tyr to HT by cytochrome P450. Adapted from (24)

When ethanol is administered with phenolic compounds it displays a dual role: ethanol present in red wine improves their bioavailability (27); it promotes an ethanol-induced rise in both Tyr and HT production following an alteration in tyramine and dopamine oxidative metabolisms. Improved Tyr bioavailability results in higher amount of HT recovery through the CYPs catalysed biotransformation.

**Dietary phenolic compounds and regulation of homocysteine**

As mentioned before, numerous studies have demonstrated the importance of naturally occurring dietary polyphenols in promoting cardiovascular health but recently, some studies have focused on the positive effects that could have against homocysteine induced CVD. It was described that there is a U-shaped relationship between alcohol consumption and homocysteine concentrations, with light to moderate consumption being associated with lower concentrations (28). Red wine consumers had lower concentrations of homocysteine compared with the beer, spirit and non-consumers and also compared with WW consumers. Therefore it was considered that red wine consumption was an independent predictor for lower homocysteine concentration(28). Moreover, research has shown that HT and homovanillyl alcohol significantly reduce homocysteine-induced cell adhesion and ICAM-1 expression in EA.hy 926 cells (a model of human endothelial cells) (29). On the contrary, Tyr and p-cumaric acid specifically only down regulate ICAM-1 expression induced by Hcy in EA.hy 926 cells and they were completely ineffective against the expression of ICAM-1 induced by TNF-alpha. These findings confirm that, phenolic compounds are able to modulate the Hcy-induced expression of adhesion molecules and likely delay plaque formation, therefore confirming the beneficial role of olive oil and wine (29). Furthermore, HT also inhibits homocysteine-induced proliferation and migration of Vascular Smooth Muscle Cells (VSMCs) reducing the expression of metal matrix proteinases (MMPs) (30). Red wine improves de vasorelaxation pattern induced by homocysteine in a porcine coronary arteries model: incubation with red wine along with homocysteine increased eNOS mRNA and protein expression (31)(32).

Considering that HHcy is a risk factor for cardiovascular diseases and wine consumption has been described as a protective factor regarding CDV, one of the aims of this study is to study the effect of wine phenolic compounds regarding homocysteine levels in subjects of high cardiovascular risk and its future implications regarding the risk of CVD.

## 2.HYPOTHESIS

The main hypothesis tested in this project is that lowering effects of wine over homocysteine plasma concentrations are due to the activity of wine polyphenols. Lower homocysteine concentrations have been associated to a reduced risk of cardiovascular disease

It is hypothesized that the moderate consumption of white wine will promote endogenous formation of Hydroxytyrosol (HT) and when white wine is supplemented with Tyrosol (TYR) capsules, the endogenous HT generation will be increased in a dose-dependent manner of the TYR ingested. Consequently, it is expected that concentrations of homocysteine will be lower when white wine is supplemented with TYR and higher when water is being administrated. Therefore, white wine polyphenols at moderate doses would improve cardiovascular risk in consequence of the reduced concentrations of homocysteine, with the largest effects obtained with wine enriched with TYR capsules.

## 3.OBJECTIVES

Main objective: to study in humans the effect of white wine polyphenols on the concentrations of homocysteine in individuals at high risk of cardiovascular disease and its clinical relevance.

Specific objectives:

- To assess the HT and TYR concentrations after the consumption of white wine in humans
- To assess the HT endogenous generation in a dose dependant manner after white wine and white wine supplemented with TYR in the form of capsules.
- To evaluate the effects of white wine on the concentrations of homocysteine compared with those elicited by white wine supplemented with TYR capsules
- To assess whether the HT endogenous generation will provide benefits on endothelial function by measuring the Reactive Hyperemia Index (RHI)

## 4.METHODS

**Subjects:**

Eligible participants should be free of any chronic degenerative disease, but at high cardiovascular risk. They will have at least three major risk factors, including smoking (>1 cig/day during the last month), hypertension (≥140/90 mmHg or antihypertensive medication), low-density lipoprotein (LDL)-cholesterol (>160 mg/dl or limit-lowering therapy), low high-density lipoprotein (HDL)-cholesterol (≤40 mg/dl in men or ≤50 mg/dl in women), overweight/obesity (body mass index≥25 kg/m2) or a family history of premature coronary heart disease (CHD). Study participants will have to follow a

controlled diet with a moderate content of antioxidants along the study. The subjects exclusion criteria are: participants with chronic diseases; participants with BMI >40 kg/m2; participants who intake antioxidant supplements; participants with multiple allergies or intestinal diseases; participants who follow special diets (vegetarian and vegan diets included); participants with any condition limiting their mobility; participants with history of hypersensitivity or intolerance to ethanol; ethanol users of >80 g/d (v) and illicit drug users; participants with an acute infection or inflammatory process in the last three months.

**Study design:**

Treatments schedule and dosage: Three treatments will be administered for 4 weeks each one, with a 3-week wash-out (WO) period pre-and between treatments following a randomized, cross-over balanced with placebo design. The order of the treatments was randomized. The different treatments are the following:

- White wine (WW): two glasses of WW (2x135 mL, 13º) in men and one glass (135mL) in women. Each 135 mL equivalent to 14 g of ethanol. It is estimated that WW will contain about 8-9 mg/l of TYR. Therefore, the dose of TYR ingested in two glasses would be 2-2.5 mg in men and 1-1.25 mg in women.
- WW+Tyrosol: two glasses of WW (2x135 mL, 13º) in men and one glass (135mL) in women in combination with capsules of 25 mg of TYR (each one to be ingested with a glass of wine at lunch or dinner). Each (135 mL) is equivalent to 14 g of ethanol. A daily dose of 27 and 26 mg of Tyrosol (equivalent to the content of 1L of wine) will be ingested in men and women, respectively.
- Water: drinking water along with meals.

In addition, study participants will follow a controlled diet with a moderate content of antioxidants along the study, in which time it will be not permitted the consumption of wine/champagne or other alcoholic drinks.

| | | Treatment | | Treatment | | Treatment |
|---|---|---|---|---|---|---|
| Order 1 | WO | WW | WO | Water | WO | WW + TYR |
| Order 2 | WO | Water | WO | WW + TYR | WO | WW |
| Order 3 | WO | WW + TYR | WO | WW | WO | Water |

Figure 5: example of a Latin square for the three treatments randomized, crossover trial

**Description of the sequence and duration of trial periods:**

The expected duration of subject participation is approximately 24 weeks (6 months): up to 3 weeks for the screening and inclusion of participants, 4 weeks for each intervention (a total of 12 weeks), with a wash-out period of 3 weeks pre- and between interventions (a total of 9 weeks). The different procedures are described in Figure 6:

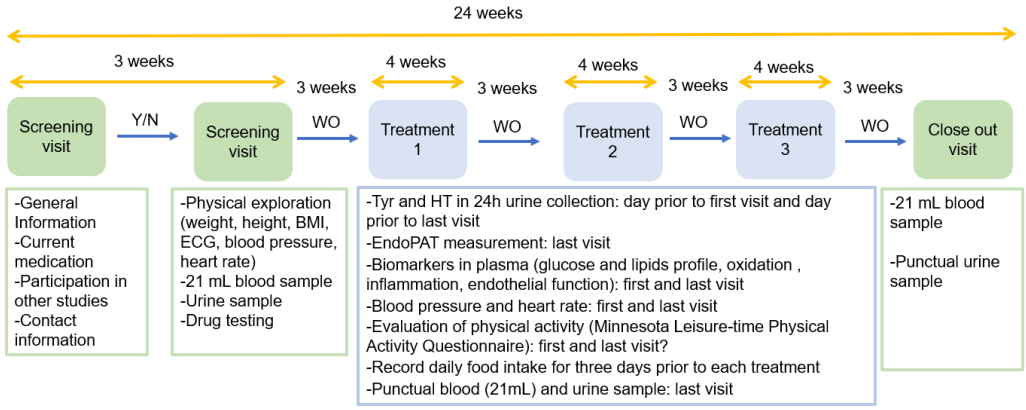



Figure 6: Clinical trial design. Abbreviations: Y/N: included or nor included: WO: Wash-out period: ECG: Electrocardiogram; BMI: Body Mass Index

**Analysis of TYR and OHTyr metabolites**

Urine samples were collected from all volunteers within 24 hours at the day prior to the beginning and the end of each intervention period. After, registering the total urine volume, two aliquots were treated with hydrochloric acid to acidify the sample and preserve wine polyphenols from oxidation. Finally, the aliquots were stored at –80 ºC until their analysis. Total Tyr and OHTyr and their metabolites in urine after each treatment were determined by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS), as previously described (33,34) using and aliquot of 500 µL. The metabolites measured were in the case of HT, HT 3'-*O*-sulfate, HT 3'-*O*-glucuronide, HT 4'-*O*-glucuronide, HT-acetate-3-o-sulfate and HVAlc-4'-*O*-glucuronide. In the case of Tyr, TYR 4'-*O*-sulfate and TYR 4'-*O*-glucuronide.

**Analysis of homocysteine**

Blood samples were collected in each visit before and after each treatment and a total of six samples (40 µL/sample) per patient were analysed. 40 µL of 1 µg/mL DL-Homocysteine-3,3,4,4-$d_4$ (ISTD) were added to all the samples. For the reduction phase, 10 µL THP tris(hydroxypropyl)phosphine) 23nM were added and for the deproteinization step 40 µL TCA (Trichloroacetic) 0.6M were added. During the derivatization step, 40 µL of 1-propanol/pyridine (3:1) and 130 µL TMP/BAC/PCF (Isooctane, Butyl acetate, Propyl chloroformate) (10/3/1) were added and lately 100 µL of $CHCl_3$ 1% PCF was added. Once samples derivatized, a liquid-liquid extraction was performed. 1mL of water and 5mL of chloroform ($CHCl_3$) were added and samples were shacked for 20 minutes.

Samples were centrifugated for 5 minutes at 3500rpm. The aqueous phase was removed, and the organic phase was evaporated under $N_2$-30° C-10/15psi for 1 hour. After evaporation 50 µL of $CHCl_3$ 1% PCF was added. The total Hcy concentration was determined by gas chromatography–mass spectrometry (GC/MS).

**Analysis of endothelial function**

Endothelial function was measured at the first and the last visit of each intervention period by monitoring endothelium-mediated changes in the digital pulse waveform known as the Peripheral Arterial Tone (PAT) signal, measured with a pair of novel modified plethysmographic probes situated on the finger index of each hand. Endothelium-mediated changes in the PAT signal are elicited by creating a downstream hyperemic response (EndoPAT 2000). Hyperemia is induced by occluding blood flow through the brachial artery for 5 minutes using an inflaTable cuff on one hand. The response to reactive hyperemia is calculated automatically by the system. A PAT ratio is created using the post and pre-occlusion values. These values are normalized to measurements from the contralateral arm, which serves as control for non-endothelial dependent systemic effects. Hiperemic reactivity has been shown to predict cardiovascular disease (35)

**Statistics**

Statistical analyses were performed using SPSS Statistics for Windows (version 18.0; SPSS Inc., Chicago, IL, USA). Descriptive statistics [mean, SD or n(%)] were used to describe the baseline characteristics of the participants and the outcome variables. Normality of continuous variables was assessed by normal probability plots and Kolmogorov-Smirnov test. Regarding normal distributed variables (Endothelial Function), to compare between baseline and outcome values within the same intervention a paired Student's t-test test was performed. To compare among treatment changes in outcome variables, analysis of covariance (ANOVA) for repeated measures was used. The Bonferroni post hoc test for multiple comparisons was used in the ANOVA analysis. It was considered significant when $p<0.05$. Regarding non-normal distributed variables, changes in HT/TYR and HCY concentrations were compared using the non-parametric Friedman test and the Wilcoxon Signed-Rank Exact Test for post-hoc analysis. To compare between baseline and outcome/final values within the same intervention a non-parametric Wilcoxon Signed-Rank Exact Test was performed. GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA) was the software used for graphical presentation.

## 5.RESULTS

### 5.1. Subjects Characteristics

A total of 20 volunteers were recruited for the study. One subject withdrew before completing the third intervention period.

Therefore, a total of 19 subjects (10 women, 9 men) completed the study. The baseline characteristics of the subjects are summarized in Table 1.

Table 1: Subjects Characteristics

| | |
|---|---|
| Age | 65,9 ± 6,2 |
| Gender | 10 women/ 10 men |
| Current smokers [n(%)] | 3 (15%) |
| Family history of premature CHD [n (%)] | 4 (20%) |
| Obesity (BMI ≥ 25kg/m2) [n (%)] | 19 (95%) |
| BMI (kg/m2) | 31,2± 0,7 |
| Type 2 Diabetes [n (%)] | 8 (40%) |
| Hypertension [n (%)] | 16 (80%) |
| Triglycerides (mg/dL) | 106,3± 69,6 |
| Total cholesterol (mg/dL) | 199,5± 32,2 |
| LDL cholesterol (mg/dL) | 124,2 ± 27,6 |
| LDL cholesterol > 130mg/dL [n (%)] | 16 (80%) |
| HDL cholesterol (mg/dL) | 53,7± 13,2 |
| HDL cholesterol <40mg/dL in men or <50mg/dL in women [n (%)] | 4 (20%) |

The majority of the subjects had obesity (95%) and hypertension (50%). A quarter had Type 2 diabetes (25%) and LDL cholesterol levels higher were higher that 130mg/dL in 80% of participants.

### 5.2. TYR and HT metabolites

Recovery of HT and TYR metabolites were analysed in 24 hour urine samples after each intervention (Figure 1, 2 and Table 2). The intervention Wine + TYR resulted in higher urinary recoveries TYR metabolites compared to water (p<0,001) and also to wine p=0,001) interventions (Figure 1). However, the difference in TYR levels after wine compared to water intervention did not result in a significant difference although a higher level of TYR was reached (p=0,156) . Regarding HT metabolites higher recoveries were observed after wine + TYR intervention compared to water (p=0,003) (Figure 2). Differences between HT urinary concentrations also reached statistically significant differences after wine + TYR intervention compared to the wine intervention (p=0,014). Nevertheless, the difference between HT concentrations after wine intervention compared to water wasn't statistically significant (p=0,313) (Figure 2).

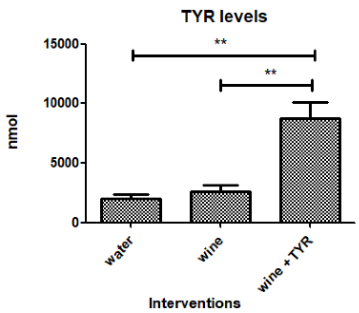



**Figure 1.** Mean and standard error of TYR urinary recovery after water, wine and wine + TYR interventions. ** = p<0.001; TYR=tyrosol;

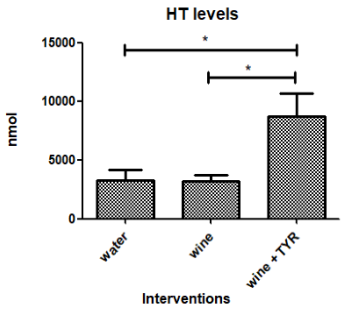



**Figure 2.** Mean and standard error of HT urinary recovery after water, wine and wine + TYR intervention. * = P<0.05; HT=Hydroxytyrosol

In order to evaluate the influence of gender on TYR and HT levels, values were adjusted for dose of TYR and no statistically significant differences between men and women were found. Men, had stadistically significant higher lelves of TYR after wine + TYR compared to water intervention (p=0,004) and also compared to wine intervention (p=0,014) (Figure 3 and Table 2).

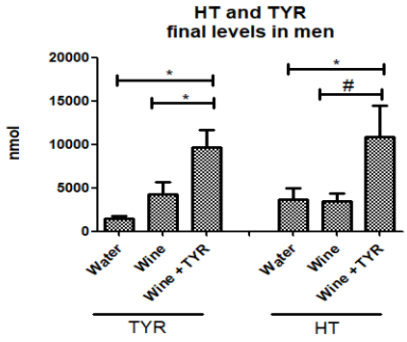



**Figure 3.** Men mean and standard error of TYR and HT urinary recovery after water, wine and wine + TYR interventions; * = P<0.05; # = p=0,064; TYR= Tyrosol; HT= hydroxytyrosol HT=Hydroxytyrosol

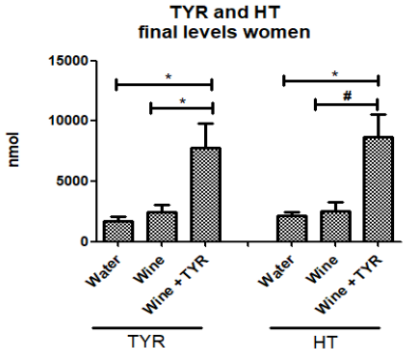



**Figure 4.** Women mean and standard error of TYR and HT urinary recovery after water, wine and wine + TYR interventions; * = P<0.05; # = p=0,064; TYR = Tyrosol; HT= Hydroxytyrosol;

Regarding HT, stadistically significant results were only obtained after wine + TYR compared to water intervention (p=0,037) and when comparing wine + TYR to wine intervention a tendency towards significance was observed (p=0,063) (Figure 3). Women obtanied statistically significant differences on the TYR recovery after wine + TYR intervention compared to water (p=0,004) and compared to wine (p=0,020) (Figure 4 and Table 2). Regarding HT, statistically significant results were only obtained after wine + TYR compared to water (p=0,043) (Figure 4). However, after wine + TYR compared to wine intervention a trend towards significance was observed (p=0,063) (Figure 4).

Table 2: Total TYR and HT recoveries. Data expressed as Mean ± SD. TYR = Tyrosol; HT = Hydroxytyrosol. *P<0,05 versus water; **P<0,001 versus water; †P<0,05 versus wine; ††P<0.001 versus wine

**TYR and HT urinary recovery**

| **Tyr men and women** | **Intervention** | **Total Tyr (nmol)** | **HT** | **Intervention** | **Total HT (nmol)** |
|---|---|---|---|---|---|
| | Water | 2010,5 ± 1594,1 | | Water | 3290,4 ± 3752,0 |
| | Wine | 2622,6 ± 2192,7 | | Wine | 3230,3 ± 1969,1 |
| | Wine + TYR | 8750,7 ± 6263,3 **†† | | Wine + TYR | 8750,7 ± 8111,7 *† |
| **Tyr men** | **Intervention** | **Total Tyr (nmol)** | **HT men** | **Intervention** | **Total HT (nmol)** |
| | Water | 1446,2 ± 906,3 | | Water | 3619,3 ± 4224,0 |
| | Wine | 4275,5 ± 4189,6 | | Wine | 3491,5± 2536 |
| | Wine + TYR | 9664,9 ± 6346,9 *† | | Wine + TYR | 108866 ± 10266,9 * |
| **Tyr women** | **Intervention** | **Total Tyr (nmol)** | **HT women** | **Intervention** | **Total HT (nmol)** |
| | Water | 1714,1 ± 1003,2 | | Water | 2120,5 ± 841,6 |
| | Wine | 2474,4 ± 1839,5 | | Wine | 2523,80 ± 1607,2 |
| | Wine + TYR | 7805,6 ± 6372,9 *† | | Wine + TYR | 8656,57 ± 4945,9 * |

Baseline levels of HT and TYR before each intervention were also compared. When comparing TYR basal levels, no statistically significant differences were observed before any of the interventions (Table 3). Regarding HT basal levels, no statistically significant differences were observed between basal levels of HT before any of the interventions Figure 8). In addition, neither women nor men presented statistically significant baseline levels of TYR or HT (Table 3).

Table 3: Baseline TYR and HT metabolites. Data expressed as Mean ± SD. TYR = Tyrosol; HT = Hydroxytyrosol. *P<0,05 versus water; **P<0,001 versus water; †P<0,05 versus wine; ††P<0.001 versus wine

**TYR and HT urinary baseline recoveries**

| **Tyr men and women** | **Intervention** | **Total Tyr (nmol)** | **HT** | **Intervention** | **Total HT (nmol)** |
|---|---|---|---|---|---|
| | **Water** | 1541,8 ± 1026,6 | | Water | 2785,7 ± 2288,3 |
| | Wine | 1824,2 ± 1713,5 | | Wine | 3292 ± 2302,2 |
| | Wine + TYR | 1950,3 ± 1379 | | Wine + TYR | 3736,4 ± 2089,2 |
| **Tyr men** | **Intervention** | **Total Tyr (nmol)** | **HT men** | **Intervention** | **Total HT (nmol)** |
| | Water | 1711,9 ± 1212,6 | | Water | 2831,8 ± 2716,2 |
| | Wine | 1842,2 ± 2067 | | Wine | 3135,3 ± 2763,5 |
| | Wine + TYR | 1923,4 ± 1387,6 | | Wine + TYR | 3002,8 ± 439,7 |
| **Tyr women** | **Intervention** | **Total Tyr (nmol)** | **HT women** | **Intervention** | **Total HT (nmol)** |
| | Water | 1371,8 ± 830,8 | | Water | 2709 ± 1554,8 |
| | Wine | 1361,8 ± 623,8 | | Wine | 3448,75 ± 1914,5 |
| | Wine + TYR | 2363,4 ± 1828,8 | | Wine + TYR | 5661,90 ± 4787,7 |

### 5.3. Endothelial Function

Differences on the endothelial function values after each intervention compared to treatment baselines are represented in Figure 5 and Table 4. None intervention resulted in a statistically significant difference on endothelial function. Nevertheless, endothelial function improved after wine + TYR intervention (p=0,274) compared to water, followed by wine (p=0,280)

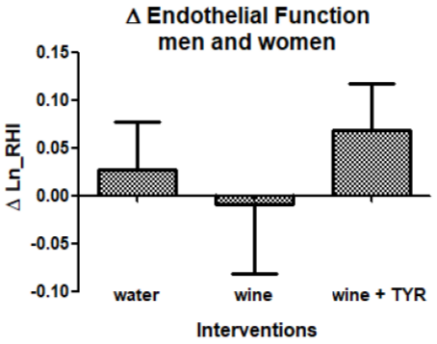



**Figure 5.** Mean and standard error of differences on Endothelial Function after water, wine and wine + TYR interventions.

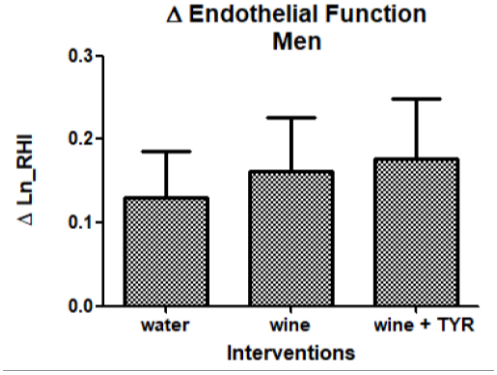



**Figure 6.** Mean and standard error of differences on the Endothelial Function after water, wine and wine + TYR interventions.

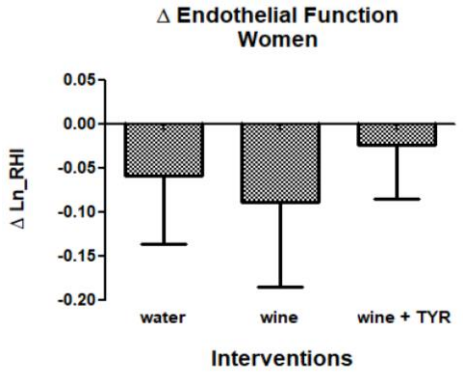



**Figure 7.** Mean and standard error of differences on the Endothelial Function after water, wine and wine + TYR interventions.

intervention (Figure 5). Age and basal heart rate were considered covariables to assess changes in endothelial function. Differential treatment effect on endothelial function are observed in men (Figure 6) compared to women (Figure 7). After the different interventions men improved the endothelial function, having the best result after wine + TYR intervention (p=0,280) (Figure 6).

However, women worsen their endothelial function after the different interventions although the best result was observed after wine + TYR intervention (Figure 7).

Baseline endothelial function before each intervention were calculated. No statistically significant differences were found on the endothelial function before each of the interventions (Table 5) and no statistically significant differences were found on the baseline levels of men and women before each of the interventions (Table 5).

Table 4: Endothelial function. Data expressed as Mean ± SD. TYR = Tyrosol; *P<0,05 versus water; **P<0,001 versus water; †P<0,05 versus wine; ††P<0.001 versus wine

**Endothelial function**

| men and Women | Intervention | Δ Ln_RHI |
|---|---|---|
| | Water | 0,02 ± 0,2 |
| | Wine | -0,009 ± 0,3 |
| | Wine + TYR | 0,06± 0,2 |
| **Men** | **Intervention** | **Δ Ln_RHI** |
| | Water | 0,13 ± 0,2 |
| | Wine | 0,16 ± 0,2 |
| | Wine + TYR | 0,17 ± 0,3 |
| **Women** | **Intervention** | **Δ Ln_RHI** |
| | Water | -0,06 ± 0,2 |
| | Wine | -0,09 ± 0,3 |
| | Wine + TYR | -0,02 ± 0,1 |

Table 5: Baseline Endothelial function. Data expressed as Mean ± SD. TYR = Tyrosol; *P<0,05 versus water; **P<0,001 versus water; †P<0,05 versus wine; ††P<0.001 versus wine

**Baseline Endothelial function**

| Men and Women | Intervention | Ln_RHI |
|---|---|---|
| | Water | 0,53 ± 0,2 |
| | Wine | 0,43 ± 0,2 |
| | Wine + TYR | 0,47 ± 0,2 |
| **Men** | **Intervention** | **Ln_RHI** |
| | Water | 0,47 ± 0,2 |
| | Wine | 0,36 ± 0,2 |
| | Wine + TYR | 0,39 ± 0,1 |
| **Women** | **Intervention** | **Ln_RHI** |
| | Water | 0,59 ± 0,22 |
| | Wine | 0,50 ± 0,2 |
| | Wine + TYR | 0,57 ± 0,3 |

### 5.4. Homocysteine

Differences on Hcy concentrations after the different interventions were calculated and are represented in Figure 8 and Table 6. A statistically significant difference was found after wine + TYR intervention (p=0.005) and, in fact, Hcy concentrations increased after wine intervention (Figure 8). Men after wine + TYR intervention did not obtain a statistically significant result although a trend toward significance was observed (p=0,064) (Figure 9).

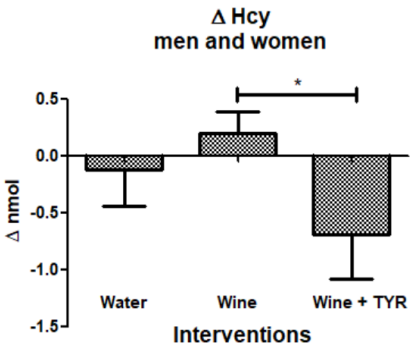



**Figure 8.** Men and women mean and standard error of Δ Hcy concentrations after water, wine and wine + TYR interventions. TYR = Tyrosol; * = p<0.05

Women reached a statistically significant reduction on Hcy concentrations after wine + TYR intervention (p=0,037) (Figure 10).

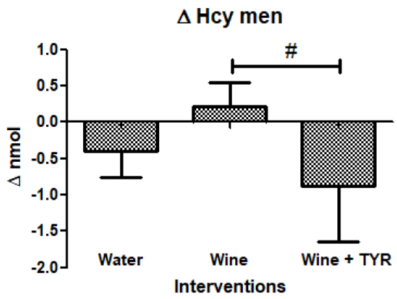



**Figure 9.** Men mean and standard error of Δ Hcy concentrations after water, wine and wine + TYR interventions. TYR = Tyrosol; # = p=0.064

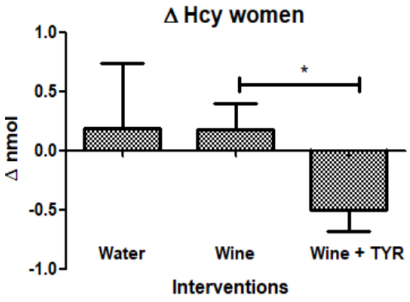



**Figure 10.** Women Mean and standard error of Δ Hcy concentrations after water, wine and wine + TYR interventions. TYR = Tyrosol; * = p<0.05

When comparing the baseline concentrations of Hcy before the different interventions, no statistically significant differences were observed before any of the interventions (Table 7). Men and women baseline Hcy concentrations were compared and no statistically significant differences were observed in men although in women, statistically significant differences were observed before wine + TYR compared to water and wine interventions (Table 7).

**Homocysteine**

| **Men and Women** | **Intervention** | **Δ Hcy (nmol)** |
|---|---|---|
| | Water | -0,12 ± 1,4 |
| | Wine | 0,19 ± 0,8 |
| | Wine + TYR | -0,69 ± 1,7 † |
| **Men** | **Intervention** | **Δ Hcy (nmol)** |
| | Water | -0,39 ± 1,1 |
| | Wine | 0,21 ± 1,0 |
| | Wine + TYR | -0,87 ± 2,4 |
| **Women** | **Intervention** | **Δ Hcy (nmol)** |
| | Water | 0,18 ± 1,6 |
| | Wine | 0,17 ± 0,7 |
| | Wine + TYR | -0,50 ± 0,5 † |

Table 6: Δ Hcy. Data expressed as Mean ± SD. TYR = Tyrosol; *P<0,05 versus water; **P<0,001 versus water; †P<0,05 versus wine; ††P<0.001 versus wine

**Homocysteine baseline concentrations**

| **Men and Women** | **Intervention** | **Hcy (nmol)** |
|---|---|---|
| | Water | 2,80 ± 1,8 |
| | Wine | 2,28 ± 1,8 |
| | Wine + TYR | 4,06 ± 2,2 |
| **Men** | **Intervention** | **P value** |
| | Water | 3,09 ± 1,7 |
| | Wine | 3,03 ± 1,7 |
| | Wine + TYR | 4,02 ± 2,3 |
| **Women** | **Intervention** | **P value** |
| | Water | 2,70 ± 1,1 |
| | Wine | 2,28 ± 1,06 |
| | Wine + TYR | 3,60 ± 0,68 *† |

Table 7: Hcy baseline concentrations. Data expressed as Mean ± SD. TYR = Tyrosol; *P<0,05 versus water; **P<0,001 versus water; †P<0,05 versus wine; ††P<0.001 versus wine

## 6.DISCUSSION

Moderate wine consumption has been associated with different health benefits in humans. Among them: cardiovascular protection, lower prevalence of metabolic syndrome, decreased incidence of hypertension, diabetes and reduced overall mortality Wine is a complex mixture of water, alcohol and several hundred microconstituents, among them TYR and HT. Wine, through the effect of alcohol on dopamine and tyramine metabolism, promotes the generation of HT in humans. In addition, in vivo conversion of TYR to HT also takes place through cytochrome P450 (36).

However, the debate of whether the beneficial effects of wine should be attributed to ethanol or the phenolic compounds has been ongoing for many years. For example, the former Lancet editor David Sharp in his article *Chateau Resveratrol* (37) pointed out that the cardiovascular advantages may stem from alcohol not wine as such and that some phenolic compounds present in wine like resveratrol, as a pharmaceutical product, were unpromising and other articles supported these views (38)(39). However, numerous studies have described the beneficial effects of wine phenols like resveratrol, TYR and HT on cardiovascular health. The main aim of this study was to describe the effect of wine phenolic compounds on the plasma homocysteine levels. Therefore, WW was chosen for its 10-fold lower concentration of phenolic compounds compared to red wine and consequently, WW acted mainly as a vehicle of alcohol. Previous findings have shown that, phenolic compounds are able to modulate the Hcy-induced endothelial dysfunction expression of adhesion molecules and likely delay plaque formation and also inhibit Hcy-induced proliferation and migration of Vascular Smooth Muscle Cells (VSMCs)(7)(10). In this study we hypothesized that Hcy concentrations would be lowered by the endogenous bioconversion of TYR from wine to HT after WW intake and consequently it could be associated with a reduced risk of CVD. In order to verify this hypothesis, a total of 20 subjects with at least 3 major cardiovascular risk factors took part in a randomized, crossover and controlled nutritional trial with three different interventions: water, WW and WW supplemented with TYR capsules (dose equivalent t about 1L of red wine).

Firstly, we compared the HT recoveries after the three interventions in order to distinguish whether an increase in HT recoveries was due to the ethanol itself, due to the bioconversion of TYR to HT or both. After wine + TYR intervention, the recoveries of HT were ~ 250% higher compared to water and wine interventions which confirmed that the bioconversion of TYR to HT was responsible for the increase of/on HT recoveries.

As mentioned before, it was hypothesized that Hcy levels would be lowered by WW polyphenols. In the present study, a statistically significant reduction on Hcy concentrations was observed in the overall group of participants after wine + TYR

intervention compared to wine intervention. Moreover, after wine + TYR intervention, both men and women, had reduced concentrations of Hcy although only women reached statistically significant results. In addition, it was observed that Hcy concentrations were non-statistically significantly higher after wine intervention both in men and women

Finally, we tested the influence of HT and TYR concentrations on endothelial function after the different interventions. Interestingly, no statistically significant improvement on endothelial function was observed after wine + TYR intervention although it resulted in better results compared to the other interventions. Neither men nor women obtained statistically significant improvements on endothelial function although men obtained better results than women. These observations are in line with findings from a study where subjects who drank between >1 drink/month and 2 drinks/day showed a higher flow-mediated dilation (FMD) compared to non-drinkers or those who drank more than 2 drinks/day, independently of the type of alcoholic beverage consumed (40). In a randomized intervention trial, 30 g of alcohol only in the form of red wine showed significant beneficial effects on endothelial function, while beer and WW had no effects (41). This study also pointed out that the beneficial effect on endothelial function was only observed 1-4h after the ingestion of red wine and this could explain why no statistically significant effects were observed in our study. However, the subjects from this study were healthy, without CVD, and therefore it makes it difficult to compare the results with our study. Thus, it seems that HT endogenous generation after wine ingestion could exert a beneficial effect on endothelial function although the effects on endothelial function still remain controversial.

When considering Hcy, the results observed in this study are in line with the results from other studies were the levels of Hcy were lowered after moderate red wine consumption. Dixon et al reported a reduced plasma Hcy levels in obese red wine consumers which could be a potential contributor to a reduced cardiovascular risk status in this subjects (28). In this study, 350 severely obese subjects were included and light to moderate red wine consumers obtained lower Hcy concentrations compared to beer, spirits and WW consumers suggesting that phenols present in wine may be responsible for this beneficial effect. In our study, a non-statistically significant increase in Hcy concentrations was observed after wine intervention. This results agree with the results obtained in Rajdl et al study (42) were they reported an increase in the Hcy levels after one month of WW consumption. However, this study lacked a placebo control group and WW intake was higher than in our study (375mL/day in man compared to 270mL/day) which could explain the statistically significant higher Hcy levels observed. In another work from Bleich et al (43) they found increased levels of Hcy in social drinkers (30g alcohol/day) after consuming beer, spirit or wine during 6 weeks. Nevertheless, they found lower

folate levels in the spirit and wine consumers group which may explain the rise in total plasma Hcy observed (43) . Similar to Beich work, Gibson et al (44) also reported an increase in Hcy levels after two weeks of moderate alcohol consumption (rather red wine or vodka) together with reduced levels of folate and vitamin B12. Taken together these results suggest that ethanol, even at moderate consumption levels, could increase Hcy plasma concentration whereas the endogenous generation of HT after WW + TYR capsules intake could counteract this effect and is be associated with a reduction in Hcy plasma concentrations. A question for the future is whether, the moderate consumption of red wine and its naturally present phenolic compounds would be able to reduce Hcy concentrations. It would be interesting that future studies compare the effects of phenolic compounds present in red wine with the effects of moderate consumption of an alcoholic beverage without phenolic compounds on Hcy plasma concentrations.

Nevertheless, we consider that some facts could have affected the results from the present study. First, drugs used for the prevention or treatment of CVD may modulate plasma homocysteine levels. Thus, a drug induced Hcy increase may counteract the desired reduction effect enhanced by phenols present in wine. In the present study, the medication taken by the subjects during the trial was considered. Firstly, B vitamins, folate medication or anti-oxidant supplements where a reason for trial exclusion and therefore they could not have affected the concentration of Hcy reported. However, anti-hypertensive drugs were used by a total of 10 subjects in this study and they have been related with increased levels of Hcy (45,46). Secondly, vitamins B12 and B6 and folic acid (FA) levels were not measured during the trial/study and they could have affected Hcy concentrations because FA and vitamins B12-B6 are important regulators of Hcy metabolism and it has been shown that lower levels of vitamin B12,B6 and folate are related with higher Hcy concentrations(47,48).. For this reason, future studies should include the measurement of vitamin B12-B6 and folate levels during the different interventions.

As it has been discussed, there's a great controversy regarding the effect of moderate alcohol consumption on the Hcy levels and there are many influences on plasma homocysteine levels that could confound results. Future studies are needed in order to clarify this controversy and specially taking into account that, relatively small decreases (i.e 3 µmol/L) on the Hcy concentrations are highly relevant on reducing the risk of myocardial infarction and stroke (49).

Here we also propose that future studies directions should consider the analysis of DYR-K1A, a serine/threonine kinase that could help to understand the effect of wine phenolic compounds on Hcy. DYR-K1A has been described as a candidate antiapoptotic factor and it has also been observed that there was a negative correlation between plasma Hcy

levels and DYR-K1A expression in a murine model of HHcy (51). In addition, overexpression of Dyrk1A increases the nuclear factor-erythroid 2-related factor 2 (NRF2) quantity and activity, a factor implicated in anti-oxidant and anti-inflammatory responses by controlling NQO1 gene expression (52). Taken together these mechanisms, it would be interesting to investigate the expression of DYR-K1A kinase in relation with the TYR/HT concentrations and also with the concentration of Hcy in subjects at high cardiovascular risk after wine and wine + TYR intake.

Strengths of the present study are the design, the comprehensive measurements, the equivalent number of women and men included in the study and the high retention rate (95%). The design of this study (crossover randomized and controlled nutritional intervention) was organized as 3 sequential interventions separated by a wash-out period. Consequently, each participant serves as its own control allowing between and within comparisons.

The study has some limitations like the low number of participants studied (although the study will be powered with the inclusion of additional subjects) and, as mentioned before, the medications that the participants took during the study that could have influenced the results.

In conclusion, to our knowledge, this is the first study to evaluate the effects on Hcy plasma concentrations and endothelial function of a novel mechanism like the endogenous HT generation after the combined intake of wine and TYR on individuals at high risk of CVD .

## 7.CONCLUSIONS

- There is an endogenous bioconversion of TYR to HT in humans after white wine consumption
- There's an increase in HT endogenous formation after the intake of white wine supplemented with Tyrosol capsules compared to the other interventions
- HT endogenous formation reduces Hcy plasma after wine + TYR capsules intake in both men and women
- No statistically significant effects on endothelial function were observed after endogenous bioconversion of TYR to HT after wine + TYR intake

## 8.ACKNOWLEDGEMENTS

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