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"TERAPIE AVANZATE BIOMEDICHE E CHIRURGICHE"

Polyphenols in red wine and common genetic variants influencing cardiovascular diseases

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PREFACE

This Ph.D. thesis is submitted as a requirement for obtaining the Ph.D. Degree at the University of Naples Federico II (Italy). It is written based on three-years research conducted by the author, Luigi Castaldo, under the supervision of Prof. Giovanni Di Minno and Alberto Ritieni at the Department of Medicine in Naples, and under the supervision of Prof. Bruna Gigante at the Karolinska Institutet in Stockholm (Sweden).

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ABSTRACT

Diet is a pivotal constituent of a healthy lifestyle. Mediterranean diet is the typical dietary pattern consumed around the Mediterranean Basin, that drew the attention of medical professionals by proving protection against chronic disease morbidity and contribute to a favorable health status. In the Mediterranean basin, the most widely consumed alcoholic beverage is wine, while coffee is the main non-alcoholic beverage.

Wine is a popular alcoholic beverage that has been consumed for hundreds of years. Benefits from moderate alcohol consumption have been widely supported by the scientific literature and, in this line, red wine intake has been related to a lesser risk for coronary heart disease (CHD). Experimental studies and meta-analyses have mainly attributed this outcome to the presence in red wine of a great variety of polyphenolic compounds such as resveratrol, catechin, epicatechin, quercetin, and anthocyanin. Resveratrol is considered the most effective wine compound with respect to the prevention of CHD because of its antioxidant properties. The mechanisms responsible for its putative cardioprotective effects would include changes in lipid profiles, reduction of insulin resistance, and decrease in oxidative stress of low-density lipoprotein cholesterol (LDL-C). In this sense, the first part of my Ph.D. project summarizes the accumulated evidence correlating moderate red wine consumption with prevention of CHD by focusing on the different mechanisms underlying this relationship.

Furthermore, in this thesis work was developed a method for simultaneous investigation of polyphenol compounds in red wine samples using ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS). In addition, total phenols content, as well as antioxidant activity in red wine samples, were also investigated.

Moreover, to expand the understanding of this current topic and evaluate the potential health benefits of moderate red wine consumption, a monocentric, open-label, non-randomized, parallel-group clinical trial was designed. Healthy volunteers will be clinically evaluated by medical staff working at the Prevention Unit of the Atherosclerosis

of the Cardiology Center Monzino (Milan, Italy), in order to evaluate the cardioprotective effect of wine (red and white) and nutraceutical formulation of resveratrol, the main active compound found in wine. Nevertheless, there were several problems approving the protocol by the Ethics Committee on the Monzino Cardiology Center. This led to a delay in the study. Currently, the protocol is being redesigned for next evaluation by Ethics Committee.

In addition, in my Ph.D. I have also investigated the qualitative and quantitative profile of the bioactive compounds of coffee by-product using UHPLC-High resolution mass spectrometry. Moreover, I studied the antioxidant and antifungal activity of this innovative source in order to use this by-product as an innovative source of high added-value ingredients.

On the other hand, I have investigated the possible associations between common genetic variants and surrogate markers of coronary artery disease. Therefore, in this part of my Ph.D. I investigated three single nucleotide polymorphisms (SNPs) in *PNPLA3* (rs738409), *TM6SF2* (rs10401969) and *GCKR* (rs1260326). These SNPs have been associated with non-alcoholic fatty liver disease (NAFLD). NAFLD and atherosclerosis-related cardiovascular diseases (CVD) share common metabolic pathways. The aim of this part was to investigate whether NAFLD-associated SNPs were also associated with markers of subclinical atherosclerosis, specifically carotid intima-media thickness (c-IMT) and the inter-adventitia common carotid artery diameter (ICCAD) in a European population, the IMPROVE study. IMPROVE study participants (n=3711) were free from clinically overt CVD but had at least three CVD risk factors. Linear regression with an additive genetic model, adjusted for age, sex and population stratification, were used to test for association of SNPs with c-IMT, ICCAD, alanine aminotransferase (ALT) and metabolic traits (body mass index BMI, glucose and lipids levels). In secondary analyses, the association of the SNPs with c-IMT and ICCAD was tested after stratification by ALT levels. No associations were observed between rs738409, rs1260326, rs10401969 and c-IMT or ICCAD. Rs738409-G and rs10401969-C were associated with ALT levels ($p < 0.001$). Moreover, rs738409-G was associated with higher BMI ($p = 0.045$). Rs1260326-T was associated with lower TG level (p

< 0.001) and with lower BMI (p = 0.028). In patients with ALT levels above 28 U/L (highest quartile), we observed an association between rs10401969-C and c-IMT measures of c-IMT_{max} and c-$IMT_{mean\text{-}max}$ (p = 0.018 and p = 0.021, respectively). In this population at high cardiovascular risk, NAFLD-associated genetic variants did not demonstrate significant effects on measures of sub-clinical atherosclerosis, except in the presence of high circulating ALT. These results support possible mediating function of impaired liver function on atherosclerosis.

LIST OF PUBLICATIONS

Paper I

Castaldo, L., Graziani, G., Gaspari, A., Izzo, L., Luz, C., Mañes, J., Rubino, M.;,Meca, G. and Ritieni A**. Study of the Chemical Components, Bioactivity and Antifungal Properties of the Coffee Husk**. 2018. *Journal of Food Research*, *7*(4), 43-54.

Paper II

Castaldo, L., Narváez, A., Izzo, L., Graziani, G., Gaspari, A., Di Minno, G. and Ritieni, A. **Red Wine Consumption and Cardiovascular Health**. 2019, *Molecules*, 24, 3626.

Paper III

Castaldo, L., Laguzzi F., Vigo, L., Baldassarre, D., Veglia, F., Tremoli, E., De Faire, U., Eriksson, P., Strawbridge, R.J., Smit, A. Aubrecht, J., Leander, K., Tuomainen,T.P., Pirro, M., Giral, P., Ritieni, A., Di Minno, G., Mälarstig, A., Gigante, B., on behalf of IMPROVE study group*. **Genetic variants in PNPLA3, GCKR and TM6SF2 genes associated with non-alcoholic fatty liver disease (NAFLD) do not associate with measures of sub-clinical atherosclerosis. Results from the IMPROVE study**. *Scientific reports,* Manuscript draft under review.

OTHER PUBLICATIONS:

1. Massa, M. Buono, S. Langellotti, A.L. Castaldo, L. Martello, A. Paduano, A. Sacchi, R. Fogliano. **Evaluation of anaerobic digestates from different feedstocks as growth media for Tetradesmus obliquus, Botryococcus braunii, Phaeodactylum tricornutum and Arthrospira maxima.** *New biotechnology*, 2017, 36, 8-16.

2. Rodríguez-Carrasco, Y., Castaldo, L., Graziani, G., Gaspari, A and Ritieni A. **Development of an UHPLC-Q-Orbitrap HRMS method for simultaneous determination of mycotoxins and isoflavones in soy-based burgers.** 2019, *Food Science and Technology*, 99: 34-42.

3. Castaldo, L. Graziani, G. Gaspari, A. Izzo, L. Tolosa, J. Rodríguez-Carrasco, Y. and Ritieni, A. **Target Analysis and Retrospective Screening of Multiple Mycotoxins in Pet Food Using UHPLC-Q-Orbitrap HRMS.** *Toxins,* 2019, 11, 434.

4. Izzo, L., Castaldo, L., Narváez, A., Graziani, G., Gaspari, A., Rodríguez-Carrasco, Y. and Ritieni, A. **Analysis of Phenolic Compounds in Commercial *Cannabis sativa* L. Inflorescences Using UHPLC-Q-Orbitrap HRMS.** *Molecules. 2020,* 25, 631;

5. Spadarella, G., Castaldo, L., Ventre, I. and Di Minno, A. **Bioequivalence and Haemophilia A in 2020: one size does not fit all.** *Journal Clinical Medicine,* Manuscript draft under review.

6. Narváez, A., Rodríguez-Carrasco, Y., Castaldo, L., Izzo, L. and Ritieni, A. **Ultra-high-performance liquid chromatography coupled to quadrupole Orbitrap high-resolution mass spectrometry for multi-residue analysis of mycotoxins and pesticides in botanical nutraceuticals.** *Toxins,* 2020, 12, 114.

7. Dini, I., Graziani, G., Fedele, F., Sicari, A., Vinale, F., Castaldo, L and Ritieni, A. **Metabolomic analysis by HRMS-Orbitrap and antioxidant activity of Olive Oil and olive leaves under different Trichoderma biostimulant treatment.** Manuscript draft ready for submission.

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CHAPTER 1 - Cardiovascular disease

1.1. Introduction

Coronary heart disease (CHD) and stroke are the leading causes of mortality, disability, and death in developed countries [1]. World Health Organization (WHO) estimated that 17.7 million people died each year from cardiovascular diseases (CVDs), which represent 31% of all deaths worldwide [2]. Most CHDs are due to atherosclerosis, a degenerative process of the arteries which is triggered by oxidative stress and chronic inflammatory status [3,4]. Smoking, arterial hypertension, hypercholesterolemia, diabetes mellitus, overweight/obesity, lack of physical activity are known to play a role in determining cardiovascular risk [5]. Moreover, meta-analyses of genome-wide association studies (GWAS) have shown that some genetic factors are related to the risk of CHD. The distribution of the CVD burden is not distributed equally across the globe and seems influenced by different factors including culture, risk factor prevalence, socioeconomic status, race/ ethnicity, and geography [6]. Preventive healthcare have been recommended as essential strategies to reduce CHD risk [7]. Generally, interventions that prevent the onset of the disease are defined as primary prevention, while secondary prevention is focused on reducing the progression of the disease by early diagnosis, treatment, and rehabilitation. Important strategies used in primary and secondary prevention include advice and therapeutic intervention for smoking cessation; to lower low-density lipoprotein cholesterol in plasma (C-LDL) as well as changing the lipid profile to within guideline levels; and controlling blood glucose levels; maintenance of normal blood pressure and providing information on unhealthy lifestyle risk factors, such as low physical activity, obesity, poor nutrition, alcohol consumption, and stress [8-10].

1.2. Genetic Factors

The etiology of cardiovascular disease is complex, and it is thought to can be influenced by a multiplicity of genetic and environmental factors [11]. Accumulating evidence suggests the existence of significant genetic influence on cardiovascular-related traits, particularly in early-onset forms [12]. Previous studies have identified several single-

nucleotide polymorphisms (SNPs) associated with known risk factors for cardiovascular disease such as blood pressure levels, fasting glucose, lipids, and obesity [13-16]. In fact, meta-analyses of genome-wide association study have identified more than 150 common variants SNPs associated with cardiovascular-related traits [17].

On the other hand, three genetic variants have been consistently associated with the risk of non-alcoholic fatty liver disease (NAFLD) and NAFLD hepatic complications. Rs738409 (C/G) in the Patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, rs10401969 (T/C) in the Transmembrane 6 superfamily member 2 (*TM6SF2*) gene and rs1260326 (C/T) in the glucokinase regulatory protein (*GCKR*) gene. In previous studies, these three genetic variants were not associated with the risk of CVD [18,19] and results of associations with cardiometabolic traits were conflicting [20]. At the same time, observational studies have shown an increased prevalence of subclinical atherosclerosis, measured by carotid intima-media thickness (c-IMT), reduced arterial distensibility and coronary artery calcium, in NAFLD patients [21,22]. Given the high prevalence of NAFLD and atherosclerosis, it is of importance to better understand the mechanisms underlying the link between NAFLD and atherosclerosis, in order to personalize cardiovascular risk assessment in the presence of NAFLD [23].

1.3. Diet and lifestyle

Cardiovascular diseases are associated with several abnormalities such as elevated blood pressure, high low-density lipoprotein (LDL), high triglyceride levels, high body mass index (BMI), and low high-density lipoprotein (HDL) [24]. Recent evidence suggests that maintaining a healthy lifestyle plays a key role in people's health status, including prevention and reduction of cardiovascular disease [25]. Increasing the levels of physical activity and smoking cessation turned out to yield good results for health [26,27]. Diet is also a pivotal constituent of a healthy lifestyle and scientific evidence have shown that diet rich in fruit, vegetables, cereals, legumes, nuts, moderate red wine consumption, and seeds are associated with low risk of cardiovascular disease and to the onset of major chronic degenerative diseases [28].

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CHAPTER 2 - The Mediterranean Diet

Mediterranean diet (MD) is the typical dietary pattern consumed around the Mediterranean Basin [1]. The MD drew the attention of medical professionals by proving protection against metabolic syndrome, type 2 diabetes, some kinds of cancers and other chronic diseases [2]. In the last decades, several epidemiological and experimental studies in different populations have established the health benefits associated with the adherence to the Mediterranean diet pattern, including a lower incidence of atherosclerotic clinical events [3,4]. Essentially, the traditional Mediterranean diet is characterized by a high intake of fruit, vegetables, cereals, legumes, nuts, and seeds; olive oil as the most common source of monounsaturated fatty acids; in low to moderate intake of fish, poultry and red meat, a low intake of dairy products (principally cheese and yogurt), light–moderate wine consumption; while coffee and fruit juices are the main non-alcoholic beverages [5,6].

2.1. Main Bioactive Compounds in the Mediterranean diet

Nutrition has key role in the prevention and reduction of a wide range of chronic diseases including cardiovascular diseases. Moreover, several compounds that are found in typical Mediterranean diet such as cereal, bean, fruit, and vegetables are involved in the protective effects against cardiovascular diseases. The above products are rich in beneficial substances include polyphenols, carotenoids, and dietary fibers [7].

Polyphenols are reactive metabolites widely distributed in plant-derived foods. The most important classes are phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) stilbenes (resveratrol), lignans, and flavonoids [8]. Flavonoids include (catechin, epicatechin, quercetin, kaempferol, anthocyanins, and procyanidins) [9]. All polyphenol compounds share a common basic structure characterized by the presence of several aromatic rings which considered to originate from the amino acid L-phenylalanine through the shikimic acid pathway [10]. Polyphenols are an important group of compounds occurring in fruits, herbs, vegetables, and beverages such as tea, red wine, and coffee are also good sources [11]. These secondary metabolites are fundamental for their contribution to the taste, color, and nutritional properties of plant-based foods [12]. Currently, scientific evidence supports an association between high consumption of plant-derived foods rich in polyphenol

compounds and a reduced risk of cardiovascular disease, including coronary heart disease and stroke [13]. The cardioprotective effects of polyphenols can be attributed to their capacity to react with reactive oxygen species (ROS) or to interfere with ROS production. Polyphenols are well recognized as potent antioxidant compounds and radical scavengers of peroxynitrite, a reactive substance produced by the reaction between nitric oxide (NO) and the superoxide anion [14]. Moreover, polyphenol compounds have attracted increasing attention also for their antimicrobial activity against a wide range of bacterial and fungal species [15].

Carotenoids are fat-soluble pigments abundant in fruits and vegetables and present to a lesser extent in dairy products, eggs, and some fish. In nature, the most common carotenoids are represented by α-carotene, β-carotene, lycopene, lutein and zeaxanthin [16]. Benefits from high fruits and vegetables consumption have been widely supported by the scientific literature and, in this line, carotenoids intake has been related to a lesser risk for CHD [14,17-19]. Carotenoids are known to possess potent antioxidant activity able to promote benefits to the cardiovascular system. This outcome could be explained by lipid-soluble nature of carotenoids able to be incorporated into lipoprotein particles during transport preventing diseases triggered by oxidative damage [20].

The so-called dietary fiber covers a wide variety of substances that belong to the broad family of carbohydrates including cellulose, hemicellulose, pectin and inulin. Dietary fiber have been found mostly in cereals, legumes, fruit, and vegetables [21]. These compounds are components of plant cell walls resistant to digestion by human enzymes but can be fermented by colonic microflora [22]. Dietary fiber is generally categorized in 2 major groups according to their solubility namely soluble and insoluble dietary fiber. Dietary fiber is known to have a wide range of important biological properties including cholesterol-lowering effect, delay gastric emptying, increase satiety, change in intracolonic pH and increase in fecal mass after anaerobic bacteria fermentation [23,24]. Scientific evidence has reported that these important compounds are responsible for reducing the risk of cardiovascular disease, obesity, and type 2 diabetes [25].

2.2. Main products in the Mediterranean Diet

Mediterranean diet is characterized by an abundance of plant foods (fruit, vegetables, cereals, legumes, nuts, and seeds), olive oil as the most common source of fat, minimally processed foods, fish and poultry consumed in low-to-moderate amounts, in general, modest amount of foods from animal sources including red meat. Moderate wine consumption; The main non-alcoholic beverages including coffee and fruit juices [26].

High intake of plant-origin foods such as fruit, vegetables, and cereals represent a hallmark of the Mediterranean diet. Plant-origin foods provide important nutrients as well as protective substances that contribute to health and general well-being [2].

Olive oil is the most common source of monounsaturated fatty acids in the Mediterranean diet, namely oleic acid. In addition to oleic acid, olive oil contains several bioactive compounds—polyphenols—with wide biological properties [27]. Hydroxytyrosol, tyrosol, and oleuropein are considered the most effective polyphenols found in olive oil [28]. Scientific evidence supports the crucial role of olive oil in the Mediterranean diet in the prevention of CHD and other chronic degenerative diseases due to its high amounts of monounsaturated fatty acids and polyphenolic fraction [29].

2.2.1. Red wine

Wine is a popular alcoholic beverage that has been consumed for hundreds of years [14]. Red wine is considered to be a very important component of the Mediterranean diet, usually consumed in moderate amounts during meals. In general, benefits from moderate red wine consumption have been widely supported by the scientific literature and, in this line, red wine intake has been related to a lesser risk for coronary heart disease [14]. Experimental studies and meta-analyses have mainly attributed this outcome to the presence in red wine of a great variety of polyphenolic compounds such as resveratrol, catechin, epicatechin, quercetin, and anthocyanin [14]. The mechanisms responsible for its putative cardioprotective effects would include changes in lipid profiles, reduction of insulin resistance, and decrease in oxidative stress of low-density lipoprotein cholesterol (LDL-C) [30]. However, moderate consumption of red wine is present in dietary habit with

recognized beneficial properties for cardiovascular health such as the Mediterranean Diet [31]. Resveratrol (3, 4, 5 trihydroxystilbene), a non-flavonoid polyphenolic compound, is considered the most effective wine compound with respect to the prevention of CHD because of its antioxidant properties [14]. The cardioprotective actions of resveratrol were initially tested in animal models. In these studies, resveratrol shown ability to protect cardiomyocytes against oxidative stress, autophagy, apoptosis, and cardiac fibrosis [32-34]. Recently, the actions of resveratrol have also been evaluated in several clinical trials. The results of these studies have shown positive effects such as antiatherogenic, anti-inflammatory, antihypertensive, antioxidant, hypolipidemic and insulin-sensitizing effects [35]. In the following paragraphs, the main bioactive components found in red wine are discussed

2.2.1.1. Non-flavonoid

Non-flavonoid phenolic components of grapes and wine include three main groups: stilbenes, benzoic and cinnamic acids [36]. The last two groups can be present as hydroxybenzoic and hydroxycinnamic acids. Benzoic acids are present in the grape as well as in oak wood and during the storage can migrate into the final wine [37].

2.2.1.1.1 Hydroxybenzoic acids

The hydroxybenzoic acids (HBAs) are phenolic metabolites with a general structure characterized by a C6-C1. In red wine, the most abundant HBAs are represented by p-hydroxybenzoic, gallic, vanillic, gentisic, syringic, salicylic and protocatechuic acids [38,39]. As reported, the different hydroxybenzoic acids may occur mainly in their free form [40]. Gallic acid is an important HBA present in high concentration in red wine. This compound is not found in grape and is probably formed by hydrolysis of tannins (condensed or hydrolysable) during the vesting period in oak wood [41]. The total amount of hydroxybenzoic acids in red wine is expected to range from undetectable to 218.0 mg/L, as shown in Table 1.

2.2.1.1.2. Hydroxycinnamic acids

Hydroxycinnamic acids are the major phenols in both grapes and wine [42,43]. Caffeic, coumaric and ferulic acids are some of the most important compounds in this polyphenol sub-class [44]. Natural hydroxycinnamic acids are not found in grape, appearing as their tartaric acid esters or diesters [45]. The main hydroxycinnamic acids of wine are p-coutaric, caftaric, and fertaric acids [46]. In nature, hydroxycinnamic acids exist in two isomeric forms but the trans-form is the most abundant in both grapes and wine [47]. Coutaric acid is mainly contained in the grape skin, while trans-caftaric and trans-fertaric acid are mainly present in the pulp [48]. The amount of hydroxycinnamic acids in different red wines was found to range from 60.0 to 334.0 mg/L, as shown in Table 1.

2.2.1.1.3. Resveratrol

Resveratrol (3, 4, 5 trihydroxystilbene), a non-flavonoid polyphenolic compound, is a common phytoalexin synthesized in response to the attack of bacteria and fungi [49]. It is present in more than 70 plant species, including berries, peanuts, cocoa and grape skin [50]. Resveratrol has two phenol rings linked to each other by a styrene double bond in its chemical structure [51]. It exists as cis- (Z) and trans- (E) isomers and both have been detected in wine at variable concentrations [52,53]. ranging from 0.1 to 7.0 mg/L and 0.7 to 6.5 mg/L, respectively [52-54]. Variability is mainly due to grape cultivar, geographic origin, oenological practices and wine type [54,55].

Several studies regarding the health benefits of trans-resveratrol in maintaining human health and preventing a wide variety of human diseases are available [56-61].

Magyar et al., [62] investigated the cardioprotective effect of low doses of resveratrol (10 mg/day) in 40 patients with stable coronary artery disease. The results showed that resveratrol intake displayed a significantly lowered LDL-cholesterol level, improved endothelial function and left ventricular diastolic function and protected against some unfavorable hemorheological changes.

Romain et al. [63] investigated the benefits of grapevine-shoot intake, a phenolic extract (Vineatrol 30) that contains considerable amounts of resveratrol (about 15,2%) on

cardiovascular system in hamsters fed with high-fat diet. Results showed that Vineatrol 30 was able to prevent aortic fatty streak deposition by increasing antioxidant and anti-inflammatory activity.

Fujitaka et al [64] investigated the effects of high dose (100 mg/day) of modified resveratrol, Longevinex, on the metabolic profile, inflammatory response and endothelial function in subjects with metabolic syndrome (MetS). Results showed that after 3 weeks, modified resveratrol specifically improved endothelial function in patients with MetS.

D'Archivio et al., [65] highlighted the possible influence of the matrix sugar content on resveratrol bioavailability, since it appears to be higher for the aglycone form in comparison to its glycosides in grape juice.

Wang et al., [66] demonstrated that trans-resveratrol is rapidly metabolized by glucuronidation and/or sulfation reactions as well as by hydrogenation of the aliphatic double bond, probably mediated by intestinal bacterial metabolism.

Regarding its bioactivity, trans-resveratrol may represent a promising dietary supplement, and currently is proposed as a therapeutic agent for many diseases [67-70].

2.2.1.2. Flavonoids

Flavonoids are plant-derived phytochemicals with antioxidant properties that account for over 85% of the phenolic components in red wine [71]. Flavonoids share a common basic structure of three-ring system with a central oxygen-containing ring (C ring) [72]. Substitution of the central pyran ring and the different oxidation degree are responsible for their chemical diversity [73]. Based on these differences, the flavonoids comprise a wide range of compounds such as flavones, flavonols, flavanols, anthocyanidins and anthocyanins[74]. Natural flavonoids can exist in its free form, aglycone or as glycoside condensed with the hydroxyl group of sugar such as glucose, galactose, rhamnose, glucuronide, xylose and arabinose [75]. They are widely distributed primarily in vegetables, seeds, nuts, spices, herbs, cocoa and grape skin. Total level of flavonoids can vary from 150.0 mg/L to 650.0 mg/L

In the previous few decades, a large amount of experimental and epidemiological investigations supports the protective effect of flavonoids on cardiovascular and chronic degenerative disease [76,77]. The cardioprotective effects ascribed to the flavonoids against atherosclerosis development might be due to the ability of flavonoids to improve the lipid profiles, reduce insulin resistance and oxidative stress especially for LDL-C, as suggested by several studies [78-81].

2.2.1.2.1. Flavones

Flavones display three functional groups such as hydroxy, carbonyl and conjugated double bonds between C2 and C3 in the flavonoid skeleton. These compounds were found in grape skin and wine in both aglycones and glycosides forms. In grapes, luteolinis the only flavone present, in levels ranging from 0.2 to 1.0 mg/L [82-84]. Flavones are known to play a wide range of important biological properties including antioxidant, anti-inflammatory and anti-tumor activity and are also used as supplements in CHD and neurodegenerative disorders [85].

Li et al., [86] investigated the cardioprotective properties of total flavones of Choerospondias axillaries intake (range from 75.0 to 300.0 mg/kg) in rat model of ischemia/reperfusion (I/R). Results indicated that flavones intake was able to reduce heart pathologic lesion and improved cardiac function by increasing antioxidative activities.

2.2.1.2.2. Flavan-3-ols

Flavanols are benzopyrans that include simple monomeric and polymeric forms which are contained in noteworthy concentrations in red wine. The most important flavanols in grape are catechin and its enantiomer epicatechin, biosynthetic precursors of proanthocyanidins, which are responsible for the structure and astringency of wine [71]. The catechin and epicatechin levels in red wine were reported to range from –50-120 mg/L [48,83,84]. Moreover, catechin levels rose up to 1000 mg/L, especially in selected old red wines [65].

Several studies indicate that flavan-3-ols may exert cardioprotective actions, which might be due to the ability of flavan-3-ols to increase nitric oxide (NO) bioactivity and decrease

superoxide production [87]. Ramirez-Sanchez et al., [88] studied the tentative effect of epicatechin on stimulating endothelial nitric oxide synthase (eNOS), an enzyme that generates the vasoprotective molecule NO in human coronary endothelial cells. The results showed that acute administration of epicatechin may induce eNOS activation in endothelial cells.

2.2.1.2.3. Flavonols

Flavonols are often characterized by a hydroxyl group in C3 (3-hydroxyflavones), thus being often named 3-hydroxyflavones. Flavonols found in red wine include aglycons such as myricetin, quercetin, kaempferol and rutin and their respective glycosides which can be glucosides, glucuronides, galactosides and diglycosides. These main flavonols can be found at a total concentration ranging from 12.7 to 130.0 mg/L [82-84]. These compounds are known to play a wide range of biological activities and are considered the main active compounds within the flavonoids group [89].

Annapurna et al., [90] investigated the cardioprotective actions of quercetin and rutin (range from 5 to 10 mg/kg) in both normal and diabetic rats. Quercetin and rutin intake showed a protective effect in I/R-induced myocardial infarction in normal as well as diabetic rats. Therefore, it was concluded that quercetin and rutin protection could be due an increased in antioxidant activity.

2.2.1.2.4. Anthocyanins

Anthocyanin is the glycosylated form of the so-called anthocyanidin. The general molecular structure of anthocyanis is based on the flavilium or 2-phenylbenzopyrilium cation, with hydroxyl and methoxyl groups present at different positions of the basic structure. The great variety of anthocyanins found in nature depends on the number and position of hydroxylated groups, and the number and the position of conjugated sugar and acyl moieties in their structure [48]. Anthocyanins, namely malvidin, cyanidin, delphinidin, petunidin, peonidin and pelargonidin, have been detected in both grape and red wine in levels ranging from 90 to 400 ng/mL [91-93]. Anthocyanins are usually found as the glucosyde form but rhamnose, xylose and galactose have also been observed as common

sugar moieties. Anthocyanins are also found with acyl substituents bound to sugars, aliphatic acids, cinnamic acids and pigments with aliphatic as well as aromatic substituents.

An ever-expanding amount of scientific evidence supports a protective role of habitual dietary intake of anthocyanins against age-related chronic conditions including CHD [94-98].

McCullough et al., [99] investigated in a cohort study of 98,469 participants the association between flavonoid intake and risk of death from CHD. The results confirm that anthocyanidins and proanthocyanidins were associated with lower CHD risk concluding that food sources rich in flavonoids should be considered for CHD risk reduction.

Similarly, Cassidy et al., [100] evaluated the relationship between intakes of different flavonoid classes and multiple inflammatory biomarkers assessed in combination as an inflammation score (IS) in a cohort study of 2,375 participants. The results showed that higher intakes of anthocyanins were inversely associated with reductions of IS score (73%).

Huang et al., [101] studied the most common red wine anthocyanins: malvidin-3-glucoside and malvidin-3-galactoside, to evaluate their effect on inflammatory response in endothelial cells. The anti-inflammatory effect was showed by malvidin-3-glucoside as well as malvidin-3-galactoside, and also a synergistic effect was found.

Anthocyanins represent promising molecules for the development of therapeutic agents to prevent chronic inflammation in many diseases.

2.2.1.2.5. Tannins

Tannins are another important subgroup of phenols of red wine that contribute to astringency and are also implicated in reactions that lead to browning, especially in white wines. They can be classified into two main classes, namely hydrolyzable and condensed tannins. The latter forms, polymers of flavan-3-ol without sugar residues, are predominant in grape and wine while hydrolyzable tannins are naturally present in oak barrels. Total content of tannins ranges from 1.1 to 3.4 g/L [93,102]. In vivo studies carried out in animals and humans suggest that tannins possess potent antioxidant, anti-inflammatory and radical scavenging activity able to promote benefits to human health [103,104].

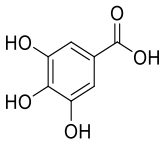
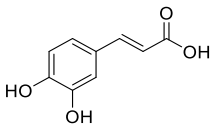
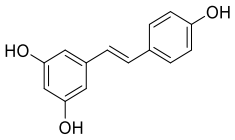
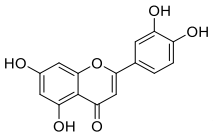
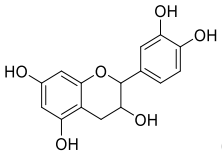
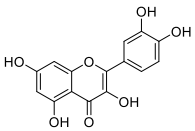
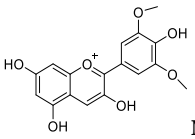
2.2.1.2.5.1. Hydrolyzable tannins

The basic unit of hydrolyzable tannins are gallic and ellagic acids usually esterified with glucose or related sugars. They are more susceptible to be hydrolyzed than condensed tannins by pH changes and enzymatic or non-enzymatic processes. Based on the type of phenolic acid present in their structure, hydrolyzable tannins can be divided in gallotannins and ellagitannins, usually found as a mixture in plant sources. The hydrolyzable tannins are not found in grapes but they are extracted from barrels wood during wine aging, which is thus proposed in literature as a marker of maturity for this type of wines. Due to differences on the aging process and the type of wood, the final content of hydrolyzable tannins can strongly vary from 0.4 to 50.0 mg/L [93,105-107].

2.2.1.2.5.2. Condensed tannins

Condensed tannins (or proanthocyanidins) are oligomers flavonoid that contribute to astringency in wine. Depolymerization of the condensed tannins under oxidative condition leads to the formation of proanthocyanidins. Flavan-3-ols and their precursor flavan-3,4-diols (leucoanthocyanidin) are the main components of condensed tannins in nature and can be found at concentrations levels from 1.2 to 3.3 g/L [93,102].

Table 1. Main representative groups of polyphenols present in red wine, ref [92,108-113]

Group	Subclass	Main representatives	Range in mg/L	Characteristic structure
Non-flavonoid	**Hydroxybenzoic acids**	**Gallic, ellagic, parahydroxybenzoic, protocatechuic, vanillic, syringic acids**	**0-218.0**	 **Gallic acid**
	Hydroxycinnamic acids	**Coutaric, caftaric, and fertaric acids**	**60.0-334.0**	 **Caffeic acid**
	Stilbenes	**Resveratrol**	**0.1-7.0**	 **Resveratrol**
Flavonoids	**Flavones**	**Luteolin**	**0.2-1.0**	 **Luteolin**
	Flavan-3-ols	**Catechin and epicatechin**	**50.0-120.0**	 **Catechin**
	Flavonols	**Myricetin, quercetin, kaempferol and rutin**	**12.7-130.0**	 **Quercetin**
	Anthocyanins	**Malvidin, cyanidin, peonidin, delphinidin, pelargonidin, petunidin**	**90.0-400.0**	 **Malvidin**

2.2.2. Coffee

Coffee is the most preferred morning beverage throughout the world due to its pleasant flavor and stimulating properties. Widespread use of coffee has been reported among the Mediterranean populations [6]. Traditionally, coffee consumption was discouraged due to a risky global profile, but it is progressively moving to a less negative position, also due to its better-known bioactive compounds content [114]. Although several case-control studies reported a higher risk of myocardial infarction in populations with high coffee intakes [115-118], scientific evidence supports an inverse relationship between moderate coffee consumption and the risk of CHD, but this outcome is under debate [119-122]. In coffee, a wide range of active compounds have been found, that may have beneficial effects in CVD protection [123]. Coffee is considered as a complex chemical mixture of compounds including carbohydrates, fibers, and lipids. In addition, coffee is also rich in other bioactive compounds such as minerals, alkaloids, polyphenols, diterpenes, and vitamins [124]. Among alkaloids, caffeine is considered the major and active ingredient found in coffee. Some other components of coffee seem to play an important role in cardiovascular protection, i.e. chlorogenic and caffeic acid [114]. These compounds are recognized as powerful antioxidants with a wide array of health benefits effects such as improves nitric oxide bioavailability, reducing LDL oxidative susceptibility, protection against diabetes mellitus and endothelial dysfunction and, increasing plasma levels of glutathione [123,125-127].

2.2.2.1. Coffee by-product

In 2018, the total production of coffee was of 169 million of 60 kg bags, and the Brazil is largest producer [128]. The coffee industry generates a huge amount of wastes and by-products, which are often poured into the environment, contributing to soil and water pollution [128]. Particularly, caffeine (12 g/kg), tannins (63 g/kg) and polyphenols have been recognized as the main substances involved in this environmental problem [129-131].

Coffee husk is a fibrous mucilaginous material obtained during the processing of coffee cherries [132], representing the main residue in the production of coffee. Several

studies have proposed alternative uses for the coffee husk such as silage, aerobic composting, animal feed, vermiculture, production of biogas, vinegar, biopesticides, enzymes, single-cell protein and probiotics [133]. However, this residue is currently returned to the soil or burned, which could be a serious threat to environmental pollution.

Recently, the use of by-products as a resource of natural food ingredients with high nutritional value has gained increasing interest because their recovery may be economically attractive [134].

In this context, coffee husks could be considered an innovative resource of compounds for the food and pharmaceutical industries, in fact, they contain high levels of important phytochemicals such as caffeine, dietary fiber and several compounds with antioxidant activity [135,136].

Furthermore, several findings support a remarkable antifungal activity exert by caffeic and caffeoylquinic acid [137,138]. Scientific evidence showed that the above compounds were able to inhibit the fungal growth by blocking the glucan synthase in the cell wall [138].

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CHAPTER 3 - Moderate alcohol consumption

Coronary heart disease (CHD) and stroke are the leading causes of mortality, disability, and death in developed countries [1]. Most CHDs are due to atherosclerosis, a degenerative process of the arteries which is triggered by oxidative stress and chronic inflammatory status [2,3]. Smoking, arterial hypertension, hypercholesterolemia, diabetes mellitus, overweight/obesity, lack of physical activity and genetic factors are known to play a role in determining cardiovascular risk [4].

Although excessive alcohol intake is associated with the development of chronic diseases and other serious problems, a wealth of data from scientific evidence support an inverse relationship between moderate alcohol consumption and the risk of CHD [5]. Moderate alcohol consumption is defined in the Dietary Guidelines for Americans 2015-2020 as up to one unit of alcohol per day for women and up to two units of alcohol per day for men [6].

Several studies provide evidence that light-moderate alcohol consumption is associated with a higher level of high-density lipoprotein cholesterol (HDL-C), lower incidence of type-2 diabetes (T2D) and a reduction of lipid oxidative stress [7-10]. Such epidemiological studies have supported that red wine consumption is more CHD-preventative in comparison to the intake of other alcoholic beverages [11]. It is uncertain whether the apparent beneficial properties for health attributed to the consumption of red wine are due solely to the presence of alcohol or in concert with antioxidant compounds other than alcohol present in red wine [12] . In addition to alcohol, red wine contains a wide range of active compounds, polyphenols, with antioxidant and anti-inflammatory properties that could contribute to protection from atherosclerotic pathologies [13].

The light-moderate drinking of red wine has been proposed as a possible explanation for the epidemiological phenomenon “French Paradox”[14], which says that France population shows a relatively lower CHD incidence/mortality rate compared with other Western countries, despite their diets contain higher amounts of total fat and saturated fatty acid.

3.1. Putative mechanisms of action

A large number of epidemiological studies and meta-analysis have consistently shown that light-moderate drinking of red wine has a protective effect against CHD [15,16]. Several plausible underlying biological mechanisms have been postulated to explain the beneficial effects of light-moderate red wine consumption as well as phenolic compounds contained in red wine on the development of CHD and atherosclerosis [17,18]. Understanding the mechanisms by which light-moderate drinking of red wine improves the cardiovascular functions is crucial for the treatment and prevention of CHD.

3.1.1. Lipid profile

Epidemiological studies have consistently shown associations between hyperlipidemia and risk of developing CHD, obesity and T2D. Light-moderate drinking of alcohol, especially red wine, is associated with beneficial changes in lipid homeostasis as shown by the results of several clinical trials and meta-analyses.

Da Luz et al. evaluated the association between moderate red wine consumption and changes in HDL-C levels and in the coronary vasculature. The study included 205 subjects (101 and 104 drinkers and abstainers, respectively) aged around 60 years. Red wine drinkers displayed an HDL-C level significantly higher than the abstainers and a protective effect on coronary lesions.

Marques-Vidal et al. [19] had similar results in a large cohort. The study included 5,409 subjects categorized as abstainers (0 drinks/week, n=1463) moderate alcohol drinkers (1–13 drinks/week, n=2972), high alcohol drinkers (14–34 drinks/week, n=867) and very high alcohol drinkers (≥35 drinks/week, n=107). The results showed that alcohol consumption increased HDL-C levels rather than polyphenols in light moderate drinkers and partly explained the cardioprotective effect displayed from alcohol consumption.

These results were corroborated by Park et al., [20] who investigated the benefits of moderate consumption of alcohol in hypertensive population with a focus on the lipid profile. The study included 2,014 participants, aged 20–69 years. The results showed that alcohol consumption was negatively associated with prevalence of low HDL-C whereas the amount of high triglycerides increased with a higher alcohol intake.

Magnus et al., [21] investigated in a cohort study of 149,729 participants the hypothesis that moderate alcohol intake exerts its cardioprotective function by increasing HDL-C levels. The results showed that increasing HDL-C level is not a relevant mechanism by which ethanol exerts its cardioprotective effect.

A recent meta-analysis [22] examined the effect of moderate alcohol consumption on lipid profile, concluding that alcohol consumption significantly increased levels of HDL-C, apolipoprotein A1, and adiponectin. Moreover, the results showed that alcohol did not significantly changed triglycerides levels.

The findings above reported support an increase in the plasma HDL-C concentration level as a result of chronic moderate alcohol consumption. Higher HDL has been consistently observed in cohort studies regarding alcohol consumption and attributed to alcohol itself. In fact, alcohol appears to be responsible for the increase of plasma HDL rather than polyphenols in wine light-moderate drinkers. Table 2 summarized these studies. The studies that showed positive changes as a consequence of light-moderate drinking of alcohol on lipid metabolism, except for HDL-C, were inconclusive especially in cross-sectional studies where some outcome had a longer half-life time than those analyzed [23]. On the other hand, the effect of light-moderate drinking of alcohol, including for red wine, on triglycerides, LDL, very low-density lipoprotein (VLDL), and lipoprotein(a) is unclear and it is still under debate.

Table 2. Summary studies assessing the relationship between moderate alcohol consumption and lipid profile.

Study model	Outcome(s)	Study characteristics	Main findings	Ref.
Drinkers vs. absteiners	HDL-C level and changes in the coronary vasculature	Benefits of moderate consumption of red wine	Drinkers displayed a significantly higher HDL-C level and a protective effect on coronary lesions	[24]
Men	HDL-C level	The study included 5,409 subjects categorized in abstainers, moderate, high and, very high alcohol drinkers	Alcohol consumption increased HDL-C levels	[19]
Hypertensive population	Lipid profile	Benefits of moderate consumption of alcohol	Alcohol consumption was negatively associated with low HDL-C	[20]
Cohort study	Cardioprotective function	Increasing HDL-C levels as the mechanism used by alcohol to exert its cardioprotective function.	Increasing HDL-C levels is not a relevant mechanism by which ethanol exerts its cardioprotective effect	[21]
Meta-analysis	Lipid profile	Effect of moderate alcohol consumption on lipid profile	Alcohol consumption increased levels of HDL-C, apolipoprotein A1, and adiponectin	[22]

3.1.2. Glucose metabolism

The cardioprotective effect of red wine consumption may partly be explained by the association between moderate red wine consumption and a lower incidence of T2D. Chiva-Blanch et al. [23] showed that moderate consumption of red wine (30 g of alcohol per day) and dealcoholized red wine decreased homeostasis model assessment of insulin resistance (HOMA-IR) and plasma insulin after 4 week in 67 men having high cardiovascular risk. These results suggest that the beneficial effects could be mediated due

to antioxidant compounds present in red wine and alcohol did not seem fundamental to exhibit such effects.

Brasnyo´ et al., [25] investigated the effects of low doses of resveratrol (2 x 5 mg/day) on glucose metabolism in nineteen T2D patients. After 4 weeks, resveratrol improved insulin resistance and increased phosphorylation of protein kinase B (AKT), which play a key role in insulin signaling interfering directly in glycogen synthesis. Therefore, it was concluded that resveratrol might be used for medicinal application.

Da Luz et al. [26] evaluated the association of moderate red wine consumption with changes in glucose levels and diabetes. The study included 205 subjects (101 and 104 drinkers and abstainers, respectively) aged around 60 years. Red wine drinkers displayed a significantly lower incidence of diabetes and lower glucose levels compared to abstainers.

A recent meta-analysis [27] of 20 cohort studies comprising 477,200 subjects confirms the U-shaped relationship between moderate amounts of alcohol consumption and risk of incident T2D for both sexes compared with lifetime abstainers. The amount of alcohol that showed higher protective effect was 22g/day for men and 24g/day for woman, while became deleterious over 60 and 50 g/day alcohol in men and women, respectively. Therefore, in this study the amount of polyphenols was not considered and the protective effect has been attributed to alcohol.

The cardioprotective effect of moderate alcohol consumption were corroborated by Mekary et al., [28] through a large prospective study including 81,827 participants on the impact of alcohol consumption and the positive association between glycemic load (GL) and the incidence of T2D. They found that higher alcohol intake (≥15 g/day) attenuates the effect of GL on T2D incidence.

Ramadori et al. [29] conducted a study with diet-induced obese and diabetic mice to evaluate the impact of approximately 79.2 ng/day intracerebroventricular infusion of resveratrol on glucose metabolism. The results showed a normalized hyperglycemia and improved hyperinsulinemia by activating SIRT 1 expressed in brain. Table 3 summarizes these studies.

These findings suggest that a light to moderate alcohol consumption, especially red wine, may be associated with improved insulin resistance and with lower incidence of

diabetes providing another potential explanation for the reduction in cardiovascular events associated with moderate alcohol intake.

Table 3. Summary studies assessing the impact of red wine consumption on glucose metabolism.

Study model	Outcome(s)	Study characteristics	Main findings	Ref.
Men	Glucose metabolism	Light-moderate alcohol consumption (red win, dealcoholized red wine, and gin)	Dealcoholized red wine decreased plasma insulin and HOMA-IR	[23]
Drinkers vs. absteiners	Glucose level and diabete	Benefits of moderate consumption of red win	Drinkers showed a lower incidence of diabetes and lower glucose levels compared to abstainers	[24]
Meta-analysis	Risk of incident T2D	Effect of moderate alcohol consumption on incidence of T2D	Light-moderate alcohol consumption decreased incidence of T2D	[27]
Cohort study	GL and the incidence of T2D.	Impact of alcohol consumption and the positive association between GL and T2D	Higher alcohol intake (≥15 g/day) attenuates the effect of GL on T2D incidence.	[28]
Obese and diabetic mice	Glucose metabolism	Impact of intracerebroventricular infusion of resveratrol on glucose metabolism	Normalized hyperglycemia and improved hyperinsulinemia mediated by activating SIRT 1 expressed in brain	[29]
T2D	Glucose metabolism	Effect of resveratrol on glucose metabolism	Resveratrol improved insulin resistance and increased AKT phosphorylation	[30]

3.1.3. Oxidative stress

Many important cardioprotective effects of wine polyphenols can be attributed to their capacity to react with reactive nitrogen species (RNS) or to interfere with RNS production. Wine polyphenols are well recognized as potent antioxidant compounds and radical

scavengers of peroxynitrite, a reactive substance produced by the reaction between NO and superoxide anion [31]. Several observational studies have indicated that the oxidation of LDL plays a crucial role in the initiation and progression of atherosclerosis [32]. The inverse association between red wine consumption and mortality from cardiovascular diseases may be explained by the capacity of red wine polyphenols to reduce LDL oxidation [33]. These findings showed that the beneficial effects on LDL oxidation could be exerted by a higher antioxidant activity of red wine compared to beverage with no polyphenolic content.

Estruch et al., [34] studied the benefits of moderate consumption of red wine compared to gin, alcoholic beverage without polyphenolic content, on lag phase time of LDL-particles. The study was conducted with forty healthy men aged 38 years, concluding that after 28 days of moderate consumption of red wine (30g/day) the lag phase time of LDL oxidation increased until 11.0 min compared to the same gin intake.

Similarly Chiva et al., [35] checked, in sixty-seven subjects at high cardiovascular risk, the effects of alcoholic and dealcoholized red wine and gin intake on plasma NO and blood pressure. After 4-week, the result showed that dealcoholized red wine was able to decrease systolic and diastolic blood pressure and increase plasma NO concentration.

Egert et al., [36] evaluated changes in markers of oxidative stress following quercetin intake in ninety-three overweight or obese subjects aged 25–65 years. Quercetin is an important flavonoid present in high amount in red wine and grape. After 6-week, 150 mg/day of quercetin supplementation significantly decreased plasma concentrations of oxidized LDL. Therefore, it was concluded that quercetin may provide protection against CHD.

Bulut et al. [37] evaluated in ten healthy males the effects of alcoholic (red wine and liquor) and non-alcoholic (mineral water and Coke) beverages consumed during a high-fat meal once a week for four weeks on circulating microparticles (MPs). Volunteers in the red wine and liquor group consumed the same amount of alcohol. The results indicated that the number of MPs increased after a single high-fat meal (increase by about 62%) but red wine consumption decreased these negative effects (increase by about 5%). Table 4 summarizes these studies.

These findings support that moderate red wine consumption may act as an antioxidant by decreasing oxidized LDL plasma levels and increasing plasma NO concentration. Scientific evidence indicates that oxidized LDL may play a major role to the onset and progression of oxidative stress-associated diseases, such as atherosclerosis [38]. Moreover, increased oxidized LDL plasma level was predictive for future myocardial infarction [39]. Nevertheless, the beneficial effects of moderate red wine consumption on reduce LDL oxidation seem to be independent on its alcohol component.

Table 4. Summary studies assessing the impact of red wine consumption on oxidative stress.

Study model	Outcome(s)	Study characteristics	Main findings	Ref.
Men	Lag phase time of LDL-particles	Benefits of moderate consumption of red win, dealcoholized red wine, and gin.	Red wine consumption showed a lag phase time of LDL oxidation increased until 11.0 min	[34]
High cardiovascular risk	Plasma nitric oxide, sistolic and diastolic pressure	Effects of alcoholic and dealcoholized red wine and gin intake on plasma NO and blood pressure	Dealcoholized red wine reduced systolic and diastolic blood pressure	[35]
Overweight or obese subjects	Concentrations of oxidized LDL	Changes in markers of oxidative stress following 150 mg/day of quercetin supplementation	Quercetin significantly decreased plasma concentrations of oxidized LDL	[36]
Men	Circulating microparticles	Benefits of moderate consumption of red win, dealcoholized red wine, and gin during a high-fat meal	Red wine consumption decreased circulating microparticles	[37]

3.2. Conclusions

In the last decades, several human and animal studies have indicated that moderate red wine consumption has beneficial effects on health. Phenolic compounds present in red wine have shown antioxidant and anti-inflammatory properties, being able to reduce insulin resistance and to exert a beneficial effect by decreasing oxidative stress. As a consequence, a clear effect on the reduction of risk factors and the prevention of cardiovascular diseases have been observed. Different mechanisms are involved in the cardioprotective effects of moderate red wine consumption: while alcohol appears to be responsible for increasing plasma HDL-C, the polyphenolic component may play a key role in the reduction of T2D incidence and LDL oxidation. In light of these considerations, a moderate intake of red wine may produce cardioprotective effects. However, more in-depth knowledge is required in order to understand the molecular basis of the potential mechanisms involved.

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CHAPTER 4 - The IMPROVE Study

The IMPROVE study is a European prospective, multicentre, observational, longitudinal cohort study, performed in seven centers in five European countries: Finland, France, Italy, the Netherlands, and Sweden. The study enrolled 3,711 subjects (age 54 to 79 years) in both men and women with at least 3 vascular risk factors (VRFs) and free from cardiovascular as well as cerebrovascular disease diagnosis before enrolment. Each participant provided two different informed consents; one for general participation in the study and one for genotyping. Moreover, at subjects were also asked to fill in a questionnaire. The protocol was designed for a study duration of 36 months, baseline measurements were performed between March 2004 and April 2005 and repeated after 15 months and 30 months.

4.1 Study objectives

The primary objective of the IMPROVE study was to evaluate the association between carotid Intima–media thickness (C-IMT) and interadventitia common carotid artery diameter (ICCAD) with future vascular events (myocardial infarction, cardiovascular death, stroke, or any intervention in the carotid, coronary, or peripheral arterial districts occurring from the 15th to the 36th month of follow-up). C-IMT and ICCAD, measured by high resolution B-mode ultrasound, are non-invasive marker of subclinical atherosclerosis identified as predictors of cardiovascular events (CVEs). A wide range of exposures, including genetic markers and other biological markers analysed in blood samples, are studied in order to identify the major determinants of C-IMT and ICCAD measurements in this European population cohort.

4.2 C-IMT and ICCAD measurements

Measurement of C-IMT and ICCAD with B-mode ultrasound are a non-invasive, sensitive, and reproducible technique widely used as markers of subclinical vascular disease and for evaluating CVD risk [1]. An even expanding amount of studies demonstrated that C-IMT and ICCAD were significantly associated with risk for myocardial infarction, stroke, death from coronary heart disease, or a combination of these events [1-4]. Moreover, these

ultrasound variables, in some studies, were independent of traditional risk factors to predict future CVD events. Currently, C-IMT values less than or equal to the 25th percentile is considered lower CVD risk. While values in the 25th to 75th percentile suggestive of unchanged CVD risk. However, values greater than or equal to the 75th percentile are considered high and indicative of increased CVD risk [5,6].

4.3. Main outcomes in IMPROVE study

In the first report of the IMPROVE study, C-IMT measurements showed an independent association with geographical gradient. Moreover, the geographical gradient for C-IMT has predicted CHD mortality even better than the 10-year score risk. The north-to-south geographical gradient remained strongly associated with C-IMT measurements even after adjustment of established vascular risk factors. These findings suggest that other mechanisms play a role. The authors concluded that other unknown contributory mechanisms namely genetic, nutritional, or environmental factors may be important in the genesis of this geographical gradient.

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GENERAL OBJECTIVE

Cardiovascular disease is influenced by a multiplicity of genetic and environmental factors. Diet is a pivotal constituent of a healthy lifestyle. In the Mediterranean basin, red wine and coffee are consumed on a regular basis. The aim of my thesis work was to develop a method for the simultaneous investigation of polyphenol compounds (*n*=17) in red wine in order to obtain useful data to use *in vivo* study. I have also evaluated the biological activities of coffee by-product in order to use this by-product as an innovative source of high added-value ingredients. On the other hand, I have assessed the possible associations between common genetic variants and surrogate markers of coronary artery disease.

Specific objectives

1. Optimization of spectrometric (Q-Orbitrap HRMS) and chromatographic (UHPLC) conditions for the simultaneous determination of polyphenol compounds (*n*=17) in red wine samples. Moreover, determination of total phenol content in red wine extract as well as antioxidant activity through the ABTS and DPPH assays. These preliminary results may become useful to develop a future *in vivo* study in order to demonstrate the potential health benefits of moderate red wine consumption.

2. Evaluate the cardioprotective effect of moderate red wine consumption in the Italian adult population: *in vivo* study.

3. Determination of bioactive compounds in coffee husk (raw material and infusion) using UHPLC-High resolution mass spectrometry and evaluation of biological activities including antioxidants and antifungal in order to use this by-product as an innovative source of high added-value ingredients.

4. Evaluation of possible associations between rs738409, rs10401969 and, rs1260326 with measures of carotid subclinical atherosclerosis in a large European cohort consisting of individuals at high risk of cardiovascular events but free of clinical CVD manifestation. In the same study population, I have also assessed the association of these three SNPs with metabolic traits.

SPECIFIC OBJECTIVE 1

The aim of this part of my thesis work was a) to develop a method for simultaneous investigation of polyphenol compounds (*n*=17) in red wine samples (*Vitis vinifera* L) using ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS); b) Determination of total phenol content in red wine extract c) antioxidant activity through the ABTS and DPPH assays. These preliminary results may become useful to develop a future *in vivo* study in order to demonstrate the potential health benefits of moderate red wine consumption.

MATERIALS AND METHODS

Standards and samples

A total of fifteen red wines samples, made from Aglianico grape variety (*Vitis vinifera* L.), were donated from company located in Campania region, Southern Italy. Wines samples were collected into 2 mL Eppendorf and stored at -80°C before to analysis.

Chemicals were from Sigma-Aldrich (St. Louis, MI, USA) and solvents, ethanol, methanol, and water (LC-MS grade) were purchased from Carlo Erba reagents (Naples, Italy).

Sample preparation

All analyses were performed on samples diluted properly with ethanol and filtered through 0.45 μm nylon filter and stored at -18°C until analysis.

Determination of the Antioxidant Activity

The scavenger activity of the samples against free radicals was determined spectrophotometrically, by two different assays as previously reported [1,2]. The antioxidant capacity based on the DPPH and ABTS free radical scavenging ability of the extract was expressed as mmol Trolox equivalents (TEAC) per liter of sample and all determinations were performed in triplicate.

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity

The ABTS assay was conducted based on the method reported by Re et al., [1] with minor modifications. Briefly, 44 μL of 2.45 mM aqueous potassium persulfate was added to 2.5 mL of aqueous ABTS (7 mM), following 16 h of incubation at room temperature in the dark. The ABTS solution was diluted with ethanol to an absorbance value of 0.75 (±0.02) at 734 nm to obtain an ABTS radical working solution. Then, 100 μL of sample was added to 1 mL of ABTS radical working solution. The absorbance was monitored after 3 minutes at 734 nm. The results were expressed as μmol Trolox equivalents (TE) per liter of sample.

2.1.4.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay was modified using methods by Brand-Williams et al., [2]. Briefly, methanolic DPPH (4 mg in 10 mL) was diluted with methanol to an absorbance value of 0.90 (±0.02) at 517 nm to obtain an DPPH radical working solution. Then, 200 μL of sample was added to 1 mL of DPPH radical working solution. The absorbance was monitored after 10 minutes at 517 nm. The results were expressed as μmol Trolox equivalents (TE) per liter of sample.

Total phenolic content (TPC)

Total phenolic content was performed according to the Folin-Ciocalteu method [3] with slight modifications. Briefly, 125 μL of extract sample was diluted in 500 μL of deionized water, then 125 μL of the Folin-Ciocalteu reagent was added to the mixture, followed by 6 min of incubation at room temperature. Afterward, 1.25 mL of 7.5% of sodium carbonate solution and 1 mL of deionized water were added in the mixture. The absorbance at 760 nm after 90 min of incubation in the dark was measured. The results were expressed as milligrams gallic acid equivalence (g GAE)/L of sample.

UHPLC-High Resolution Mass Spectrometry (HRMS-Orbitrap) Analysis of Polyphenolic Compounds in red wine extract samples

Polyphenolic profile was analyzed by Ultra High Pressure Liquid Chromatograph (UHPLC, Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, Ma, USA) equipped with a degassing system, a Quaternary UHPLC pump working at 1250 bar, and an autosampler device. Chromatographic separation of polyphenols was performed with a thermostated (T = 25 °C) Kinetex 2.6 μm Biphenyl (100 x 2.1 mm, Phenomenex) column. Injection volume was of 2 μL. The mobile phase consisted of a binary solution: water (phase A) and methanol (phase B), both mobile phases contained 0.1% of formic acid. A gradient elution program was applied as follows: an initial 5% B, increased to 30% B in 1.3 min and anew to 100% B in 8 min. The gradient was held for 2 min at 100% B, and reduced to 5% B in 2 min. The flow rate of 0.2 mL/min. Afterward, the gradient switched back to 5% in 2 min, and another 2 min for column re-equilibration at 5%. The UHPLC system was coupled to a Q-Exactive Orbitrap mass spectrometer (UHPLC, Thermo Fischer Scientific, Waltham, Ma, USA). An ESI source (Thermo Fisher Scientific, Waltham, MA, USA) was operated in negative ion mode (ESI^-) setting two scan events (Full ion MS and All ion fragmentation, AIF) for all compounds of interest. Full scan data were acquired at a resolving power of 35,000 FWHM (full width at half maximum). Ion source parameters were: spray voltage -2.8 kV, capillary temperature 310 °C, S-lens RF level 50, sheath gas pressure (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, auxiliary gas heater temperature 350 °C. Value for automatic gain control (AGC) target was set at 3×10^6 , a scan range of *m*/*z* 90–1000 was chosen and the injection time was set to 200 ms. Scan-rate was set at 2 scans/s. Data analysis and processing has been performer using the Xcalibur software, v. 3.1.66.10.

For the scan event of AIF, the parameters were set as follows: mass resolving power of 17,500 FWHM at 200 ms; scan time = 0.10 s. The collision energy was varied in the range of 10–45 eV to obtain representative product ion spectra. Data processing were performed by the Quan/Qual Browser Xcalibur software, v. 3.1.66.10 (Xcalibur, Thermo Fisher Scientific). Detection was based on calculated exact mass with a mass error below 5 ppm and on retention time of the molecular ion; while regarding the fragments on the intensity

threshold of 1000 and a mass tolerance of 5 ppm. Quantitative results were obtained working in scan mode with HRMS exploiting the high selectivity achieved in full-scan mode, whereas MS/HRMS information was used for confirmatory purposes.

RESULTS AND DISCUSSION

Identification of Polyphenols Compounds in red wine samples though UHPLC-Q-Orbitrap HRMS

Identification of individual flavonoids and non-flavonoids were conducted through UHPLC-Q-Orbitrap HRMS. By combination of MS and MS/MS spectra, a total of 17 different polyphenolic compounds were identified from different samples of red wine samples.

In table 5 are shown all mass parameters including adduct ion, theoretical and measured mass (*m*/*z*), accuracy and sensitivity. Experiments were achieved in ESI^- mode. All studied analytes exhibited better fragmentation patterns producing the quasi-molecular ion $[M-H]^-$.

Table 5. Chromatographic and spectrometric optimized parameters including retention time, adduct ion, theoretical and measured mass (*m*/*z*), accuracy and sensibility for the investigated analytes (*n*=17)

Compound	Retention time (min)	Chemical Formula	Adduct ion	Theoretical mass (m/z)	Measured mass (m/z)	Accuracy (Δmg/kg)	LOD (mg/L)	LOQ (mg/L)
Gallic acid	2.37	C7H6O5	$[M-H]^-$	169.01425	169.0147	2.66	0.0005	0.0014
Protocathecuic acid	3.53	$C_7H_6O_4$	$[M-H]^-$	154.02715	154.02717	0.1101	0.0007	0.002
Catechin	7.65	$C_{15}H_{14}O_6$	$[M-H]^-$	289.07176	289.07224	1.6605	0.0015	0.0046
Chlorogenic acid	8.13	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0878	353.08798	0.5098	0.0012	0.0036
Caffeic acid	8.25	$C_9H_8O_4$	$[M-H]^-$	179.03498	179.03455	-2.4018	0.0007	0.002
Epicatechin	8.51	$C_{15}H_{14}O_6$	$[M-H]^-$	289.07176	289.07196	0.6919	0.0014	0.0043
Luteolin-7-O-glucoside	9.23	$C_{21}H_{20}O_{11}$	$[M-H]^-$	447.09328	447.09366	0.8499	0.0008	0.0025
***p*-Coumaric acid**	9.3	$C_9H_8O_3$	$[M-H]^-$	163.04001	163.03937	-3.9254	0.0006	0.0018
Rutin	9.78	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.14611	609.14624	0.2134	0.0012	0.0035
Ferulic acid	9.88	$C_{10}H10O_4$	$[M-H]^-$	193.05063	193.05016	-2.4346	0.0018	0.0054
Quercetin-3-glucoside	9.93	$C_{20}H_{20}O_{12}$	$[M-H]^-$	463.0882	463.08862	0.907	0.0017	0.0052
Kaempferol-3-O-glucoside	10.36	$C_{21}H_{20}O_{11}$	$[M-H]^-$	447.09323	447.0936	0.8276	0.0008	0.0025
Quercetin	11	$C_{15}H_{10}O_7$	$[M-H]^-$	301.03538	301.03508	-0.9966	0.0021	0.0064
Luteolin	11.21	$C_{15}H_{10}O_6$	$[M-H]^-$	285.04046	285.0405	0.1403	0.0004	0.0012
Kaempferol	11.56	$C_{15}H_{10}O_6$	$[M-H]^-$	285.04046	285.04086	1.4033	0.0005	0.0014
Apigenin	11.85	$C_{15}H_{10}O_5$	$[M-H]^-$	269.04555	269.04572	0.6319	0.0004	0.0011
Resveratrol	15.23	$C_{14}H_{12}O_3$	$[M-H]^-$	196.08936	196.08941	0.3098	0.0006	0.0018

Quantification of the polyphenols in red wine extract by high-resolution mass spectrometry (HRMS-Orbitrap)

A Q Exactive Orbitrap LC-MS/MS has been used for the mass spectrometry analysis. An ESI source operating in negative (ESI-) ion mode was used for all the analyzed compounds. Data regarding the polyphenols content in the analyzed red wine samples are shown in Table 6. The results were expressed
as the average content (mg/L) and concentration range.

Among the non-flavonoid polyphenolic compound, the resveratrol was quantified at concentrations range between 2.8 to 4.6 mg/L, with a mean value of 3.2 mg/L. The resveratrol concentration found in assayed samples was higher than that usually assessed in red wine (mean value 1.7 mg/L) [4], and comparable to the level reported in a recent study that has investigated the resveratrol content in Aglianico red wines [5]. Moreover, resveratrol has been associated with numerous positive properties and health benefit [6].

Regarding non-flavonoids, in the here analyzed samples hydroxybenzoic acids (gallic and protocatechuic acid) and hydroxycinnamic acids (chlorogenic, caffeic, *p*-coumaric and ferulic acid) were investigated. Hydroxybenzoic and hydroxycinnamic acids represented the 25.6 and 15.5% of total polyphenols found in red wine, respectively. Gallic and protocatechuic acids were found as the most commonly detected non-flavonoids ranging from 26 to 320 and 22.2 to 61.2 mg/L, with a mean value of 45.2 and 36.0 mg/L, respectively.

Flavonoids, namely flavones, flavonols, and flavanols including their main aglycones and glycosides have been quantified in red wine samples. Flavanols including catechin and epicatechin were the most flavonoids found in assayed samples, amounting from 47.0 to 52.8% and from 17.6 to 20.6%, respectively. These results were consistent with those reported previously [7,8]. Regarding flavones, apigenin was the most common compound found in the samples, at average content of 31.4 mg/kg (range from 4.9 to 69.5 mg/L).

Quercetin showed to be the more relevant flavonol, quantified between 15.2 to 15.8 mg/L, with a mean value of 15.5. Moreover, the most common flavonol found in red wine

was rutin at a mean content of 9.0 mg/L (range from 8.8 to 8.9 mg/L) and quercetin-3-glucoside detected in levels ranging from 5.5 to 6.6 mg/L (mean value 6.1 mg/L).

Table 6. Polyphenols content in the analyzed red wine samples (n=17). Results are expressed as average and range comparing. The table showed the recent surveys reporting the polyphenol concentration in red wine samples [5,7,9-16].

SAMPLE		**Average (mg/L)**	**Range (mg/L)**	**literature (mg/L)**
NON-FLAVONOIDS				
Hydroxybenzoic acids				
	Gallic acid	45.2	35.3 - 145.3	26 – 320
	Protocathecuic acid	36.0	22.2 - 61.2	88.0
	SUM	81.2		
Hydroxycinnamic acids				
	Chlorogenic acid	2.3	1.9 - 2.9	0.23 - 2.1
	Caffeic acid	9.4	6.6 - 2.9	3 – 18
	p-Coumaric acid	30.4	17.9 - 42.3	7.5 – 22
	Ferulic acid	7.2	1.6 - 12.8	19.0
	SUM	49.3		
Stilbenes				
	Resveratrol	3.2	2.8 - 4.6	0.7 - 6.5
FLAVONOIDS				
Flavonol				
	Rutin	9.0	8.9 - 8.8	0.5 – 10.8
	Quercetin-3-glucoside	6.1	5.5 - 6.6	50 - 100
	Kaempferol-3-O-glucoside	0.1	0.02 - 0.1	6.6 - 10.2
	Quercetin	15.5	15.2 - 15.8	5 – 53
	Kaempferol	0.0	0.03 - 0.1	18.0
	SUM	30.6		
Flavones				
	Luteolin-7-*O*-glucoside	0.0	0.002 - 0.02	-
	Luteolin	0.1	0.01 - 0.1	0.2 - 1
	Apigenin	31.4	4.9 - 69.5	-
	SUM	31.5		
Flavanols	Catechin	65.5	40.3 - 92.5	27–191
	Epicatechin	56.0	41 - 95.2	21.4–128
	SUM	121.5		

Total phenolic content (TPC) and Antioxidant activity

Phenolics are reactive metabolites widely spread in the vegetal kingdom and represent the most studied phytochemicals due to their bioactivity. In fact, these important secondary metabolites are known to have a wide range of important biological properties including antioxidant and anti-inflammatory activity [17,18].

In this work, the amount of total phenolic content of red wine was investigated. Table 7 shows the result reported as average content (± SD) and concentration range and expressed as g GAE/L of sample.

The TPC mean value found in red wine extracts was 3.2 g GAE/L of sample, in a concentration range from 2.7 to 4.1 g GAE/L. These results are comparable to the findings reported in a recent study that has investigated the TPC content in Aglianico red wines [19].

DPPH radical and ABTS radical cation assays were used for evaluation of free radical-scavenging properties of red wine extract. The antioxidant capacity of samples was measured against a Trolox standard and expressed as TEAC (Trolox equivalent antioxidant capacity). The TEAC values ranged from 19.2 ± 1.9 and 21.3 ± 2.3 mmol Trolox/L, respectively for ABTS and DPPH assay. The results obtained with ABTS and DPPH assays showed a remarkable antioxidant activity of polyphenolic extract of the analyzed samples.

Table 7. Total polyphenol compounds expressed as g GAE/L and antioxidant activity expressed as mmol Trolox/L of red wine

	TPC		**ABTS**		**DPPH**	
Sample	**Average (g GAE/L)**	**Range (g GAE/L)**	**Average (mmol trolox/L)**	**Range (mmol trolox/L)**	**Average (mmol trolox/L)**	**Range (mmol trolox/L)**
Red wine	3.2 ± 0.6	2.7 - 4.1	19.2 ± 1.9	17.5 - 21.5	21.3 ± 2.3	18.5 - 22.4

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SPECIFIC OBJECTIVE 2

To evaluate the cardioprotective effect of moderate wine (red and white) consumption and resveratrol intakes in the Italian adult population in order to know their therapeutic potential in CVD protection.

MATERIAL AND METHODS

Study design

Monocentric, open-label, non-randomized, parallel-group clinical trial will be conducted on healthy volunteers clinically evaluated by medical staff working at the Prevention Unit of the Atherosclerosis of the Cardiology Center Monzino (Milan, Italy).

The prespecified primary endpoints will be the change in plasma levels of oxidized LDL [determined by ELISA assay], individual antioxidant capacity (IAC). Moreover, urinary levels of 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-isoprostaglandin F2α (8-isoPGF2α) [determined by mass spectrometry] were evaluated. Secondary endpoints will be the changes in plasma levels of C-reactive protein (CRP); endothelial function [determined by laser doppler evaluation of reactivity to acetylcholine and sodium nitroprusside]; urinary levels of 11-dehydro thromboxane B2 (11-DH-TXB2) [determined by mass spectrometry] will be evaluated.

Inclusion criteria will be as follows: male sex, 20 to 65 years old, free from cardiovascular disease and moderate wine drinker (150 mL/day). The major exclusion criteria will be as follow: absence of renal dysfunction (creatinine clearance below 30 mL/min) and chronic liver disease or transaminase levels above normal value (glutamic oxaloacetic transaminase (GOT) 1-40 IU/L, glutamic pyruvic transaminase (GPT) 1-40 IU/L, gamma-glutamyl transferase (GGT) 0-35 IU /THE) and high intake of antioxidant products (e.g., vitamins) and/or with vasodilatory action.

A total of 30 men will be enrolled, of whom abstainers n=10; moderate red wine drinkers n=10; moderate white wine drinkers n=10. The study design consisted of a run-in period (7 days), and the intervention period (28 days).

Intervention groups:

- Red wine;
- White wine;
- Resveratrol.

The study design will expect a wash-out period (7 days) of any alcoholic beverage or other beverage or food with a high content of polyphenols or other antioxidants. During the intervention period (28 days) each wine drinker subject will be given 150 mL of wine (red or white) to replace the wine usually consumed or given tablets containing 5 g of resveratrol.

Results

Nevertheless, there were several problems approving the protocol by the Ethics Committee on the Monzino Cardiology Center. This led to a delay in the study. Currently, the protocol is being redesigned for next evaluation by Ethics Committee.

SPECIFIC OBJECTIVE 3

The objectives of this study were a) to investigate the qualitative and quantitative profile of the bioactive compounds in coffee husk (raw material and infusion) using UHPLC-High resolution mass spectrometry b) the antioxidant activity through the ABTS and DPPH assays c) Determination of total, soluble, and insoluble fiber and d) the antifungal activity in solid and liquid medium (MIC-MFC).

MATERIALS AND METHODS

Standard and samples

Two types of coffee husk derived from coffee Arabica (coffee husk 1 and coffee husk 2), which will be called from now CH1 and CH2, were obtained from Café do Brasil S.p.A. (Naples, Italy). CH1 and CH2 were obtained by dry and semi-dry processing of coffee cherries, respectively.

Chemicals were from Sigma-Aldrich (St. Louis, MI, USA) and solvents, ethanol, methanol, and water (LC-MS grade) were purchased from Carlo Erba reagents (Naples, Italy).

Samples preparation

The procedure adopted for the preparation of coffee husk infusion (CHI) was as follows: 100 mL boiling water was poured onto 4 g of the coffee husk, covered and left to infuse for 4 min. After filtration with Whatman No. 1 paper, the volume and dry matter yield were measured. Subsequently, infusions were filtered through a 0.2 μm nylon filter and immediately frozen and stored at -18°C until analysis. For the preparation of coffee husk polyphenol extract (CHPE), each sample was weighed (0.5 g), reduced to a fine powder in a blender and extracted with 25 mL of CH_3OH:H_2O (80:20 *v*/*v*) by vortexing for 2 min followed by 10 min in an ultrasonic bath and 20 min on an horizontal shaker at 250 rpm. The solution was centrifuged at 4500 g for 5 min and the supernatant was transferred to a 50 mL tube and the extraction repeated on the pellet with further 25 mL of 80% aqueous CH_3OH, supernatants were combined, filtered through a 0.2 μm nylon filter and stored at -

18°C until analysis.

Total Antioxidant Activity

The free radical scavenging activities of the CHIs and CHPEs were evaluated using ABTS and DPPH assays described above [1,2]. The results were expressed as mmol Trolox equivalents (TEAC) per kg of sample and all determinations were performed in triplicate.

Determination of Total, Soluble, and Insoluble Fiber

The amount total dietary fiber (TDF), insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) was calculated according to a gravimetric enzymatic method AOAC Official Method 991.43 as the weight of residue minus the weight of protein and ash [3].

UHPLC-High Resolution Mass Spectrometry (HRMS-Orbitrap) Analysis of Polyphenolic Compounds

Qualitative and quantitative profile of polyphenols including caffeine has been obtained using Ultra High Pressure Liquid Chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000 a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (T=25°C) Kinetex 1.7 μm Biphenyl (100 x 2.1 mm) column. Injection volume was of 2 μL. Eluent phase was formed as follow: phase A (H_2O in 0.1% formic acid), phase B (methanol in 0.1% formic acid). All metabolites were eluted using a 0.2 mL/min flow rate with a gradient programmed as follows: 0 min - 5 % of phase B, 1.3 min - 30% of phase B, 9.3 min - 100% of phase B, 11.3 min - 100% of phase B, 13.3 min - 5% of phase B, 20 min - 5% of phase B.

For the mass spectrometry analysis, a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) has been used. An ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) operating in negative ion mode (ESI-) for all the analyzed compounds and in positive (ESI+) ion mode for caffeine. Ion source parameters in (ESI-) mode were: spray voltage -2.8 kV, sheath gas (N_2 >95%) 45, auxiliary gas (N_2 >95%) 10,

capillary temperature 275°C, S-lens RF level 50, auxiliary gas heater temperature 305°C.

Value for automatic gain control (AGC) target was set at 1×10^6, with a resolution of 70,000 FWHM (full width at half maximum), and a scan rate in the range between 100 and 1000 *m*/*z* in Full MS/Scan mode. Ion source parameters in (ESI+) mode used for alkaloids analysis were: spray voltage 3.3 kV, sheath gas (N_2 >95%) 45, auxiliary gas (N_2 >95%) 13, sweep gas 7, capillary temperature 275°C, S-lens RF level 50, auxiliary gas heater temperature 305°C. The AGC target value was of 1×10^6, the resolution set at 70,000 FWHM, with a scan rate in the range between 100 and 1000 *m*/*z* in the Full MS mode.

The accuracy and calibration of the Q Exactive Orbitrap LC-MS/MS was checked daily using a reference standard mixture obtained from Thermo Fisher Scientific. The linearity of the method was assessed at both low (LOQ-5 mg/kg) and high (5 mg/kg-120 mg/kg) concentration ranges, using six concentration levels in each calibration range. Calibration curves for all compounds were prepared in triplicate. Sensitivity was evaluated by limits of detection (LODs) and limits of quantitation (LOQs). Data analysis and processing has been performer using the Xcalibur software, v. 3.0.63 (Xcalibur, Thermo Fisher Scientific).

The peaks for the studied compounds in the samples were confirmed by comparing the retention times of the peak with those of standards as well as by recognizing both the precursor and product ions with a mass error below 5 ppm.

Microorganisms and Culture Conditions

The strains of *P. camemberti* CECT 2267, *P. expansum* CECT 2278, *P. roqueforti* CECT 2905, *A. parasiticus* CECT 2681, *A. niger* CECT 2088, *F. moniliformis* CECT 2982, *F. verticillioides* CECT 20926, and *F. graminearum* CECT 20490 were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). The strain of A. flavus ITEM 8111 was obtained by the Agro Food Microbial Culture Collection of the Institute of Science of Food Production (Bari, Italy), and were preserved in sterile 30 % glycerol at -80 °C. Therefore, they were maintained in PDB broth at 25 °C and inoculated in PDA plates.

Antifungal Activity Tests on Solid Medium

Agar well diffusion method used to evaluate the antimicrobial activity of the water and methanol extracts of coffee husks samples was performed in according with the work of Madhyastha et al., [4]. Ten microliters of extract were added on sterile discs (6-mm Whatman No. 1, Madrid Spain). All strains were maintained in PDA for 7 days at 30 °C, and the spores were obtained at the time of analysis using 1 mL of distilled water added on the agar surface. After that, 100 μL of a spore suspension was introduced in plate containing 10 mL of liquid PDA at 45 °C. Once the medium has solidified, the discs were placed on the agar surface. After refrigeration at 4 °C for 6 h, the plates were incubated 7 days at 30 °C. After the fungal growth, the measurement of the inhibition halo diameter was carried out, being considered positive for antifungal activity halos larger than 8 mm around the disc.

Determination of minimum inhibitory concentration and minimum fungicidal concentration (MIC-MFC)

The test was performed in liquid medium and 96 well clear polystyrene microplate, using the modified method of Siah et al., [5]. A volume of 100 μl of water and methanol extracts of coffee husks at concentrations of 0.1, 0.5, 1, 5, 10 50, 100, 250 and 500 μg/mL was added in the wells. The wells were inoculated with the mycotoxigenic fungi using 100 μl of a 10^5 spores/mL suspension in PDB. The negative control consisted of inoculated medium without any treatment. The microplates were incubated at 25°C, on a rotary shaker at 140 rpm for 7 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the coffee husk extract, that prevents visible fungal growth. Four replicates of each micro-assay were carried out and the experiment was repeated three times. For the Minimum Fungicidal Concentration (MFC) assay, the concentration corresponding to the MIC and higher were sub-cultured on PDA plates. The MFC is the lowest extract concentration in which a visible growth of the subculture was prevented after 3 days of incubation at 25 °C.

Statistics and Data Analysis

The experiments were done in triplicate. The results are given as mean standard deviation (SD). One-way analysis of variance (ANOVA) was applied to the data using IBM SPSS (version 21). A difference was considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Determination of TDF, IDF and SDF

Scientific evidence suggests that a higher intake of dietary fiber and phytochemicals is associated with a lower risk of cardiovascular diseases and cancer [6]. Moreover, dietary fiber intake in Western countries should increase to help prevent degenerative diseases [7]. Dietary fiber found in raw materials and infusions were evaluated in the assayed samples. Table 8 shows the results here obtained.

In the here analyzed samples, TDF showed a mean value of 367.45 and 396.04 g/kg for dry and wet CH, respectively. These results are according to the values reported in literature (from 245 to 430 g/kg) [8]. In Infusion samples, TDF was detected at mean values of 0.98 and 0.64 g/L for CH2 and CH2, respectively.

As shown in Table 1, IDF was found with a mean value of 326.35 and 350.26 g/kg for dry and wet CH, respectively. Insoluble dietary fiber was not found in infusion samples.

As far as SDF was quantified with a mean value of 41.26 and 46.67 for dry and wet CH, respectively. In infusion, the SDF quantified with a mean value of 0.98 and 0.64 g/L.

Data recorded highlighted a high content of dietary fiber in both types of samples. Dietary fibers are known to have a wide range of important biological properties including lowers blood pressure and serum cholesterol, improves glycemia and insulin sensitivity, prevention and mitigation of cardiovascular disease [9,10].

Therefore, CHs could be considered as a potential novel source of dietary fiber intended for infusion or nutraceutical formulations.

Table 8. Comparison of DF content in CHIs and dry matter of CH.

	Samples	CH1	CH2
TDF	Dry matter g/kg	367.45±2.49	396.04±3.18
	Infusion g/L	0.98±0.12	0.64±0.53
IDF	Dry matter g/kg	326.35±1.49	350.26±1.89
	Infusion g/L	ND	ND
SDF	Dry matter g/kg	41.26±0.49	46.67±0.22
	Infusion g/L	0.98±0.08[a]	0.64±0.22[b]

Total Antioxidant Activity

The results of antioxidant activity evaluated through ABTS and DPPH assays are reported in Table 9 and expressed as mmol Trolox/kg. The results obtained showed a remarkable antioxidant activity of analyzed samples. No statistically significant difference was detected among different types of CHI in both antioxidant assays.

Coffee husk seems to have higher antioxidant activity compared to other coffee by-products. In fact, several findings have reported contents of 21.2 mmol Trolox/kg in silverskin; 20.4 mmol Trolox/kg in spent waste coffee; 18.4 mmol Trolox/kg in cherry husk and 15.3 mmol Trolox/kg in coffee pulp [8].

Table 9. Antioxidant activity in CHI samples. The results are expressed in mmol Trolox/kg

	CHI1		CHI2	
Sample	**mmol Trolox/kg**		**mmol Trolox/kg**	
	ABTS	**DPPH**	**ABTS**	**DPPH**
CHI	28.58±0.52	26.89±0.59	28.17±0.59	26.61±0.59

Identification of the Bioactive Compounds in Coffee Husks and in Infusion by High-resolution Mass Spectrometry (HRMS-Orbitrap)

A Q Exactive Orbitrap LC-MS/MS has been used for the mass spectrometry analysis. An ESI source operating in Full MS/Scan and PRM mode, both in negative (ESI-) and in positive (ESI+) ion mode was used for all the analyzed compounds. Qualitative data regarding the phenolic compounds of coffee husk extracts are shown in Table 3. In this work,

the bioactive compounds have been identified by using MS/MS experiments; accurate mass measurements and precursor ion scan. The isotopic pattern and accurate masses were selected to establish the elemental composition. The strategies for identification of polyphenols were based on observed MS/MS spectra than those in the literature. Table 10 reported the 30 compounds identified using triple-quadrupole MS/MS and Orbitrap experiments, in addition to theoretical and measured mass, accuracy, molecular formula, retention times, collision energy and the fragment ions exploited for the identification.

Table 10. Peak Numbers, Compound name, formula, Ionization mode, Theoretical and Measured (*m*/*z*), Accuracy, fragment ion, collision energy and retention time

Peak	Compound	Formula	Ionization mode	Theoretical (*m*/*z*)	Measured (*m*/*z*)	Accuracy (Δmg/kg)	Fragment ions (*m*/*z*)	CE (V)	RT (min)
1	Trigonelline	$C_7H_7NO_2$	$[M-H]^+$	138.05496	138.05514	1.30	11.006.006	30	1.44
2	Gallic Acid	$C_7H_6O_5$	$[M-H]^-$	169.01425	169.01470	2.66	125.02428; 169.01406	20	2.37
3	Protocatecuic Acid	$C_7H_6O_4$	$[M-H]^-$	153.01933	153.01953	1.31	10.902.933	20	5
4	3-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.08781	353.08817	1.02	191.05609; 179.03481; 135.04508	20	7.06
5	(+)-catechin	$C_{15}H_{14}O_6$	$[M-H]^-$	289.07176	289.07186	0.35	245.08187; 203.07126; 205.05025	20	7.79
6	5-*p*-Coumaroyl quinic Acid	$C_{16}H_{18}O_8$	$[M-H]^-$	337.09289	337.09338	1.45	191.05598; 173.04520; 163.03989	20	7.94
7	5-*O*-caffeoyl quinic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.08781	353.08796	0.42	191.05609; 179.03478	20	8.25
8	Caffeic acid	$C_9H_8O_4$	$[M-H]^-$	179.03498	179.03507	0.50	13.504.503	20	8.37
9	Theobromine	$C_7H_8N_4O_2$	$[M-H]^+$	181.07200	181.07208	0.44	110.07130; 163.06142; 135.06652;	30	8.55
10	procyanidin A type trimer	$C_{45}H_{36}O_{18}$	$[M-H]^-$	863.18290	863.18502	2.46	57.512.382	25	8.57
11	Gentisic Acid	$C_7H_6O_4$	$[M-H]^-$	153.01933	153.01949	1.05	10.902.936	20	8.66
12	Theofillyne	$C_7H_8N_4O_2$	$[M-H]^+$	181.07200	181.07209	0.50	69.04483; 124.05061; 96.05561	30	8.67
13	(−)-epicatechin	$C_{15}H_{14}O_6$	$[M-H]^-$	289.07176	289.07218	1.45	245.08163; 205.05034	20	8.7
14	4-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.08781	353.08826	1.27	173.04537;191.05589; 179.03484	20	8.93

15	procyanidin B type trimer	$C_{45}H_{38}O_{18}$	$[M-H]^-$	865.19854	865.20035	2.09	577.13519; 287.05609; 289.07185	40	8.93
16	4-*O*-feruloylquinic acid	$C_{17}H_{20}O_9$	$[M-H]^-$	367.10346	367.10370	0.65	19.105.609	20	9.33
17	*p*-Coumaric Acid	$C_9H_8O_3$	$[M-H]^-$	163.04007	163.04037	1.84	11.905.014	20	9.56
18	5-*O*-feruloylquinic acid	$C_{17}H_{20}O_9$	$[M-H]^-$	367.10346	367.10357	0.30	191.05600; 173.04537	30	9.6
19	3,4-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.11950	515.11982	0.62	353.08744; 173.04527; 179.03477; 335.07697	25	9.93
20	quercetin-3-*O*-rutinoside	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.14611	609.14685	1.21	30.103.514	30	10
21	3,5-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.11950	515.11986	0.70	17.903.486	30	10.06
22	quercetin-3-*O*-glucoside	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.08820	463.08888	1.47	30.103.465	32	10.16
23	Ferulic acid	$C_{10}H_{10}O_4$	$[M-H]^-$	193.05063	193.05070	0.36	149.00352; 134.03721; 178.02727	20	10.16
24	4,5-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.11950	515.11983	0.64	35.308.725		10.49
25	Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	$[M-H]^-$	447.09328	447.09363	0.78	28.504.044	30	10.6
26	Isorhamnetin 3-*O*-Rutinoside	$C_{28}H_{32}O_{16}$	$[M-H]^-$	623.16176	623.16241	1.04	31.505.104	30	10.64
27	Caffeine	$C_8H_{10}N_4O_2$	$[M-H]^+$	195.08765	195.08780	0.77	138.06617; 110.0713;	25	10.74
28	3-*O*-Feruloyl-4-*O*-caffeoylquinic acid	$C_{26}H_{26}O_{12}$	$[M-H]^-$	529.13515	529.13538	0.43	367.10296; 335.07703	25	10.85
29	4-*O*-Caffeoyl-5-*O*-feruloylquinic acid	$C_{26}H_{26}O_{12}$	$[M-H]^-$	529.13515	529.13594	1.49	367.10318; 335.07699	25	11.41
30	Naringenin	$C_{15}H_{12}O_5$	$[M-H]^-$	271.06120	270.106156	1.33	151.00355; 119.05012; 271.06118	20	12.08

The discrimination among isomers of CGA was done using their fragmentation pattern in MS^2 and their relative intensity, in accordance with the hierarchical scheme of identification described by Clifford et., (2003) [11]. For the peaks 4, 7 and 14 mass spectral analysis showed the same molecular ion negatively charged $[M-H]^-$ at *m*/*z* 353.08781. The isomers produced different mass spectra in-source fragmentation of *m*/*z* 353. 08781 which

allowed distinguish the different compounds.

These 3 isomers were differentiated by the MS^2 fragmentation pattern. The 4-CQA (peak 14) showed a MS^2 base peak at *m*/*z* 173.04537 ([quinic acid-H-H_2O]) due to the dehydration of the quinic acid ion, a feature of cinnamoyl group bonded to the quinic acid moiety at the position 4. The 3-CQA (peak 4) and 5-CQA (peak 7) showed the same MS^2 base peak at *m*/*z* 191.05609 ([quinic acid-H]) and they could be distinguished by comparing the relative intensity of the secondary ion from caffeoyl moiety ([caffeic acid-H]) at *m*/*z* 179.03498 [12]. Peak 6 showed MS^2 base peak at *m*/*z* 191.05609 ([quinic acid-H]).

Peaks 16 and 18 with $[M-H]^-$ ions at *m*/*z* 367.10346 indicating the presence of feruloylquinic acids. 4-*O*-feruloylquinic acid showed a single fragment MS^2 base peak at *m*/*z* 191.05605. Peak 18 generated fragment ion at *m*/*z* 191.05605 with additional minor ion at *m*/*z* 173.04537 in keeping with that previously described for Peak 16.

Peaks 19, 21, and 24 with $[M-H]^-$ ions at *m*/*z* 515.1195 were identified as di-CQA isomers according to the formula $C_{25}H_{23}O_{12}^-$. Dicaffeoylquinic acid isomers showed peaks at *m*/*z* 353 and 515, which confirmed the losses of two caffeic acid units. The analysis in the LTQ-Orbitrap confirmed their presence showing the deprotonated molecule $[M-H]^-$ (*m*/*z* 515), the ion [M-H- 162]$^-$ (*m*/*z* 353), corresponding to the loss of a caffeic acid unit and the deprotonated caffeic acid (*m*/*z* 179). Peaks 28 and 29 showing a fragmentation pattern characterized by predominant ion $[M-H]^-$ at *m*/*z* 529.13515 this daughter ion is indicative of an *O*-caffeoyl-*O*-feruloyl quinic acid conjugated compound. Peaks 28 and 29 showed a MS^2 base peak at *m*/*z* 367.10296 and the fragmentation spectra showed an additional fragment ion at *m*/*z* 335.07703. This would suggest that these compounds are the 3-*O*-feruloyl-4-*O*-caffeoylquinic acid isomers.

The ionization in the positive mode generated the protonated molecule $[M+H]^+$ for alkaloids. Theobromine and theophylline are methyl xanthines, which are intermediates of caffeine metabolism. Peak 9 and 12 were differentiated by MS^2 fragmentation pattern. The fragmentation pathway was characterized by base peak at *m*/*z* 110.07130 and 69.04483, this suggests the loss of $C_3H_3NO_2$ (cyanoformate) and $C_4H_4N_2O_2$ (diisocyanatoethane), respectively. Trigonelline (Peak 1) *m*/*z* 138.05496 shows a fragmentation pattern with the

parent ion-producing a single fragment at *m*/*z* 110.06006. Caffeine (Peak 27) in MS^2 fragmentation shows the base daughter ion at *m*/*z* 138.06617 corresponded to loss of C_2H_3NO methyl carbamoyl.

For cinnamic and benzoic acids the spectra showed the deprotonated molecule $[M-H]^-$. For ferulic, caffeic, protocatechuic, gentisic, *p*-coumaric and gallic acid loss of CO_2 was observed, giving as a characteristic ion the $[M-H-44]^-$ [13]. Peak 5 and 13 showed the same MS^2 base peak at *m*/*z* 245.08187 this suggests the loss of CH_2CHOH group [14].

For flavonol *O*-glycosides such as quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, luteolin-7-*O*-glucoside and isorhamnetin 3-*O*-rutinoside the spectra generated with a collision energy (CE) between -30 and -32 V revealed both the deprotonated molecule $[M-H]^-$ of the glycoside and the ion corresponding to the deprotonated aglycone $[A-H]^-$. For Peak 30 the fragmentation pathway was characterized by a base peak at *m*/*z* 151.00355 and 119.05012 characteristic for naringenin and its derivatives [13]. For peaks 10 and 15 loss of catechin residue was observed, obtaining the base daughter ion $[M-H-288]^-$ at *m*/*z* 575.12382 and 577.13519, respectively.

Quantification of Major and Minor Compounds Found in Coffee Husks and in Infusion

The CGAs identified and quantified in CHPEs and CHIs showed in Table 11 and the results are expressed as mg/kg. In CHPEs the results showed amounts of CGA ranging from 1019.19 to 1564.68 for CHPE1 and CHPE2, respectively. The infusion contains an amount of CGA ranging from 370.22 to 608.11 for CHI1 and CHI2, respectively.

In according with the work of Mullen et al., the 5-O-caffeoylquinic acid was the major CGA present in all samples investigated, and no statistically significant differences were found among samples of the same type were observed [15].

Moreover, dicaffeoylquinic acid isomers, 3-*O*-Feruloyl-4-*O*-caffeoylquinic acid and, 4-*O*-Caffeoyl-5-*O*-feruloylquinic were not detected in CHIs.

Table 11. Quantification of Major Compounds in CHPEs and CHIs

Compound	CHPE 1 mg/kg	CHPE 2 mg/kg	CHI 1 mg/kg	CHI 2 mg/kg	LOD µg/kg	LOQ µg/kg
3-*O*-caffeoylquinic acid	24.41±0.09	53.34±0.88	8.34±0.19	19.01±0.93	0.07	0.2
5-*p*-Coumaroylquinic Acid	35.12±0.19	61.55±0.76	8.82±0.12	20.23±0.43	0.07	0.2
5-*O*-caffeoylquinic acid	654.72±2.49	1121.7±5.22	283.32±0.12	502.0±3.59	0.07	0.2
4-*O*-caffeoylquinic acid	7.62±0.04	11.12±0.49	<LOQ	<LOQ	0.07	0.2
4-*O*-feruloylquinic acid	15.21±0.11	14.72±0.77	5.32±0.12	5.34±0.12	0.07	0.2
5-*O*-feruloylquinic acid	170.32±3.29	171.82±1.43	64.42±0.43	61.53±0.34	0.07	0.2
3,4-*O*-dicaffeoylquinic acid	22.82±0.53	20.21±0.14	<LOQ	<LOQ	0.07	0.2
3,5-*O*-dicaffeoylquinic acid	62.42±0.23	65.51±1.12	<LOQ	<LOQ	0.07	0.2
4,5-*O*-dicaffeoylquinic acid	18.28±0.12	34.41±1.23	<LOQ	<LOQ	0.07	0.2
3-*O*-Feruloyl-4-O-caffeoylquinic acid	8.62±0.13	8.84±0.22	<LOQ	<LOQ	0.07	0.2
4-*O*-Caffeoyl-5-O-feruloylquinic acid	0.4±0.09	1.46±0.02	<LOQ	<LOQ	0.07	0.2
Total major compounds	1019.19	1564.68	370.22	608.11		

The quantitative data on the alkaloid compounds found in the CHPEs and CHIs are reported in Table 12. In according to literature data, the results obtained in the samples here analyzed showed that caffeine and trigonelline were the predominant alkaloids [15].

Table 12. Quantification of alkaloids in CHPEs and CHIs.

Compound	CHPE 1 mg/kg	CHPE 2 mg/kg	CHI 1 mg/kg	CHI 2 mg/kg	LOD µg/kg	LOQ µg/kg
Trigonelline	1825.72±3.39	1559.00±5.49	558.02±1.55	550.45±1.39	0.011	0.02
Theobromine	5.33±0.21	4.42±0.19	2.15±0.03	1.19±0.03	0.008	0.03
Theofillyne	15.04±0.09	8.6±0.24	4.82±0.22	3.05±0.11	0.008	0.03
Caffeine	1327.39±5.41	1334.5±4.23	506.32±0.29	553.25±1.28	0.009	0.02
Total alkaloids	3173.48	2906.52	1066.49	1107.94		

The total amount of caffeine and trigonelline found in CHPEs showed a two-to-three-fold increase when compared to infusions samples. The caffeine content in coffee husk in literature ranged from 1,300 to 8,200 mg/kg, values significantly higher than the **here** analyzed samples [15-17]. Theophylline and theobromine (methylxanthines, intermediates of the caffeine metabolism) were few represented both in CHPEs and CHIs.

Table 13 shown the quantitative data of minor flavonoid compounds present in the

samples. Phenolic acids, flavan-3-ols, and flavonoids were detected and quantified in the CHPEs whereas in CHIs, were found only six phenolic compounds (protocatechuic acid, caffeic acid, epicatechin, rutin, isorhamnetin 3-O-rutinoside, and naringenin).

Table 13. Quantification of minor compounds in CHPEs and CHIs.

Compound	CHPE 1 mg/kg	CHPE 2 mg/kg	CHI 1 mg/kg	CHI 2 mg/kg	LOD µg/kg	LOQ µg/kg
Gallic Acid	3.22±0.1	1.90±0.01	<LOQ	<LOQ	0.07	0.2
Protocatecuic Acid	48.94±0.2	37.42±0.78	10.62±0.4	5.05±0.3	0.07	0.2
(+)-catechin	1.55±0.1	0.82±0.01	<LOQ	<LOQ	0.06	0.18
Caffeic acid	6.12±0.1	18.33±0.07	0.75±0.01	2.14±0.1	0.06	0.18
procyanidin A type trimer	4.74±0.1	0.62±0.02	<LOQ	<LOQ	0.06	0.18
Gentisic Acid	77.13±1.1	27.37±0.33	<LOQ	<LOQ	0.06	0.18
(−)-epicatechin	24.95±0.3	7.97±0.2	0.19±0.1	0.13±0.1	0.06	0.18
procyanidin B type trimer	12.52±0.1	3.27±0.1	<LOQ	<LOQ	0.06	0.18
p-Coumaric Acid	0.41±0.1	4.56±0.1	<LOQ	<LOQ	0.07	0.2
quercetin-3-*O*-rutinoside	95.82±1.1	114.95±5.1	10.95±0.1	9.85±0.1	0.07	0.2
quercetin-3-*O*-glucoside	69.87±0.9	67.06±0.9	<LOQ	<LOQ	0.07	0.2
Ferulic acid	10.24±0.2	ND	<LOQ	<LOQ	0.07	0.2
Luteolin-7-*O*-glucoside	10.27±0.4	5.74±0.1	<LOQ	<LOQ	0.06	0.18
Isorhamnetin 3-*O*-Rutinoside	6.67±0.3	5.23±0.1	0.65±0.02	0.57±0.2	0.07	0.2
Naringenin	1.32±0.2	1.94±0.1	0.14±0.1	0.18±0.1	0.06	0.18
Total minor compounds	373.6	297.1	23.3	17.9		

Water and Methanol Coffee Husk Extracts Antifungal Activity, MIC-MFC

Coffee is the most preferred morning beverage throughout the world due to its pleasant flavour and stimulating properties. Natural preservatives have been developed as an alternative source of novel antimicrobials in encountering food-borne pathogens. In recent years, coffee has been associated with antimicrobial activity against several different bacteria and fungi [18].

The active components or the antimicrobial mechanism of coffee are not fully elucidated yet. Coffee is composed of several compounds such as water, caffeine, lipids, tannic acids, mineral substances, theobromine, organic acids, alkaloids, and several vitamins, that could be responsible for its antimicrobial effects. Possible candidates include complex mixtures of phenolic compounds and Maillard reaction polymers such as

melanoidins [18,19].

The antimicrobial mechanism of action of the phenolic compounds is not clear. They can express their antimicrobial activity by changing the cytoplasmic membrane's structure, disrupting the active transport, the proton motive force and/or the electron flow. Moreover, the mechanism for phenolic toxicity may include enzymatic inhibition by the oxidized compounds, probably through interaction with sulfhydryl groups or through more non-specific reactions with proteins [20].

In Table 14 are evidenced the data related to the antifungal activity on solid medium of the water and methanol extracts of the two coffee husks samples. In CHIs samples, antifungal activity against the assayed mycotoxigenic fungi was not found., whereas both methanol extracts presented an antifungal activity quantified with a diameter clearing zone of 8 mm on the three strains of Penicillium, and on A. *flavus* and A. *niger*. The antifungal activity evidenced by the methanol extracts of the two coffee husks samples was quantified through the Minimum inhibitory Concentration and the Minimum Fungicidal Concentration (MIC-MFC) evaluation calculated using the 96-well microplates assay.

As evidenced in Table 15 the methanol extract of sample 1 presented a MIC variable from 6.3 to 25 g/L, considering that the most sensible strains to this natural antimicrobial complex were P. *expansum* and P. *roqueforti* with 6.3 and 12.5 g/L. The MFC data observed for the antimicrobial complex produced through the methanol extract of the sample 1, ranged from 6.3 to 50 g/L, confirming that the strains most sensible to this treatment was the P. *expansum*. The MIC data evidenced for the methanol extracts of the sample 2, ranged from 6.0 to 50 g/L. The highest sensibility of this extract was observed on the strain of P. *expansum*, whereas the lowest sensibility was observed on the strain of A. *flavus* and A. *niger*. The data evidenced for the MFC of the sample two-methanol extract ranged from 12.5 to 50 g/L.

Since the results showed an important antifungal activity on the P. *expansum* strains, the coffee husk extracts could be considered as good candidates for the biocontrol of mycotoxigenic fungi tested in food and feed chain.

Table 14. Antifungal activity in solid medium evidenced by Coffee husk 1 and Coffee husk 2 water and methanolic extracts on the mycotoxigenic fungi tested. Calculation of antifungal activity: 8 mm diameter clearing zone (+).

Fungi	CH1		CH2	
	Water extract	Methanol extract	Water extract	Methanol extract
P. camemberti CECT 2267	−	+	−	+
P. expansum CECT 2278	−	+	−	+
P. roqueforti CECT 2905	−	+	−	+
A. parasiticus CECT 2681	−	−	−	−
A. flavus ITEM 8111	−	+	−	+
A. niger CECT 2088	−	+	−	+
F. moniliformis CECT 2982	−	−	−	−
F. verticilliodes CECT 20926	−	−	−	−
F. graminearum CECT 20490	−	−	−	−

To the best of my knowledge, this is the first study that reports the antifungal activity of the coffee husk extracts studied against the mycotoxigenic fungi. However, other authors have investigated the antimicrobial activity of coffee and coffee by-products extract against several bacteria such as Streptococcus, Bacillus, Legionella species and other enterobacteria species [18-20].

Table 15. Minimum inhibitory concentration and minimum fungicidal concentration (MIC-MFC) evidenced by coffee husk methanolic extracts on the mycotoxigenic fungi tested.

Fungi	CHE1		CHE2	
	MIC	MFC	MIC	MFC
P. camemberti CECT 2267	25	50	25	50
P. expansum CECT 2278	6.3	6.3	6	25
P. roqueforti CECT 2905	12.5	25	12.5	12.5
A. flavus ITEM 8111	25	25	50	50
A. niger CECT 2088	25	50	50	50

Coffee samples were in general less active against Gram-negative bacteria than Gram-positive, except for E. faecalis. These different activities may be attributable to structural differences in cell wall composition. Gram-negative bacteria possess an LPS component in their outer membrane that makes them more resistant to antibacterial agents [19]. Moreover, the melanoidins, high-molecular-weight compound found in coffee, showed antimicrobial activity against Gram-negative. At MIC value, At MIC value, melanoidins

have been able to break the cell membrane, which was independent of the bacterial transmembrane potential [21]. Rurián et al., concluded that antimicrobial activity of coffee depends primarily on the roasting of the coffee beans and more specifically on the roasted ingredients mediating its effects.

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SPECIFIC OBJECTIVE 4

Evaluation of possible associations between rs738409, rs10401969 and, rs1260326 with measures of carotid subclinical atherosclerosis in a large European cohort consisting of individuals at high risk of cardiovascular events but free of clinical CVD manifestation. In the same study population, we have also assessed the association of these three SNPs with metabolic traits.

MATERIALS AND METHODS

Study population

We performed our study in a large European cardiovascular cohort including individuals at high risk of cardiovascular events (acronym: IMPROVE (Carotid **I**ntima **M**edia Thickness and IMT-**PR**ogression as Predictors of **V**ascular **E**vents in a High-Risk European Population). The IMPROVE study was previously described [1]. Briefly, between 2004 and 2005, 3,711 participants, free from CVD, but with at least three conventional atherosclerosis risk factors, were recruited in five European countries. Study participants reported their lifestyle habits, previous and current diseases, and medications and underwent a physical examination. Blood samples were withdrawn and stored at -80°C in the biobank. Height (m) and weight (kg) were used to estimate body mass index (BMI; kg/m^2). Hypertension was defined as blood pressure higher than 140/90 mmHg at the visit and/or if self-reported and/or in the presence of treatment; diabetes was defined if blood glucose ≥7.0 mmol/L and/or if self-reported and/or in the presence of treatment with glucose-lowering drugs or insulin. Smoking was defined either as never and former or as current smoking.

Plasma total cholesterol (TC), LDL-cholesterol (LDL-C), triglycerides (TG), glucose level and ALT were measured using standard enzymatic methods from fasting blood samples [1].

Ultrasonographic measures

Carotid artery ultrasonographic scan was recorded at baseline measuring four consecutive segments at the far wall of the left and right carotid artery in three angles (anterior, lateral and posterior). Data from the eight segments measured in each patient were averaged to estimate the c-IMT$_{mean}$, c-IMT$_{max}$ and c-IMT$_{mean-max}$. The inter-adventitia common carotid artery diameter (ICCAD) was measured in a plaque free area of the second centimetre of the common carotid proximal to the bifurcation. Data are expressed in mm. Details on protocol, validation and precision of carotid ultrasound measurements were previously described [2].

Genotyping

Genomic DNA from IMPROVE study participants was genotyped with two genotyping arrays, the CardioMetaboChip 200K and the Immunochip, each one analysing approximately 200,000 genetic variants [3]. Standard quality control procedures for genetic data were conducted on the individual genotyping chips as well as the combined chip (CardioMetabo-Immuno) [4]. Multidimensional scaling (MDS) components were calculated using PLINK version 1.07 [5] (using default settings) to identify possible non-European ethnicity and used to adjust for population stratification. SNPs were excluded for deviation from Hardy-Weimberg equilibrium ($p < 0.0000001$), call rate < 95% or minor allele frequency (MAF) < 1%. Subjects with call rate < 95%, cryptic relatedness, ambiguous sex or identified as outliers by MDS were also excluded. Genotype data for rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T) were extracted from the CardioMetabo-Immuno chip after the quality control procedures described above.

After exclusion of study participants with no visualized carotid artery (n = 7) and missing genotypes (n = 356), a total of 3,347 subjects were analysed in the present study. Given the low allele frequency of the effect allele (EA) for rs10401969, heterozygous CT and homozygous CC (n = 393 and n = 19, respectively) were pooled in the analysis.

Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and categorical variables as percentages.

Linear regression models were used to assess the association between the studied SNPs, rs738409 (C/G), rs1260326 (C/T) and rs10401969 (T/C), and c-IMT measures (c-IMT_{mean}, c-IMT_{max} and c-$IMT_{mean-max}$), ICCAD, ALT and metabolic traits (BMI, TC, LDL-C, TG and glucose level). Individuals with type 2 diabetes were excluded from the analysis of glucose levels. C-IMT measures and ICCAD were log transformed when pertinent to achieve a normal distribution. Analyses were performed assuming an additive model of inheritance. Results are reported as beta (β) coefficient and standard error (SE). Estimates were adjusted for age, sex, and multidimensional scaling dimensions (MSD). Three MSD components were found to be informative (MSD1, MSD2, MSD3) and introduced in the analytical model, as previously reported [6]. In secondary analyses, ALT (U/L) levels were categorized in quartiles (1st quartile (Q), Q1: ≤ 16, n = 876; Q2: > 16 - ≤ 21, n = 929; Q3: > 21 - ≤ 28, n = 801; Q4:> 28, n = 741). The association of the three SNPs with c-IMT and ICCAD levels in the different ALT quartiles was then estimated by linear regression, after adjustment for age, gender, and MSD1-3.

Multiple testing correction was not applied for ultrasonographic measures because they are strongly correlated. However, in the main analysis we corrected for multiple comparison considering 5 independent tests (c-IMT and ICCAD, lipids, ALT, BMI and glucose). All statistical tests were two-sided and a $p < 0.01$ was then considered statistically significant. Statistical analysis was performed using STATA 12 (STATA corp LP, College Station, Texas, USA).

RESULTS

Clinical characteristics

Clinical, biochemical and ultrasonographic characteristics of the study participants by rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T) genotypes are presented in Table 16. Study participants carrying the GG genotype at rs738409 had higher ALT levels and lower c-IMT and ICCAD as compared to the CC and CG genotype groups; those carrying the TC/CC genotype of rs10401969 had higher ALT levels, c-IMT and ICCAD as compared

to the CC group. Finally, TT carriers at rs1260326 had lower BMI, ALT levels and c-IMT and ICCAD as compared to the CC/CT carriers.

Table 16. Clinical characteristics, biochemical and ultrasonographic measurements in the IMPROVE study population stratified by rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T).

		rs738409			rs10401969		rs1260326		
	N	CC	CG	GG	TT	CT+CC	CC	CT	TT
Men, n (%)	3347	1070 (47.9)	482 (48.9)	46 (42.6)	1385 (47.2)	221(53.6)	556 (52.0)	712 (45.0)	389 (53.4)
Age, years	3347	65.0 (59.8-67.3)	64.2 (59.6-67.1)	62.21 (58.6-66.6)	64.3 (59.5-67.2)	66.3 (60.8-67.2)	64.6 (59.5-67.1)	65.0 (60.1-67.4)	63.8 (59.4-63.3)
BMI, (kg/m^2)	3346	26.8 (24.2 9.3)	26.9 (24.6-29.7)	26.7 (24.1-30.9)	26.8 (24.2-29.4)	26.8 (24.6-29.8)	27.28 (24.7-30.2)	26.7 (24.2-29.1)	24.5 (23.8-28.9)
Cardiovascular risk factors, n (%)									
Current smoke	3347	351 (15.6)	135 (13.7)	16 (14.8)	448 (15.6)	54 (13.1)	163 (15.2)	223 (14.4)	116 (15.9)
Diabetes mellitus Type 2	3294	570 (25.7)	267 (27.5)	31 (29.8)	751 (26.0)	117 (28.7)	333 (31.7)	382 (25.11)	153 (21.2)
Hypertension	3347	1787 (79.28)	789 (80.1)	85 (78.7)	2310 (78.7)	351 (85.2)	881 (82.3)	1218 (78.63)	562 (77.2)
Biochemical measurements									
TC	3341	5.4 (4.7-6.2)	5.4 (4.7-6.2)	5.6 (4.5-6.4)	5.5 (4.7-6.2)	5.2 (4.6-6.0)	5.3 (4.6-6.2)	5.4 (4.7-6.2)	5.6 (4.9-6.3)
LDL-C	3347	3.5 (2.8-4.2)	3.5 (2.8-4.2)	3.6 (2.7-4.5)	3.5 (2.8-4.2)	3.4 (2.9-4.1)	3.5 (2.8-4.2)	3.5 (2.8-4.2)	3.64 (3.0-4.3)
HDL-C	3341	1.2 (1.0-1.5)	1.2 (1.0-1.4)	1.2 (1.0-1.4)	1.2 (1.0-1.5)	1.2 (1.0-1.4)	1.2 (1.0-1.5)	1.2 (1.0-1.5)	1.2 (1.0-1.5)
TG	3341	1.3 (0.9-1.9)	1.3 (0.9-1.9)	1.3 (0.9-1.7)	1.3 (0.9-1.9)	1.2 (0.9-1.7)	1.2 (0.9-1.7)	1.3 (0.9-1.8)	1.4 (1.0-2.1)
Glucose	3341	5.5 (4.9-6.3)	5.5 (5.0-6.4)	5.4 (4.9-6.4)	5.5 (4.9-6.3)	5.6 (5.1-6.6)	5.6 (5.1-6.5)	5.5 (5.0-6.3)	5.4 (4.8-6.0)
ALT	3347	20 (16.0-27.0)	21 (17.0-29.0)	23.5 (17.5-34.5)	20 (16 - 27)	22 (17.0–30.0)	21 (17.0-29.0)	20 (16.0–27.0)	20 (17.0–27.0)
Ultrasonographic measures (mm)									
C-IMT$_{mean}$	3346	0.85 (0.7-1.0)	0.85 (0.7-1.0)	0.81 (0.7-0.9)	0.85 (0.7-1.0)	0.87 (0.8-1.0)	0.86 (0.7-1.0)	0.85 (0.7-1.0)	0.84 (0.7-1.0)
C-IMT$_{max}$	3346	1.85 (1.4-2.5)	1.93 (1.4-2.6)	1.84 (1.4-2.3)	1.85 (1.4-2.5)	1.93 (1.4-2.5)	1.93 (1.4-2.6)	1.85 (1.4-2.5)	1.84 (1.4-2.4)
C-IMT$_{mean-max}$	3347	1.19 (1.0-1.4)	1.20 (1.0-1.4)	1.13 (1.0-1.3)	1.18 (1.0-1.4)	1.23 (1.1-1.4)	1.22 (1.0-1.4)	1.19 (1.0-1.4)	1.17 (1.0-1.4)
ICCAD	3347	7.72 (7.2-8.3)	7.82 (7.2-8.4)	7.55 (7.1-8.0)	7.73 (7.2-8.3)	7.82 (7.2-8.4)	7.85 (7.3-8.4)	7.71 (7.2-8.3)	7.64 (7.2-8.2)

Data are expressed as median (interquartile ranges) for continuous variables or number (percent) for categorical variables.

Abbreviations: BMI: body mass index; ALT: Alanine aminotransferase; TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol. Unit of measure: ALT: U/L; BMI: kg/m^2; TC, LDL-C, TG and Glucose: mmol/L.

Association of rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T) with c-IMT and ICCAD

No associations were observed between the effect allele (EA) rs738409-G, rs10401969-C and rs1260326 –T (vs the respective common allele) with C-IMT and ICCAD, as shown in Table 17.

Table 17. Association of rs738409 (C/G), rs10401969 (T/C), and rs1260326 (C/T) with c-IMT measures and ICCAD

Ultrasonographic measures	N	SNP	EA	Model 1			Model 2		
				β	SE	*p*	β	SE	*p*
		rs738409	G	-0.002	0.0029	0.443	-0.001	0.003	0.82
c-IMT $_{mean}$	**3346**	rs10401969	C	0.012	0.005	0.025	0.001	0.004	0.77
		rs1260326	T	-0.004	0.002	0.06	0.003	0.002	0.18
		rs738409	G	0	0.006	0.963	0.002	0.005	0.64
c-IMT $_{max}$	**3346**	rs10401969	C	0.014	0.009	0.108	0	0.008	0.97
		rs1260326	T	-0.01	0.004	0.013	0.001	0.004	0.81
		rs738409	G	-0.003	0.003	0.353	-0.001	0.003	0.66
c-IMT $_{mean-max}$	**3347**	rs10401969	C	0.014	0.005	0.006	0.003	0.005	0.48
		rs1260326	T	-0.005	0.002	0.02	0.003	0.002	0.17
		rs738409	G	0	0.001	0.499	0.002	0.001	0.17
ICCAD	**3347**	rs10401969	C	0.006	0.002	0.015	0.001	0.002	0.73
		rs1260326	T	-0.005	0.001	<0.001	-0.001	-0.001	0.27

Abbreviations: N: number of subjects; EA: effect allele. Model 1 univariate analysis. Model 2 adjusted for age, sex and MSD 1-3.
All c-IMT variables and ICCAD were logarithmically transformed before statistical analysis.

Association of rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T) with metabolic traits

Table 18 shows the results of the association of EA for each SNPs with ALT and metabolic traits.

In multivariate analysis, rs738409-G and rs10401969-C were associated with ALT circulating levels (β = 0.029, SE = 0.006, $p < 0.001$ and β = 0.040, SE = 0.009, $p < 0.001$, respectively), while no association was observed for rs1260326-T.

When we tested the association of the three SNPs with metabolic traits, only rs1260326-T was found to be associated with TG (β = 0.105, SE = 0.020, $p < 0.001$) and BMI (β = -0.224, SE = 0.102, $p = 0.028$).

Table 18. Association of the effect allele at rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T) with metabolic traits.

Metabolic traits	N	SNP	EA	Model 1			Model 2		
				β	SE	*p*	β	SE	*p*
		rs738409	G	0.031	0.006	<0.001	0.029	0.006	<0.001
ALT	3347	rs10401969	C	0.042	0.01	<0.001	0.04	0.009	<0.001
		rs1260326	T	-0.012	0.004	0.009	-0.008	0.004	0.062
		rs738409	G	0.296	0.136	0.029	0.265	0.132	0.045
BMI	3346	rs10401969	C	0.181	0.225	0.421	-0.038	0.219	0.816
		rs1260326	T	-0.569	0.101	<0.001	-0.224	0.1022	0.028
		rs738409	G	-0.025	0.036	0.489	-0.026	0.034	0.441
TC	3341	rs10401969	C	-0.13	0.059	0.028	-0.042	0.057	0.465
		rs1260326	T	0.122	0.027	<0.001	0.027	0.027	0.312
		rs738409	G	-0.008	0.032	0.803	-0.007	0.031	0.831
LDL-C	3347	rs10401969	C	-0.096	0.053	0.069	-0.022	0.051	0.659
		rs1260326	T	0.064	0.024	0.008	-0.027	0.024	0.255
		rs738409	G	-0.018	0.026	0.474	-0.022	0.025	0.388
TG	3341	rs10401969	C	-0.044	0.042	0.3	-0.03	0.042	0.482
		rs1260326	T	0.114	0.019	<0.001	0.105	0.02	<0.001
		rs738409	G	0.024	0.025	0.344	0.027	0.023	0.233
Glucose	2426	rs10401969	C	0.087	0.041	0.035	0.033	0.038	0.376
		rs1260326	T	-0.087	0.018	<0.001	-0.031	0.017	0.076

Abbreviations: N: number of subjects; EA: effect allele, ALT: Alanine aminotransferase; BMI: body mass index; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides. Unit of measure: ALT: U/L; BMI: kg/m^2; TC, LDL-C, TG and Glucose: mmol/L
Model 1: univariate analysis
Model 2: adjusted by age, sex and MDS 1-3
ALT and TG were logarithmically transformed before statistical analysis because of skewed distributions.

Association of rs738409 (C/G), rs10401969 (T/C) and rs1260326 (C/T) with c-IMT and ICCAD after stratification by ALT levels

Tables 19 and 20 shows c-IMT measures and ICCAD across rs738409, rs10401969, and rs1260326 genotype groups stratified by ALT quartiles.

As shown in Supplementary Table II, rs10401969-C was positively associated with c-IMT$_{max}$ and c-IMT$_{mean-max}$ (p = 0.018 and 0.021, respectively) in the highest ALT quartile.

No significant associations between rs738409 and rs1260326 and c-IMT and ICCAD were observed in any of the ALT quartiles (data not shown).

Table 19. Distribution of c-IMT and ICCAD measures across rs738409 (C/G), rs10401969 (T/C), and rs1260326 (C/T) genotype groups in the different ALT quartiles.

Quartile	N		rs738409			rs10401969		rs1260326		
			CC (N=623)	CG (N=227)	GG (N=26)	TT (N=786)	CT+CC (N=90)	CC (N=265)	CT (N=433)	TT (N=178)
I	876	IMT_{mean}	0.85 (0.74-0.99)	0.87 (0.73-1.01)	0.78 (0.73-0.95)	0.85 (0.74-1.00)	0.85 (0.70-0.97)	0.86 (0.75-0.99)	0.85 (0.75-1.00)	0.83 (0.72-0.98)
		IMT_{max}	1.85 (1.45-2.55)	1.94 (1.45-2.61)	1.8 (1.39-2.31)	1.88 (1.45-2.59)	1.84 (1.39-2.42)	1.93 (1.45-2.55)	1.85 (1.45-2.59)	1.85 (1.39-2.41)
		$IMT_{mean-max}$	1.21 (1.04-1.41)	1.22 (1.04-1.48)	1.13 (1.00-1.36)	1.21 (1.04-1.42)	1.22 (1.04-1.41)	1.23 (1.05-1.42)	1.22 (1.05-1.42)	1.17 (1.02-1.42)
		ICCAD	7.68 (7.17-8.27)	7.8 (7.20-8.30)	7.27 (6.92-7.89)	7.68 (7.15-8.24)	7.81 (7.22-8.34)	7.79 (7.20-8.25)	7.64 (7.15-8.30)	7.64 (7.10-8.15)
			CC (N=632)	CG (N=274)	GG (N=23)	TT (N=827)	CT+CC (N=102)	CC (N=279)	CT (N=413)	TT (N=237)
II	929	IMT_{mean}	0.85 (0.74-0.98)	0.85 (0.76-0.99)	0.88 (0.74-1.04)	0.85 (0.75-0.98)	0.84 (0.74-1.03)	0.86 (0.74-1.02)	0.85 (0.75-0.97)	0.84 (0.74-0.98)
		IMT_{max}	1.85 (1.45-2.50)	1.88 (1.39-2.42)	1.93 (1.48-2.42)	1.85 (1.45-2.42)	1.84 (1.39-2.61)	1.85 (1.45-2.61)	1.85 (1.48-2.42)	1.85 (1.39-2.41)
		$IMT_{mean-max}$	1.18 (1.05-1.39)	1.16 (1.05-1.41)	1.22 (1.00-1.54)	1.18 (1.05-1.39)	1.2 (1.07-1.45)	1.2 (1.05-1.45)	1.18 (1.05-1.37)	1.16 (1.03-1.38)
		ICCAD	7.67 (7.18-8.24)	7.79 (7.28-8.34)	7.89 (7.11-8.30)	7.69 (7.21-8.27)	7.8 (7.22-8.38)	7.81 (7.27-8.35)	7.69 (7.21-8.27)	7.6 (7.17-8.24)
			CC (N=546)	CG (N=235)	GG (N=20)	TT (N=691)	CT+CC (N=110)	CC (N=253)	CT (N=373)	TT (N=175)
III	801	IMT_{mean}	0.85 (0.74-0.98)	0.86 (0.73-1.01)	0.87 (0.75-0.94)	0.85 (0.73-1.00)	0.87 (0.76-1.03)	0.86 (0.74-1.00)	0.85 (0.73-0.99)	0.85 (0.74-1.02)
		IMT_{max}	1.84 (1.39-2.41)	1.85 (1.39-2.51)	1.8 (1.42-2.59)	1.84 (1.39-2.41)	1.85 (1.48-2.51)	1.85 (1.45-2.5)	1.76 (1.39-2.32)	1.85 (1.35-2.5)
		$IMT_{mean-max}$	1.2 (1.02-1.42)	1.18 (1.03-1.41)	1.2 (1.07-1.33)	1.19 (1.02-1.40)	1.23 (1.06-1.49)	1.22 (1.03-1.42)	1.18 (1.02-1.38)	1.2 (1.03-1.45)
		ICCAD	7.68 (7.19-8.33)	7.78 (7.22-8.29)	7.8 (7.33-8.12)	7.73 (7.20-8.27)	7.78 (7.20-8.46)	7.84 (7.31-8.48)	7.64 (7.14-8.16)	7.64 (7.21-8.29)
			CC (N=453)	CG (N=249)	GG (N=39)	TT (N=631)	CT+CC (N=110)	CC (N=273)	CT (N=330)	TT (N=178)
IV	741	IMT_{mean}	0.84 (0.73-0.99)	0.85 (0.76-1.00)	0.78 (0.71-0.88)	0.83 (0.73-1.00)	0.88 (0.78-1.01)	0.86 (0.76-1.00)	0.85 (0.73-1.02)	0.82 (0.74-0.95)
		IMT_{max}	1.84 (1.36-2.50)	1.94 (1.45-2.59)	1.67 (1.20-2.22)	1.78 (1.35-2.50)	2.13 (1.67-2.61)	1.93 (1.45-2.51)	1.85 (1.35-2.59)	1.74 (1.39-2.31)
		$IMT_{mean-max}$	1.19 (1.02-1.40)	1.23 (1.04-1.44)	1.1 (0.98-1.28)	1.18 (1.01-1.39)	1.26 (1.11-1.48)	1.23 (1.05-1.43)	1.19 (1.01-1.44)	1.16 (1.02-1.36)
		ICCAD	7.87 (7.34-8.44)	7.92 (7.33-8.46)	7.56 (7.11-8.09)	7.87 (7.32-8.42)	7.84 (7.35-8.51)	7.97 (7.35-8.56)	7.8 (7.34-8.41)	7.72 (7.21-8.31)

Data are reported as median and IQR. Minor allele frequency (%) for each quartile (Q): rs738409-G Q1:16, Q2:17, Q3:17, Q4:22; rs10401969-C Q1:5, Q2:5, Q3:7, Q4:8; rs1260326-T Q1:45, Q2:47, Q3:45, Q4:44.
Abbreviations: N number of subjects

Table 20. Association of rs10401969 (T/C) with measures of c-IMT and ICCAD in the different ALT quartiles.

Quartile	**N**	**Model 1**	**Model 2**

		Ultrasonographic measures	β	SE	*p*	β	SE	*p*
I	876	c-IMT $_{mean}$	0.003	0.013	0.765	-0.004	0.009	0.667
		c-IMT $_{max}$	-0.01	0.019	0.574	-0.021	0.011	0.224
		c-IMT $_{mean-max}$	0.002	0.011	0.847	-0.006	0.01	0.559
		ICCAD	0.005	0.005	0.309	0.002	0.004	0.697
II	929	c-IMT $_{mean}$	0.003	0.009	0.704	-0.008	0.008	0.317
		c-IMT $_{max}$	0	0.168	1	-0.015	0.016	0.33
		c-IMT $_{mean-max}$	0.006	0.01	0.558	-0.007	0.009	0.432
		ICCAD	0.005	0.005	0.275	-0.001	0.004	0.866
III	801	c-IMT $_{mean}$	0.012	0.009	0.177	0.003	0.008	0.756
		c-IMT $_{max}$	0.015	0.017	0.378	-0.002	0.016	0.894
		c-IMT $_{mean-max}$	0.016	0.01	0.092	0.005	0.009	0.575
		ICCAD	0.005	0.005	0.254	0	0.004	0.958
IV	741	c-IMT $_{mean}$	0.024	0.01	0.015	0.016	0.009	0.077
		c-IMT $_{max}$	0.051	0.018	0.004	0.039	0.016	0.018
		c-IMT $_{mean-max}$	0.031	0.01	0.003	0.022	0.009	0.021
		ICCAD	0.006	0.005	0.227	0.001	0.004	0.769

Abbreviations: N number of subjects.
Model 1 univariate analysis
Model 2 adjusted for age, sex and population structure
All c-IMT variables and ALT were logarithmically transformed before statistical analysis

DISCUSSION

The main finding of our study is that rs738409, rs10401969 and rs1260326, three genetic variants consistently associated with NAFLD, were not associated with measures of sub-clinical atherosclerosis (c-IMT and ICCAD) in European individuals at high CV risk. Our results confirm and extend previous observations showing an association of these three variants with ALT circulating levels and suggest that atherosclerosis burden might be higher in the presence of rs10401969-C and high ALT levels. Consistent with this, our data support the hypothesis that an impaired liver function might contribute to or be associated with more severe atherosclerotic disease [1,2].

Rs738409 (C/G) maps at the Patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, encoding an enzyme with lipid acyl hydrolase activity. Rs738409-G causes an amino acid substitution (I148M) in a *PNPLA3* side chain, able to modify the catalytic properties of the enzyme leading to triglyceride accumulation in the liver [3]. The increased hepatic fat accumulation observed in subjects carrying rs738409-G allele may explain the elevated ALT levels [4,5].

Studies performed in NAFLD patients have provided controversial results on the association between rs738409 (C/G) and measures of subclinical atherosclerosis [6,7]. In young NAFDL patients, rs738409-GG, was associated with a greater severity of carotid plaques, thicker c-IMT and with c-IMT progression [6]. On the other hand, rs738409-GG was found to be associated with c-IMT measures only in subjects with metabolic syndrome and NAFLD [7] and was not associated with c-IMT measures in a Chinese cohort of 4,300 middle-aged men and women [8]. Our findings do not support an association of this SNP either with measures of subclinical atherosclerosis or with metabolic traits, thus supporting the hypothesis that this SNP is not related to CVD, but to NAFLD.

Rs10401969 maps to a locus at Chr19p13.11 [9], known to be of importance in the regulation of lipid metabolism [10]. Previous studies have suggested that Transmembrane 6 superfamily member 2 (*TM6SF2*), a gene expressed in the small intestine and in the liver and involved in regulation of lipid absorption and metabolism [11], is the causative gene within the Chr19p13.11 locus [12]. Rs10401969 is in strong linkage disequilibrium (LD) in European populations with a nonsynonymous mutation where presence of rs58542926-G replaces a glutamate with lysine at residue 167 (E167K) [13]. This amino-acid change causes misfolding and an accelerated degradation of *TM6SF2* that results in reduced lipid absorption and hepatic metabolism. This predisposes to liver steatosis and related hepatic complications and at the same time maintains a favourable, non-atherogenic lipid profile consistent with the observation that this mutation exerts a protective effect towards CV events [9,14-19].

Our results suggest that in the presence of high circulating ALT levels, which represent an indicator of liver damage, presence of the rs10401969-C has a modest

association with c-IMT measures. High circulating ALT levels are not specific for NAFLD and may be elevated in a variety of clinical conditions. ALT levels were associated with an increased risk of CVE in a Dutch population after adjustment for determinants of the metabolic syndrome and other CV risk factors [20] and with c-IMT in Korean patients with different degree of NAFLD [21]. Our results confirm and extend previous knowledge on the intertwine between NAFLD and atherosclerosis and may suggest that elevated circulating ALT levels in patient at high risk for NAFLD and CVD (such as patients with metabolic syndrome) concomitant presence of might be regarded as a risk indicator for the risk of CVD, and may prompt additional investigations even in the absence of clinically evident CVD.

Rs1260326-T at *GCKR* causes an amino-acid change (P446L) in the glucokinase regulatory protein associated with NAFLD risk [22]. GCKR modulates the activity of glucokinase, an enzyme involved in the regulation of glucose metabolism and storage in the liver. Presence of the amino-acid substitution has been reported to be associated with a favourable effect on glucose and insulin metabolism, but with an increased biosynthesis of lipids in the liver [23]. This SNP has been formerly associated with an increased c-IMT in patients with metabolic syndrome [24], but was not associated with c-IMT measures in individuals without myocardial infarction or type 2 diabetes [25]. Moreover, rs780094 in the *GCKR* locus, in strong LD with rs1260326, was also associated with a significant increase in c-IMT in men but not in women in general population Japanese cohort [26]. Our data do not support the association of this variant with measures of subclinical atherosclerosis.

This study has several strengths: a large sample size, a wide range of important vascular risk factors, consistent methodology for a large number of ultrasonographic variables acquisition and measurement; methods of carotid image acquisition and all measurement of c-IMT variables and ICCAD were standardized and the sonographers enrolled in the study were trained and certified in all involved center. Limitations of our study are mainly related to the lack of assessment of liver pathology, that was only graded by ALT levels. Moreover, other factors possibly modifying ALT were not assessed.

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GENERAL CONCLUSION

In this Ph.D. thesis, a comprehensive analysis of the red wine bioactivity and polyphenols characterization through UHPLC-Q-Orbitrap spectrometry measurement were carried out. Our results highlighted the high content of bioactive compounds as well as antioxidant capability of the red wine. Therefore, it is desirable to continue expanding the understanding of the current topic through *in vivo* study, in order to demonstrate the potential health benefits of moderate red wine consumption.

On the other hand, coffee husk bioactivity and polyphenols characterization using UHPLC-High resolution mass spectrometry were investigated. Coffee husk represents a great source of bioactive compounds showing high antioxidant and antifungal activity. This study indicates the possible exploiting the health-promoting potential of this coffee by-product in order to develop natural food ingredients, herbal infusions, and supplements with functional properties. Moreover, the demonstrated antifungal activities suggests that it has a promising potential as a natural food ingredient to extend and stabilize the shelf life of foods.

Finally, the possible associations between rs738409, rs10401969 and, rs1260326 with measures of carotid subclinical atherosclerosis in a large European cohort consisting of individuals at high risk of cardiovascular events but free of clinical CVD manifestation were assessed. This study shows no association among genetic variants associated with NAFLD and measures of subclinical atherosclerosis, except in the presence of high circulating ALT. These results support the possible mediating function of impaired liver function on atherosclerosis.