UNIVERSITAT DE BARCELONA

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Abstract

Chronic diseases are the leading cause of death in the world. Several of these conditions are related to the cardiovascular or cognitive system whose pathophysiology is closely linked to alterations in inflammatory processes. There are some modifiable factors that can help prevent or promote the development of these diseases, and nutrition is one of them. Mediterranean diet (MedDiet) stands out as one of the dietary patterns with more health benefits. This plant-based diet includes a balanced intake of macronutrients, and it also provides micronutrients and bioactive compounds, including vitamins and polyphenols, which are recognized for their beneficial effects. Therefore, this dissertation aimed to investigate the role of three naturally occurring components of the MedDiet - fatty acids, polyphenols, and vitamin B12 - on three main oucomes: metabolic and inflammatory pathways (objective 1), cardiovascular (objective 2) and cognitive health (objective 3). All the research was conducted using data from the PREDIMED and PREDIMED-Plus studies, two randomized clinical trials that included an older Mediterranean population at high cardiovascular disease (CVD) risk.

To achieve the first objective of this thesis, we analyzed the plasma fatty acid composition of participants in the PREDIMED and PREDIMED-Plus trials. The first findings demonstrated that the bioavailability of saturated fatty acids, and especially palmitic acid, was impaired in diets with high consumption of fruits and vegetables. Furthermore, alterations in the estimated activities of the enzymes that metabolize fatty acids were associated with cardiometabolic risk factors. Fatty acid metabolism has a great impact on health, as depending on their degree of saturation they can exhibit anti-inflammatory or pro-inflammatory properties. We found that increases in saturated fatty acids, and specifically palmitic acid, were associated with higher levels of the inflammatory molecule interleukin-6. Vitamin B12, on the other hand, was inversely associated with this inflammatory molecule and also with C-reactive protein. In addition, wine consumption measured as its biomarker tartaric acid presented an inverse relationship with circulating levels of adhesion molecules associated with atherosclerosis.

In light of these findings, we turned into the second objective of this dissertation, starting by exploring the influence of wine on cardiovascular health. Wine consumption was associated with lower concentrations of LDL-cholesterol in postmenopausal women, a population at increased risk of CVD. Regarding other risk factors of CVD, a panel of urinary phenolic compounds discriminant for type-2 diabetes was described, and among them dihydrocaffeic acid and genistein diglucuronide were found to be protective. Considering the low bioavailability of polyphenols and high metabolism by the gut microbiota, a novel method was used to identify and quantify microbial phenolic metabolites (MPM) in urine. A positive association was found between them and cardiovascular health, particularly for urolithin B glucuronide and LDL-cholesterol.

MPM have potential positive benefits on cognitive health; however, to exert these effects, they must overcome another challenge: crossing the blood-brain barrier. Permeability through this layer that protects the central nervous system is a requirement for compounds to exert direct biological effects on cognitive health, but the literature has only demonstrated that a select few can penetrate. In our studies, we found that MPM were associated with better global cognition, especially protocatechuic acid and enterolactone glucuronide. MPM were particularly beneficial for frontal lobe functions, and vanillic acid glucuronide showed the strongest association. Lastly, the vitamin B12 on cognition was evaluated according to the adherence to MedDiet. Only in participants with high adherence vitamin B12 was associated with better memory, while no link was observed with low adherence.

Overall, these findings highlight the role of dietary choices in supporting healthy aging and preventing chronic diseases in the context of an older Spanish population at high risk of CVD.

Abbreviations

AHA	*American Heart Association*
BMI	*Body mass index*
CVD	*Cardiovascular disease*
CRP	*C-reactive protein*
DASH	*Dietary approaches to stop hypertension*
D5D	*δ-5-desaturase*
D6D	*δ-6-desaturase*
DHA	*Docosahexaenoic acid*
EPA	*Eicosapentaenoic acid*
FAME	*Fatty acid methyl esters*
F&V	*Fruits and vegetables*
GC-FID	*Gas chromatography coupled to a flame ionization detector*
HDL-c	*High-density lipoprotein cholesterol*
HPLC	*High-performance liquid chromatography*
ICVH	*Ideal cardiovascular health*
ICAM	*Intercellular adhesion molecule*
IL-6	*Interleukin-6*
LDL-c	*Low-density lipoprotein cholesterol*
MS	*Mass spectrometry*
MedDiet	*Mediterranean diet*
MCFA	*Medium-chain fatty acids*
MetS	*Metabolic syndrome*
MPM	*Microbial phenolic metabolites*
MCP-1	*Monocyte chemotactic protein 1*
MUFA	*Monounsaturated fatty acid*
PUFA	*Polyunsaturated fatty acid*
ROS	*Reactive oxygen species*
SFA	*Saturated fatty acid*
SCD	*Stearoyl-CoA desaturase*

TNF-α	*Tumour necrosis factor alpha*
TLR-4	*Toll-like receptor 4*
T2D	*Type-2 diabetes*
VCAM	*Vascular cell adhesion molecule*

Contents

INTRODUCTION

1. Introduction

1.1. Preventive nutrition

Chronic diseases affect more than half of the adult population and are responsible for approximately 90% of deaths in Spain[1]. These conditions, also referred to as non-communicable diseases, are long-term health conditions that persist over an extended period and typically progress slowly. Therefore, chronic diseases are a global health challenge, affecting millions of people and posing a significant burden on healthcare systems worldwide[2].

Nutrition plays a pivotal role in the development and progression of chronic diseases. Unhealthy eating habits are significant contributors to the development of chronic diseases, whereas healthy nutritional habits can prevent the onset in these diseases[3]. The concept of preventive nutrition refers to the proactive and strategic use of dietary choices and nutritional practices to reduce the risk of developing chronic diseases, promote overall health, and enhance well-being[4]. Preventive nutrition, therefore, emphasizes the role of nutrition in disease prevention rather than solely treating health issues after they have developed.

For many years, epidemiological studies with large cohorts have focused on assessing the effects of diet on long-term health. These studies have yielded dietary patterns that are associated with beneficial health characteristics of different geographical origins. In general, these patterns are plant-based and incorporate limited protein and fat intake. Among the most clearly defined patterns are the Dietary Approaches to Stop Hypertension (DASH), Nordic diet, Traditional Asiatic diet, and the Mediterranean diet (MedDiet)[5–8].

1.1.1. Mediterranean diet

The MedDiet has been shaped over thousands of years through the exchange of people, cultures, and foods from countries around the Mediterranean basin, resulting in a rich

culinary heritage. The MedDiet was initially defined in the 1960s and described the food pattern followed in Crete, much of the rest of Greece, and the south of Italy[9,10]. Compared to other regions, the population of the Mediterranean area had lower levels of chronic diseases such as coronary heart disease and specific types of cancers, as well as higher adult life expectancy during that period[9].

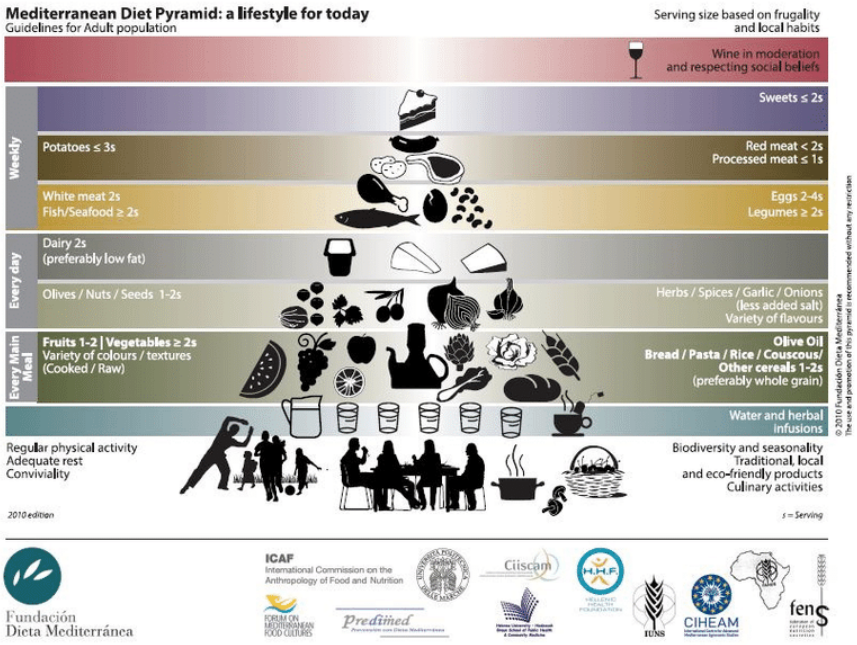

Figure 1.1.1: Mediterranean diet pyramid.

The MedDiet is a plant-based dietary pattern characterized by a high consumption of fruits, vegetables, legumes, nuts, whole grains, and olive oil as the principal source of fats. It also includes high to moderate intakes of fish and seafood, moderate consumption of dairy products, poultry, and eggs (zero to four per week), low consumption of red meat, and moderate intake of wine consumed with meals[11].

This dietary pattern has been associated to numerous health effects over the years. Numerous studies have presented compelling evidence regarding the advantages of the MedDiet in relation to chronic illnesses, which encompass cardiovascular disease (CVD) and its associated risk factors such as obesity, hypertension, and dyslipidemia[12]. Additionally, the MedDiet has shown promising effects in mitigating neurodegenerative disorders potentially contributing to cognitive preservation and overall brain health[13].

1.1.2. Inflammation

Inflammation has been implicated in the pathology of several diseases, such as CVD, neurodegenerative diseases, and certain cancers. Various types of cells are responsible for the production of proinflammatory and antiinflammatory factors[14]. Whereas acute inflammation plays a protective role in combating infections and repairing damaged tissues, a prolonged imbalance in the secretion of proinflammatory molecules leads to a status of chronic inflammation with harmful health effects.

Chronic inflammation is a recognized key driver of the development and progression of CVD[15]. In the early stages of atherosclerosis, endothelial cells become activated in response to tissue injury or infection caused by stimuli that include inflammatory cytokines. Over time, this process leads to endothelial dysfunction[16]. This upregulates adhesion molecules in the vascular endothelium and circulating leukocytes, promoting the recruitment of inflammatory molecules in response to inflammatory stimuli. This contributes to the formation of atherosclerotic plaques[17]. Once the plaque evolves, the presence of foam cells and other immune cells trigger the ongoing inflammatory response conferring instability to the plaque. If the rupture of the plaque occurs, its contents are released into the bloodstream, which can lead to the formation of blood clots, ultimately increasing the risk of a stroke[17]. Chronic inflammation has also been associated with heart failure, partly due to the endothelial dysfunction, but also through activation of neurohormonal systems that trigger myocardial inflammation[18,19].

In recent years, neuroinflammation has emerged as a potential contributor to degenerative diseases as Alzheimer's disease. Proinflammatory molecules in the central nervous system are primarily produced by microglia, which are the central nervous system's primary immune cells. The constant production of inflammatory cytokines results in a permanent activation of these cells that ultimately leads to the impairment of the microglia and promotes processes of amyloidosis, neuronal death, and neurodegeneration[20,21].

Several epidemiological and clinical trials have suggested the beneficial effect of the MedDiet in reducing inflammatory biomarkers, such as interleukines or C-reactive protein (CRP)[22,23]. Furthermore, following the MedDiet also demonstrated a positive impact in decreasing serum indicators of atherosclerotic plaque stability when compared to a low-fat diet[24]. Certain constituents within its dietary composition, like polyphenols and omega-3 fatty acids, possess potent anti-inflammatory properties, potentially contributing in the regulation of the inflammatory processes associated with several chronic diseases[25,26]. The impact of the MedDiet on inflammatory pathways at the genetic level has been scarcely studied, yielding no significant results[27]. However, specific components included in this dietary pattern, such as monounsaturated fatty acid (MUFAs) or phenolic compounds, influence the expression of genes related to inflammatory and atherosclerotic processes[28].

1.1.3. Cardiovascular disease

CVDs rank as the primary cause of death globally, as an estimated 17.9 million people die from this cause each year, accounting for 32% of total global deaths[29]. CVD encompasses an array of disorders affecting the heart and blood vessels, including conditions such as coronary heart disease, cerebrovascular disease, rheumatic heart disease, and various other related conditions[29]. Even though the cause of CVD is not fully understood, certain individual characteristics can act as risk factors that increase the likelihood of experiencing it through increasing the risk of inflammation and weakening the immune system. These factors include smoking, hypertension, hypercholesterolemia, diabetes mellitus, obesity, sedentary lifestyle, and unhealthy diets[30,31].

Numerous epidemiological studies have linked adherence to the MedDiet with a lower risk of suffering from CVD[32–37]. Two meta-analyses that included observational studies and clinical trials concluded that MedDiet reduced the incidence of CVD, stroke, and several metabolic risk factors[32–34]. In the EPIC study, that included a European cohort, MedDiet adherence was associated with lower risk of coronary heart disease and a reduction of all-cause mortality in coronary patients[35,36]. There is consistent

evidence indicating that the MedDiet leads to improvements in CVD risk factors, such as blood lipids or hypertension[38,39]. A meta-analysis found that MedDiet was associated with lower total cholesterol and a small reduction in low-density lipoprotein cholesterol (LDL-c) and triglycerides[40]. There is also strong evidence that the MedDiet is effective in reducing blood pressure, both systolic and diastolic[41,42].

The largest clinical study conducted to assess its effects was the PREDIMED trial, which took place in Spain and involved 7,447 participants. This trial unequivocally demonstrated the MedDiet's superiority over a low-fat diet in primary CVD prevention. The incidence of CVD was 30% lower in the groups that followed the MedDiet, whether supplemented with extra virgin olive oil or nuts, compared to the control group[37]. Considering these results, it was decided to conclude the study since the MedDiet had clearly proven to be superior to the low-fat diet in preventing CVD, and it was considered unethical to continue with a control group not following the MedDiet recommendations. After the success of the PREDIMED trial, in 2013 a second study was initiated, the PREDIMED-Plus trial, to continue investigating the benefits of the MedDiet. The PREDIMED-Plus trial is currently an on-going study. However, the results found so far in substudies that used a subsample of the cohort are promising. Salas-Salvadó et al. found that MedDiet combined with an intensive lifestyle intervention was beneficial for weight loss and improvements in the metabolic syndrome (MetS) components after 12 months of follow-up[43]. In the same period of time, the intervention was able to induce changes in the gut microbiota[44] and improved high-density lipoprotein cholesterol (HDL-c) and triglyceride metabolism compared to MedDiet without physical activity[45].

1.1.4. Cognitive health

Dementia affects over 55 million people worldwide and every year the number increases with 10 million new cases[46]. Dementia comprises a group of neurodegenerative diseases that affect memory and mental capacity, interfering with daily life and leading to an impaired cognitive function beyond what can be expected from natural ageing. Among them, Alzheimer's disease is the most common, as

it accounts for 60-70% of the total cases of dementia[47]. While the causes of these diseases at a molecular level are not fully understood, some risk factors that predispose individuals to develop them are known. Within these factors, we can distinguish non-modifiable ones, such as age or depression, as well as an extensive list of modifiable factors that include smoking, physical activity, hypertension, diabetes, obesity, social isolation, alcohol consumption, and unhealthy diets[46].

As it was previously mentioned (see section 1.1.1), inflammation and processes related to atherosclerosis and the formation of arterial plaques could play an important role in neurodegenerative disease[48]. Mitochondrial dysfunction is also commonly found in patients with dementia, as it results in the accumulation of toxic reactive oxygen species (ROS). Indeed, mitochondria can regulate inflammatory pathways, and its malfunction can initiate inflammatory processes[49].

Evidence indicates that higher adherence to the MedDiet is associated with better cognitive function. To date, the majority of studies conducted have been observational studies, and various meta-analyses have demonstrated the benefits of this dietary pattern on cognitive health, including cognitive impairment[50] and lower risk of Alzheimer's disease[51,52]. Regarding intervention studies, few research has been conducted. In the PREDIMED study, Valls-Pedret et al. reported an improvement in global, frontal, and memory functions in the groups that followed a MedDiet[53]. In another substudy of the PREDIMED trial, participants in the MedDiet groups obtained better scores in cognitive tests indicating cognitive impairment and risk of dementia[54]. Another clinical trial that included participants with normal and impaired cognition found that after four weeks MedDiet improved biomarkers related to Alzheimer's disease and cerebral perfusion in the healthy subjects[55]. A cross-sectional study reported that MedDiet was protective against cognitive decline and mediotemporal atrophy, and suggested that the observed benefits could be related to a decrease of amyloidosis and tau-pathology[56].

In summary, a MedDiet pattern could protect against cognitive decline and neurodegenerative diseases, especially in older adults with a higher risk. Nevertheless, more studies are needed to clarify this relationship and elucidate the mechanisms.

1.2. Nutrients and bioactive compounds present in the Mediterranean diet

Several individual components of the MedDiet have proved to exert beneficial effects on health. The consumption of multiple servings of fruits and vegetables is encouraged, which provides reduced caloric burden and intake of several micronutrients with antioxidant properties[57]. It is also recommended the consumption of whole grains as a source of fiber, related to antiinflammatory properties and inhibition of fat absorption[58]. Olive oil and nuts, the main sources of healthy fats of the MedDiet, not only provide omega-9 and omega-3 fatty acids, but are also rich in compounds that are attributed to having multiple health benefits, as polyphenols, vitamins, or phytoesterols[59,60]. Another source of healthy fats in the MedDiet is fish, which contains omega-3 fatty acids and other bioactive compounds such as vitamin B12[61].

Consequently, the MedDiet incorporates a variety of foods with advantageous properties for the prevention of chronic diseases, and it may be the synergy of all these components that contribute to its highly beneficial dietary profile. The following chapter provides an explanation of the nutrients found in the MedDiet that are the focus of this thesis: fatty acids, polyphenols, and vitamin B12.

1.2.1. Fatty acids

Fatty acids are the primary components of lipids, a major energy source that can also facilitate the transportation of fat-soluble vitamins and other lipid-soluble compounds throughout the body. Fats and oils are present in a wide variety of foods, both of plant and animal origin, including nuts, dairy products, meats, or fish[62].

Chemically, fatty acids are comprised of carboxylic acids with aliphatic chains, which may be saturated or unsaturated, straight or branched. The hydrocarbon chain varies from 2 to 36 carbon atoms and typically have an even number of carbon atoms[63]. The length and degree of saturation are highly variable among different fatty acids, and

these factors determine their chemical and physical properties. These characteristics also play a crucial role in their effects on health[62].

Fatty acids in the organism can originate either exogenously or endogenously, depending on the type. Fatty acids are produced through the synthesis of two or three carbon precursors, facilitated by acyl carrier protein, NADPH, and acetyl-CoA carboxylase[63,64]. Elongation occurs through the utilization of malonyl-CoA in the microsomal system and acetyl-CoA in the mitochondrial system. Furthermore, their degradation via β-oxidation in the mitochondria results in the release of energy[63,64].

Humans are able to synthesize MUFAs from saturated fatty acid (SFAs) of endogenous or dietary origin due to the action of the enzyme stearoyl-CoA desaturase (SCD) or δ-9-desaturase[65], which introduces a double bond in the δ-9 position of the aliphatic chain. Specifically, this enzyme is responsible for the transformation of palmitic (C16:0) and stearic acid (C18:0) into palmitoleic (C16:1 n-7) and oleic acid (C18:1 n-9), respectively[66]. The importance of this biotransformation lies in the fact that palmitoleic and oleic acids are the major components of phospholipids membranes and cholesterol esters[67]. Further desaturation of oleic acid into polyunsaturated fatty acid (PUFAs) is not possible in humans, as we lack of the necessary enzymes to catalyze the reaction[68]. Therefore, linoleic (C18:2 n-6) and α-linolenic (C18:3 n-3) acids are classified as essencial fatty acids, as they can only be obtained through dietary sources. Further elongation and saturation of linoleic and linolenic acids to synthesize other long chain fatty acids is performed by δ-6-desaturase (D6D), δ-5-desaturase (D5D), and δ-4-desaturase, and δ-6- and δ-5-elongase[69–71]. However, the activity and efficiency of these enzymes can be affected by cardiometabolic alterations, such as type-2 diabetes (T2D) or obesity, and age[72,73].

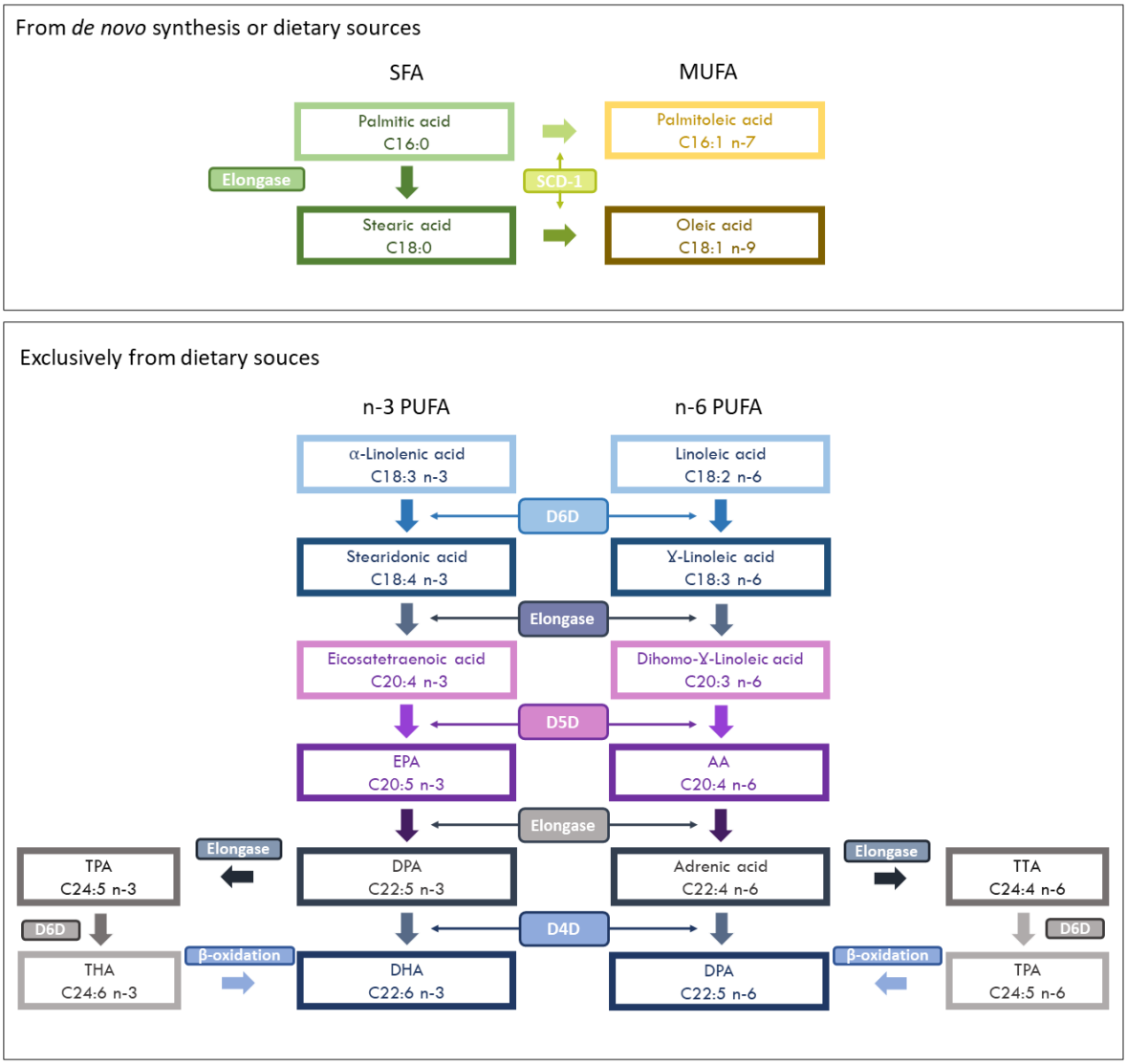

Figure 1.2.1: Fatty acids metabolism[69–71].
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; TTA, tetracosatetraenoic acid; TPA, tetracosapentaenoic acid; THA, tetracosahexaenoic acid; DHA, docosahexaenoic acid; SCD, stearoyl coenyme A desaturase; D6D, δ-6-desaturase; D5D, δ-5-desaturase; D4D, δ-4-desaturase.

Since the effects of fatty acids vary greatly depending on their chemical structure, in the following section, we have classified them according to their level of saturation[62].

1.2.1.1. Saturated Fatty Acids

SFAs lack double bonds in their aliphatic chain, and their properties such as melting point and lipophilicity increase with chain length[74,75]. Short-chain fatty acids (SCFA)

have 1-6 carbon atoms, while medium-chain fatty acids (MCFA) have 7-12 carbon atoms[76,77]. SCFA and MCFA cannot form micellar structures and are not part of cell membranes[78]. SCFA are primarily produced by the fermentation of dietary fiber in the colon by gut microbiota[79,80]. Food sources of exogenous SCFA and MCFA include milk, dairy products, coconut oil, palm kernel oil, butter, and margarine[81,82].

The most abundant long-chain SFAs in nature are myristic (C14:0), palmitic (C16:0), and stearic acid (C18:0) along with the MCFA lauric acid (C12:0)[83]. In Europe, the major dietary sources of SFAs are meat products, dairy products, and fats and oils[84]. These SFAs not only serve as an energy source but also as components of cell membranes. In addition, palmitic acid can also influence cellular functions by acting as precursor molecules for the biosynthesis of ceramide and sphingolipids, which modifies membrane fluidity[85].

The impact of SFAs, especially long-chain SFAs, on health outcomes has yielded mixed results. Consumption of saturated fats has been associated with chronic diseases such as T2D and CVD[86,87]. The mechanisms underlying these effects are related to inflammation and the activation of molecules that trigger inflammation like toll-like receptor 4 (TLR-4) and nuclear factor kB[88,89]. However, not all SFAs have the same impact on cardiometabolic health. Lauric, myristic, and palmitic acids have been found to increase total cholesterol levels, while stearic acid has shown no effect or more favorable effects on LDL-c[90,91]. Stearic acid may pose a lower risk to cardiovascular health based on its influence on lipoproteins[92]. However, its role in other CVD markers is still unclear[93]. Palmitic acid, on the other hand, is known for its negative effects, including inducing of proinflammatory cytokines and its association with obesity, CVD, T2D, and cancer[94,95]. Similar findings have been observed for lauric and myristic acids, which exhibit similar effects on inflammation and lipoproteins[96,97].

1.2.1.2. Monounsaturated Fatty Acids

MUFAs contain one carbon-carbon double bond and are typically in liquid form at room temperature. The main dietary sources of MUFAs are olive oil, avocados, peanuts, nuts, canola oil, and seeds, even though some of them can also be synthesized

endogenously. Oleic acid is the main representative of this group, followed distantly by palmitoleic and vaccenic acid (C18:1 n-7)[98]. MedDiet is particularly rich in MUFAs, specially oleic acid, due to its high intake of olives and olive oil. It has been suggested that the cardioprotective effects of the MedDiet are partly attributed to MUFAs[99].

Diets rich in oleic acid have been effective in reducing cardiovascular risk factors, such as total cholesterol, LDL-c, and non-HDL-c, although to a lesser extent than PUFAs[100]. Oleic acid has also demonstrated positive effects on insulin metabolism and reducing cholesterol levels[101,102]. In vitro studies have supported these findings, suggesting oleic acid's ability to inhibit cholesterol and fatty acid synthesis enzymes and initiate angiogenesis[103,104]. Additionally, oleic acid exhibits anti-inflammatory properties by modulating inflammatory pathways[105,106]. The MedDiet supplemented with extra virgin olive oil, a source of oleic acid, has been associated with anti-inflammatory effects[24,107]. Oleic acid may also influence cardiovascular health through modulating the intestinal microbiota, promoting beneficial bacteria and lowering cardiovascular risk[108]. Higher intake of MUFAs has been associated with reduced mortality and lower CVD risk[109,110]. Overall, oleic acid and MUFAs have potential benefits for cardiovascular health.

1.2.1.3. Polyunsaturated Fatty Acids

PUFAs play a crucial role in maintaining optimal health. These fatty acids are characterized by having multiple double bonds in their chemical structure, which gives them a flexible and fluid nature. PUFAs are classified into two main categories: omega-3 and omega-6 fatty acids. The precursors α-linoleic and linolenic acid cannot be produced by the human body and therefore must be obtained through dietary sources[98]. Omega-3 fatty acids can be found in fatty fish such as salmon or sardines, as well as in walnuts and flaxseeds. Omega-6 fatty acids, on the other hand, are abundant in vegetable oils and seeds. Through a series of metabolic reactions, linolenic acid is converted into eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA), two of the most studied PUFAs of the omega-3 series due to their numerous health effects[111]. This pathway shares the same enzymes as the metabolism

of linoleic acid, leading to direct competition for the metabolism of omega-6 and omega-3 PUFAs. In Western diets, the typical dietary ratio of linoleic to α-linolenic acid is approximately 7 to 20. This unbalanced ratio may contribute to the poor conversion of α-linolenic acid to EPA in humans and severely limits the conversion to DHA[98].

Omega-3s have been associated with numerous health benefits, including supporting cardiovascular health by reducing blood triglyceride levels, lowering blood pressure, and decreasing the risk of heart disease[112]. Moreover, EPA and DHA are critical for brain health and cognitive function, as DHA is a major component of brain tissue[113,114]. Adequate intake of EPA and DHA has been linked to improved memory, concentration, and a reduced risk of cognitive decline[115,116]. Additionally, EPA and DHA are essential for fetal brain development during pregnancy and early childhood[117]. Omega-3 fatty acids may also contribute to alleviate symptoms of depression and anxiety, and support eye health[118,119].

Omega-6 fatty acids are essential for normal growth and development, as well as for the proper functioning of various body systems. They are involved in the production of hormones, cell signaling, and inflammatory responses. They are also essential for maintaining the integrity of cell membranes[120]. However, while omega-6 fatty acids are necessary for the body, it is important to maintain a balanced ratio with omega-3 fatty acids. An imbalance between omega-6 and omega-3 fatty acids, often prevalent in Western diets, has been linked to increased inflammation and an elevated risk of chronic diseases, including CVD and certain inflammatory conditions[121].

1.2.2. Polyphenols

Polyphenols are a class of naturally occurring compounds that are commonly found in plants. They are distinguished by their unique chemical structure, which includes one or multiple phenol rings[122]. Because of their distinctive chemical characteristics, the content of polyphenols in foods can be assessed using the reliable and simple Folin-Ciocalteu method, which is based on a redox reaction[123].

According to their chemical structure, they can be classified in two groups: flavonoids and non-flavonoids[122]. Flavonoids are characterized by their C6-C3-C6 structure, and include the subcategories of flavonols, flavanols, flavones, flavanones, isoflavones, and anthocyanins. Non-flavonoid polyphenols, on the other hand, encompass a diverse range of compounds that usually have one or two rings united by hydrocarbon chains, such as phenolic acids, lignans, stilbenes, tyrosols, and others.

Polyphenols are widely distributed in a variety of foods, including fruits, vegetables, whole grains, tea, coffee, cocoa, and other plant-based products[124]. These foods serve as the basis of the MedDiet, and as such, polyphenols may play a role in the beneficial health effects associated with this dietary pattern.

Details of published evidence on the fundaments of the Folin-Ciocalteu method and its application to assess the phenolic content in foods associated with the MedDiet can be found in the publication entitled ”The chemistry behind the Folin-Ciocalteu method for the estimation of (poly)phenol content in food. Total phenolic intake in a Mediterranean dietary pattern” (Publication 1).

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Review

The Chemistry Behind the Folin−Ciocalteu Method for the Estimation of (Poly)phenol Content in Food: Total Phenolic Intake in a Mediterranean Dietary Pattern

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ABSTRACT: The Folin−Ciocalteu assay is a reference method for the quantification of total (poly)phenols in food. This review explains the fundamental mechanism of the redox reaction on which the method is based and looks at some of the practical considerations concerning its application. To accurately estimate the antioxidant capacity of (poly)phenolic compounds, a thorough knowledge of their structural characteristics is essential, as the two are closely associated. Therefore, to help researchers interpret the results of the Folin−Ciocalteu method, this review also summarizes some of the main phenolic structural features. Finally, we have used the Folin−Ciocalteu method to estimate the total phenolic intake associated with high adherence to a Mediterranean diet, ranked as one of the healthiest dietary patterns, which is characterized by a high consumption of (poly)phenol-rich food such as wine, virgin olive oil, fruits, vegetables, whole grains, nuts, and legumes.

KEYWORDS: *antioxidant, total phenolic content, bioactive compounds, structure−activity relationship, virgin olive oil, wine*

1. INTRODUCTION

The Mediterranean diet is characterized by high consumption of fruits, vegetables, whole grains, legumes, and olive oil, moderate intake of wine, fish, and poultry, and low consumption of red meat and dairy products. Ranked as the healthiest diet in the world by the U.S. News and World Report,[1] it also has an added value of sustainability, being typically based on locally produced, traditional, and seasonal foods.[2] The health benefits of the Mediterranean diet are partly attributed to the effects of (poly)phenols,[3] the daily intake being around 800−900 mg. Apart from coffee, a principal source of dietary phenols, the diet of Mediterranean countries is distinguished from the dietary habits of northern Europe by the consumption of wine, olives, and virgin olive oil, all rich in (poly)phenols.[4]

In the field of phenolic analysis, the Folin−Ciocalteu (F−C) assay was initially applied to study wine, but it has since become the reference method to determine and quantify phenolic compounds in a wide variety of foods and biological samples due to its simplicity and reproducibility.[5,6] Despite its popularity, the F−C test is not specifically designed for phenolic compounds, as the reagent could be reduced by other nonphenolic compounds also present in the sample, with the risk of content overestimation.[7,8] Numerous methods exist to gauge the overall phenolic content and antioxidative potential of fruits and vegetables, relying on chemical reactions that encompass the transfer of hydrogen atoms (HATs) or single electrons (SETs). For instance, the oxygen radical absorbance capacity (ORAC) test is HAT based, while the F−C and ferric reducing antioxidant power (FRAP) assays involve SET reactions. On the other hand, Trolox equivalent antioxidant capacity (TEAC) assays incorporate elements of both SET and HAT mechanisms. It should be noted that the values obtained from these various measurements often diverge, especially when comparing the results of SET and HAT assays. The disparities can be attributed to several factors: the underlying mechanisms, the use of different reference standards (such as gallic acid, Trolox, quercetin, etc.) to express antioxidant activity, the varying sensitivities of compounds to each test, and the complex nature of food matrices, which frequently cause interferences and matrix effects.[9] These discrepancies have been untangled in a recent publication combining data from various food indexes and electrochemical studies in a global approach.[10] In another comparative study, the antioxidant capacity of plant extracts measured by various methods was linked to the concentration of phenolic compounds as determined by the F−C technique. The results of the DPPH and ABTS assays were found to be strongly correlated with those of the F−C method ($R = 0.939$ and 0.966, respectively). Similarly, a robust correlation was observed between the ferric-reducing potential, as determined by the FRAP assay, and the total phenolic content ($R = 0.906$).[11] The selection of the F−C assay over other techniques is usually based on its reputation for reliability, having a long history of use and acceptance in the scientific community. Moreover, it is relatively cost effective compared to other methods, rendering it accessible for researchers with limited budgets. As the F−C assay is sensitive and can quantify a wide range of phenolic compounds,

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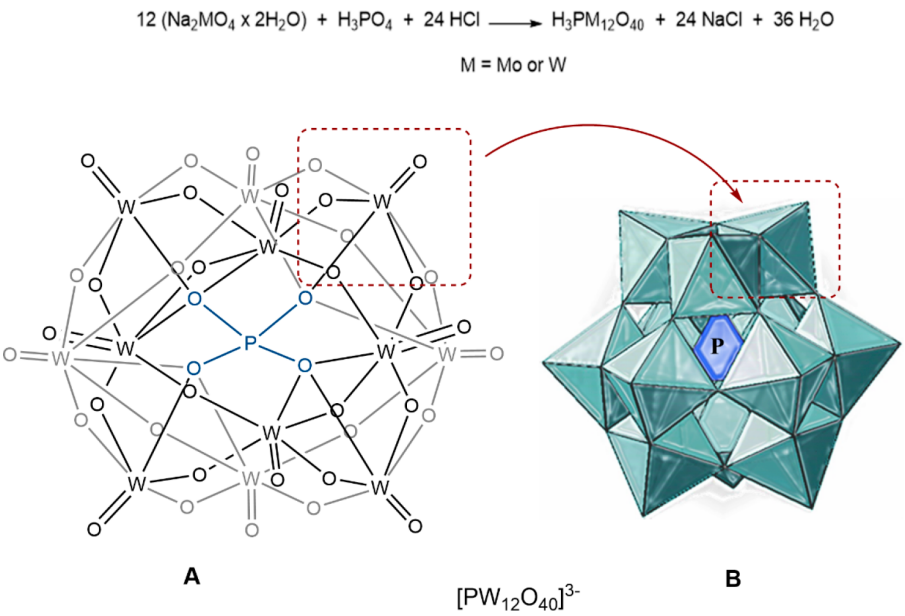

Figure 1. Preparation of the Folin−Ciocalteu reagent. α-Keggin structure of the anionic derivative $[PW_{12}O_{40}]^{3-}$ (A). Polyhedral model form (B).[19]

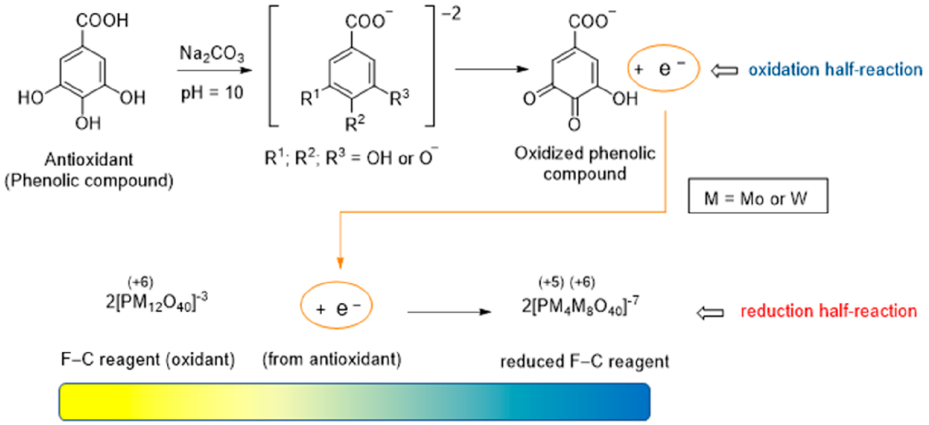

Figure 2. General redox reaction in the Folin−Ciocalteu assay. Metal complex species according to Munteanu.[7]

it is suitable for analyzing complex phenolic mixtures found in fruits, vegetables, and other foods. Additionally, it can be easily integrated into various laboratory setups and is compatible with common laboratory equipment.

The oxidizing F−C reagent reacts with reducing agents (antioxidants) to form a soluble, vividly blue complex, although its structure and the mechanism of its reactivity with phenolic compounds have not been fully determined.[12] The diverse class of chemical compounds known as (poly)phenols, which are among the most significant plant antioxidants, is distinguished by the presence of a phenol functional group, which consists of a hydroxyl (−OH) group directly attached to an aromatic ring. The study of the structure−activity relationships of the main dietary phenolics in the F−C reaction has found that the antioxidant activity of phenolic compounds is strongly affected by their structural features.

The aim of this review is to summarize the chemistry behind the F−C assay, focusing on the reagent itself, the redox reaction that takes place during the assay, as well as the relationship between the structural elements of the main dietary phenolic compounds and their antioxidant capacity. Moreover, the total phenolic intake associated with high adherence to a Mediterranean diet[13] quantified by F−C analysis is assessed.

2. FOLIN−CIOCALTEU REAGENT

Although the F−C reagent is readily available on the market, it can also be prepared following the original protocol[14] by boiling a mixture made of sodium tungstate ($Na_2WO_4{\cdot}2H_2O$, 100 g), sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL), and water (700 mL) for 10 h (Figure 1). The process generates a yellow solution composed of the complex compounds, phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and phosphotungstic acid ($H_3PW_{12}O_{40}$). Lithium sulfate (150 g, $Li_2SO_4{\cdot}4H_2O$) is added after boiling to reduce the formation of precipitates. If the reagent turns green because of contaminating reductants, its quality can be restored by adding a few drops of bromine or a small amount of 30% hydrogen peroxide.[15]

The precise chemical structure of the F−C reagent is unknown; however, it is described as a complex mixture of

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phosphotungstic and phosphomolybdic acids that is reduced throughout the assay to produce a blue chromophore with a maximum absorbance at 765 nm.[14−16] In 1933, Keggin solved the structure of the acid $H_3PW_{12}O_{40}$ using powder X-ray diffraction (see Figure 1).[17] The α-Keggin structure of the anionic derivatives of phosphotungstic and phosphomolybdic acids has the general formula $[XM_{12}O_{40}]^{n-}$,[18] where X is the heteroatom (in the F−C reagent, X is pentavalent phosphorus P(V)), M is the addendum atom (molybdenum, Mo, and/or tungsten, W), and O is oxygen. The structure has tetrahedral symmetry and is comprised of one phosphorus surrounded by four oxygen atoms (depicted in blue in Figure 1). The 12 octahedral MO_6 units that surround the core heteroatom are connected by the nearby oxygen atoms.

3. REDOX REACTION IN THE FOLIN−CIOCALTEU ASSAY

The F−C method is based on an electron-transfer reaction in which the antioxidant species acts as the electron donor and the F−C reagent acts as the oxidant (see Figure 2).

The reduction of the anionic derivatives of phosphotungstic and phosphomolybdic acids by antioxidants causes a color shift from yellow to blue, and the magnitude of the color shift when the reaction is complete is directly proportional to the reducing activity of the phenolic compounds. The reducing capacity of an antioxidant is frequently measured as gallic acid equivalents (GAE).[8] In more detail, the transfer of electrons from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in an alkaline solution creates blue complexes that are detected spectroscopically at about 760 nm. (Poly)phenols react with the F−C reagent only under basic conditions (pH of 10, adjusted by a sodium carbonate solution). Although the exact chemical composition of the F−C reaction is unknown, a series of reversible one- or two-electron reactions promoted by the phenolic compounds at basic pH change the initial yellow F−C reagent ($H_3PMo_{12}O_{40}$ + $H_3PW_{12}O_{40}$) to blue species, which may be ($PMoW_{11}O_{40}$),[4−8] $(PM_{12}O_{40})^{7-}$ (M = Mo or W),[7] or Mo_8O_{23} + W_8O_{23}.[20] It is assumed that molybdates are more easily reduced than tungstates in heteropoly salts; hence, the electron-transfer reaction takes place between the phenolic compound and Mo(VI), and some of the Mo^{6+} in the complex are reduced to Mo^{5+} by accepting an electron from the phenolic antioxidant (Figure 2).

4. PRACTICAL CONSIDERATIONS REGARDING THE FOLIN−CIOCALTEU ASSAY

The F−C assay is a useful method for determining the antioxidant activity of phenolic compounds as it is easy to use, consistent, and reliable. Nonetheless, the reaction conditions should be chosen carefully as the accuracy of the test is influenced by factors such as pH, temperature, and reaction duration. As interference issues in the F−C assay strongly depend on the food matrix and the variable reducing capacity of nonphenolic compounds, there are no simple guidelines. Nevertheless, some authors have studied the use of different methods to clean up the interference substances and alternative F−C reacting conditions to limit TPC overestimation.[21,22]

4.1. Standard for Calibration. Tannic acid has long been used as a reference for calibration curves when determining the total phenolic content (TPC) of wines.[5] However, because the content of tannic acid can differ among wines and spirits, Singleton et al.[5] substituted it for GAE as a reference for reporting F−C results. The gallic acid added to the wine was quantitatively recovered, and a mixture of natural phenolic compounds of various classes produced an absorbance equal to the total of their individual contributions, indicating that chemical deviances from Beer's rule were largely absent in the F−C system. Depending on the thickness of the optical cuvette, the minimum limit of quantification is 3 mg GAE/L. Although gallic acid is now routinely used as a standard for calibration curves, equivalents of catechin,[23,24] tannic acid,[25] chlorogenic acid,[26] caffeic acid,[27] and ferulic acid[28] have also occasionally been used, requiring standardization of the reported results.[29] Caffeic acid,[30] gallic acid,[31] and hydroxytyrosol (HTyr)[32] calibration curves were utilized to measure phenolic compounds in virgin olive oil extracts. Twelve different extra virgin olive oils were analyzed using various methods, and their phenolic content was statistically compared using two-tailed paired *t* tests. Results from the F−C assay (expressed as HTyr/20 g of oil) before and after acid hydrolysis were statistically similar to acid hydrolysis−HPLC results (HTyr + tyrosol).[32]

4.2. pH in the Folin−Ciocalteu Assay. Phenolic compounds only react with the F−C reagent in basic conditions. A sodium carbonate solution is added to the mixture containing the sample and the acidic F−C reagent to bring the pH level to approximately 10, avoiding excessive alkalinity. Sodium hydroxide and sodium cyanide have also been successfully used for this purpose.[5] A comparable approach based on the generation of phosphomolybdenum blue using a reagent without tungstate was described for the evaluation of antioxidant capacity in an acidic medium at a high temperature,[33] but this alternative method has not been tested with a wide range of antioxidants.[34]

4.3. Temperature and Time of Sample Incubation. In the F−C assay, the sample must be incubated with the reagent for 1 h, after which the absorbance is measured at 760 nm at room temperature. The blue color is quite stable at room temperature, so measurement of the standard, blank, and sample set at 760 nm after 6 h gives similar results to those after 1 h, albeit the standard deviation is higher.[5] The color may emerge more rapidly at a warmer temperature, but higher temperatures (>40 °C) cause the color to disappear more quickly.

4.4. Solvent Used in the Folin−Ciocalteu Assay. The conventional F−C reagent can only be used with water-soluble antioxidants,[29] and the reaction media is treated with lithium sulfate to prevent the precipitation of sodium complexes.[14] Thus, for the simultaneous analysis of lipophilic and hydrophilic antioxidants, the F−C method was modified and standardized using an isobutanol and water medium with sodium hydroxide.[35] Although this alternative procedure is not routinely applied, it has been successfully used to test hydrosoluble compounds such as ascorbic, gallic, caffeic, ferulic, and rosmarinic acids, Trolox, quercetin, catechin, glutathione, and cysteine as well as lipophilic antioxidants like butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone, lauryl gallate, and β-carotene. There is a need for further studies to evaluate the F−C method with other lipophilic antioxidants.

5. STRUCTURE−ACTIVITY RELATIONSHIPS OF (POLY)PHENOLS IN THE FOLIN−CIOCALTEU ASSAY

To ascertain the impact of the highly variable phenolic structures on the results of the F−C assay, in this section, we explore how the structural properties of the major dietary phenolic compounds are related with their reducing capacity and,

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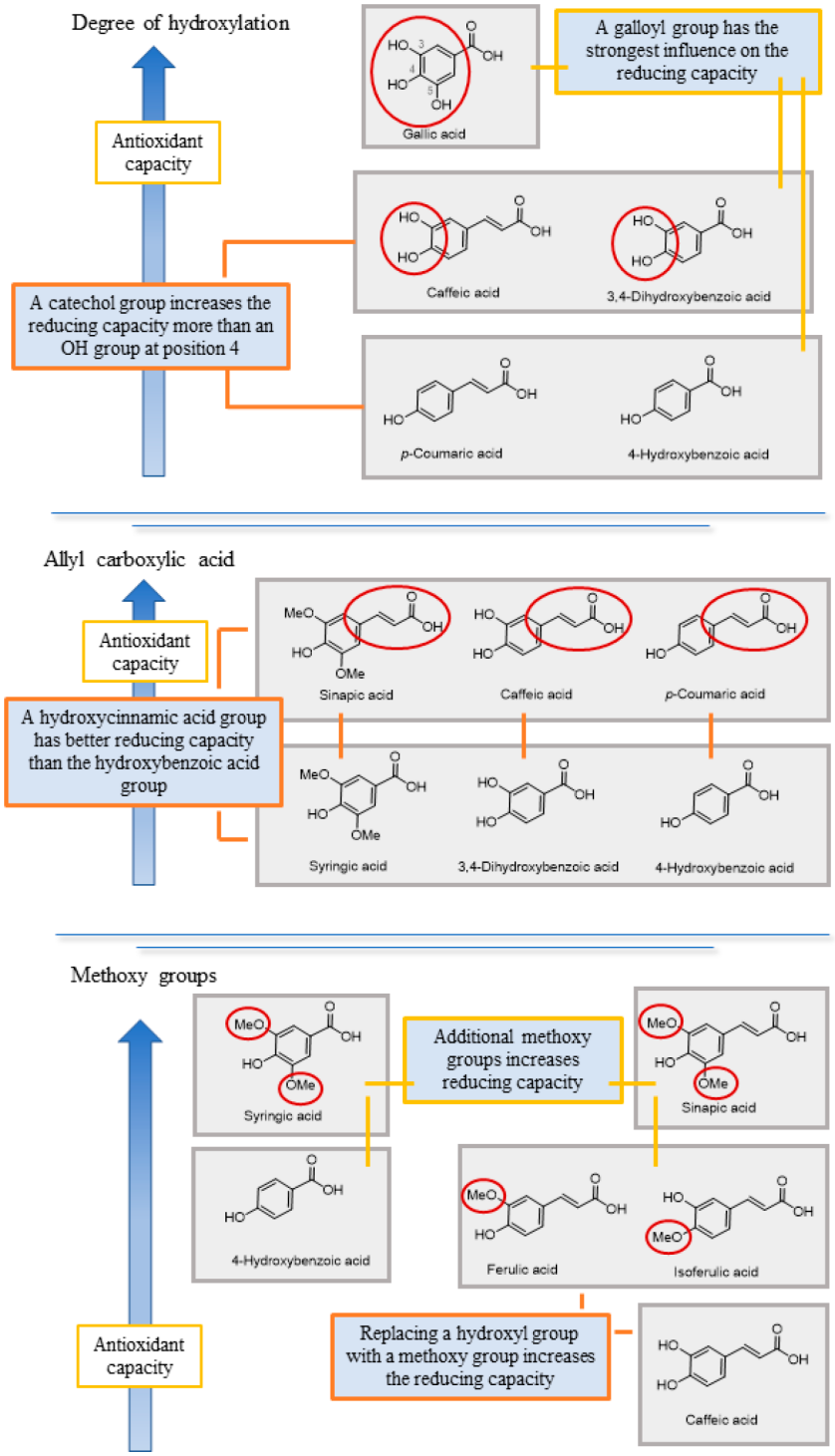

Figure 3. Key factors in the reducing capacity of phenolic acid derivatives: degree of hydroxylation, allyl carboxylic acid, and methoxy groups.

consequently, their antioxidant ability. Several studies have employed the F−C method to assess the antioxidant activity of samples containing a broad range of structurally diverse phenolic compounds, whereas more in-depth research on phenolic structure−activity relationships has focused mainly on phenolic acids and flavonoids.

5.1. Phenolic Acids. The ability of phenolic acids to scavenge free radicals depends on the quantity and position of the hydroxyl and methoxy groups in their molecules (Figure 3).[36,37] The galloyl group has the most positive effect on phenolic reducing capacity, which explains why gallic acid, a 3,4,5-trihydroxybenzoic acid, is the strongest antioxidant in the phenolic acid group. Additionally, compounds with a catechol

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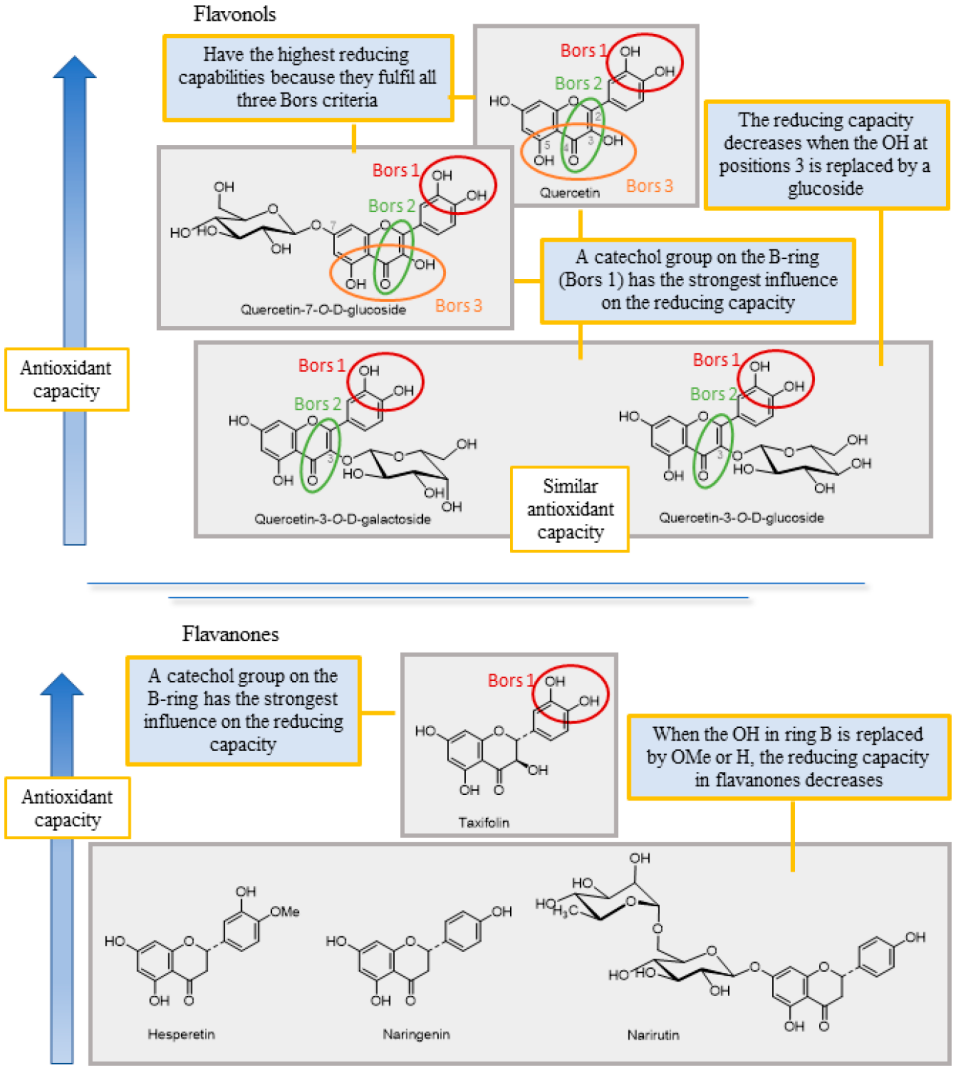

Figure 4. Key factors in the reducing capacity of flavonols and flavanones.

group rather than a single hydroxyl group at position 4 have higher reducing capacities, which is the case of caffeic acid in comparison with *p*-coumaric acid and 3,4-dihydroxybenzoic acid in comparison with 4-hydroxybenzoic acid.[10,38] A likely explanation is that the stabilization of the phenoxyl radical by an intramolecular hydrogen bond enhances antioxidant activity.[39]

Despite having the same number of hydroxyl and methoxy groups in the same position, hydroxycinnamic acids have a stronger reducing capacity than hydroxybenzoic acids, probably because the former have higher resonance stabilization.[37,38] Hence, a higher reducing capacity is found for sinapic acid versus syringic acid, caffeic acid versus 3,4-dihydroxybenzoic acid, and *p*-coumaric acid versus 4-hydroxybenzoic acid.

Additionally, the response of phenolic compounds in the F−C assay is improved if they bear a methoxy group instead of hydrogen atoms at the corresponding positions. This accounts for the slightly higher values obtained for syringic acid compared to 4-hydroxybenzoic acid and for sinapic acid compared to ferulic and isoferulic acids.[37,38] Furthermore, in hydroxycinnamic acids, replacing a hydroxyl group with a methoxy group (an electron donor) can improve the radical scavenging activity and boost the reducing capacity, explaining why ferulic acid has more reducing power than caffeic acid.[36,37,39,40]

5.2. Flavonoids. Three structural properties, based on Bors criteria, have been postulated to explain the antioxidant capacity of flavonoids[41] (Figure 4). The presence of a catechol group on the B ring (Bors 1) increases the stability of the resulting antioxidant radical; a 2,3 double bond conjugated to a 4-oxo group on the C ring (Bors 2) allows electron delocalization; the presence of OH groups at positions 3 and 5 in combination with a 4-oxo group facilitates electron delocalization via hydrogen bonds (Bors 3). Previous studies have found that the number and placement of OH groups in flavonoids, particularly those on the B ring, and glycosylation affect the F−C assay results.[10,37,42] The flavonoids without a hydroxyl group (e.g., *trans*-chalcone, flavone, and isoflavone) have no radical scavenging capacity.[36] As expected, flavonols and flavanols have stronger reducing capabilities than other flavonoids, followed by some flavanones. Of the three Bors criteria, Bors 1 is thought to have the greatest influence on the reducing capacity, because flavanols only fulfill Bors 1 despite having equivalent reducing power to the flavonols.[38]

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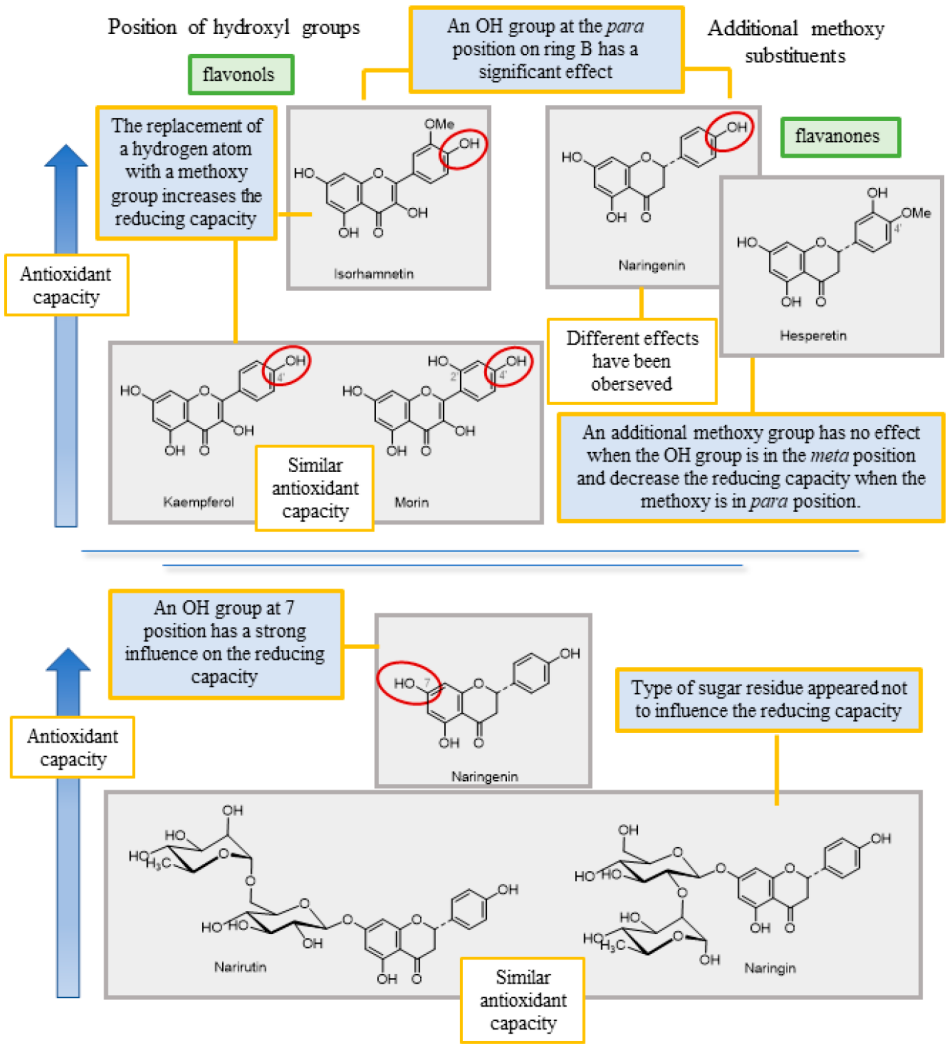

Figure 5. Key factors in the reducing capacity of flavonoids: positions of the hydroxyl groups and the presence of additional methoxy substituents.

In the flavonol subgroup, the catechol group on the B ring (fulfilling Bors 1) has the greatest influence on the flavonol reducing capacity, which explains the high values found for quercetin, quercetin-7-*O*-D-glucoside, quercetin-3-*O*-D-galactoside, and quercetin-3-*O*-D-glucoside (Figure 4). As it meets all three Bors criteria, quercetin is the most powerful reducing agent, followed by quercetin-7-*O*-glucoside. The reducing capacity is lower in quercetin-3-*O*-D-glucoside and quercetin-3-*O*-D-galactoside because the OH at position 3 is replaced by a sugar residue, a weaker electron-donating group than OH. However, as their values were not significantly different, it is assumed that the type of sugar residue does not influence the reducing capacity.[38] In flavanones, the presence of a catechol group (Bors 1) has the strongest effect on their reducing abilities, which explains why taxifolin outperforms hesperetin, narirutin, and naringenin.[38]

Generally, OH groups in the ortho and para positions appear to confer greater reducing capacity than those in the meta position due to the stabilization of the phenoxyl radical by intramolecular hydrogen bonds (Figure 5).[37,39,43] However, in flavonols, the presence of a hydroxyl group at position 4′ was found to have a substantial effect whereas a hydroxyl group at position 2′ had a minimal effect, explaining why there was no significant difference in the reducing capacity between morin and kaempferol. Replacing a hydrogen atom with a methoxy group increases the reducing ability, hence the higher value of isorhamnetin compared to kaempferol,[38] whereas replacing a hydroxyl group with a methoxy group has the reverse effect (Cai et al., 2006;[36] Ma and Cheung, 2007;[37] Shahidi et al., 1992[39]). Despite having an extra methoxy group on the B ring, according to Platzer, the reducing ability of hesperetin does not differ significantly from that of naringenin because the hydroxyl group is in the meta position.[38] However, Ma et al. reported that the presence of a methoxy group instead of a hydroxyl group at position 4′ decreases the reducing power of hesperetin compared to naringenin as the resulting methoxy-substituted phenoxy radical cannot be stabilized by intramolecular hydrogen bonding.[37]

The presence of a hydroxyl group at position 7 also has a substantial impact, hence the noticeably greater reducing ability of naringenin compared to narirutin.[44] As in flavonols, the reducing capacity of flavanones appears to be unaffected by the type of sugar residue, which explains the identical values found for narirutin and naringin.[38]

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Table 1. Average Total Phenolic Content (TPC), TPC by Portion, and the Main (Poly)phenols in Foods Consumed in the Mediterranean Diet[a]

	Average TPC	TPC by portion per day	Portion/ quantity of food per day	Main (poly)phenol type	Principal examples
Fruit	Oranges: 278.59 mg/100 g FW[c] Apples: 200.96 mg/100 g FW[c] Cherries: 174.90 mg/100 g FW[c]	Oranges: 668.62 mg Apples: 482.30 mg Cherries: 419.76 mg	240g (3 portions of 80g each)	Phenolic acids	Hydroxycinnamic acids • Caffeoylquinic acid • *p*-Coumaroylquinic acid
				Flavonoids	Anthocyanins (cherries) • Cyanidin 3-*O*-rutinoside • Cyanidin 3-*O*-glucoside
					Flavanones • Hesperetin • Naringenin Flavanols • (-)-Epicatechin • Procyanidin dimer B2
Vegetables	Spinach: 115.2 (steam), 55.6 (boiled), and 122.5 (uncooked) mg/100 g FW Onions: 102.83 mg/100 g FW[c] Lettuce (red): 114.00 mg/100 g FW[c] Lettuce (green): 65.92 mg/100 g FW[c] Tomato, whole, raw: 45.06 mg/100 g FW[c] Potatoes: 25 (cooked) and 35 (uncooked) mg/100 g FW	Spinach: 460 mg (steam) and 222.4 (boiled) mg Onions: 411.32 mg Lettuce (red): 456.00 mg Lettuce (green): 263.68 mg Tomato: 180.24 mg Potatoes: 100 mg (cooked)	400g	Phenolic acids	Hydroxycinnamic acids • Caffeoylquinic acid
				Flavonoids	Flavonols • Rutin • Quercetin
Wine	Wine [Red]: 215.48 mg/100 ml[c]	Men: 430.96-646.44 mg Women: 215.48-430.96 mg	Women: 100-200 mL Men: 200-300 mL	Phenolic acids	Hydroxycinnamic acids
				Flavonoids	Flavanols • (+)-Catechin • Procyanidins Anthocyanins
Legumes	Kidney beans: 630 (cooked) and 890 (uncooked) mg FAE[b]/100 g FW Lentils: 493.7 (boiled), 528.9 (pressure-cooked) and 908.0 (uncooked) mg/100 g FW Green mung beans: 520 (cooked) and 690 (uncooked) mg FAE[b]/100 g FW Chickpeas: 130 (cooked) and 210 (uncooked) mg FAE[b]/100 g FW	Kidney beans: 390 mg (cooked) Lentils: 306 mg (boiled) and 327 mg (pressure-cooked) Green mung beans: 322 mg (cooked) Chickpeas: 80.6 (cooked)	62g (150g x3 a week)	Phenolic acids	Hydroxycinnamic acids • *p*-Coumaric acid • Ferulic acid
				Flavonoids	Flavanols • (+)-Catechin 3-*O*-glucose Flavonols • Kaempferol 3-*O*-glucoside
Nuts	Chestnut: 2756.67 mg/100 g FW[c] Walnut: 1574.82 mg/100 g FW[c] Pistachio 1420.00 mg/100 g FW[c] Peanut: 406.29 mg/100 g FW[c] Almond: 287.09 mg/100 g FW[c]	Chestnut: 358.36 mg Walnut: 204.72 mg Pistachio 184.6 mg Peanut: 52.81 mg Almond: 37.32 mg	13g (30g x3 a week)	Phenolic acids	Hydroxybenzoic acids: • Ellagic acid • Gallic acid
				Flavonoids	Flavanols • (-)-Epigallocatechin • (+)-Catechin Isoflavonoids • Daidzein
				Lignans	Secoisolariciresinol
Whole grain bread/ pasta/rice	Bread, whole grain flour: 215.75 mg/100 g FW[c] Pigmented rice, whole grain: 202.6 (cooked) and 409.7 (uncooked) mg FAE[b]/100 g FW Non-pigmented rice, whole grain: 87.2 (cooked) and 99.4 (uncooked) mg FAE[b]/100 g FW Whole wheat pasta: 84.4 (cooked) and 152.9 (uncooked) mg FAE[b]/100 g FW	Bread, whole grain flour: 116.50 mg Pigmented rice, whole grain: 109.4 mg (cooked) Non-pigmented rice, whole grain: 47.0 mg (cooked) Whole wheat pasta: 45.5 mg (cooked)	54g (75g x5 a week)	Phenolic acids	Hydroxycinnamic acids • Ferulic acid
				Lignans	Lariciresinol
Sofrito	25.17 mg/100 g FW	11.08 mg	44g (103g x3 a week)	Phenolic acids	Hydroxycinnamic acids • Chlrorogenic acid • *p*-Coumaric acid • Ferulic acid
				Flavonoids	Flavannoes • Naringenin Flavonols • Rutin • Quercetin
Oil	Olive, oil, extra virgin: 55.14 mg/100 g FW	11.02 mg	20g (FDA and EFSA recommendation)	Other (poly)phenols	Tyrosols: • 3,4-DHPEA-EA • 3,4-DHPEA-EDA

[a]Portions were defined according to the recommendations of the Mediterranean diet[46] and in raw food; the TPC data were obtained from the Phenol Explorer Database.[48,58] [b]mg/100 g FW using equivalents of ferulic acid. [c]Polyphenol Explorer Database overall data.

In summary, the structure−activity relationship of phenolic antioxidants in the F−C assay has been explored in phenolic acids, flavonols, and flavanones but not flavanols. The antioxidant activity of primary dietary phenolic compounds can be predicted based on their structural properties. While the F−C assay results are mostly explained by how many Bors criteria are met, the degree of hydroxylation and the locations of the hydroxyl and methoxy groups are key variables in the reducing capacity when none of the Bors principles are applicable.

6. TOTAL PHENOLIC INTAKE WITH HIGH ADHERENCE TO A MEDITERRANEAN DIET

A high adherence to a Mediterranean diet is associated with more beneficial health outcomes compared to a low adherence due to a higher intake of total (poly)phenols as well as specific phenolic compounds such as flavonoids, anthocyanins, and

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lignans.[45] Table 1 lists the main food sources of (poly)phenols and the amount of (poly)phenols consumed when following a Mediterranean diet according to the validated MEDAS (14-point Mediterranean Diet Adherence Screener) questionnaire.[46] The TPC data were obtained from Polyphenol Explorer Database, which is based on average values from published studies.[47,48] However, to obtain the TPC of foods that are typically consumed after cooking, the original literature sources were examined.

According to the MEDAS questionnaire, fruits constitute one of the main sources of (poly)phenols for the Mediterranean population, with oranges being the fruit consumed with the highest TPC, followed by apples and cherries.[46] Considering that three portions of fruit per day are recommended in the Mediterranean dietary pattern, which is equivalent to 240 g, these foods provide 419.76−668.62 mg of (poly)phenols per day. Phenolic acids are commonly found in fruits, the most significant being hydroxycinnamic acids such as caffeoylquinic acid and *p*-coumaroylquinic acid. Fruits also contain three types of flavonoid compounds: flavanones (e.g., hesperetin and naringenin), flavanols (e.g., (−)-epicatechin and procyanidin dimer B2), and anthocyanins (e.g., cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside in cherries). Anthocyanins are responsible for the red, purple, and blue colors of many fruits.

The TPC in vegetables is highly variable depending on the vegetable and if they are fresh or cooked. Among the most frequently consumed, those with the highest content of (poly)phenols measured with the F−C assay are spinach, onions, red and green lettuce, and, finally, tomatoes and potatoes. Spinach is commonly consumed raw in salads or boiled/steamed, and its TPC varies depending on the preparation method.[49] In contrast, potatoes are usually boiled, resulting in a reduction of TPC from 35 to 25 mg/100 g FW.[50] Following the recommended consumption of 400 g of vegetables per day, the average daily total phenolic intake from this source would range from 100 to almost 460 mg. The main phenolic compounds present in these vegetables are phenolic acids such as caffeoylquinic acids (chlorogenic acid) and flavonols such as kaempferol and quercetin.

The Mediterranean diet is characterized by a moderate consumption of wine, mainly red, with meals. The TPC in red wine depends on the type of grapes used and the wine making process, among other factors, but on average, it is 215.48 mg/100 mL. In the Mediterranean dietary pattern,[2] red wine represents one of the main sources of phenolic compounds, providing a daily intake of 215.48−430.96 mg for women and 430.96−646.44 mg for men. The main (poly)phenols in red wine are hydroxycinnamic acids and flavonoids such as anthocyanins and flavanols ((+)-catechin and procyanidins).

Legumes, particularly beans, are recognized as a very good source of (poly)phenols, although there is a notable difference between cooked and uncooked legumes. In uncooked kidney beans, the TPC can reach up to 870 mg per 100 g compared to about 630 mg per 100 g after boiling. Consuming 62 g (150 g × 3 a week) of kidney beans provides 390 mg of phenolic compounds.[51] In second place are lentils, which have the highest TPC among legumes when analyzed in raw form (908 mg/100 g fresh weight (FW)), the amount also decreasing drastically after cooking, with boiled lentils containing 493.7 mg/100 g FW and pressure-cooked lentils 529 mg/100 g FW.[52] Green mung beans are another valuable source of (poly)-phenols, a portion of 62 g providing 322 mg of phenolic compounds when cooked. The TPC of chickpeas is slightly lower, decreasing by 35.6% after cooking.[51] According to the guidelines of the Mediterranean diet, legumes, particularly kidney beans, lentils, and green mung beans, constitute one of the primary sources of total phenolic intake, despite their reduction by cooking. (+)-Catechin 3-*O*-glucose and kaempferol 3-*O*-glucoside are flavanols found in legumes, which are also a good source of phenolic acids such as hydroxycinnamic acids (*p*-coumaric and ferulic acids).

Among nuts, the highest TPC is found in chestnuts, followed closely by walnuts and pistachios, with a lower content in peanuts and almonds. The main (poly)phenols in nuts are hydroxybenzoic acids (e.g., ellagic acid and gallic acid), lignans (e.g., secoisolariciresinol), flavonoids (e.g., flavanols such as (−)-epigallocatechin and (+)-catechin), and isoflavonoids (e.g., daidzein).

In the Mediterranean diet, there is a preference for whole grain foods, which have a higher content of bioactive compounds such as fiber and (poly)phenols, over refined foods. Bread, rice, and pasta made with whole grains are good sources of lignans (lariciresinol) and hydroxycinnamic acids such as ferulic acid. Cooking was found to reduce the average TPC in pigmented rice by about 50% (from 410 to 203 mg ferulic acid equivalents (FAE)/100 g FW) but had no significant effect on the average TPC of nonpigmented rice, which remained quite constant (87.2 mg FAE/100 g FW).[53] A 54 g portion of whole wheat pasta, despite the reduction of TPC after cooking (57.7%), provides 45.5 mg of (poly)phenols per day.[54]

Sofrito, a traditional sauce in Mediterranean cuisine prepared by sautéing onions, garlic, and tomatoes in olive oil, is reported to contain 25.17 mg of phenolic compounds per 100 g of FW.[45] Among these compounds are various types of phenolic acids, including hydroxycinnamic acids such as chlorogenic acid, *p*-coumaric acid, and ferulic acid.

Extra virgin olive oil (EVOO) is the main source of fat in the Mediterranean diet. The recommended daily intake of EVOO, according to the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), is 20 g per day, which would provide approximately 11.02 mg of (poly)phenols.[55] The specific types of (poly)phenols found in EVOO are tyrosols and secoiridoids such as 3,4-DHPEA-EA and 3,4-DHPEA-EDA. It is worth noting that the TPC in EVOO varies according to factors such as the olive variety and stage of ripeness, the production process, and storage conditions.[56,57]

7. CONCLUSIONS

Although the precise chemical composition of the F−C reagent is unknown, the F−C assay is based on the reduction of a yellow phosphotungstate−phosphomolybdate complex by antioxidants (reductants) to a blue chromogen. The reducing capacities of the major dietary phenolic compounds can be predicted based on their structural features. As the structure of phenolic compounds conditions their antioxidant power, the results of the F−C assay will depend on the content of individual (poly)phenols in the sample.

The F−C assay has been widely used in studies to measure the TPC in foods or extracts and is regarded as a reference method in this regard. The Mediterranean diet is characterized by the consumption of many (poly)phenol-rich foods, such as fruits, vegetables, legumes, wine, and nuts, which could be partly responsible for its demonstrated health benefits. However, it is important to note that the F−C assay measures the TPC, and not all phenolic compounds have the same bioactivity or health impact. Therefore, more research is needed to understand the

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health effects of specific phenolic compounds in the Mediterranean diet.

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Notes

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ABBREVIATIONS USED

EVOO, extra virgin olive oil; FAE, ferulic acid equivalents; F−C, Folin−Ciocalteu; FW, fresh weight; GAE, gallic acid equivalents; TPC, total phenolic content

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Polyphenols have been associated with a wide variety of positive health effects. Dietary polyphenols have been associated with lower risk of all-cause mortality, reduced CVD risk, protection against certain types of cancer, or improved cognitive functions[125–129]. However, most of the studies relied on food assessments of polyphenols intake, which are susceptible to subjectivity and measurement errors[130]. Due to the high interindividual variability in the absorption process[131], it is more accurate to measure them in biological samples. To assess them as a whole, and not individually, the fastest and more efficient method is the Folin-Ciocalteu assay[132]. The challenges of applying this methodology on biological samples such as urine and plasma to measure the phenolic content and its association with inflammation, chronic diseases, and mortality were reviewed in Publication 2, entitled "Total (poly)phenol analysis by the Folin-Ciocalteu assay as an antiinflammatory biomarker in biological samples".

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REVIEW ARTICLE

Total (poly)phenol analysis by the Folin-Ciocalteu assay as an anti-inflammatory biomarker in biological samples

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ABSTRACT

The Folin-Ciocalteu method is a well-established and widely used assay for measuring total (poly) phenol content in food/plant products. In recent years, there has been growing interest in applying this method to human samples due to its simplicity and efficacy. However, biological matrices such as blood and urine contain several interference substances that must be eliminated beforehand. This mini-review summarizes the current state of knowledge regarding the use of the Folin-Ciocalteu assay to measure total phenolic content in human urine and blood samples, as well as the preceding cleaning methods to remove interferences. Higher total (poly)phenol levels measured by the Folin-Ciocalteu method have been associated with a decrease in mortality and several risk variables. We focus on the application of this sustainable assay as a biomarker of poly(phenol) intake and its potential use as an anti-inflammatory biomarker in clinical laboratories. The Folin-Ciocalteu method, with a clean-up extraction step, is a reliable tool for determining total (poly)phenol consumption. Here, we also recommend using the Folin-Ciocalteu assay as means to measure anti-inflammatory activity.

KEYWORDS

Antioxidant capacity; biological samples; phenol intake; healthy aging

Background

The Folin-Ciocalteu (F–C) photometric assay is one of the most common procedures used to determine phenolic compounds, although it was initially developed to detect and quantify tyrosine and tryptophane in proteins, the F–C reagent reacting with the phenolic groups in these two amino acids (Folin and Ciocalteau 1927). The F–C assay was subsequently modified to analyze the (poly)phenol content of red wine (Singleton, Rossi Jr., and Rossi Jr. 1965) and is now considered the reference method for phenolic compound quantification and determination in a wide variety of matrices (Wang et al. 2019; Khosravi et al. 2020; Jiang et al. 2022). Additionally, it is increasingly being applied in human biological samples (blood and urine) as a way of estimating total (poly)phenol intake (Arenas and Trinidad 2017; Laveriano-Santos et al. 2022).

Although the precise chemical composition of the F–C reagent is undefined, it is known to contain a mixture of phosphomolybdic and phosphotungstic acids, which upon reduction produce a blue chromophore with maximum absorption at 765 nm. The assay is based on an electron-transfer reaction between the F–C reagent (the oxidant) and an antioxidant species (the electron donor) (Figure 1). The extent to which the reagent changes color after the electron extraction depends on the reducing activity of the antioxidant compounds. It is frequently measured as gallic acid equivalents (GAE), as gallic acid has shown the highest absorbance compared to other compounds as chlorogenic or neochlorogenic acid (Kyoung Chun and Kim 2004). The F–C assay has numerous advantages as a methodology to assess the antioxidant activity of phenolic compounds, including simplicity, reproducibility, and robustness. However, since the test is sensitive to pH, temperature, and reaction time, the reaction conditions need to be carefully selected to obtain reliable results (Singleton, Orthofer, and Lamuela-Raventós 1999). As phenolic compounds only react with the acidic F–C reagent in basic conditions, because a deprotonated OH group in the phenolic ring is required, a sodium carbonate solution is added to the mixture of samples and reagent to increase the pH to approximately 10, avoiding excessive alkalinity. Samples must be incubated with the F–C reagent for 90-120 min at room temperature. Up to six hours at room temperature, the blue color remains consistent, and the results obtained are comparable to those obtained after just one hour, although there is an increased standard deviation. Even though color may emerge more rapidly at high temperatures, at >40 °C the color also disappears more quickly (Singleton, Orthofer, and Lamuela-Raventós 1999). The conventional F–C reagent is only applicable to water-soluble antioxidants (Singleton, Rossi Jr., and Rossi Jr. 1965).

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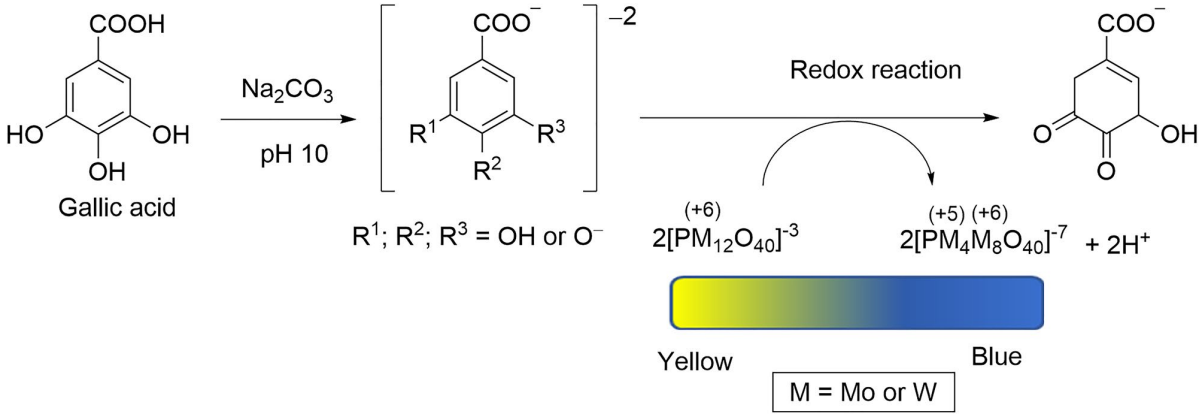

Figure 1. The redox reaction and color variation in the Folin–Ciocalteu assay, together with the metal complex species identified by Munteanu (Munteanu and Apetrei 2021).

Among the most important antioxidants found in plants, (poly)phenols constitute a large and diverse class of organic compounds that contain a phenol functional group consisting of a hydroxyl (–OH) group bonded directly to an aromatic ring. More than 9000 phenolic structures have been described, the most abundant being phenolic acids, flavonoids, stilbenes and lignans; flavonoids and phenolic acids account for 55-60% and 30-40% of dietary (poly)phenols, respectively (Rothwell et al. 2013; Tresserra-Rimbau et al. 2013).

When compared to self-reported information from food frequency questionnaires or dietary recalls, biomarkers of nutritional intake have various advantages for epidemiological and clinical studies (Marshall 2003) as they yield more objective and precise data. Quite recently, the F–C method has been used in blood and urine samples as a biomarker of phenolic intake, although a clean-up procedure is necessary to avoid interferences (Roura et al. 2006). Higher levels of urinary (poly)phenols measured by the F–C assay have been correlated with a decrease in mortality related to cardiovascular disease and lower DNA oxidative damage (Pedret et al. 2012; Zamora-Ros et al. 2013), possibly due to the antioxidant and anti-inflammatory properties of phenolic compounds.

Here we provide an updated overview of the use of the F–C assay in urine and plasma as an objective tool for the analysis of total (poly)phenol intake. The limitations and interferences associated with the method, modifications of the extraction procedures used for sample clean-up, and the potential of the assay for use as an anti-inflammatory biomarker in biological samples are also explored.

Limitations and interferences in the F-C method

Since 1965, the F–C assay has been widely used to analyze plant-based food, but its application in biological samples started only in the 1990s (Serafini, Maiani, and Ferro-Luzzi 1998; Maskarinec et al. 1999). Overestimation of the total (poly)phenol content is a major concern when applying the F–C test in biological matrices due to the effect of non-phenolic reducing agents in the samples (Blasco et al. 2005), such as aromatic amines, certain amino acids, high sugar concentrations, citric acid, or ascorbic acid (Ainsworth and Gillespie 2007). Tryptophan, indoles, purines, guanine, xanthine, and uric acid are also reported to react with the F-C reagent to yield molybdenum blue (Singleton, Orthofer, and Lamuela-Raventós 1999). As reported by Lamuela-Raventós, urine contains reductant substances that may potentially interfere with the F–C reagent (see Table 1) (Lamuela-Raventós 2018). Vitamin C, found in urine at a maximum concentration of 100 mg/L, is reactive with the F–C reagent. In contrast, organic acids, such as oxalic, citric, and tartaric acid, as well as folic acid, which has maximum levels in urine equivalent to those of vitamin C, and hippuric acid (10 mg/L) do not react in the F–C assay. Even though the maximum concentration of Fe (II) and some amino acids (Phe, Tyr, Gkut, Arg) in urine is only 1 mg/L, they are detected in the F–C assay. Moreover, the F–C reagent reacts with dopamine (0.4 mg/L), norepinephrine or noradrenaline (0.08 mg/L), and epinephrine or adrenaline (0.02 mg/L).

Table 1. Reductant substances that may potentially interfere with the F–C reagent.

Compounds with reducing capacity (maximum levels in urine)	F–C Assay
Vitamin C (100 mg/L)	+
Organic acids: oxalic, citric and tartaric acids (100 mg/L)	–
Folic acid (100 mg/L)	–
Hippuric acid (10 mg/L)	–
Fe (II) (1 mg/L)	+
Amino acids: Phe, Tyr, Glut, Arg (1 mg/L)	Weak
Dopamine (0.4 mg/L)	+
Norepinephrine or noradrenaline (0.08 mg/L)	+
Epinephrine or adrenaline (0.02 mg/L)	+

Application in biological samples

Accurate assessment of phenolic intake based on self-reported dietary records and food composition tables can be hindered by the subjectivity of the data and the impact on content by factors such as product variety and degree of ripeness, as well as food processing techniques. Therefore, the development of biomarkers that can be measured in blood and

Table 2. Studies performed in human urine samples using the Folin–Ciocalteu assay.

Authors	Cleaning method	Substances removed	Participants	Results expressed as	Validation	Ref.
Roura et al. (2006)	SPE	Water-soluble compounds (sugar, iron, organic acids, amino acids, vitamins and hippuric acid)	36 healthy adults	mg catechin/g of creatinine	Linearity Precision	(Roura et al. 2006)
Medina-Remón et al. (2009)	SPE	Water-soluble compounds (sugar, iron, organic acids, amino acids, vitamins and hippuric acid)	60 adults at high cardiovascular risk	mg GAE/g creatinine	Linearity Sensitivity Accuracy Precision Stability	(Medina-Remón et al. 2009)
Kendall et al. (2009)	None	None	36 healthy young adults	mg gallic acid/L	Not detailed	(Kendall et al. 2009)
Hussein et al. (2009)	As Medina-Remón et al.	As Medina-Remón et al.	49 adolescents	mg GAE/g creatinine	As Medina-Remón et al.	(Hussein et al. 2009)
Medina-Remón et al. (2011)	As Medina-Remón et al.	As Medina-Remón et al.	589 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Medina-Remón et al. 2011)
Zamora-Ros et al. 2011	As Medina-Remón et al.	As Medina-Remón et al.	928 older adults	mg GAE/g creatinine	As Medina-Remón et al.	(Zamora-Ros et al. 2011)
Pedret et al. 2012	As Medina-Remón et al.	As Medina-Remón et al.	81 healthy adults	mg GAE/g creatinine	As Medina-Remón et al.	(Pedret et al. 2012)
Vinson et al. (2012)	SPE	Non-phenolic interferences	8 healthy adults	µmol/24h urine	Not detailed	(Vinson et al. 2012)
Zamora et al. (2013)	As Medina-Remón et al.	As Medina-Remón et al.	807 older adults	mg GAE/24h urine	As Medina-Remón et al.	(Zamora-Ros et al. 2013)
Rabassa et al. (2015)	As Medina-Remón et al.	As Medina-Remón et al.	652 old adults	mg GAE/24h urine	As Medina-Remón et al.	(Rabassa et al. 2015)
Urpi-Sarda et al. (2015)	As Medina-Remón et al.	As Medina-Remón et al.	811 old adults	mg GAE/24h urine	As Medina-Remón et al.	(Urpi-Sarda et al. 2015)
Wruss et al. (2015)	None	None	35 healthy subjects	(+)-catechin equivalents in mg/L	Not detailed	(Wruss et al. 2015)
Guo et al. (2016)	As Medina-Remón et al.	As Medina-Remón et al.	573 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Guo et al. 2016)
Rabassa et al. 2016	As Medina-Remón et al.	As Medina-Remón et al.	368 old adults	mg GAE/24h urine	As Medina-Remón et al.	(Rabassa et al. 2016)
Medina-Remón et al. (2017)	As Medina-Remón et al.	As Medina-Remón et al.	1139 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Medina-Remón et al. 2017)
Guo et al. (2017)	As Medina-Remón et al.	As Medina-Remón et al.	573 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Guo et al. 2017)
Hinojosa-Nogueira et al. (2017)	SPE	Water-soluble compounds (sugar, iron, organic acids, amino acids, vitamins and hippuric acid)	228 children	mg GAE/g creatinine	Not detailed	(Hinojosa-Nogueira et al. 2017)
Halder et al. (2017)	As Medina-Remón et al.	As Medina-Remón et al.	20 men	mg GAE/mmol creatinine	As Medina-Remón et al.	(Haldar et al. 2019)
Hurtado-Barroso et al. (2018)	As Medina-Remón et al.	As Medina-Remón et al.	22 healthy men	mg GAE/mmol creatinine	As Medina-Remón et al.	(Hurtado-Barroso et al. 2018)
Hurtado-Barroso et al. (2019)	As Medina-Remón et al.	As Medina-Remón et al.	22 healthy men	mg GAE/mmol creatinine	As Medina-Remón et al.	(Hurtado-Barroso et al. 2019)
Laveriano-Santos et al. (2020)	As Medina-Remón et al.	As Medina-Remón et al.	1194 adolescents	mg GAE/g creatinine	As Medina-Remón et al.	(Laveriano-Santos et al. 2020)
Laveriano-Santos et al. (2022)	As Medina-Remón et al.	As Medina-Remón et al.	1151 adolescents	mg GAE/g creatinine	As Medina-Remón et al.	(Laveriano-Santos et al. 2022)

SPE, solid-phase extraction; GAE, gallic acid equivalents.

urine is essential for more precise estimates of (poly)phenol intake and to establish the health effects of these dietary constituents (Roura et al. 2006). Most of the studies employed a commercial F-C reagent available from Honeywell (NC, USA).

Urine samples

Studies applying the F–C assay to analyze total (poly)phenol content in human urine samples are listed in Table 2. To the best of our knowledge, the first such study was that of Roura et al. in 2006, in which an initial clean-up step using a solid-phase extraction cartridge ensured that undesired water-soluble compounds were eliminated from the samples. The reliability of the method was validated in a study of 36 volunteers who consumed either a (poly)phenol-rich or a (poly)phenol-free diet (Roura et al. 2006). However, the most optimized and commonly used methodology in this field was developed by Medina-Remón et al. who increased efficiency and sustainability by using 96-well microtiter plates (Alexander Medina-Remón et al. 2009). Thus, interfering substances from spot-urine were removed by

Table 3. Studies analyzing human blood samples using the F-C assay.

Authors	Cleaning /extraction method	Substances removed	Participants	Results expressed as	Validation	Ref.
Serafini, Maiani, and Ferro-Luzzi (1998)	Protein precipitation and extraction. Hydrolysis with HCl acid Metaphosphoric acid (MPA) 0.75 mol/L	Proteins, lipids, and hydrolyzed conjugates.	10 healthy subjects	quercetin equivalents mg/L	Recovery Limit of detection reproducibility	(Serafini, Maiani, and Ferro-Luzzi 1998)
Maskarinec et al. (1999)	50 mL of plasma were mixed with 50 ml of 95% ethanol followed by vortex and centrifugation for 5 min at 11003 g	Protein precipitation with ethanol	33 females	micromole/L quercetin equivalents	Not detailed	(Maskarinec et al. 1999)
Arendt et al. (2005)	As in Serafini et al.	As in Serafini et al.	78 healthy subjects	mg catechin equivalents/L	As Serafini et al.	(Arendt et al. 2005)
Müller et al. (2010)	As in Serafini et al.	As in Serafini et al.	70 healthy subjects	mg catechin equivalents/L	As Serafini et al.	(Müller et al. 2010)
Andersson et al. (2010)	Method used by Maskarinec et al. (1999)	Ethanol precipitation	22 females	micromole/L quercetin equivalents	Reproducibility CV < 10%	(Andersson et al. 2010)
Wruss et al. (2015)	None	None	35 healthy subjects	(+)-catechin equivalents in mg/L	As Serafini et al.	(Wruss et al. 2015)
Arenas and Trinidad (2017)	As in Serafini et al.	As in Serafini et al.	10 healthy subjects	micromole/L GAE	As Serafini et al.	(Arenas and Trinidad 2017)

GAE, gallic acid equivalents.

solid-phase extraction on Oasis((R)) MAX 96-well plate cartridges before performing the F–C assay, and gallic acid was used as the standard. The phenolic content in urine measured by this quick and easy methodology was demonstrated to be a reliable biomarker of (poly)phenol intake.

In 2011, as part of the InCHIANTI study, Zamora et al. corroborated that total (poly)phenols measured in spot morning urine correlated with levels in 24h urine (Zamora-Ros et al. 2011). Since then, many studies in elderly or young and healthy populations have applied this procedure either in spot or 24h-urine to assess (poly)phenol intake and associated health outcomes. In elderly cohorts, including those of the PREDIMED and InCHIANTI trials, individuals with higher urinary excretion of (poly)phenols showed improvements in cardiovascular health parameters, such as glucose and triglyceride levels, blood pressure, or body weight (Guo et al. 2016; Guo et al. 2017; A. Medina-Remón et al. 2011). In the InCHIANTI study, Zamora et al. also found a negative association between (poly)phenol excretion and mortality in older adults (Zamora-Ros et al. 2013). In the same cohort, a lower risk of substantial cognitive decline was observed in individuals with higher urinary (poly)phenols after three years of follow-up, as well as reduced frailty (Rabassa et al. 2015; Urpi-Sarda et al. 2015). Furthermore, Rabassa et al. showed that higher levels of urinary (poly)phenols were associated with a lower risk of physical performance decline (Rabassa et al. 2016).

In a cross-sectional study performed in healthy individuals, DNA oxidative stress biomarkers were inversely correlated with the phenolic content in urine, suggesting that phenolic compounds may attenuate oxidative damage (Pedret et al. 2012). Haldar et al. showed that the intake of a (poly)phenol-rich curry, assessed using the F-C assay in urine, improved glucose homeostasis in 20 healthy males (Haldar et al. 2019). This approach has also been used in studies of younger populations. A clinical trial with 49 male adolescents demonstrated that urinary (poly)phenols were an accurate biomarker of intake (Hussein et al. 2009). Laveriano et al. reported that urinary polyphenols were associated with a better cardiovascular profile in a cohort of Spanish adolescents. Interestingly, they found differences between the sexes, as cardiovascular health in boys was more strongly associated with phenolic excretion than in girls (Laveriano-Santos et al. 2020; Laveriano-Santos et al. 2022).

Sample cleaning with solid phase extraction has been modified or alternatives have been proposed. For example, Vinson et al. employed a Polyclar solid phase procedure, whereas Kendall et al. did not perform any cleaning of the urine samples (Kendall et al. 2009; Vinson et al. 2012). In other studies, samples were only submitted to centrifugation and the supernatant was used to quantify the phenolic compounds (Wruss et al. 2015). However, these approaches do not eliminate the potential interfering substances.

Blood samples

Plasma has also been used as a matrix to measure (poly) phenols, though less frequently (see Table 3). As in urine, a preceding clean-up step is required, which eliminates plasma protein interferences. Hydrolysis has been used to separate the (poly)phenols from the lipids, followed by protein precipitation using metaphosphoric acid to remove plasma proteins (Serafini, Maiani, and Ferro-Luzzi 1998). This methodology was shortened by Arendt et al. who only centrifuged the plasma samples before adding metaphosphoric acid to precipitate the proteins (Arendt et al. 2005). In both studies, circulating levels of (poly)phenols were found to increase after the consumption of alcohol-free wine. The same procedure was used in plasma samples to examine the reducing capacity of the F–C reagent and (poly)phenol levels after consumption of white tea and a pomace drink, respectively (Müller et al. 2010; Arenas and Trinidad 2017). In a clinical trial with women who consumed fruits and vegetables, Maskarinek et al. used ethanol to precipitate plasma proteins before performing the F–C assay; surprisingly, the intervention did not increase total (poly)phenols (Maskarinec et al. 1999). This method was replicated in another study, which observed a decrease in (poly)phenols immediately after exercise (Andersson et al. 2010).

Interestingly, Wruss et al. did not use any clean-up process to eliminate plasma proteins, submitting the samples only to centrifugation before performing the F–C assay. The participants of the study consumed apple juice and the total phenolic content in plasma was determined after 10 h to assess (poly)phenol pharmacokinetics (Wruss et al. 2015).

Potential future perspectives

Many pathologies studied in relation to (poly)phenol intake involve inflammatory processes that initiate or worsen the disease. However, the association between total (poly)phenols in biological samples measured by the F–C assay and inflammation has been scarcely studied. A study on healthy men found that dietary (poly)phenols were associated with a better response in vascular and plasmatic inflammatory biomarkers (Hurtado-Barroso et al. 2018; Hurtado-Barroso et al. 2019). Another clinical trial has assessed this relationship, finding that a higher total phenolic content in urine was associated with decreased inflammatory biomarkers in an older Mediterranean population (Medina-Remón et al. 2017). Numerous clinical trials have observed that phenolic intake is associated with better inflammatory status (Chai et al. 2019; Lockyer et al. 2017; Del Bo' et al. 2021). Moreover, Arancibia et al. demonstrated that total (poly) phenols excreted in urine measured by the F–C assay can serve as a reliable biomarker of anti-inflammatory diets, particularly in women, supporting the inverse relationship between total polyphenol intake and inflammation (Arancibia-Riveros et al. 2023). Altogether, these data suggest that the total phenolic content determined by the F–C assay could be used as a biomarker of inflammation. Considering the simplicity and low cost of the F–C test compared to assays that measure inflammatory molecules, it could be an effective alternative for routine use in any clinical laboratory.

Conclusions

The Folin-Ciocalteu assay is a well-known and efficient method for measuring the total phenolic content in plant-based foods and beverages. More recently, due to its speed and simplicity, it has been applied in human urine and blood samples, which are previously submitted to a cleaning procedure to eliminate interferences. The assay is easy to perform, economical, and sustainable, making it ideal for application in routine laboratory analysis as a biomarker of total (poly)phenol intake and potentially of inflammatory status.

The ability to measure total phenolic compounds in biological samples provides valuable information for assessing the impact of dietary interventions and disease prevention strategies. It can also provide valuable insights into the relationship between polyphenol intake, mortality, inflammation, and chronic diseases.

Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Disclosure statement

The authors declare no conflict of interest.

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Abbreviations

F-C	Folin-Ciocalteau
GAE	gallic acid equivalents
SPE	solid-phase extraction.

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1.2.2.1. Microbial phenolic metabolites

Dietary polyphenols are known for their low bioavailability[131]. Polyphenols frequently arrive in the small intestine bound to glycoside molecules, requiring liberation to facilitate absorption[122]. Those polyphenols that bypass this process reach the colon, where they can undergo metabolism by gut microbiota before absorption[133]. This newly formed microbial phenolic metabolites (MPM) can reach the bloodstream and target organs, therefore, it may be these compounds that exert the biological effects, rather than dietary polyphenols. In addition, circulating polyphenols can be modified in the liver by phase II enzymes that conjugate them with methyl, glucuronide, or sulphate groups, enhancing their solubility[134].

Therefore, the production of the type and quantity of MPM largely depends on the composition of each individual's microbiota[135]. In fact, the relationship between intestinal microbiota and polyphenols is bidirectional, as the phenolic compounds consumed through the diet are prebiotics that serve as substrates for intestinal bacteria and can modify the microbial composition[133].

To date, only few studies have assessed the relationship between MPM and health outcomes. Certain compounds that might have microbial origins have been assessed in relation to inflammation and CVD risk factors, yet the predominant focus of studies has not been on phenolic compounds derived exclusively from the microbiota. For instance, phenolic acids, which can be generated by the gut microbiota, have demonstrated anti-inflammatory effects and the potential to enhance glucose and lipid profiles[136]. Similarly, enterolignans have been associated with protective effects on CVD factors, such as glucose metabolism or weight. However, the majority of studies have examined these compounds primarily from a dietary perspective[137,138]. Regarding metabolites exclusively produced by the gut microbiota, a clinical trial reported that urolithin, an microbial ellagic acid, decreased LDL-c depending on the phenotype of the individual[139]. In the PREDIMED trial, urinary MPM, and particularly hydroxybenzoic glucuronide, were associated with a lower risk of T2D[140].

Regarding their impact on cognition, MPM encounter an additional challenge—they must traverse the blood-brain barrier to exert their effects. A review on the available literature on the role of MPM in the gut-brain axis, including their origin in the gut microbiota, the crossing of the blood-brain barrier and their effects on cognition can be found in the publication entitled ”From the gut to the brain: the long journey of phenolic compounds with neurocognitive effects” (Publication 3, under revision in *Nutrition Reviews)*.

Narrative Review

From the gut to the brain: the long journey of phenolic compounds with neurocognitive effects

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Abstract

The human gut microbiota is a complex community of microorganisms that play a crucial role in maintaining overall health. Recent research has shown that gut microbes also have a profound impact on brain function and cognition, leading to the concept of the gut-brain axis. One way in which the gut microbiota can influence the brain is through the bioconversion of polyphenols to other bioactive molecules. Phenolic compounds are a group of natural plant metabolites widely available in the human diet, which have anti-inflammatory and other positive effects on health. Recent studies have also suggested that some gut microbiota-derived phenolic metabolites may have neurocognitive effects, such as improving memory and cognitive function. The specific mechanisms involved are still being studied, but it is believed that phenolic metabolites may modulate neurotransmitter signalling, reduce inflammation, and enhance neural plasticity. Therefore, to exert a protective effect on neurocognition, dietary polyphenols or their metabolites must reach the brain, or act indirectly by producing an increase in bioactive molecules such as neurotransmitters. Once ingested, phenolic compounds are subjected to various processes (e.g., metabolization by gut microbiota, absorption, distribution) before they cross the blood-brain barrier (BBB), perhaps the most challenging stage of their trajectory. Understanding the role of phenolic compounds in the gut-brain axis has important implications for the development of new therapeutic strategies for neurological and psychiatric disorders. By targeting the gut microbiota and its production of phenolic metabolites, it may be possible to improve brain function and prevent cognitive decline. In this article, we review the current state of knowledge on the endogenous generation of phenolic metabolites by the gut microbiota and how these compounds can reach the brain and exert neurocognitive effects.

Keywords: Dietary polyphenols, neurodegeneration, microbial metabolites, gut microbiota, blood brain barrier.

1. Introduction

The human gut microbiota has a wide-reaching impact on health [1]. One of the ways this complex community of microorganisms benefits the host is by enhancing the bioavailability of food components such as polyphenols, which are metabolized into simpler, more easily absorbed products [2]. Conversely, polyphenols can act as prebiotics, modifying the composition of microbial colonies, which also has benefits for human health [2,3].

Dietary polyphenols, plant secondary metabolites with anti-inflammatory and antioxidant effects, frequently reach the small intestine bound to glycoside molecules, requiring liberation to be absorbed [4,5]. Most polyphenols bypass this process and arrive to the colon, where they may act as substrates for the microbiota [2]. A wide variety of reactions are involved in the microbial transformation of polyphenols, including ring fission, hydrolysis, demethylation, reduction, decarboxylation, dihydroxylation, and isomerization [6]. Once in the bloodstream, phenolic compounds (PC) can be distributed to most tissues, including the brain [7]. Furthermore, circulating polyphenols can undergo modifications in the liver through phase II enzymes, which conjugate them with methyl, glucuronide, or sulfate groups, thereby increasing their solubility [8]. Although phenolic metabolites can affect the brain indirectly, for example, by increasing levels of neurotransmitters such as dopamine, in this review, we focus on those that are able to cross the blood-brain barrier (BBB) and exert direct neurocognitive effects [9]. The function of this important physiological barrier is to prevent harmful substances from reaching the brain [10], but it can also keep out xenobiotics, including polyphenols. Therefore, the ability to penetrate the BBB largely conditions the effects of PC on the central nervous system

(CNS) [11]. Whereas the generation of phenolic metabolites by the microbiota has been extensively studied, research on their BBB permeability has only recently gained attention.

The first function described for the BBB was the protection of the brain from uncontrolled diffusion of substances from the blood. It is now understood to be a complex and dynamic interface that regulates the exchange of substances between the blood and the CNS [12]. Specifically, the functions of the BBB are to: (i) shield the brain from potential blood-borne toxins; (ii) meet the metabolic demands of the brain; and (iii) regulate the homeostatic environment in the CNS for proper neuronal function [13,14]. Brain microvascular endothelial cells (EC), pericytes, astrocytes, tight junctions, neurons, and the basal membrane construct physically tight brain capillaries in the BBB [15]. Importantly, the EC express multiple substrate-specific transport systems that control the movement of nutrients, energy metabolites, and other essential molecules from the blood to the brain and the removal of metabolic waste products from the brain's interstitial fluid into the blood [14,16,17]. Non-essential molecules, such as PC that prevent oxidative stress, can also cross the barrier and exert beneficial effects on the brain [11]. As Figure 1 illustrates, phenolic bioavailability in the brain is affected by physicochemical traits, including molecular weight, hydrophobicity, and lipophilicity [11,18], as well as physiological factors, such as efflux transporters (e.g., P-glycoprotein) [19]. In this novel research field, studies have been carried out using a wide range of *in silico*, *in vitro*, and *in vivo* approaches [20–22].

This review summarizes the available evidence for the ability of phenolic metabolites to cross the BBB, their endogenous origin, and direct cognitive effects on the brain. The results of *in silico*, *in vitro*, and *in vivo* studies are organized according to the phenolic group (flavonoids, ellagitannins, lignans, phenolic acids, and others).

2. Methods

The search of literature included the following keywords: “gut-brain axis” AND “polyphenols”, “microbial phenolic compounds”, “microbiota” AND “polyphenols”, “polyphenols” AND “blood-brain barrier”, “microbial phenols” AND “cognition” OR “neurodegeneration” OR “Alzheimer’s disease” OR “dementia.” The search sources included PubMed and Scopus databases; no time restrictions were imposed. Titles and abstracts were screened, and relevant full-article texts were extracted, reviewed, and downloaded to Mendeley.

3. Flavonoids

Dietary flavonoids are mostly metabolized into phenolic acids by the gut microbiota. Isoflavonoids such as genistin and daidzein, as well as their aglycones genistein and daidzein, are almost exclusively found in soybeans and soy-derived products (e.g., tofu, soymilk, miso soup), which typically contain 0.01-0.3% isoflavonoids [23,24] [25]. Following intake, they are digested by intestinal glucosidases and absorbed as aglycones. Intestinal bacteria use reductase enzymes to convert the isoflavone daidzein to equol and *O*-desmethylangolensin. The main metabolites found in biological fluids after the consumption of soy-derived food are shown in Table S1.

Equol is regarded as a phytoestrogen due to its structural similarity to 17β-estradiol and is thought to exert its effects through the estrogen signal transduction pathway [26]. While all animal species investigated so far produce equol, only one-third to one-half of humans have this ability (depending on the race), as they harbour equol-producing microbes. These individuals may therefore be the only ones to fully benefit from soy or isoflavone ingestion [27]. Furthermore, it has been proposed that dietary fat consumption reduces the ability of gut microbiota to produce equol [25]. Non-invasive urine analysis was employed to quantify systemic exposure to isoflavonoids and compliance in a soy intervention

study, with isoflavonoid excretion in overnight urine and spot urine accurately reflecting circulating plasma isoflavonoid levels in healthy postmenopausal women [23]. A larger daidzein dose was associated with increased urine excretion of equol in postmenopausal women, whether ingested in fermented or non-fermented soymilk [28]. In this context, a study of three generations of Japanese-Americans living in Hawaii found that older women produced more equol following a soy challenge, but there was no change in total isoflavonoid excretion after a standardized dosage of soymilk [29].

A study employing *in silico* and *in vitro* techniques demonstrated that equol passively crosses both the gut and the BBB [21]. The computational prediction of equol as a BBB penetrating molecule was supported by the *in vitro* results, which showed a high permeability, with -*log*P_e values of 39.7 ± 0.14. To date, no further evidence related to this finding has been published.

Several mechanisms of action have been described by which equol improves brain health. *In vitro* studies showed that equol increases glial cell migration, an essential process for brain development [30], and possesses neuroprotective properties that counteract the cytotoxicity of β-amyloid plaques generated in a cellular model of Alzheimer's disease (AD) [31]. It has also been reported that equol enhances hippocampus and synaptic plasticity in rats [32,33]. In addition, this phytoestrogen promoted brain metabolic activity in an animal model of human menopause [34]. Other studies performed in rats showed that equol reduced oxidative stress and protected against cerebral ischemia and neuronal apoptosis [35,36]. The few studies on the cognitive effects of equol in humans are restricted to the context of menopause management through its estrogenic effects. A randomized clinical trial demonstrated that a phytoestrogen formulation containing equol preserved cognitive function during menopause [37,38].

4. Ellagitannins

Ellagitannins and ellagic acid are found in nuts and some fruits, such as pomegranates and berries, and are generally poorly absorbed. In the intestinal tract, ellagitannins are metabolized by the gut microbiota to ellagic acid, and ellagic acid is hydrolysed into urolithins [39] which besides health benefits have potential application as dietary biomarkers [40]. The conversion of ellagic acid and ellagitannins to urolithins in the gut varies greatly between individuals, which has been associated with differences in the colon microbiota [41]. Urolithins and ellagic acid derivatives identified after the intake of ellagitannin-rich foods are shown in Table S2.

Urolithins have been identified in human biological samples in intervention studies with ellagitannin-rich foods such as red raspberries, pomegranates, and walnuts. Among the numerous metabolites found in blood or urine samples [42,43], the compounds originating from ingested ellagitannins were mainly phase II metabolites of the colon-derived urolithins A and B (urolithin A, urolithin A-*O*-glucuronide and urolithin B-*O*-glucuronide) [44]. Quantitative examination of 24 h plasma and 48 h urine revealed that urolithins were not detectable following dietary washout until 24 h after red raspberry ingestion, indicating they were produced by microbial conversion of ellagic acid. Similarly, another study found that concentrations of urolithin A-*O*-sulfate and urolithin A-*O*-glucuronide in both plasma and urine were highest at 24 h after the intake of red raspberries [45]. Phase II metabolites of the colon-derived urolithins A and B were still being excreted in urine 32–48 h after raspberry ingestion, indicating a high persistence in the body, although their overall urinary recovery was only 7.0% of intake [42]. After the daily administration of pomegranate extract to colorectal cancer patients for 15 days [46], urolithin derivatives were identified in plasma, urine, and colon tissues. Individual and total metabolite levels were higher in normal versus malignant colon tissues.

In individuals with metabolic syndrome following a nut-enriched diet, urinary levels of urolithins A and B were also shown to increase significantly. Additionally, urolithins C and D were detected, as well as a complex combination of urolithin-conjugated forms, indicating significant phase II metabolism following absorption [47]. Eight urolithin metabolites were detected in breast milk after walnut ingestion; although excreted in nanomolar amounts, they may enhance the anti-inflammatory and health benefits of breastfeeding for infants [48].

To date, animal studies have shown that urolithins are widely distributed in a range of tissues, including the brain [49], which may indicate an ability to cross the BBB. *In silico* prediction of BBB permeability indicated that urolithin A, urolithin B, and their methyl derivatives fulfilled theoretical criteria required for BBB penetration [50]. Importantly, this study reported that methylation increases the lipophilicity of hydroxyl groups, which enhances BBB penetrability, unlike the increased hydrophilicity caused by sulfation or glucuronidation in the intestine and liver. As for other types of urolithins, like C or D, no studies have been conducted thus far to assess their permeability through the BBB.

Promising effects on neurocognitive health have been reported for urolithin A in recent years. According to numerous *in vivo* and preclinical studies, urolithin A is involved in several molecular mechanisms that prevent cognitive deterioration. It reduces the formation of ß-amyloid plaques [51,52], decreases apoptosis, and enhances neurogenesis [53,54]. In addition, urolithin A promotes mitophagy, which is impaired in AD patients, activates hyperphosphorylation of tau, and reduces the toxicity of ß-amyloid plaques, both recognized as fluid biomarkers of AD [55,56]. Ahsan et al., however, reported that instead of mitophagy, urolithin A induced autophagy, which attenuated ischemic neuronal death [57]. A clinical trial carried out in 9 individuals demonstrated that, by activating mitophagy, urolithin A improves mitochondrial gene expression [58]. In a clinical

trial evaluating the effect of the Mediterranean diet on brain health in 284 adults, Kaplan et al. observed that higher concentrations of urolithin A in urine were associated with a lower hippocampal occupancy score, an indicator of brain-related atrophy [59].

To date, the cognitive effects of urolithin B have been less studied than those of urolithin A. In activated microglia, Lee et al. showed that it exerts anti-inflammatory and antioxidant effects through the inhibition of nitric oxide and proinflammatory cytokines, and an increase in anti-inflammatory cytokines in the brain [60]. Another study reported that urolithin B reduced apoptosis and the production of reactive oxygen species (ROS) in neuroblastoma cells [61]. Similarly, Chen et al. found that urolithin B inhibited apoptosis and activated regulated pathways in favor of neuronal survival [62]. Another proposed molecular mechanism for urolithin B involves the inhibition of monoamine oxidase (MAO), which in high concentrations promotes neurological disorders [63]. However, clinical studies are needed to corroborate that these effects also occur *in vivo* in humans.

5. Lignans

The mammalian lignans enterodiol and enterolactone are generated in the colon by bacteria acting on lignan plant precursors, such as secoisolariciresinol diglycoside, which are abundant in fiber-rich foods such as cereals, with lower amounts found in vegetables and fruits [64]. These microbial metabolites are regarded as phytoestrogens due to their estrogen-like biological properties, although they have less estrogenic activity than isoflavones [65]. The formation of lignans has been extensively researched *in vitro*, but few investigations have focused on their presence in bodily fluids following food consumption (Table S3).

Flaxseed is one of the best sources of dietary lignans. In a clinical study, blood enterolactone concentration was doubled by adding ground flaxseed and flaxseed oil to one or two daily meals [66]. Besides enterolactone and enterodiol, hydroxylated derivatives

are also found in human urine after the ingestion of flaxseed [67]. The availability of lignans in flaxseed is enhanced by crushing or grinding the whole seeds, likely due to improved accessibility for the microbiota to interact with the lignans [68]. However, no significant differences in urinary lignan excretion were found after the ingestion of raw versus processed flaxseed, suggesting that the amount consumed is probably more significant than its form. The metabolism of lignan precursors contained in flaxseed was found to be time- and dose-dependent, but was not influenced by processing [69]. Plasma lignan levels peaked 9 h after the flaxseed was consumed, and remained stable for 24 h. After one week of supplementation, plasma and urinary lignan concentrations increased considerably compared to baseline, and plasma concentrations stabilized by the eighth day, indicating they can be sustained by a once-daily dose of flaxseed.

Sesame seeds and rye grain also have a high concentration of lignans and are therefore an alternative to flaxseed as rich sources of enterodiol and enterolactone precursors [70,71]. Regular consumption of rye bread increases serum concentrations and urinary excretion of enterolactone much more than eating refined wheat cereal [71]. Non-targeted metabolite profiling proved to be an effective method to identify marker urinary metabolites, such as enterolactone glucuronide, that distinguish between the consumption of whole grain rye bread and white wheat bread [72].

An *in silico* and *in vitro* study demonstrated that enterolactone passively crosses the BBB [21]. First, computational prediction software identified enterolactone as a molecule that could penetrate the BBB. This finding was in line with *in vitro* results, showing high permeability with $-log P_e$ values of 3.52 ± 0.20. However, further studies in animals or in cerebrospinal fluid (CSF) are needed to demonstrate the ability of this compound to cross the barrier. Regarding enterodiol, although predicted to be BBB permeable by *in silico* assays, when tested *in vitro* it was found to exhibit low passive penetration [21].

While dietary lignans have been associated with neurological benefits, the role of the metabolite enterolactone has been less studied. This enterolignan has shown neuroprotective properties in *in vitro* studies, as it suppresses nitric oxide production and proinflammatory cytokines in murine microglia [21]. Köse et al. found that it can inhibit carbonic anhydrase, acetylcholinesterase (AchE) and butyrylcholinesterase (BchE), enzymes involved in the development of AD [73]. Regarding research in humans, a few clinical and observational studies have reported a positive association between dietary lignans and cognitive performance [74–76]. However, to our knowledge, no trial has been performed in humans to assess the specific relationship between enterolactone in biological samples and neurocognition, despite promising results from Sun et al., who found that higher urinary concentrations were associated with fewer sleep disorders [77].

6. Phenolic acids

Hydroxyphenylacetic, hydroxybenzoic and hydroxycinnamic acids

Coffee, fruits, wholegrains, and nuts are the most abundant dietary sources of phenolic acids [78,79], and numerous studies have identified phenolic metabolites in urine and plasma following their consumption. There are different bacterial actions that can lead to the production of these metabolites, including *O*-deglycosylation, C-ring fission, hydrolysis or dihydroxylation. Studies focusing on coffee and orange juice are described below, and the metabolites found after ingestion of other foods, such as cocoa products, nuts, red raspberry, and tomato, are listed in Table S4.

The metabolites of chlorogenic acid, a hydroxycinnamic acid derivative, were analysed in human plasma and urine following coffee consumption. The most common metabolites were phase II derivatives of reduced forms of hydroxycinnamic acids: sulphated and methyl derivatives, and to a lesser extent, glucuronidated conjugates [80]. Overall, 0-24 h urinary excretion of chlorogenic acids ranged from 15.7 to 29.1% of intake,

demonstrating that, in addition to being subject to substantial metabolism, chlorogenic acids in coffee are well absorbed [81,82]. A study quantifying urinary phenolic acid metabolites after coffee consumption found the greatest inter- and intra-individual variance in those produced by the intestinal microbiome [83]. After acute coffee ingestion, at least six chlorogenic acid compounds were identified intact in human plasma, although urine does not appear to be their primary excretion pathway, which instead may be through digestive fluids such as bile [84].

Caffeic, ferulic, isoferulic, caffeoylquinic, vanillic, sinapic, *p*-hydroxybenzoic, and *p*-coumaric acids, as well as some of their dihydro derivatives, were the predominant metabolites detected in urine following coffee consumption, with a considerable inter-individual variance [84,85]. After 1 h of coffee intake, high C_{max} values (maximum concentration in the body) were found for sulfates of caffeic, ferulic, and caffeoylquinic acid lactones, indicating that they were absorbed in the small intestine. However, dihydroferulic acid and its *O*-sulfate and dihydrocaffeic acid-*O*-sulfate had a second T_{max} (the time of maximum concentration) at 4 h of ingestion, suggesting absorption in the large intestine and possible catabolism by colonic bacteria [82].

The health benefits associated with orange juice consumption are attributed not only to absorbable and circulating flavanone metabolites but also to gut microbial metabolites. After an acute intake of orange juice, several microbial phenolic acid derivatives, including free phenolics and phase II sulfate, glucuronide, and methyl metabolites, were identified in plasma and/or urine samples (Table S4) [86,87]. Among the main phenolic catabolites found in plasma were methoxycinnamic acid, (hydroxy-methoxyphenyl)propanoic acid, (methoxyphenyl)propanoic acid (hydroxyphenyl)propanoic acid, and (phenyl)propanoic acid derivatives, as well as substantial amounts of phenylacetic and hippuric acids [87,88]. Phenolic acids represented

about 20% of the phenolic metabolites in systemic circulation after orange juice consumption. In 24 h urine, hydroxycinnamates (ferulic-*O*-glucuronides), hydroxyphenylpropanoic acids ((methoxyphenyl)propanoic acid-*O*-sulfate), hydroxyphenylacetic acids (hydroxyphenylacetic acid-*O*-sulfate), hydroxybenzoic acids and hippurates were identified [86–88].

Mixed results have been found regarding the ability of phenolic acids to cross the BBB and reach the brain. The permeability of caffeic acid (a hydroxycinnamic acid) has been assessed in human and animal studies. The detection of caffeic acid in CSF could indicate that once absorbed into the blood stream it can reach brain cells [89]. No correlation was found between levels in CSF and plasma, suggesting that the transportation of caffeic acid through the BBB is not the result of simple or facilitated diffusion. In addition, the authors propose that conjugation reactions may enhance permeability, and that transient conjugation or de-conjugation of polyphenols takes place in close vicinity to the BBB. In a BBB cell model, the similarity of apparent permeability (P_{app}) coefficient values between apical-to-basolateral and basolateral-to-apical transport suggested that the uptake of caffeic acid was close to the efflux rate [90]. However, according to another study, caffeic acid cannot pass through the BBB due to its low permeability coefficients, but is adsorbed in cerebral blood vessels [91]. Moreover, a parallel artificial membrane permeability assay for the BBB showed the permeability values of caffeic acid were below the lower limit [92]. Its inherent difficulties in crossing the BBB have led studies to target caffeic acid and attempt to increase its transport efficiency [93]. Application of PEGylation technology, in which poly(ethylene glycol) (PEG) was covalently attached to caffeic acid, effectively improved BBB permeability, which was attributed to the inhibition of P-glycoprotein [93].

Based on the results of numerous *in vitro* studies, ferulic acid metabolites can be described as having negligible BBB permeation [91,92,94,95], with only one *in silico* simulation study predicting permeability [96]. A proposed explanation is low paracellular or transcellular passive diffusion due to hydrophilicity [92]. The involvement of an active efflux mechanism that pumps compounds back to the apical side of the BBB model has also been described [94]. PEGylation clearly improved the transport efficiency of ferulic acid but to a lesser extent than other compounds [93], an outcome difficult to justify in terms of chemical structure, since ferulic acid is more lipophilic than caffeic acid.

Based on the chemical properties of gallic acid (Log*P* 0.89), it was suggested that this microbial metabolite of epigallocatechin may be available to the brain, as its lipophilicity is close to optimal (Log*P* 1.5–2.7) [97]. The BBB permeability coefficient of gallic acid measured using a model kit of mouse EC [98] was reported to be 21.97 ± 1.92 ($\times 10^{-6}$ cm/s), with a transport efficacy of 6.52 ± 0.5 (%, 30 min), values close to those of caffeine and higher compared to other substances. However, a limitation of this study is that only one reference compound of high permeability (caffeine) was used, and none of medium or low permeability, which renders the results inconclusive. It is also important to emphasize that in this study the permeation capacity of gallic acid was altered by the co-presence of epigallocatechin (18.68 ± 1.56 × 10^{-6} cm/s). A recent study determined that the P_{app} of gallic acid was null, due to the absence of peaks in an analysis of the brain solution of the BBB kit by liquid chromatography coupled to mass spectrometry (LC−MS/MS), which suggests this compound is not permeable [91].

A study performed in rats found that protocatechuic acid penetrated the BBB with a brain distribution ratio (AUC_{brain}/AUC_{blood}, area under the curve) of 0.09 ± 0.02 [99]. In support of these results, the apical-to-basolateral P_{app} values (4.369 ± 1.410 × 10^{-5} cm/s) recently reported in a transmembrane transport study indicated it was easily absorbed. However,

the P_{app} basolateral-to-apical values were very similar ($4.132 \pm 0.288 \times 10^{-5}$ cm/s) and the efflux ratio was 0.946, suggesting that penetration of protocatechuic acid was counteracted by its ready transport to the apical region [90]. Protocatechuic acid was detected in rat brains 2 h after the administration of a strawberry extract. However, it was not observed in the brain solution of the BBB kit, so its permeability coefficient could not be determined [91]. Further evidence for BBB impermeability is that the metabolite was not detected in the brain after intravenous administration to rats [100]. The authors hypothesized that protocatechuic acid is predominantly methylated into vanillic acid, as vanillic acid is present in larger quantities [91].

The BBB permeability of vanillic acid has been mostly studied *in vitro.* It has been proposed that vanillic acid improves BBB stability and cerebral blood flow [101]. In contrast with an *in vitro* study which reported that vanillic acid is unable to cross the BBB [102], Shimazu et al. found it was permeable [91]. Nevertheless, only small amounts were detected in the brain, and the authors hypothesized that it may also be adsorbed into cerebral blood vessels. Another study reported that vanillic acid sulfate, a phase-II metabolite of vanillic acid, could cross the BBB, although the P_{app} value was low [9].

Homovanillic acid is not an indicator of the exposure of neurons to dietary phenols, as it can be formed endogenously in the brain. Specifically, it is the result of several reactions catalyzed by catecholamines released by the brain, such as adrenaline, noradrenaline, and dopamine, and catalyzed by brain MAO and catechol-*O*-methyltransferase [89]. An animal study showed that the organic anion transporter 3, which is expressed in the abluminal membrane of the BBB, is involved in the efflux transport of homovanillic acid from the brain [103].

It has been reported that 4-hydroxyphenyl acetic acid cannot cross the BBB due to: i) limited *in silico* BBB permeation attributed to a negative charge at physiological pH

(experimental pKa = 4.25); ii) no apical-to-basolateral permeation in humans and rats in *in vitro* BBB models, and iii) high basolateral-to-apical permeability [104]. Another hypothesis suggests that 4-hydroxyphenyl acetic acid has a strong tendency to bind to albumin, which may limit its brain penetration [105]. 3-Hydroxyphenyl acetic acid, on the other hand, has been detected in the CSF, suggesting it may cross the BBB or blood-CSF barrier [89]. However, reports indicate that it can also be formed endogenously in the brain [106–108], and it has been described as a metabolite of oxidative deamination of tyramine [89].

The cognitive effects of caffeic acid have been widely investigated. *In vitro* studies have shown that caffeic acid can reverse tau hyperphosphorylation [109], restore dopaminergic neurotransmission to alleviate oxidative damage [110,111], and inhibit ß-amyloid deposition [112]. Nevertheless, most of this research has been conducted in experimental animal models of AD. Due to its antioxidant properties, caffeic acid increased the cognitive function of a murine model of AD by restoring the cholinergic function and reducing AChE activity [113]. These results were supported by Vera-Castro et al., who found that caffeic acid modulated the activity of purinergic and cholinergic enzymes in rats with streptozotocin-induced diabetes [111]. Similar findings were reported in a study with isolated rat brains incubated with caffeic acid, which reduced the activity of enzymes associated with cognitive impairment, such as AChE, BChE, and ATPase [114]. In mice with lipopolysaccharide-induced depression induced to depression with lipopolysaccharide, caffeic acid reduced the expression of inflammatory cytokines and oxidative stress in the brain, suggesting it could be beneficial against neuroinflammation [115]. This finding was supported by Sun et al., who reported a decrease in local inflammation, as well as a lower accumulation of ß-amyloid plaques in the hippocampus in experimental models of neurodegeneration [116,117]. The anti-inflammatory activity was also key to attenuating motor deficits in a mouse model of Parkinson's disease [117]. Caffeic acid has been

proposed to attenuate ageing-related cognitive impairment through up-regulation of hippocampal neurogenesis, as it restored the neurodegeneration caused by D-galactose in rats [118]. Little research on the effect of caffeic acid on the human brain has been carried out. Although benefits of coffee on cognitive health have been reported, they cannot be attributed solely to caffeic acid due to the presence of other known neuroprotective substances such as caffeine [119] [120,121].

Vanillic acid has been described as a potent antioxidant capable of inhibiting AChE and BChE, as well as reducing nitric oxide, malonaldehyde, and the activities of enzymes related to oxidative stress in hippocampal cells [122]. *In vitro* results suggest it may also exert neuroprotective effects by stimulating mitochondrial biogenesis and modulating the expression of genes involved in neuronal differentiation [123]. Siddiqui et al. showed that vanillic acid significantly increased neurite growth and reduced the expression of inflammatory molecules in hippocampal neurons with induced inflammation [124]. These beneficial effects of vanillic acid on the brain have been proven in *in vivo* experimental models. In mice with induced neurodegeneration, vanillic acid improved spatial learning and memory, and was associated with a decrease in AChE, cortisone, and inflammatory molecules [125]. In other studies with animal models of cognitive impairment, vanillic acid decreased the accumulation of ß-amyloid plaques, neuronal apoptosis, neuroinflammation and oxidative stress [126,127]. Khoshnam et al. demonstrated its anti-inflammatory effects in rats, which showed reduced levels of proinflammatory cytokines in the hippocampus [128]. However, vanillic acid did not confer any protection against global cerebral ischemia in adolescent rats [129]. In a rat model of Huntington disease, Bains et al. found that vanillic acid improved the motor and cognitive functions affected by this pathology through reducing oxidative stress, neuroinflammation, and mitochondrial dysfunction [130]. Although the neuroprotective properties of vanillic acid are relatively

well characterized, very few studies have attempted to demonstrate these effects in humans. Its phase-II metabolite, vanillic acid glucuronide, was associated with better cognitive functions in individuals at high cardiovascular risk [131]. However, more studies in humans are needed to corroborate the benefits of vanillic acid for cognitive health.

Phenyl-γ-valerolactones

Cocoa and tea are the main dietary sources of flavan-3-ols, an important group of flavonoids that are extensively metabolised in the colon to phenyl-γ-valerolactones (Table S5). In this context, the metabolic activity of gut microbiota was studied in regular and infrequent consumers of chocolate after the administration of dark chocolate [132]. Although 5-(dihydroxyphenyl)-γ-valerolactone, and hydroxy (dihydroxyphenyl)-valeric acid metabolites were found in both regular and infrequent consumers of chocolate, an increase in sulfate, glucuronide, and methyl-sulfate conjugates of 5-(dihydroxyphenyl)-valeric acid was only observed in the regular chocolate consumers, suggesting that the metabolic response of the microbiota was conditioned by the frequency of consumption.

Green tea is another good source of flavan-3-ols. The urinary metabolic profile after the consumption of this beverage showed several valerolactone conjugates with glucuronide, sulfate, and methyl groups, suggesting that microbial metabolism is largely responsible for the bioavailability of green tea flavan-3-ols [133]. Although evidence suggests that green tea catechins are partially metabolized in the distal section of the gastrointestinal tract, specifically the ileum [134], significant amounts appear to move to the colon where they are degraded by the activity of the colonic microflora. After being absorbed and passing through the circulatory system, the amounts of phenolic acid metabolites eliminated in urine were equivalent to roughly 40% of flavan-3-ol intake [135].

Several flavan-3-ol metabolites were identified in biological fluids after the consumption of a ready-to-drink green tea [136]. In urine, by far the most abundant were polyhydroxyphenyl-γ-valerolactones generated by colonic microflora, their average concentrations being 10-fold higher than those of flavan-3-ol conjugates. Black tea metabolites in human urine have been comprehensively structurally identified and quantified [137], and their pharmacokinetics have been determined in plasma [138]. Within 2-4 h of consumption, both conjugated and unconjugated catechins appeared in plasma, followed by a variety of microbial metabolites. Concentrations of the conjugated gut microbial metabolites remained high for up to 25 h after drinking black tea. Although a study on the metabolism of raspberry polyphenols only detected glucuronide forms of phenylvalerolactones and phenylcinnamic acids in plasma [44], the data for conjugated pyrogallol and 5-(dihydroxyphenyl)-γ-valerolactone from black tea indicate sulfation is predominant over glucuronidation.

An *in vitro* study on the BBB permeability of 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and its conjugated forms reported that it reached the brain parenchyma [139]. A comprehensive study that included *in silico* prediction, *in vitro* microvascular EC, and *in vivo* assays in rats, demonstrated that 5-(hydroxyphenyl)-γ-valerolactone-sulfate could reach the brain [20]. Corral-Jara et al. used a bioinformatics approach to demonstrate that this metabolite potentially binds to transcription factors and modulates signaling pathways involved in preserving brain endothelial vascular cells [140]. Phenyl-γ-valerolactone metabolites, including 5-(hydroxyphenyl)-γ-valerolactone-sulfate, can reduce ß-amyloid plaques by modulating proteolysis and upregulating autophagy in neuronal cells [141]. The beneficial effects of phenyl-γ-valerolactone metabolites in relation to ß-amyloid plaques have also been observed *in vivo*, as they reduced neuroinflammation and prevented the formation of ß-amyloid oligomers in a mouse model [142]. These

preclinical results are promising but have yet to be confirmed in humans, with no studies conducted to date.

7. Stilbenes

Little is known about the stilbene microbial metabolites such as dihydroresveratrol (Table S6), which is produced by hydrogenation of the aliphatic double bond of *trans*-resveratrol in the gut. After the intake of red wine (250 mL), grape juice (1 L), or tablets, each containing equal amounts of *trans*-resveratrol, dihydroresveratrol was undetectable in human plasma in its free forms [143], but it was detected and quantified in human urine after a real-life dose (250 mL) of wine. Gut microbial metabolism of resveratrol was also verified by the presence of glucuronide and sulfate conjugates of dihydroresveratrol in plasma and urine after the moderate consumption of red wine. Although free dihydroresveratrol was not found in any sample [144], in the subsequent long-term study (28 days), the unconjugated form was observed [145].

A study in mice failed to detect dihydroresveratrol and its sulfate and glucuronide forms in the brain [146]. On the other hand, a clinical trial with individuals diagnosed with mild to moderate AD showed that both resveratrol and its main phase-II metabolites penetrated the BBB and exerted effects on the CNS [147]. Nevertheless, individuals with AD may experience altered BBB permeability, potentially leading to increased permeability as a result of neuroinflammation [148]. Therefore, further research is needed to obtain more conclusive data about the ability of these microbial derivatives to cross the BBB.

8. Other polyphenols

Hydroxytyrosol

After olive oil consumption by humans, tyrosol and hydroxytyrosol (derived from the hydrolysis of oleuropein) were excreted in the urine mainly as glucuronide conjugates

with concentrations according to the dose consumed, ranging from 20 to 22% for tyrosol and 30 to 60% for hydroxytyrosol [149]. Both a single-dose ingestion (50 ml) and short-term consumption (one week, 25 mL/day) of virgin olive oil resulted in an increase in both PC in 24 h urine ($P < 0.05$), with urinary tyrosol appearing to be a more accurate biomarker of virgin olive oil intake [150]. In plasma samples, several phenolic metabolites and their free forms were identified and quantified 60 and 120 min after the consumption of 30 ml of virgin olive oil rich in PC (Table S7) [151].

The food matrix was observed to have a substantial impact on hydroxytyrosol pharmacokinetics, as the oily nature of foods emerged as a relevant factor in enhancing its bioavailability [152]. In particular, plasma concentrations 30 min after oral ingestion of extra virgin olive oil (3.79 ng/mL) were significantly higher compared to the control plasma, the oil being highlighted as the best dietary source of hydroxytyrosol [152]. Regarding its bioavailability, the intake of extra virgin olive oil, as well as fortified refined olive, flax, and grapeseed oils with 5 mg of hydroxytyrosol, provided significantly higher urinary contents compared with basal urine, whereas hydroxytyrosol metabolites (hydroxytyrosol acetate, dihydroxyphenylacetic acid, tyrosol, and homovanillic alcohol) showed no significant changes.

Yu-Tse Wu et al. showed that although hydroxytyrosol has a short half-life and half-residence time, it can penetrate the BBB [95], results that were confirmed by the detection of hydroxytyrosol in brain tissue [22]. According to these studies, this compound reaches the brain in micromolar concentrations, far from the amount necessary for a clinical effect. This limitation prompted Mursaleen et al. to create micellar nanocarriers of hydroxytyrosol to increase its BBB permeability and enhance its therapeutic effects [153]. The presence of hydroxytyrosol in the brain can be endogenous, due to the metabolism

of dopamine, but this is a minor metabolic pathway and of limited importance under normal conditions [154].

Several studies have demonstrated that hydroxytyrosol provides a neuroprotective effect when it reaches the brain. An *in vitro* study showed that it reduces the levels of MAO inhibition products linked to Parkinson's disease [155]. In addition, hydroxytyrosol has a beneficial effect against AD, as it prevents the aggregation of ß-amyloid plaques, reduces oxidative stress, and stabilizes fibrils and oligomers [156–158]. Furthermore, hydroxytyrosol reverted the mitochondrial energetic deficit in a cellular model [159]. Other pathologies involving neuroinflammation may also benefit from hydroxytyrosol. Studies performed in rats showed that it induces the expression of anti-inflammatory molecules and reduces apoptosis [160,161], therefore ameliorating neuropathic pain and improving stroke outcomes. Unfortunately, to date, very few clinical trials have investigated the therapeutic role of hydroxytyrosol in neurodegenerative diseases.

9. Conclusion

Cumulative evidence highlights that the gut microbiota plays a key role in the relationship between polyphenols and cognition through the gut-brain axis, since it transforms dietary polyphenols into biologically active metabolites able to cross the BBB and exert benefits for cognitive health. Promising results have been obtained for these microbial phenolic metabolites, many of which can modulate mechanisms involved in neurodegenerative disease pathogenesis, for example, by reducing oxidative stress, apoptosis or neuroinflammation (Figure 2).

However, although encouraging, the findings in this field reported to date have two main limitations. The first is that most studies have focused on the protective properties of

parent polyphenols and little attention has been given to assessing the effects of microbial phenolic metabolites, even though these can circulate and reach target tissues more readily. The second is that although crossing the BBB is a prerequisite for dietary polyphenols to act on the brain, the permeability of microbiota-derived phenolic metabolites has been scarcely investigated. To date, the only PC shown to have this capacity are equol, urolithin A and B, enterolactone, caffeic and vanillic acid, 5-(hydroxyphenyl)-γ-valerolactone-sulfate, and hydroxytyrosol.

Given that microbial derivatives are well-placed to be therapeutic targets for neurological disorders, understanding the role of the gut microbiota in their metabolism could lead to the development of novel treatments. Therefore, more research is necessary to determine which of the most abundant systemic metabolites penetrate the BBB and what effect they have on the brain.

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Figures

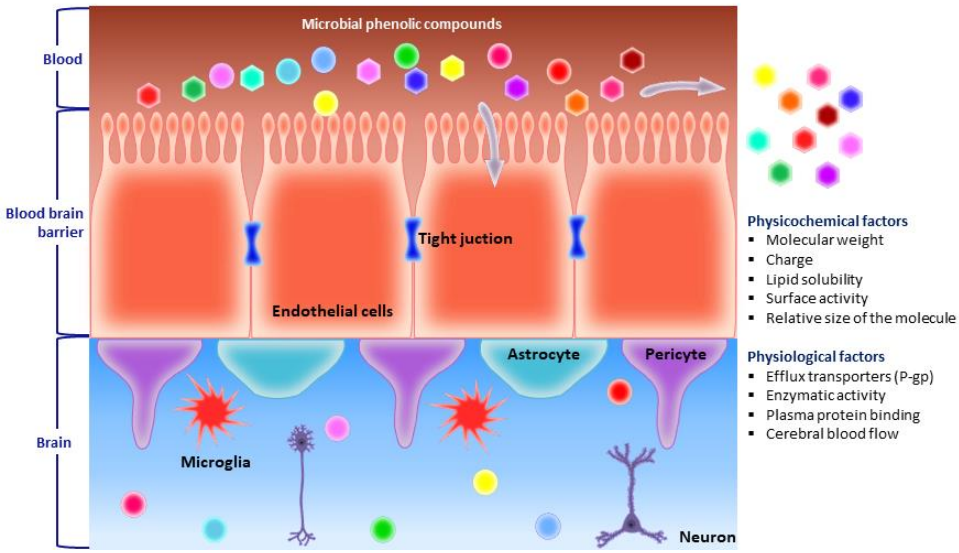

Figure 1. Summary of blood-brain barrier permeability of microbial phenolic metabolites.

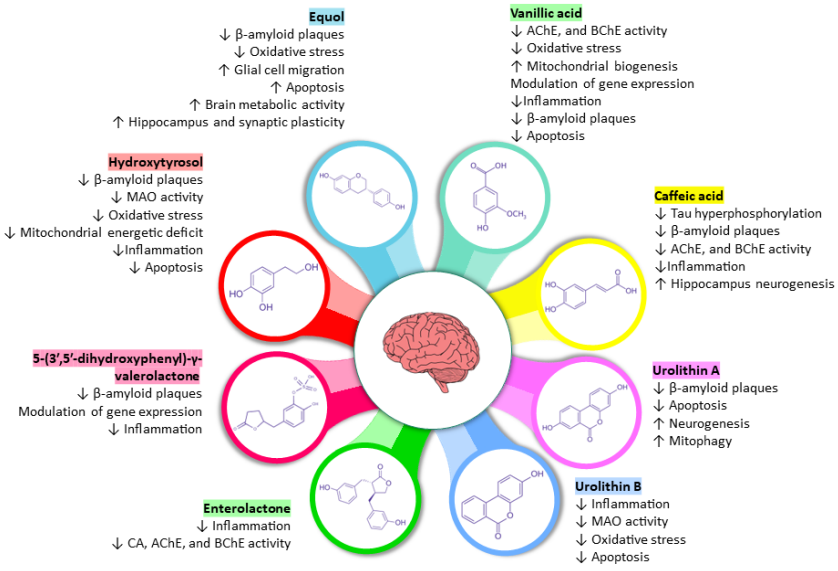

Figure 2. Summary of the molecular mechanisms of phenolic compounds in the brain. AChE, acetylcholinesterase; BChE, butylcholinesterase; CA, carbonic anhydrase; MAO, monoamine oxidase.

Supporting information

Table S1. Main metabolites found in biological fluids after the intake of isoflavonoid-rich foods.

Table S2. Urolithins and ellagic acid derivatives found after the intake of ellagitannin-rich foods.

Table S3. Mammlian lignans found after the intake of lignan-rich foods.

Table S4. Phenolic acid metabolites found in biological fluids after the intake of cocoa products, coffee, tomato, and orange juice.

Table S5. Main microbial metabolites found in biological fluids after the intake of flavan-3-ol-rich foods.

Table S6. Microbial resveratrol metabolites found after the intake of ellagitannin-rich foods.

Table S7. Main microbial metabolites found in biological fluids after the intake of olive oil and other oils.

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1.2.3. Vitamin B12

Vitamin B12, also known as cobalamin, is an essential water-soluble vitamin that plays a crucial role in various physiological processes[141]. It is involved in DNA synthesis, red blood cell production, nerve function, and the metabolism of amino acids and fatty acids[142]. Two key enzymes, methionine synthase and methylmalonyl-CoA mutase, depend on vitamin B12 for their functions[143]. Methionine synthase converts homocysteine into methionine, a vital amino acid for protein synthesis and various physiological processes[144]. Methylmalonyl-CoA mutase aids in breaking down specific amino acids like valine, isoleucine, methionine, and threonine, ensuring their proper metabolism[145]. These enzymes also contribute to DNA synthesis, with methionine synthase supplying essential components and methylmalonyl-CoA mutase regulating DNA replication and cell division[146].

Among the B vitamins, B12 is the only one that requires intrinsic factor, a protein secreted by the stomach lining, for efficient absorption in the small intestine[147]. Good dietary sources of vitamin B12 include animal products such as meat, fish, dairy products, and eggs[148]. Vegetarians and vegans, who avoid animal-derived foods, are at a higher risk of vitamin B12 deficiency and may need to supplement or include fortified foods in their diet[148].

Deficiency in vitamin B12 can impair myelin synthesis, leading to demyelination and nerve damage. This can manifest as neurological symptoms, such as numbness, tingling, muscle weakness, difficulty with coordination, and even cognitive impairment. In severe cases, it can result in irreversible nerve damage[149].

Additionally, vitamin B12 is involved in the conversion of fatty acids into their active forms, which are crucial for nerve cell membrane integrity and function[150]. Fatty acids are integral components of cell membranes, including those of nerve cells. Proper membrane structure and fluidity are essential for efficient nerve signal transmission[151].

Moreover, vitamin B12 helps regulate the levels of the amino acid homocysteine. Elevated levels of homocysteine have been associated with increased risk of nerve

damage and neurodegenerative disorders, and its pathological accumulation is known has hyperhomocysteinemia[152]. By participating in the conversion of homocysteine to methionine, vitamin B12 helps maintain optimal levels of this amino acid, contributing to nerve health[153].

HYPOTHESIS AND AIMS

2. Hypothesis and aims

Hypothesis

The general hypothesis of this work is that nutrients and bioactive compounds naturally present in the Mediterranean diet, including fatty acids, polyphenols, and vitamin B12, confer health benefits to an older population at high risk of CVD.

Aims

In this study, we aim to examine the potential relationship of nutrients and bioactive compounds inherent to the Mediterranean diet, specifically focusing on fatty acids, polyphenols, and vitamin B12. We explore how these compounds influence different health aspects susceptible to the effects of aging in an older Spanish population at high risk of CVD.

To achieve this aim, the following specific objectives were proposed:

Objective 1: To study the relationship of fatty acids, polyphenols, and vitamin B12 with metabolic and inflammatory pathways.

1.1 To examine the association between dietary patterns and the bioavailability of fatty acids (Publication 4).
1.2 To assess how endogenous metabolism of fatty acids may vary their role on metabolic health (Publication 5).
1.3 To investigate the potential link between fatty acids, polyphenols, and vitamin B12 with circulating inflammatory biomarkers (Publication 6, 7 and 8).

Objective 2: To explore how cardiovascular health is influenced by these bioactive compounds.

2.1 To assess if wine consumption, a polyphenol-rich beverage, measured as urinary tartaric acid, is related to the cardiovascular health of post-menopausal women (Publication 9).

2.2 To evaluate the associations of polyphenols and their microbial metabolites on cardiovascular risk factors and T2D (Publication 10 and 11).

Objective 3: To study the role of polyphenols and vitamin B12 on cognitive decline.

3.1 To assess the relationship of polyphenols produced by the gut microbiota with cognitive decline (Publication 12 and 13).

3.2 To evaluate the link between vitamin B12 and cognition according to the adherence to the MedDiet (Publication 14).

METHODS AND RESULTS

3. Methods and Results

3.1. Summary of the experimental methodology

3.1.1 Analysis of total fatty acids

Total fatty acids present in plasma samples were analyzed using gas chromatography coupled to a flame ionization detector (GC-FID) following an adapted method from Bondia-Pons et al.[154]. Previously, fatty acids were converted into fatty acid methyl esters (FAME) through a derivatization process to enhance their stability and volatility. FAME were injected into the GC-FID system, and an inert gas, hydrogen, was used to carry the sample through the polar capillary column. The fatty acids were quantified using the internal calibration method, with C13:0 methyl ester employed as the internal standard.

3.1.2 Analysis of tartaric acid

Urinary tartaric acid was determined by liquid chromatography-electrospray ionization-mass spectrometry (MS) in an High-performance liquid chromatography (HPLC) system coupled to a triple quadrupole mass spectrometer API 3000 following the methodology described by Regueiro et al.[155]. Urine samples were previously diluted in acidified water and filtered by 0.2 μm Nylon filters. Chromatographic separation was achieved using a reversed-phase C18 column and the acidic mobile phases of 0.5% formic acid in water and in acetonitrile, followed by a post-column addition of acetonitrile to improve the ionization. Deuterated tartaric acid was used as internal standard and quantification was accomplished through calibration curves of standard solutions.

3.1.3 Analysis of phenolic compounds

Identification and quantification of phenolic compounds and their metabolites in urine was performed by HPLC coupled to linear trap quadrupole-Orbitrap high-resolution MS following an adapted method described by Laveriano-Santos

et al.[156]. Urine samples were diluted, centrifuged, and acidified before undergoing a step of clean-up by solid-phase extraction to separate the phenolic compounds from undesired substancies. Chromatographic separation was achieved in a reversed-phase column F5 with the mobile phases 0.05% formic acid in water and in acetonitrile. Phenolic compounds were quantified through calibration curves with their respective standards, and the glucuronidated and sulfated metabolites were quantified using the standard corresponding to their aglycone.

3.1.4 **Analysis of inflammatory biomarkers**

Circulating inflammatory molecules were measured using two different methods. Initially, ELISA was used to analyze e-Selectin, p-Selectin, soluble vascular cell adhesion molecule (VCAM), soluble intercellular adhesion molecule (ICAM), interleukin-6 (IL-6) in plasma with a commercial kit (BLK and PelkinElmer Elast Amplification System)[24]. Thereafter, plasma molecules VCAM, ICAM, IL-6, tumour necrosis factor alpha (TNF-α) and monocyte chemotactic protein 1 (MCP-1) were measured using xMAP technology on the Luminex platform (Luminex Corporation, Austin, TX, USA)[157]. High-sensitive CRP was determined in serum by particle-enhanced immunonephelometry.

3.2. Metabolic and inflammatory pathways

The bioavailability of nutrients, especially those involved in metabolic pathways, as fatty acids, can modulate the intensity and duration of inflammatory responses. The first part of the present objective focused on assessing the influence of diets rich in fruits and vegetables (F&V), such as the MedDiet, on the bioavailability of different types of fats measured as fatty acids in plasma (Publication 4).

Circulating fatty acids are representative of the individual's diet, however they can also be affected by the endogenous metabolism carried out by desaturase enzymes. Therefore, this objective also aimed to investigate the altered activities of desaturases and their association with individual CVD components (Publication 5).

Lastly, the associations of specific dietary compounds, including fatty acids, wine polyphenols, and vitamin B12, with inflammatory biomarkers were explored to study their relationship with inflammatory pathways (Publication 6, 7, and 8).

3.2.1. Publication 4

Fruit and vegetable consumption is inversely associated with plasma saturated fatty acids at baseline in Predimed plus trial

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Supplementary Material available in Annex section.

Abstract

Aims: To assess the relationship between fruit and vegetable (F&V) consumption and plasma fatty acids and their subtypes.

Methods: Plasma fatty acids were assessed in a cross-sectional analysis of a subsample of 240 subjects from the PREDIMED-Plus study. Participants are categorized into four groups of fruit, vegetable, and fat intake according to the food frequency questionnaire. Plasma fatty acid analysis is performed using GC. Associations between fatty acids and F&V consumption were adjusted for age, sex, physical activity, body mass index (BMI), total energy intake, and alcohol consumption.

Results: Plasma SFAs are lower in groups with high F&V consumption, especially when fat intake is high. Total fatty acids and n-6 PUFAs tend to be lower in high consumers of F&V only in the high-fat intake groups.

Conclusions: F&V consumption is associated with lower plasma SFAs when fat intake is high. These findings suggest that F&V consumption may have different associations with plasma fatty acids depending on their subtype and on the extent of fat intake.

RESEARCH ARTICLE

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Fruit and Vegetable Consumption is Inversely Associated with Plasma Saturated Fatty Acids at Baseline in Predimed Plus Trial

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Scope: Plasma fatty acids (FAs) are associated with the development of cardiovascular diseases and metabolic syndrome. The aim of our study is to assess the relationship between fruit and vegetable (F&V) consumption and plasma FAs and their subtypes.

Methods and Results: Plasma FAs are assessed in a cross-sectional analysis of a subsample of 240 subjects from the PREDIMED-Plus study. Participants are categorized into four groups of fruit, vegetable, and fat intake according to the food frequency questionnaire. Plasma FA analysis is performed using gas chromatography. Associations between FAs and F&V consumption are adjusted for age, sex, physical activity, body mass index (BMI), total energy intake, and alcohol consumption. Plasma saturated FAs are lower in groups with high F&V consumption (-1.20 mg cL^{-1} [95% CI: [-2.22, -0.18], *p*-value = 0.021), especially when fat intake is high (-1.74 mg cL^{-1} [95% CI: [-3.41, -0.06], *p*-value = 0.042). Total FAs and n-6 polyunsaturated FAs tend to be lower in high consumers of F&V only in the high-fat intake groups.

Conclusions: F&V consumption is associated with lower plasma saturated FAs when fat intake is high. These findings suggest that F&V consumption may have different associations with plasma FAs depending on their subtype and on the extent of fat intake.

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1. Introduction

The Mediterranean diet is recognized as a healthy dietary pattern proven to provide protection against cardiovascular diseases.[1] This dietary pattern is characterized by high intakes of fruits, vegetables, legumes, nuts, whole grains, and olive oil, some of which make up good sources of healthy fats.[2,3]

Dietary fat is a major energy source, but it can play different roles in health depending on its fatty acid (FA) composition.[4–8] Monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) have been linked to several health benefits, such as de-

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crease in metabolic syndrome incidence[9] and improvement in cardiovascular status.[10,11] Special attention has been paid to n-3 PUFAs due to their anti-inflammatory properties.[12,13] Conversely, in the past decades, diets rich in saturated FAs (SFAs) were associated with higher cardiometabolic and type 2 diabetes risk.[14–16] Overall, replacing saturated fat with unsaturated fat has been shown to be an effective strategy to prevent coronary heart disease[17,18] and reduce overall cardiovascular risk.[14,19,20]

Absorption of dietary fat takes place in the duodenum and proximal jejunum, where emulsified triglycerides arrive in the form of lipid droplets, are hydrolyzed by pancreatic lipases to form FAs and monoglycerides, and the lipolytic products are solubilized by bile acids into mixed micelles. The micelles deliver FAs and monoglycerides to the lipid bilayer of enterocytes, and these molecules are absorbed by simple diffusion, to be reconstituted into triglycerides in the cytosol.[21] Additionally, there are other minority mechanisms involved in FA uptake, mainly long-chain FAs, based on protein transporters.[22]fhd

Various dietary components modulate intestinal fat absorption, including fiber, proteins, carbohydrates, and cholesterol.[21] In particular, fiber is known to significantly reduce nutrient bioavailability,[23] and there is evidence that it can decrease postprandial triglyceride concentrations when consumed in a mixed meal.[24] Fruits and vegetables (F&Vs) are rich sources of fiber; therefore, these foods may affect dietary lipid bioavailability.[25] However, no large studies have investigated whether F&V consumption may affect the plasma concentrations of the different FA classes.

The purpose of this study was to evaluate the association of F&V consumption with plasma concentrations of total FAs and their subtypes in an older Mediterranean population with metabolic syndrome.

2. Experimental Section

2.1. Study Design

This study was a cross-sectional analysis of baseline data from the PREDIMED-Plus study, an ongoing 6-year multicenter, randomized, parallel-group clinical trial designed to assess the effect of an energy-restricted Mediterranean diet, combined with physical activity and behavioral support, on hard cardiovascular events. A total of 6874 participants were recruited and randomized into the trial in 23 Spanish centers from September 2013 to December 2016.[26]

Eligible participants were males (aged 55–75 years) and females (aged 60–75 years) with overweight (body mass index [BMI] ≥25) or obesity (BMI ≥30) who met at least three metabolic syndrome criteria according to the International Diabetes Federation and the American Heart Association and National Heart, Lung, and Blood Institute.[27] The participant selection and the description of the study sample was described previously.[28] Details on the study protocol could be found at http://predimedplus.com.

2.2. Ethics Statement

The study was conducted according to the ethical standards' guidelines of the Declaration of Helsinki and all procedures in-

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volving human participants and patients were approved by the Institutional Review Boards of the participating centers. The clinical trial was registered in the ISRCTN of London, England with the number 89898870 on July 24, 2014. Written informed consent was obtained from all participants.

2.3. Sample Selection

Dietary intake was assessed using the validated, semi-quantitative 143-item PREDIMED-Plus food frequency questionnaire (https://www.predimedplus.com/wp-content/uploads/2020/07/FFQ-PREDIMED-Plus.pdf).[28] A total of 240 participants with extreme values of F&V and fat intake were randomly selected and classified into the following groups: 1) low F&V consumption (first decile) and low-moderate fat (first quartile) intake (reference); 2) low F&V consumption (first decile) and high fat intake (fourth quartile); 3) high F&V consumption (tenth decile) and low-moderate fat intake (first quartile); and 4) high F&V consumption (tenth decile) and high fat intake (fourth quartile).

2.4. Covariates

Trained personnel collected baseline data on age; sex; prevalence of diabetes, hypercholesterolemia, and hypertension; BMI; and smoking habit as previously described.[28] From the validated food frequency questionnaire, consumption of alcohol (g day^{-1}) and energy intake (kcal day^{-1}) were also estimated. We estimated the overall quality of diet based on the 17-item energy-reduced Mediterranean diet score.[29]

2.5. Sample Size Calculation

This study was performed in a sub-cohort of 240 participants that had been previously selected for another work of our group.[30] As the design and sample size was suitable for this study, we decided to use the same sub-cohort.

2.6. Derivatization and Analysis of Total Plasma Fatty Acids

2.6.1. Reagents and Standards

Sodium methylate, boron trifluoride in methanol (14% w/v) and *n*-hexane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride was purchased from Panreac Quimica SLU (Barcelona, Spain) and anhydrous sodium sulphate from Scharlab (Barcelona, Spain).

Tridecanoic acid methyl ester (C13:0), used as internal standard, was purchased from Sigma-Aldrich. Supelco 37 component FAME mix and PUFA No.2 (Animal source), used for peak identification, were purchased from Merck (Darmstadt, Germany). Standards were stored in powder form at –20 °C and protected from light.

2.6.2. Sample Preparation

Plasma samples used for FA analyses were stored at −80 °C, prior to analysis. Plasma FAs were determined by fast gas chromatography with a previous derivatization to their corresponding FAMEs.[31] Briefly, 20 µL of the internal standard were added to 100 µL of plasma sample. One milliliter of sodium methylate (0.5% w/v) was directly added and heated to 100 °C for 15 min. After cooling, samples were esterified with 1 mL of boron trifluoride-methanol reagent, also at 100 °C, for 15 min. Once the tubes were cooled, FAMEs were isolated by adding 500 µL of n-hexane. After shaking for 1 min, 1 mL of a saturated sodium chloride solution was added. Finally, the tubes were centrifuged for 10 min at 3000 rpm. After drying with anhydrous sodium sulphate, the clear n-hexane top layer was transferred into an automatic injector vial equipped with a volume adapter of 300 µL.

2.6.3. Gas Chromatographic Conditions

Fast GC analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of FAMEs was carried out on a capillary column (40 m × 0.18 mm I.D. x 0.1 µm film thickness), coated with an RTX-2330 stationary phase of 10% cyanopropyl phenyl—90% byscyanopropyl polysiloxane from Restek (Bellefonte, USA).

Operating conditions were as follows: the split-splitness injector was used in split mode with a split ratio of 1:50. The injection volume of the sample was 1 µL. The injector and detector temperatures were kept at 250 °C and 300 °C, respectively. The temperature program was as follows: initial temperature 110 °C, increased at 52 °C min^{-1} to 195 °C and held at this temperature for 6 min, and then increased at 25 °C min^{-1} until 230 °C and held for 6.5 min (total run time: 16.03 min). Hydrogen was used as the carrier gas, at a constant pressure of 26 psi that refers to a linear velocity of 40 cm s^{-1} at 110 °C. Data acquisition and processing were performed with the Shimadzu-Chemstation software for GC systems. Results were presented as plasma FA concentrations (mg cL^{-1}).

2.7. Statistical Analyses

The data were analyzed using the available complete PREDIMED-Plus database, dated August 10, 2017.

Baseline characteristics of the participants were presented as means ± standard deviations (SD) for continuous variables and percentages for categorical variables. To determine possible between-group differences in baseline characteristics, we used one-way ANOVA for continuous variables and χ^2-test for categorical variables. Total and specific subtypes of fat intake were expressed as median (interquartile range). For continuous variables, significant differences between groups were analyzed using Bonferroni post-hoc test for one-way ANOVA.

Nine participants who reported extreme total energy intakes were excluded from the analysis (>3500 Kcal day^{-1} for females and >4000 Kcal day^{-1} for males).[32]

Multivariable adjusted linear regression models were used to assess differences between groups and plasma FAs. Three different adjustment models were applied. Model 1 was minimally adjusted for age (continuous) and sex. Model 2 was additionally adjusted for physical activity (quartiles) and BMI (continuous).

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Table 1. Participant characteristics according to dietary F&V consumption and fat intake.

Characteristics	All	Low F&V		High F&V		*p*-value
		Low-moderate fat	Very high fat	Low-moderate fat	Very high fat	
No. of participants	228	57	58	60	53	
Age (y)	66.1 ± 4.4	65.9 ± 4.52	66.3 ± 3.61	65.8 ± 5.07	66.2 ± 4.25	0.936
Females, n (%)	106 (46.1)	26 (44.1)	26 (44.8)	32 (53.3)	22 (41.5)	0.604
Type-2 diabetes mellitus, n (%)	53 (23.2)	10 (17.5)	11 (19)	21 (35.0)	11 (20.8)	0.157
Hypercholesterolemia, n (%)	154 (67.5)	39 (68.4)	42 (72.4)	40 (66.7)	34 (64.2)	0.792
Hypertension, n (%)	198 (86.4)	48 (84.2)	53 (91.4)	53 (88.3)	44 (83.0)	0.496
BMI (kg/m^2)	32.7 ± 3.53	32.1 ± 3.03	32.7 ± 3.64	32.4 ± 3.76	33.6 ± 3.53	0.115
Current smoker, n (%)	37 (16.2)	8 (14.0)	10 (17.2)	6 (10.0)	13 (24.5)	0.200
Leisure-time physical activity, MET (min/week)	2499 ± 2406	1649 ± 1855^{a}	2276 ± 1890^{a,b}	3064 ± 3248^{b}	3019 ± 2368^{b}	0.003
Total cholesterol (mg/dL)	193.2 ± 2.8	194.6 ± 35.7^{a,b}	209.09 ± 36.6^{b}	188.91 ± 38.6^{a}	192.6 ± 31.3^{a,b}	0.014
LDL (mg/dL)	128.1 ± 8.3	135.0 ± 124.2	142.3 ± 122.2	115.9 ± 35.5	134.6 ± 125.7	0.613
HDL (mg/dL)	47.5 ± 0.9	49.2 ± 9.1	48.8 ± 13.3	48.7 ± 11.9	47.4 ± 11.6	0.867
VLDL (mg/dL)	29.3 ± 1.1	28.0 ± 12.7^{a,b}	35.5 ± 20.7^{b}	25.8 ± 14.0^{a}	30.3 ± 14.6^{a,b}	0.015
AST (U/L)	23.01 ± 0.6	23.1 ± 11.3	23.7 ± 8.6	22.7 ± 4.9	23.0 ± 5.4	0.933
ALT (U/L)	26.5 ± 1.0	25.6 ± 12.9	27.4 ± 15.0	24.5 ± 7.6	27.8 ± 10.0	0.429
GGT (U/L)	35.6 ± 2.1	38.6 ± 30.2	41.3 ± 31.0	31.2 ± 19.2	29.8 ± 15.9	0.053
ALP (U/L)	77.0 ± 2.6	76.5 ± 28.5	80.2 ± 43.3	71.9 ± 22.7	70.0 ± 16.6	0.383

F&V, fruit and vegetable; BMI, body mass index; MET, metabolic equivalent of task; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; AST, aspartate aminotransferase; ALT (alanine transaminase); GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; Values are percentages for categorical variables and means ± SD for continuous variables; *p*-values were calculated by analysis of variance–one factor was used for continuous variables and the χ^2-test for categorical variables, $p < 0.05$ considered significant; Different lower-case letters indicate a significant difference among groups.

Model 3 was additionally adjusted for total energy intake (continuous) and alcohol consumption (categorized as low-moderate and high). To accommodate the use of some categorical variables, we estimated parameters using weighted least squares with robust standard errors.

Statistical analyses were performed for individual FAs and for their subtypes of FAs according to their saturation degree and series: SFAs (C14:0, C16:0, and C18:0), MUFAs (C16:1 n-9, C16:1 n-7, C18:1 n-9, C20:1 n-9, and C24:1 n-9), PUFAs (C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6, C22:5 n-6, C22:5 n-3 and C22:6 n-3), n-6 PUFAs (C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C22:4 n-6, and C22:5 n-6), and n-3 PUFAs (C18:3 n-3, C20:5 n-3, C22:5 n-3, and C22:6 n-3). The differences between groups were expressed as mean changes (95% confidence intervals, CI).

Statistical analyses were performed using Stata 16.0 (StataCorp LP, Tx. USA). Results were considered statistically significant when *p*-values were <0.05 for bilateral contrast.

3. Results

After excluding nine participants with implausible energy intakes, 228 were included in the present analysis (three samples were unavailable for analysis). The four groups were well balanced, as there were no significant differences between age, sex and cardiovascular risk factors. BMI was also comparable in the four groups, considering that 70% of participants had obesity and the rest had overweight. By study design, there was a wide prevalence of other cardiovascular risk factors, such as hypertension, hypercholesterolemia, diabetes, and smoking. Participants with lower consumption of F&V and fat intake were less physically active than those who consumed more F&V. Regarding biochemical data, low density lipoprotein (LDL), high density lipoprotein (HDL) and the liver enzymes alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) did not differ between groups. Nevertheless, the high fat intake and low F&V consumption group presented higher concentrations of total cholesterol and very low density lipoprotein (VLDL) than those with low fat intake and high F&V consumption (**Table 1**).

The average consumption of F&V in the first decile was 289 g day^{-1}, whereas in the tenth decile it was 1295 g day^{-1}. As for fat intake, the first quartile had an average intake of 67 g day^{-1} and the fourth quartile 141 g day^{-1}. Groups with low-moderate fat intake were comparable in total fat, SFA, MUFA and PUFA intake. The groups categorized as high fat intake were also comparable in total fat, SFA and MUFA intake, but they differed in PUFA intake (**Table 2**). Inter-group differences in food and nutrient intake are detailed in Table S1, Supporting Information.

3.1. Total Fatty Acid Concentrations in Plasma

The predominant FA subtypes in plasma were PUFAs, followed by MUFAs and SFAs. The main FAs in the SFA, MUFA and PUFA groups were palmitic acid (16:0), oleic acid (C18:1 n-9) and linoleic acid (C18:2 n-6), respectively.

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Table 2. Types of fat intake according to F&V consumption and fat intake.

	Low F&V		High F&V		*p*-Value
	Low-moderate fat	Very high fat	Low-moderate fat	Very high fat	
Total fat (g day^{-1})	64.9 ± 15.6[a]	140 ± 14.6[b]	69.7 ± 12.6[a]	142 ± 16.9[b]	<0.001
SFA (g day^{-1})	17.7 (4.5)[a]	36.9 (7.2)[b]	17.8 (4.7)[a]	34.5 (7.1)[b]	<0.001
MUFA (g day^{-1})	32.4 (8.8)[a]	72.8 (11.0)[b]	33.7 (7.8)[a]	75.5 (12.4)[b]	<0.001
PUFA (g day^{-1})	9.9 (3.6)[a]	22.5 (6.1)[b]	12.2 (4.0)[a]	25.3 (7.8)[c]	<0.001
F&V	277 ± 69.1[a]	300 ± 52.7[a]	1265 ± 299[b]	1328 ± 270[b]	<0.00

F&V, fruit and vegetable; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; Values are expressed as median (interquartile range); *p*-values were calculated by analysis of variance–one factor, $p < 0.05$ considered significant, after adjustment for multiple comparisons with the Bonferroni method; Different lower-case letters indicate a significant difference among groups.

3.2. High F&V Versus Low F&V Consumption

Table 3 discloses plasma FA concentrations according to F&V consumption and total dietary fat intake. Individuals with high F&V consumption tended to present with lower plasma total FA concentrations and significantly lower concentrations of SFAs (-1.20 mg cL^{-1} [95% CI: -2.22, -0.18], *p*-value = 0.021), including palmitic acid (-0.92 mg cL^{-1} [-1.71, -0.13], *p*-value = 0.023), compared with those consuming low amounts of F&V. No differences were observed between the low and high F&V consumption groups for MUFAs, total PUFAs and n-3 PUFAs. Accordingly, oleic and linoleic acid concentrations were similar. The specific group of n-6 PUFAs tended to be lower, but their major representative linoleic acid, showed no difference with high consumption of F&V.

3.3. High F&V Versus Low F&V Consumption Within the Low-moderate Fat Intake Group

The comparison between high and low F&V consumption groups when the intake of fat was low-moderate showed no differences in the plasma concentrations of total FAs, SFAs, MUFAs nor PUFAs (n-6 and n-3 series), or the individual FAs palmitic, oleic, linoleic and alpha-linolenic (**Table 3**).

3.4. High F&V versus Low F&V Consumption Within the High Fat Intake Group

Concerning the high fat intake group, individuals with higher consumption of F&V tended to present with lower concentrations of total FAs than those with low F&V consumption. However, different patterns were observed when focusing on each subtype of FA. High F&V consumption was associated with significantly lower concentrations of total SFA and a clear trend towards lower concentrations of palmitic acid in comparison with low F&V consumption (-1.74 mg cL^{-1} [-3.41, -0.06] and -1.29 mg cL^{-1} [-2.59; 0.01], *p*-values = 0.042 and 0.052, respectively). As for MUFA concentrations, no differences were found between the low and high F&V consumption groups. Likewise, oleic acid did not differ depending on F&V consumption. On the other hand, plasma PUFA concentrations tended to be lower when the consumption of F&V was high, although no significant statistical difference was observed. Separate analyses of PUFAs subclasses showed that n-6 FA concentrations were significantly lower when F&V consumption was high (-1.81 mg cL^{-1} [-3.50, -0.12], *p*-value = 0.036), whereas n-3 PUFAs remained unaffected. When considering the essential FAs linoleic and alpha-linolenic, no differences were found between the two groups (**Table 3**).

Table S2, Supporting Information includes the comparisons of all the individual FAs quantified.

4. Discussion

In this sub-analysis of the PREDIMED-Plus trial, the consumption of F&V was significantly associated with lower plasma SFA concentrations in individuals with high fat intake, but not in those with low-moderate fat intake. To the best of our knowledge, the current study is the first to show the different relationships between F&V consumption and plasma FAs according to their subtypes.

Plasma FAs have been widely used to assess the influence of diet on plasma lipids, as several studies have reported that most plasma FAs reflect dietary intake.[33–38] Recently, Furtado et al. (2019) performed a detailed comparison between dietary FA intake and the corresponding FA proportions in plasma lipid fractions, demonstrating that FAs in total plasma perform reasonably well as biomarkers that reflect long-term dietary intake of this class of macronutrients.[39]

The PREDIMED-Plus trial was conducted in a Spanish Mediterranean population, which stands out for its sizable consumption of fruits, vegetables, and sources of healthy fats.[2] According to the World Health Organization (WHO), the recommended range for PUFA intake is 6–11% of total energy intake, whereas SFA should not exceed 10%.[40] Mean PUFA and SFA intakes in the participants of this sub-study were 6.3% and 9.8% of total energy, respectively; thus, aligning with the WHO guidelines. Regarding F&V, participants reported a mean consumption of 792 g day^{-1}, which surpasses the WHO recommendation of minimum 600 g day^{-1}.[41]

F&V consumption has been related to a lower risk of cardiovascular diseases.[42] In fact, improving the lipid profile is a potential mechanism of action involved in the cardioprotective effect of F&V.[43–45] In this sense, our study shows that the groups with higher consumption of F&V tended to present with lower total FA concentrations in plasma. Interestingly, this relationship was not homogeneous for all FA classes. SFAs and their principal repre-

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Table 3. Multivariable-adjusted differences between extreme deciles of F&V consumption for total plasma FAs, specific subtypes and individual FAs (mg cL^{-1}), by categories of total fat intake.

		High F&V vs. Low F&V	*p*-value	High F&V vs. Low F&V (low-moderate fat)	*p*-value	High F&V vs. Low F&V (high fat)	*p*-value
TOTAL FAs	Mean (mg cL^{-1})	44.10 vs. 46.98		43.03 vs. 45.21		45.31 vs. 48.72	
	ß [CI]-model 1	−2.82 [-5.92; 0.28]	0.075	−2.19 [-6.68; 2.30]	0.335	−3.51 [-7.87; 0.85]	0.113
	ß [CI]-model 2	−2.77 [-5.91; 0.36]	0.082	−2.77 [-7.23; 1.67]	0.219	−3.09 [-7.52; 1.35]	0.170
	ß [CI]-model 3	−3.00 [-6.19; 0.19]	0.065	−2.28 [-7.54; 2.97]	0.391	−4.52 [-9.68; 0.65]	0.086
SFAs	Mean (mg cL^{-1})	12.61 vs. 13.79		12.36 vs. 13.21		12.89 vs. 14.36	
	ß [CI]-model 1	−1.15 [-2.14; -0.16]	0.023	−0.81 [-2.23; 0.61]	0.264	−1.50 [-2.89; -0.11]	0.034
	ß [CI]-model 2	−1.16 [-2.18; -0.15]	0.025	−1.04 [-2.51; 0.42]	0.160	−1.41 [-2.83; 0.01]	0.052
	ß [CI]-model 3	−1.20 [-2.22; -0.18]	0.021	−1.12 [-2.90; 0.66]	0.215	−1.74 [-3.41; -0.06]	0.042
MUFAs	Mean (mg cL^{-1})	13.61 vs. 14.73		13.25 vs. 14.45		14.02 vs. 15.01	
	ß [CI]-model 1	−1.07 [-2.35; 0.21]	0.102	−1.13 [-3.01; 0.76]	0.239	−1.06 [-2.88; 0.75]	0.248
	ß [CI]-model 2	−1.06 [-2.35; 0.23]	0.107	−1.35 [-3.24; 0.55]	0.161	−0.97 [-2.76; 0.82]	0.287
	ß [CI]-model 3	−0.93 [-2.22; 0.36]	0.156	−1.22 [-3.47; 1.03]	0.285	−1.07 [-3.20; 1.06]	0.321
PUFAs	Mean (mg cL^{-1})	17.75 vs. 18.33		17.29 vs. 17.43		18.27 vs. 19.22	
	ß [CI]-model 1	−0.59 [-1.67; 0.48]	0.278	−0.26 [-1.75; 1.24]	0.735	−0.94 [-2.48; 0.59]	0.226
	ß [CI]-model 2	−0.55 [-1.63; 0.54]	0.321	−0.38 [-1.77; 1.00]	0.585	−0.71 [-2.30; 0.89]	0.382
	ß [CI]-model 3	−0.86 [-2.00; 0.29]	0.143	0.06 [-1.48; 1.61]	0.934	−1.70 [-3.57; 0.17]	0.075
n-6 PUFAs	Mean (mg cL^{-1})	16.10 vs. 16.78		15.69 vs. 15.92		16.56 vs. 17.62	
	ß [CI]-model 1	−0.68 [-1.68; 0.30]	0.172	−0.33 [1.70; 1.04]	0.635	−1.06 [-2.46; 0.34]	0.136
	ß [CI]-model 2	−0.63 [-1.63; 0.37]	0.214	−0.41 [-1.69; 0.87]	0.526	−0.83 [-2.27; 0.60]	0.253
	ß [CI]-model 3	−0.95 [-2.01; 0.12]	0.081	0.02 [-1.40; 1.44]	0.976	−1.81 [-3.50; -0.12]	0.036
n-3 PUFAs	Mean (mg cL^{-1})	1.65 vs. 1.56		1.60 vs. 1.51		1.71 vs. 1.60	
	ß [CI]-model 1	0.09 [-0.06; 0.25]	0.239	0.07 [-0.14; 0.29]	0.498	0.12 [-0.13; 0.36]	0.349
	ß [CI]-model 2	0.08 [-0.07; 0.24]	0.285	0.03 [-0.17; 0.23]	0.772	0.13 [-0.15; 0.40]	0.361
	ß [CI]-model 3	0.09 [-0.06; 0.24]	0.243	0.04 [-0.17; 0.25]	0.686	0.11 [-0.22; 0.43]	0.507
Palmitic acid (C16:0)	Mean (mg cL^{-1})	9.36 vs. 10.26		9.18 vs. 9.79		9.56 vs. 10.73	
	ß [CI]-model 1	−0.88 [-1.64; -0.12]	0.024	−0.57 [-1.66; 0.53]	0.307	−1.20 [-2.28; -0.12]	0.029
	ß [CI]-model 2	−0.90 [-1.69; -0.11]	0.026	−0.76 [-1.89; 0.38]	0.191	−1.14 [-2.24; -0.04]	0.043
	ß [CI]-model 3	−0.92 [-1.71; -0.13]	0.023	−0.87 [-2.25; 0.51]	0.215	−1.29 [-2.59; 0.01]	0.052
Oleic acid (C18:1 n-9)	Mean (mg cL^{-1})	12.43 vs. 13.30		12.01 vs. 12.87		12.90 vs. 13.72	
	ß [CI]-model 1	−0.82 [-1.95; 0.31]	0.156	−0.76 [-2.35; 0.82]	0.341	−0.88 [-2.55; 0.78]	0.295
	ß [CI]-model 2	−0.82 [-1.97; 0.32]	0.158	−0.96 [-2.56; 0.63]	0.234	−0.79 [-2.43; 0.85]	0.341
	ß [CI]-model 3	−0.78 [-1.93; 0.38]	0.186	−0.90 [-2.82; 1.02]	0.355	−0.85 [-2.78; 1.08]	0.384
Linoleic acid (C18:2 n-6)	Mean (mg cL^{-1})	11.94 vs. 12.20		11.49 vs. 11.41		12.44 vs. 12.97	
	ß [CI]-model 1	−0.27 [-1.05; 0.51]	0.500	−0.02 [-1.11; 1.08]	0.978	−0.53 [-1.59; 0.53]	0.325
	ß [CI]-model 2	−0.23 [-1.02; 0.56]	0.567	−0.09 [-1.11; 0.94]	0.867	−0.37 [-1.45; 0.72]	0.505
	ß [CI]-model 3	−0.54 [-1.38; 0.31]	0.210	0.20 [-0.95; 1.35]	0.729	−0.99 [-2.31; 0.32]	0.137
Alpha-linolenic acid (C18:3 n-3)	Mean (mg cL^{-1})	0.14 vs. 0.13		0.13 vs. 0.13		0.16 vs. 0.15	
	ß [CI]-model 1	0.01 [-0.02; 0.03]	0.531	<0.01 [-0.03; 0.04]	0.864	0.01 [-0.03; 0.05]	0.522
	ß [CI]-model 2	0.01 [-0.01; 0.03]	0.484	←0.01 [-0.03; 0.03]	0.803	0.02 [-0.02; 0.06]	0.310
	ß [CI]-model 3	0.01 [-0.02; 0.03]	0.654	−0.01 [-0.05; 0.02]	0.401	0.03 [-0.02; 0.06]	0.210

F&V, fruit and vegetable; FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ß, difference between groups; CI, confidence interval; Model 1—adjusted for age and sex. Model 2—Model 1 additionally adjusted for physical activity, and BMI. Model 3—Model 2 additionally adjusted for total energy intake and alcohol consumption. *p*-values < 0.05 were considered significant.

sentative, palmitic acid, disclosed lower concentrations when the consumption of F&V was higher. MUFAs and PUFAs remained unaffected, however, as well as their main representatives oleic and alpha-linoleic acid. Taken together, these results suggest that the consumption of F&V is inversely associated with SFAs but it is not related to other FA subtypes. Growing evidence reports the relationship between SFA and harmful health outcomes, such as coronary heart disease or inflammation.[46,47] Palmitic acid,

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which is widely used in the food industry, is thought to be involved in the development of obesity, diabetes, cancer and even in increased mortality risk.[48,49] Therefore, F&V consumption may contribute to a healthier FA profile which might lead to an improvement of overall health.

Whisner et al. (2019) conducted a randomized controlled trial with a similar design that combined low-fat and high-fat meals with and without dietary fiber and found different effects on endothelial function according to the amount of fat and fiber consumed.[50] Similarly, Djuric et al. (2006) found different effects on plasma micronutrients of high F&V consumption depending on the intake of fat.[51] Indeed, in the present stratified analysis we observed that the association of F&V consumption with plasma SFA concentrations depends on the amount of fat intake. On one hand, the low-moderate fat intake group did not show any association between plasma FA concentrations and F&V consumption. Only when fat intake was high, we observed lower total FA concentrations with high F&V consumption. As explained above, plasma SFA concentrations were significantly lower when F&V consumption was higher, supporting the notion that this subgroup of FAs is affected by F&V to a higher extent than other FA classes. Palmitic acid also showed an inverse association when fat intake was high, although not significant, probably due to the reduction in the sample size with the statistical analysis when comparing only individuals with high fat intake. These results show that FA concentrations are associated with F&V consumption in different magnitudes depending on the amount of total dietary fat, with lower SFAs associated with a high fat intake with high consumption of F&V, but no difference when fat intake was lower. It is remarkable that F&V are specifically related to plasma FAs when fat intake is high, as population with high dietary fat intake is exposed to a greater risk of developing obesity and related diseases.[52] Therefore, when assessing the influence of F&V over fat intake it is important to consider this aspect.

Unexpectedly, plasma PUFA concentrations tended to be lower when F&V consumption was high, even though this association was not significant, and further analyses showed that this tendency was only ascribable to n-6 PUFAs. Interestingly, we did not observe any difference for linoleic or alpha-linolenic acid, both essential FAs that cannot be synthesized endogenously. Other n-6 PUFAs, such as gamma-linolenic acid (C18:3 n-6) or arachidonic acid (C20:4 n-6), for which significant lower concentrations were associated with high F&V consumption, mainly come from endogenous metabolism rather than dietary intake, as they are rare in foods.[53,54] Thus, inter-individual metabolic variability might play a major role in plasma PUFA concentrations and the differences found in this study may not be attributed only to F&V consumption.

These associations between F&V consumption and plasma FAs might be explained by the well-known high content of fiber of these foods. Fiber is known to interfere in nutrients absorption[25]; thus, it may play an important role in modifying the uptake of FAs according to their characteristics and the amount of fat intake.

The main strength of this study is that it involved participants at baseline of the PREDIMED-Plus study; therefore, the results reflect real-life conditions. We performed a comprehensive analysis of plasma FAs and their subtypes that allowed us to detect different patterns depending on the degree of saturation and species of each FA. Our study also has limitations. Causality could not be determined due to the cross-sectional design. Second, culinary techniques or co-intake of different nutrients could not be determined from the food frequency questionnaires. Finally, the concentrations of some FAs may be related to endogenous metabolism besides dietary intake.

In conclusion, higher F&V consumption was associated with lower plasma concentrations of SFAs and with a tendency to lower total FAs in plasma when intake of fat was high. The findings from the current study support the idea that F&V consumption may influence plasma FAs differently depending on their subtype, as it was associated with differences in plasma SFAs to a higher extent than with any other FA group. Further randomized controlled trials are needed to confirm the reported results and definitively establish the role of fiber and F&V consumption in modulating plasma FA concentrations, as well as their implications in cardiovascular health.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

J.S.-S. reported receiving research support from the Instituto de Salud Carlos III, Ministerio de Educación y Ciencia, Departament de Salut Pública de la Generalitat de Catalunya, the European Commission, the USA National Institutes of Health; receiving consulting fees or travel expenses from Danone, California Walnut Commission, Eroski Foundation, Instituto Danone, Nestle, and Abbott Laboratories, receiving nonfinancial support from Hojiblanca, Patrimonio Comunal Olivarero, the California Walnut Commission, Almond Board of California, La Morella Nuts, Pistachio Growers and Borges S.A; serving on the board of and receiving grant support through his institution from the International Nut and Dried Foundation and the Eroski Foundation; and grants and personal fees from Instituto Danone; Serving in the Board of Danone Institute International. D.C. reported receiving grants from Instituto de Salud Carlos III. R.E. reported receiving grants from Instituto de Salud Carlos III, Fundación Dieta Meditarránea and Cerveza y Salud and olive oil for the trial from Fundación Patrimonio Comunal Olivarero and personal fees from Brewers of Europe, Fundación Cerveza y Salud, Interprofesional del Aceite de Oliva, Instituto Cervantes in Albuquerque, Milano and Tokyo, Pernod Ricard, Fundación Dieta Mediterránea (Spain), Wine and Culinary International Forum and Lilly Laboratories; non-financial support from Sociedad Española de Nutrición and Fundación Bosch y Gimpera; and grants from Uriach Laboratories. E.R. reports grants, personal fees, non-financial support and other from California Walnut Commission, during the conduct of the study; grants, personal fees, non-financial support and other from Alexion; personal fees, non-financial support and other from Ferrer International and Danone, personal fees and other from Amarin, outside the submitted work. R.M.L.-R. reports personal fees from Cerveceros de España, personal fees and other from Adventia, other from Ecoveritas, S.A., outside the submitted work. S.K.N. reported being a volunteer member of the group Plant-Based Canada. The rest of the authors have declared that no competing interests exist. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Author Contributions

I.D.-L. and M.M.-M. contributed equally to the work. R.M.L.-R., Á.H. and M.M.-M. conceptualized this study; I.D.-L. performed the investigation and the formal analysis. I.D.-L. and M.M.-M. wrote the original draft. A.T.-R, Á.H., J.J.M., M.Á.M.-G., J.S.-S., D.C., M.F., J.A.M., Á.M.A.-G., J.W., J.V. (Jesús Vioque), D.R., J.L.-M., M.R.B.-L., J.L., J.L.S.-M., A.B.-C., J.A.T., V.M.-S., X.P., M.D.-R., P.M.-M., J.V. (Josep Vidal), C.V., L.D., M.S.-M., Z.V.-R., S.K.N., J.V.S., O.C., I.A., J.V.-L., R.C.-M., A.A., L.P., A.G.-R., R.C., A.M.G.-P., J.M.S.-L., C.R., M.A.M., C.S., V.R.-P., M.A.Z., I.S., S.E., N.B., M.M., E.R., R.E., M.C.L.-S., and R.M.L.-R. reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement

There are restrictions on the availability of data for the PREDIMED-Plus trial, due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following the project purposes. Requestors wishing to access the PREDIMED-Plus trial data used in this study can make a request to the PREDIMED-Plus trial Steering Committee chair: jordi.salas@urv.cat. The request will then be passed to members of the PREDIMED-Plus Steering Committee for deliberation. Clinical Trial Registry number and website where it was obtained (if applicable).

The PREDIMED-Plus study was registered at the ISRCTN of London, England: 89898870.

Keywords

dietary fats, Mediterranean diet, MUFA, PREDIMED-Plus, PUFA

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3.2.2. Publication 5

Relationship between estimated desaturase enzyme activity and metabolic syndrome in a longitudinal study

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Supplementary Material available in Annex section.

Abstract

Aims: To study the relationship between plasma desaturase estimated activities and the MetS, as well as their relationship with individual components of the MetS.

Methods: We conducted a longitudinal study of 148 participants recruited at random from the PREDIMED trial (Hospital Clinic site). At baseline and after 1 year of follow-up, desaturase estimated activities were estimated from product/precursor ratios of individual plasma fatty acids. Logistic regressions were used to assess the relationship of desaturase estimated activities MetS, adjusted for potential confounders.

Results: Estimated D5D activity was associated with lower risk of MetS, whereas SCD-16 and SCD-18 were negatively associated with MetS status. SCD-16, SCD-18, and D6D were positively associated with triglycerides, SCD-18 was inversely associated with HDL-c. Estimated D6D activity was found to be associated with increases in diastolic blood pressure. In contrast, D5D was negatively associated with triglycerides, diastolic blood pressure and waist circumference.

Conclusions: The present longitudinal study suggests that estimated SCD-16, SCD-18, and D6D have a negative impact in MetS and its components, whereas D5D may have beneficial effects for metabolic health.

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Relationship between estimated desaturase enzyme activity and metabolic syndrome in a longitudinal study

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Desaturase enzyme activities (DEA) are associated with several metabolic diseases. The aim of the present study was to assess the relationship between estimated plasma DEA and the metabolic syndrome (MetS), as well as their relationship with individual components of the MetS. We conducted a longitudinal study of 148 participants recruited at random from the PREDIMED trial (Hospital Clinic site). At baseline and after 1 year of follow-up, DEA were estimated from product/precursor ratios of individual plasma fatty acids. Logistic regressions were used to assess the relationship of estimated DEA MetS, adjusted for potential cofounders. Estimated Δ5 desaturase (D5D) activity was associated with lower risk of MetS, whereas stearoyl-CoA (SCD)-16 and SCD-18 were negatively associated with MetS status. SCD-16, SCD-18, and Δ6 desaturase (D6D) were positively associated with triglycerides, SCD-18 was inversely associated with HDL-cholesterol. Estimated D6D activity was found to be associated with increases in diastolic blood pressure. In contrast, D5D was negatively associated with triglycerides, diastolic blood pressure and waist circumference. The present longitudinal study suggests that estimated SCD-16, SCD-18, and D6D have a negative impact in MetS and its components, whereas D5D may have beneficial effects for metabolic health.

KEYWORDS

fatty acids, desaturases, gas chromatography, PREDIMED, metabolic syndrome, Mediterranean diet

Introduction

The prevalence of metabolic syndrome (MetS) has increased in the last three decades and the global prevalence has been estimated to be about 1 quarter of the world population (1). The prevalence in Spain reached 30% in 2012 (2), and this number is estimated to increase in approximately 94,000 cases every year (3). MetS is defined as a set of criteria that, when grouped together, represent a risk for developing cardiovascular disease (CVD) and type 2 diabetes (T2D), such as elevated blood triglycerides (TG) or glucose (4). The development of MetS can be promoted by unmodifiable risk factors, including genetics or aging, but also by modifiable lifestyle habits, such as physical activity or diet (5). The incidence of MetS is particularly higher in men aged over 45 years with an educational level below university studies. Healthier lifestyle therapies in the management of MetS focus on reducing weight, sedentarism, and improving the diet. It has been reported that the incidence of MetS can be reduced with a higher adherence to the Healthy Lifestyle Score, which includes never smoking, moderate to high physical activity, higher adherence to Mediterranean diet, or moderate alcohol consumption, among others (6). Other studies recommend to reach a greater adherence to Mediterranean diet to reduce its development (3).

The traditional Mediterranean diet has been recognized as protective against the development of MetS and other chronic diseases, such as T2D, CVD, and hypertension (7, 8). This healthy dietary pattern is characterized by a high intake of fruits, vegetables, legumes, nuts, and whole grains, and olive oil (9). The Mediterranean diet provides a high content of healthy fats that mostly come from olive oil and favors a better lipid profile.

The plasma fatty acid (FA) profile is considered a more reliable biomarker of dietary fat intake than food frequency questionnaires (FFQ) (10), but it may also be affected by non-dietary factors, such as endogenous metabolism. FAs can be synthesized, elongated, or desaturated in reactions catalyzed by the enzymes stearoyl-CoA desaturase (SCD-1), Δ6 desaturase (D6D), and Δ5 desaturase (D5D) (11), as shown in **Figure 1**. Altered desaturase enzyme activities (DEA), calculated with the ratios of the FAs that intervene in the reaction, are associated with cardiometabolic risk factors, such as T2D, obesity and MetS (12, 13). However, studies assessing the effect of estimated DEA on MetS and its individual components remain scarce. We hypothesized that altered ratios of estimated DEA would be associated with MetS and the individual components that constitute it. Thus, the aim of this substudy was to assess the relationship of estimated DEA with MetS and its components after 1 year of follow-up in a Mediterranean population.

Abbreviations: AA, arachidonic acid; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; D5D, Δ5 desaturase; D6D, Δ6 desaturase; DEA, desaturase enzyme activities; EPA, eicosapentaenoic acid; FA, fatty acids; FAME, fatty acid methyl ester; FID, flame ionization detector; FFQ, food frequency questionnaire; GC, gas chromatography; HDL-c, high-density lipoprotein cholesterol; MetS, metabolic syndrome; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids; SCD, stearoyl-CoA desaturase; SBP, systolic blood pressure; TG, triglycerides; T2D, type 2 diabetes.

Materials and methods

Study design

The PREDIMED (PREvención con DIeta MEDiterránea) study was a 5-year large, parallel-group, multicenter, randomized, controlled, clinical trial conducted in Spain from October 2003 to December 2010 with the aim of assessing the effect of a Mediterranean diet on the primary prevention of CVD.[1] In summary, 7,447 participants aged 55–80 years at high cardiovascular risk were included. Eligible participants were men and women with T2D, dyslipidemia, hypertension, overweight/obesity or family history of premature CVD. Exclusion criteria included sever chronic illnesses, alcohol or drug abuse, and BMI > 40 kg/m^2. A detailed description of methods and participants has been published elsewhere (14).

For the current analysis, we used a randomly selected subsample of participants from the PREDIMED-Hospital Clinic recruitment center. To estimate DEA, a total of 148 participants with available data on plasma FA profiles at baseline and after 1 year of follow-up were included.

The protocol was approved by the Research Ethics Committees at the Hospital Clinic recruiting center and all participants signed a written informed consent form.

Covariate assessment

A validated semi-quantitative 137-item FFQ was collected by trained dietitians to assess dietary intake at baseline and after 1 year (15). Nutrient intakes were calculated from Spanish food composition tables (16). One female participant who reported implausible energy intakes (>3,500 and <500 Kcal/day for females, and >4,000 and <800 Kcal/day for males) was excluded from the analysis (17). Mediterranean diet adherence was assessed with a 14-item questionnaire with a value of 0 or 1 for each dietary component (18).

Trained personnel carried out anthropometric measurements at baseline and 1-year follow-up. Physical activity was assessed with a validated Spanish version of the Minnesota physical activity questionnaire (19). The anthropometric measurements used in this study were body

1 http://www.predimed.es

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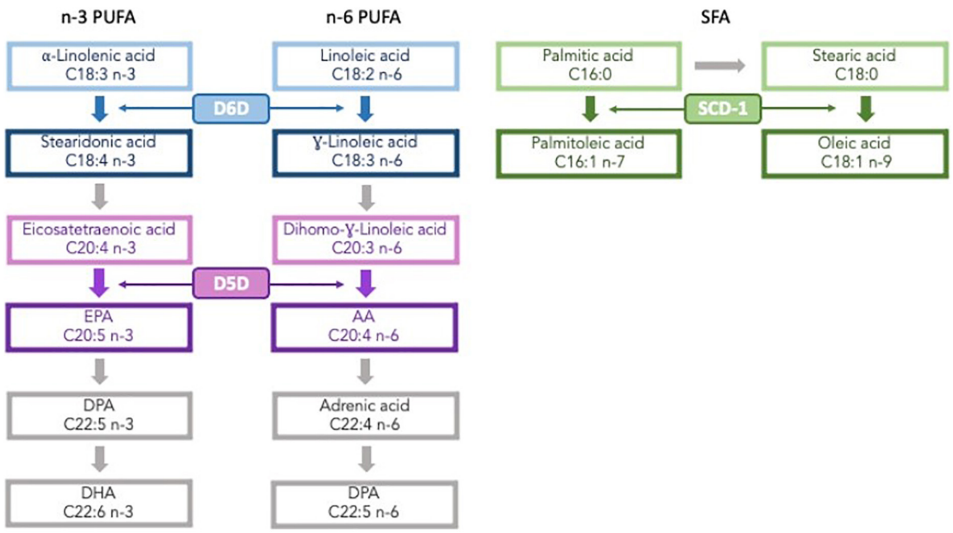

FIGURE 1
Fatty acid synthetic pathway. PUFA, polyunsaturated fatty acids; D6D, D^6 desaturase; D5D, D^5 desaturase; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; SFA, saturated fatty acids; SCD, stearoyl coenzyme A desaturase.

mass index (BMI), calculated as weight in kg/height^2 in m^2, and waist circumference (WC). Diastolic and systolic blood pressure (DBP and SBP, respectively) was measured in triplicate with a validated semi-automatic sphygmomanometer after a minimum of 5 mins rest in a seated position.

Laboratory measurements

Blood samples were collected after an overnight fast, coded, and stored at –80C° until analysis. Biochemical analyses [glucose, triglycerides, total cholesterol, and high-density lipoprotein cholesterol (HDL-c)] were performed by standard enzymatic procedures. The FA profile in plasma was determined in total lipids by fast gas chromatography with a flame ionization detector (GC-FID) with a previous derivatization to the corresponding FA methyl esters (FAMEs) (20). Fast analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with an FID and Shimadzu AOC-20i Autoinjector. Separation of FAMEs was carried out on a capillary column (10 m × 0.10 mm i.d.), coated with an SGE-BPX70 cross-linked stationary phase (70% cyanopropyl polysilphenylene-siloxane, 0.20 μm film thickness) from SGE (SGE Europe Ltd., United Kingdom). Methyl ester peaks were identified by comparison of their relative retention times with the standards Supelco 37 component FAMEs mix and PUFA No. 2 (Animal source), purchased form Merck (Darmstadt, Germany). Results were expressed as relative percentages of total FAs.

Estimation of desaturase activities

Plasma FA levels at baseline and changes after 1-year of follow-up are detailed in **Supplementary Table 1** according to MetS status.

DEA were estimated as the product/precursor ratios of FAs in plasma according to the following: SCD-16 = C16:1 *n* – 7/C16:0, SCD–18 = C18:1 *n* – 9/C18:0, D6D = C18:3 *n* – 6/C18:2 *n* – 6, and D5D = C20:4 *n* – 6/C20:3 *n* – 6 (11).

Definition of metabolic syndrome

For the present work we applied the definition of MetS proposed by six major organizations and societies (IDF, NHLBI, AHA, WHF, IAC, and IASO) (21). Accordingly, participants who presented 3 of 5 of the following risk factors were included in the MetS group: elevated TG (>150 mg/dL or drug treatment for elevated TG), reduced HDL-c (< 40 mg/dL in men and <50 mg/dL in women), elevated blood pressure (SBP > 130 and/or DBP > 85 mmHg, or antihypertensive drug treatment), elevated fasting glucose (>100 mg/dL or drug treatment of elevated glucose), and elevated WC (>102 cm for men and >88 for women).

Statistical analysis

Baseline characteristics of the participants with and without MetS are presented as means ± SD for continuous variables

TABLE 1 Baseline characteristics of participants according to MetS[1].

Characteristics	All participants	No MetS (n = 47)	MetS (n = 101)	p-value[2]
Age (years)	66.9 ± 5.9	65.5 ± 6.0	67.5 ± 5.8	0.050
Female (%)	83 (56.1)	20 (42.6)	63 (62.4)	0.024
Weight (kg)	74.1 ± 11.5	71.8 ± 10.5	75.1 ± 11.9	0.106
BMI (kg/m^2)	28.9 ± 3.7	27.5 ± 3.2	29.5 ± 3.8	0.002
T2D (%)	109 (73.7)	25 (53.2)	84 (83.2)	<0.001
Dyslipidaemia (%)	101 (68.2)	39 (83.0)	62 (61.4)	0.009
Hypertension (%)	96 (64.9)	27 (57.5)	69 (68.3)	0.197
Current smoker (%)	22 (14.9)	5 (10.6)	17 (16.8)	0.527
Education level (%)				0.667
Low	95 (64.2)	29 (61.7)	66 (65.4)	
Medium and high	53 (35.8)	18 (38.3)	35 (34.7)	
Physical activity (METS-min/day)	278.4 ± 256.3	341.0 ± 285.8	249.3 ± 237.3	0.043
Mediterranean adherence, score	3 ± 4	3 ± 4	3 ± 4	0.882

MetS, metabolic syndrome; T2D, type 2 diabetes; METS, metabolic equivalents.
[1]Continuous variables are shown as means ± SDs, and categorical variables are shown as percentages.
[2]T-tests were used for continuous variables, and a chi-square test was used for categorical variables.

TABLE 2 Dietary intake for all participants and according to MetS at baseline[1].

	All participants	No MetS (n = 47)	MetS (n = 101)	p-value[2]
Energy (kcal/day)	2515.6 ± 541.1	2633.0 ± 470.3	2461.0 ± 564.9	0.072
Carbohydrates (g/day)	273.9 ± 80.3	288.96 ± 77.7	266.0 ± 81.0	0.121
Proteins (g/day)	105.1 ± 20.6	108.9 ± 22.4	103.3 ± 19.6	0.122
Total fat (g/day)	103.8 ± 26.3	107.3 ± 21.8	102.2 ± 28.1	0.271
SFA (g/day)	28.0 ± 8.6	29.5 ± 8.7	27.3 ± 8.4	0.139
MUFA (g/day)	49.4 ± 13.6	50.1 ± 11.2	49.0 ± 14.6	0.663
PUFA (g/day)	17.9 ± 7.0	19.5 ± 7.6	17.1 ± 6.6	0.047
Cholesterol (mg/day)	395.4 ± 104.6	415.9 ± 109.8	385.8 ± 101.3	0.104
Fiber (g/day)	30.4 ± 8.0	31.6 ± 8.1	29.9 ± 7.9	0.225
Alcohol (g/day)	9.3 ± 13.9	10.8 ± 16.9	8.6 ± 12.3	0.373

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[1]Variables are shown as means ± SDs.
[2]Differences between groups calculated by T-tests.

and percentages for categorical variables. T-tests were used to assess differences in continuous variables and Chi-Square tests were used for categorical values. T-tests were also used to assess differences in plasma FA profile between participants with and without MetS, as well as within-group differences between baseline and 1-year of follow-up.

Baseline values and 1-year changes of estimated DEA were normalized and scaled in multiples of 1-SD with Blom inverse normal transformation (22). Changes in estimated DEA and MetS components were calculated as a 1-year value minus the baseline value.

The associations between the prevalence of MetS and estimated DEA were assessed with a logistic regression analysis to calculate the odds ratios (OR) and 95% confidence interval (CI) adjusting for potential confounders (sex, age, physical activity, BMI, smoking status, educational level, and total energy intake) and stratifying for sex. Multinomial logistic regression was employed to assess the relative risk ratio (RRR) of 1-year changes in MetS status and in estimated DEA, also stratifying for sex, and incorporating the intervention group into the adjustment models.

Multivariable adjusted linear regression models were used to assess differences between estimated DEA per 1-SD increase and MetS components (TG, HDL-c, DBP, SBP, glucose, and WC). The adjustment model for potential confounders included: sex, age, physical activity, smoking status, educational level, total energy intake, and BMI (except for WC). In addition, related medication was added in the adjustment model for each MetS component: TG and HDL-c were further adjusted for cholesterol-lowering drugs; DBP and DSP were further adjusted for antihypertensive medication; glucose was further adjusted for insulin and other

hypoglycemic drugs; SCD-16 and SCD-18 were further adjusted for PUFA intake. The longitudinal analysis considering 1-year changes in estimated DEA and MetS components was carried out using the same models, further adjusted for the intervention groups.

For all analyses, two-sided significance was determined at a $p < 0.05$. Analyses were performed with Stata 16.0 (Stata-Corp LP, TX, USA).

Results

General characteristics

Table 1 shows the baseline characteristics of the 148 participants according to MetS status. Approximately two thirds of the participants had MetS, whereas 47 volunteers were considered to not suffer from this syndrome. Among the participants with MetS, the majority were women (62.4%), whereas among those without MetS, the majority were men (57.4%). As expected, more participants with MetS suffered from T2D (83.2%), had higher BMI (29.5 ± 3.8 kg/m^2) and performed less physical activity (249 ± 237.3 METS-min/day). Surprisingly, a higher percentage of participants without MetS had dyslipidaemia (82.98%).

Dietary intake of all participants and stratified for MetS are in Table 2. The mean energy intake was 2,515.6 ± 541.1 kcal/day and the most consumed type of fat were monounsaturated fatty acids (MUFA). The two groups of participants with and without MetS were well-balanced and there were no differences in any nutrient except for polyunsaturaded fatty acids (PUFA) intake, as those with MetS reported significantly lower consumption (17.1 ± 6.6 g/day).

Associations of desaturase activities and metabolic syndrome at baseline

The OR of having MetS according to estimated DEA per 1-SD increase at baseline are shown in Table 3. The logistic regression model showed that in all participants higher rates of D5D activity were associated with lower incidence of MetS [OR = 0.53 (95% CI: 0.36; 0.79), p-value = 0.002]. Higher SCD-18 activity tended to lower the incidence of MetS, although it did not achieve statistical significance [OR = 1.54 (95% CI: 0.96; 2.58), p-value = 0.096].

TABLE 3 Odds ratio associated with having MetS with DEA according to sex at baseline per 1-SD increase (n = 148).

	OR (CI 95%)	p-value[1]
SCD-16	1.08 (0.73; 1.62)	0.691
SCD-18	1.54 (0.96; 2.58)	0.096
D6D	1.18 (0.80; 1.73)	0.397
D5D	0.53 (0.36; 0.79)	0.002

MetS, metabolic syndrome; DEA, desaturase enzyme activities; SCD, stearoyl-CoA desaturase; D6D, D^6 desaturase; D5D, D^5 desaturase. Odds ratio (95% confidence interval) by logistic regression analysis. $p < 0.05$.
[1]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, and total energy intake. SCD-16 and SCD-18 were further adjusted for PUFA intake.

The associations between estimated DEA per 1-SD and MetS components after full adjustment are shown in Table 4. Higher estimated SCD-18 and D6D activity were positively associated with higher concentrations of TG [β = 40.50 (95% CI: 23.00; 58.02) per 1-SD increase, p-value = 0.001, and β = 15.45 (95% CI: 1.57; 29.33) per 1-SD increase, p-value = 0.029, respectively]. Higher rates of estimated SCD-18 activity were also associated with lower HDL-c [β = −4.02 (95% CI: −6.20; −1.85) per 1-SD increase, p-value = < 0.001]. Lastly, DBP and WC were inversely associated with estimated D5D activity [β = −1.79 (95% CI: −3.10; 0.48) per 1-SD increase, p-value = 0.008, and β = −0.94 (95% CI: −1.82; 0.06) per 1-SD increase, p-value = 0.037].

Associations of desaturase activities and metabolic syndrome after 1 year of follow-up

The relationship between 1-year changes in MetS status and estimated DEA per 1-SD increase is presented in Table 5. The multinomial regression model showed that increases in estimated D5D activity were associated with MetS improvement [OR = 2.04 (95% CI: 1.18; 3.56), p-value = 0.011], whereas increases in estimated SCD-18 activity were associated with a lower probability of improving MetS status [OR = 0.46 (95% CI: 0.29; 0.71), p-value = 0.001]. In addition, higher estimated SCD-16 activity was associated with an increased risk of worsening MetS status [OR = 2.15 (95% CI: 1.18; 3.94), p-value = 0.013].

Table 6 shows the associations between 1-year changes in DEA per 1-SD increase and MetS components. Changes in estimated SCD-16 and SCD-18 activity were positively associated with higher TG [β = 18.08 (95% CI: 7.02; 29.13) per 1-SD increase, p-value = 0.002, and β = 34.88 (95% CI: 19.97; 49.79) per 1-SD increase, p-value = 0.001, respectively]. Increases in the rate of estimated SCD-18 were associated with decreases in HDL-c concentrations [β = −1.53 (95% CI: −2.62; −0.44) per 1-SD increase, p-value = 0.006], whereas increases in levels of estimated D6D activity were associated with higher DBP [β = 1.70 (95% CI: 0.44; 2.97) per 1-SD increase, p-value = 0.009]. Finally, we observed a decrease in TG and DBP when estimated D5D activity increased [β = −13.09 (95% CI: −24.99; −1.19) per 1-SD increase, p-value = 0.031, and β = −1.52 (95% CI: −2.78; −0.26) per 1-SD increase, p-value = 0.018, respectively].

TABLE 4 Multivariable-adjusted regression for estimated DEA per 1-SD increase and MetS components at baseline.

	SCD-16		SCD-18		D6D		D5D	
	β (CI 95%)	*p*-value	β (CI 95%)	*p*-value	β (CI 95%)	*p*-value	β (CI 95%)	*p*-value
TG[1]								
	14.41 (−0.49; 29.31)	0.058	40.50 (23.00; 58.02)	<0.001	15.45 (1.57; 29.33)	0.029	−11.62 (−30.38; 7.13)	0.223
HDL-c[1]								
	0.68 (−1.68; 3.04)	0.570	−4.02 (−6.20; −1.85)	<0.001	−0.66 (−2.91; 1.60)	0.565	0.85 (−1.19; 2.90)	0.410
DBP[2]								
	1.12 (−0.34; 2.58)	0.131	0.96 (−0.51; 2.44)	0.198	1.18 (−0.51; 2.88)	0.170	−1.79 (−3.10; 0.48)	0.008
SBP[2]								
	0.05 (−3.17; 3.26)	0.976	0.71 (−1.97; 3.38)	0.602	2.05 (−0.67; 4.78)	0.138	−2.16 (−4.82; 0.49)	0.109
Glucose[3]								
	4.02 (−2.31; 10.36)	0.211	7.37 (−0.47; 15.20)	0.065	6.24 (−2.17; 14.65)	0.145	4.39 (−1.21; 9.99)	0.123
WC[4]								
	0.17 (−0.74; 1.09)	0.711	0.01 (−0.94; 0.95)	0.986	−0.03 (−0.96; 0.89)	0.944	−0.94 (−1.82; -0.06)	0.037

MetS, metabolic syndrome; DEA, desaturase enzyme activities; SCD, stearoyl-CoA desaturase; D6D, D^6 desaturase; D5D, D^5 desaturase; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; DBP, diastolic blood pressure; SBP, systolic blood pressure; WC, waist circumference; ß, difference between groups; CI, confidence interval.
[1]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, and cholesterol-lowering medication. SCD-16 and SCD-18 were further adjusted for PUFA intake.
[2]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, and antihypertensive medication. SCD-16 and SCD-18 were further adjusted for PUFA intake.
[3]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, insulin and hypoglycemic medication. SCD-16 and SCD-18 were further adjusted for PUFA intake.
[4]Adjusted for age, sex, smoking status, educational level, physical activity, and total energy intake. SCD-16 and SCD-18 were further adjusted for PUFA intake.

TABLE 5 Multinomial logistic regression for changes in MetS status and estimated DEA ratios after 1 year of follow-up per 1-SD increase.

	Maintained MetS status	MetS status improvement		MetS status worsening	
		RRR (CI 95%)	*p*-value[1]	RRR (CI 95%)	*p*-value[1]
SCD-16	Ref.	0.78 (0.46; 1.33)	0.365	2.15 (1.18; 3.94)	0.013
SCD-18	Ref.	0.46 (0.29; 0.71)	0.001	0.63 (0.35; 1.16)	0.136
D6D	Ref.	0.98 (0.66; 1.45)	0.923	1.23 (0.71; 2.11)	0.462
D5D	Ref.	2.04 (1.18; 3.56)	0.011	0.76 (0.33; 1.78)	0.535

MetS, metabolic syndrome; DEA, desaturase enzyme activities; SCD, stearoyl-CoA desaturase; D6D, D^6 desaturase; D5D, D^5 desaturase. Relative risk ratio (95% confidence interval) by multinomial logistic analysis. $p < 0.05$.
[1]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, and intervention group. SCD-16 and SCD-18 were further adjusted for PUFA intake.

Discussion

In the present longitudinal substudy of the PREDIMED trial, we observed that higher estimated activities of SCD-16, SCD18, and D6D had an adverse effect on MetS status and its components after 1 year of follow-up. In contrast, estimated D5D activity showed a protective effect against MetS and its components, particularly TG and DBP. To our knowledge, this is the first study to assess the effect of estimated DEA with MetS and its components after 1 year of follow-up in a Mediterranean population.

The activity of these desaturases is known to be related to metabolic health. Differences in the plasma FA profile and estimated DEA have been previously described between metabolically healthy and unhealthy individuals (23, 24). On this basis, Svendsen et al. proposed that these enzymatic activities could serve as novel biomarkers of metabolic health (13, 25). This is in accordance with the results of the present study, as we found that estimated DEA were associated with MetS at baseline and after 1 year of follow-up.

The analysis of plasma estimated DEA related to FA metabolism showed a beneficial effect of estimated D5D activity on the prevalence of MetS. These results are consistent with previous studies that report a positive influence of D5D on cardiovascular health. For example, higher D5D activity has been favorably associated with stroke risk factors, T2D, and abdominal obesity (26–28). D5D is the rate-limiting enzyme that catalyzes the transformation of eicosatetraenoic and dihomo-gamma-linoleic acid into eicosapentaenoic acid (EPA) and arachidonic acid (AA), respectively. Therefore, lower D5D activity leads to the accumulation of precursors and other intermediate FAs that increase cardiometabolic risk, such as gamma-linoleic or dihomo-gamma-linoleic acid (29). Despite all these findings, Mayneris-Perxachs et al. did not observe any association between D5D and the odds of having MetS in a cross-sectional sub-analysis with baseline data of the PREDIMED study (30). However, they did find that D6D and

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TABLE 6 Multivariable-adjusted regression for 1-year changes in estimated DEA per 1-SD increase and MetS components.

	SCD-16		SCD-18		D6D		D5D	
	β (CI 95%)	*p*-value	β (CI 95%)	*p*-value	β (CI 95%)	*p*-value	β (CI 95%)	*p*-value
TG[1]								
	18.08 (7.02; 29.13)	0.002	34.88 (19.97; 49.79)	<0.001	1.71 (−11.35; 14.76)	0.796	−13.09 (−24.99; −1.19)	0.031
HDL-c[1]								
	−0.85 (−2.40; 0.71)	0.282	−1.53 (−2.62; −0.44)	0.006	−0.19 (−1.49; 1.11)	0.776	−0.45 (−1.70; 0.80)	0.477
DBP[2]								
	1.16 (−0.02; 2.35)	0.055	0.68 (−0.83; 2.19)	0.374	1.70 (−0.44; 2.97)	0.009	−1.52 (−2.78; −0.26)	0.018
SBP[2]								
	0.68 (−1.49; 2.85)	0.537	0.03 (−2.50; 2.55)	0.984	1.52 (−0.51; 3.56)	0.141	−2.25 (−4.64; 0.13)	0.064
Glucose[3]								
	2.41 (−4.97; 9.78)	0.520	1.00 (−5.17; 7.17)	0.750	1.99 (−4.55; 8.53)	0.548	−1.80 (−8.84; 5.23)	0.613
WC[4]								
	0.56 (−0.36; 1.48)	0.234	1.28 (0.40; 2.17)	0.005	0.12 (−0.75; 1.00)	0.783	−0.59 (−1.46; 0.28)	0.181

MetS, metabolic syndrome; DEA, desaturase enzyme activities; SCD, stearoyl coenzyme A desaturase; D6D, D^6 desaturase; D5D, D^5 desaturase; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; DBP, diastolic blood pressure; SBP, systolic blood pressure; WC, waist circumference; ß, difference between groups; CI, confidence interval.
[1]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, intervention group, and cholesterol-lowering medication. SCD-16 and SCD-18 were further adjusted for PUFA intake.
[2]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, intervention group, and antihypertensive medication. SCD-16 and SCD-18 were further adjusted for PUFA intake.
[3]Adjusted for age, sex, smoking status, educational level, BMI, physical activity, total energy intake, intervention group, insulin, and hypoglycemic medication. SCD-16 and SCD-18 were further adjusted for PUFA intake.
[4]Adjusted for age, sex, smoking status, educational level, physical activity, total energy intake, and intervention group. SCD-16 and SCD-18 were further adjusted for PUFA intake.

SCD-18 were adversely associated with MetS, which is consistent with our results. The activity of D6D, the key enzyme in the conversion of linoleic and alpha-linoleic acid, and SCD-1, which catalyzes the synthesis of MUFA from saturated fatty acids (SFA), is inhibited by PUFA intake (31). In this regard, evidence obtained in clinical trials has shown that diets with high intakes of PUFA down-regulate SCD-1 activity (32), particularly PUFA resulting from fish consumption (33). However, in relation with our findings, including PUFA intake as a confounder variable in the analyses of SCD-16 and SCD-18 minimally altered the results, suggesting that their associations with MetS and its components were not dependent on PUFA intake. Overall, our findings confirm the results of previous studies in which elevated D5D and reduced D6D and SCD-1 activities positively impacted cardiometabolic risk factors (24, 34).

SCD-16, SCD-18, and D6D are generally known to exert negative effects on metabolic health and other CVD risk factors. SCD-16 and SCD-18 have also been positively associated with BMI, blood pressure, and total cholesterol (35, 36), which is in accordance with our results. Other studies have found that D6D is related to higher TG, blood pressure, BMI and total cholesterol (37, 38). Moreover, D6D has showed a positive association with inflammatory biomarkers, such as ICAM-1 or C-reactive protein (37, 39), which suggests that this enzyme has a negative effect on metabolic health due to the activation of inflammatory pathways.

In contrast, estimated D5D activity has been favorably associated with MetS components, as it has been to related to higher HDL-c, lower blood pressure, and lower BMI (36, 40). Several mechanisms may explain the associations found between estimated DEA and MetS components. PUFA synthesized by D5D and D6D can modulate the expression of transcription factors that participate in lipogenesis and FA oxidation, such as PPAR. In addition, these FA also produce eicosanoids, which are inflammatory mediators that play major roles in lipogenesis or insulin resistance (41). Taken together, these results suggest that D5D products are involved in antiinflammatory responses and upregulation of transcription factors that lead to a better lipid profile and decreased CVD risk, whereas D6D and SCD products may have the opposite effect.

The main strength of the present study is its longitudinal nature, as this is considered the most rigorous method to establish a cause-effect relationship. Other strengths include the analysis of biological samples. Among the limitations of the study is that all the participants were >55 years and at high risk of CVD, thus the results may not be representative of other populations. Additionally, the sample size was relatively small compared to similar studies.

The present study shows that in a Mediterranean population of over 55 years and at high cardiovascular risk, estimated SCD-16, SCD-18, and D6D activities were adversely associated with MetS, whereas D5D was associated with a protective effect. Among the components that constitute the MetS, TG, HDL-c, DBP, and WC were adversely affected by estimated activities of SCD-16, SCD-18, and D6D. In contrast, D5D was associated with beneficial changes in TG and DBP. Therefore, our results contribute to the hypothesis that FA metabolism influences metabolic health and desaturases dysregulations may

be indicative of metabolic alterations. Further research is needed to confirm the current findings in the general population.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets presented in this article are not readily available because there are restrictions on the availability of data for the PREDIMED trial, due to the signed consent agreements around data sharing. Requestors wishing to access the PREDIMED-dataset generated and/or analyzed during the current study can make a request to the PREDIMED trial Steering Committee chair. Requests to access these datasets should be directed to RL-R, lamuela@ub.edu.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committees at the Hospital Clinic recruiting center and all participants signed a written informed consent form. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ID-L: conceptualization, investigation, formal analysis, and writing – original draft. CA-R: methodology and writing – original draft. AT-R, RC, and ZV-R: writing – review and editing. SC-B: methodology and writing – review and editing. ER, MF, and RE: investigation and writing – review and editing. ML-S: formal analysis and writing – review and editing. RL-R: conceptualization, investigation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author ER reports grants, personal fees, non-financial and other from the California Walnut Commission while the study was carried out; grants, personal fees, non-financial support and other from Alexion; and personal fees and other from Amarin, outside the submitted work. Author RL-R reports personal fees from Cerveceros de España, personal fees and other from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. Author RE reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain), and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Alburquerque, United States), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain), and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.991277/full#supplementary-material

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3.2.3. Publication 6

Changes in plasma total saturated fatty acids and palmitic acid are related to pro-inflammatory molecule IL-6 concentrations after nutritional intervention for one year

Inés Domínguez-López, Camila Arancibia-Riveros, Rosa Casas, Anna Tresserra-Rimbau, Cristina Razquin, Miguel Á Martínez-González, Frank B Hu, Emilio Ros, Montserrat Fitó, Ramon Estruch, M Carmen López-Sabater, and Rosa M Lamuela-Raventós

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Supplementary Material available in Annex section.

Abstract

Aims: To analyze the association of plasma fatty acids with inflammatory biomarkers in a PREDIMED trial subsample after one year of intervention.

Methods: In a one-year longitudinal study of 91 participants of the PREDIMED trial (Barcelona-Clinic center), plasma fatty acids and inflammatory biomarkers were analyzed using GC-FID and ELISA kits, respectively.

Results: In baseline plasma, a multivariable-adjusted ordinary least squares regression model showed that n-3 PUFAs concentrations were inversely associated with concentrations of soluble ICAM-1 and E-selectin, whereas the level of the most abundant SFA, palmitic acid, was directly associated with concentrations of IL-6. After one year of nutritional intervention, changes of plasma diet-derived total SFAs and palmitic acid were directly associated with changes in IL-6, respectively, after correction for multiple testing.

Conclusions: Our findings suggest that SFAs of dietary origin, especially palmitic acid, are directly involved in the increase of IL-6 in plasma.

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Changes in plasma total saturated fatty acids and palmitic acid are related to pro-inflammatory molecule IL-6 concentrations after nutritional intervention for one year

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ABSTRACT

Systemic inflammation is associated with an increased risk of non-communicable diseases, such as cardiovascular diseases and diabetes. Circulating fatty acids (FA) are known to be related to these conditions, possibly through their role in inflammation, although different types of FAs can have opposite effects on inflammatory mediators. The aim of the present study was to analyze the association of plasma FAs with inflammatory biomarkers in a PREDIMED trial subsample after one year of intervention. In a one-year longitudinal study of 91 participants of the PREDIMED trial (Barcelona-Clinic center), plasma FAs and inflammatory biomarkers were analyzed using gas chromatography and ELISA, respectively. In baseline plasma, a multivariable-adjusted ordinary least squares regression model showed that n-3 polyunsaturated FAs concentrations were inversely associated with concentrations of soluble intercellular adhesion molecule-1 (sICAM-1) and E-selectin, whereas the level of the most abundant saturated FA, palmitic acid, was directly associated with concentrations of interleukin-6 (IL-6) (β = 0.48 pg/mL, 95% CI: 0.03, 0.93 per 1-SD increase, p-value = 0.037). After one year of nutritional intervention, changes of plasma diet-derived total saturated FAs and palmitic acid were directly associated with changes in IL-6 (β = 0.59 pg/mL [95% CI: 0.28, 0.89] per 1-SD, p-value = 0.001; β = 0.64 pg/mL, 95% CI: 0.31, 0.98, p-value = 0.001), respectively, after correction for multiple testing. Our findings suggest that saturated FAs of dietary origin, especially palmitic acid, are directly involved in the increase of IL-6 in plasma.

Abbreviations: ACE, angiotensin-converting enzyme; CDV, cardiovascular disease; CRP, C-reactive protein; DNL, de novo lipogenesis; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; E-Sel, E-selectin; FA, fatty acid; FAME, fatty acid methyl ester; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; LPS, lipopolysaccharide; MedDiet, Mediterranean diet; MUFA, monounsaturated fatty acid; NSAID, non-steroidal anti-inflammatory drug; PPAR-Y, peroxisome proliferator-activated receptor y; PUFA, polyunsaturated fatty acid; sP-Sel, soluble P-selectin; SFA, saturated fatty acids; TLR-4, toll-like receptor 4; T2D, type 2 diabetes; VCAM-1, vascular cell adhesion molecule-1.

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1. Introduction

The Mediterranean diet (MedDiet) is characterized by a high consumption of extra virgin olive oil (with a high monounsaturated to saturated fat ratio), fruits, vegetables (excluding potatoes), legumes, whole grains, and tree nuts; a moderate-to-high consumption of fish, but low consumption of dairy products, red and processed meats and sweets. A huge accrual of epidemiological evidence and trials support that this traditional food pattern should be considered as an ideal healthy model worldwide, in part because it combines foods rich in antioxidants and nutrients with anti-inflammatory effects [1,2]. In this context, biological plausibility is strongly supported by the fact that different studies have reported that adherence to the MedDiet and/or consumption of its main components is inversely associated with systemic inflammation [3], and lower circulating levels of adhesion molecules, such as P-Selectin (P-Sel) or E-Selectin (E-Sel) [4,5], interleukin 6 (IL-6), TNF-α and C-reactive protein (CRP) [6,7], and white blood cell and platelet counts [8]. On the other hand, reduced adherence to the MedDiet has been directly associated with a worse profile of plasma inflammatory biomarkers [9] generated by immune cells, such as macrophages, lymphocytes and natural killer T cells, and dendritic and mast cells, which infiltrate the lesions caused by the inflammatory process and accelerate disease development [10,11].

Inflammation plays a key role in the pathophysiology of a wide range of diseases, including arthritis, asthma, atherosclerosis [12], autoimmune diseases [13], cancer [2], diabetes [14], and obesity [15], and anti-inflammatory foods and diets have a potential therapeutic role in these conditions [16]. Among the key components of the MedDiet are monounsaturated fats, particularly oleic acid provided by olive oil and olives, and fish and seafood products, which contain omega 3 polyunsaturated fatty acids (n-3 PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reported to have an anti-inflammatory effect through different metabolic pathways [17,18]. In contrast, saturated fatty acids (SFAs) derived mainly from meat and dairy sources, and, therefore, with lower intakes in the MedDiet, have been directly associated with inflammatory processes. The aim of the present study was to prospectively analyze the relationship between changes in plasma FAs and inflammatory biomarkers in a subsample of the PREDIMED study after one year of follow-up.

2. Methods

2.1. Study design

A prospective cohort analysis was carried out using baseline and one year data from the PREDIMED (PREvención con DIeta MEDiterránea) study, a large, parallel-group, multicenter, randomized, controlled, five-year clinical trial designed to assess the effect of MedDiets enriched in olive oil or nuts on cardiovascular disease (CVD) incidence (http://www.predimed.es) [19]. It was conducted in Spain from October 2003 to December 2010 and included 7447 participants at high cardiovascular risk. Eligible participants were men (55–80 years) and women (60–80 years) with type 2 diabetes (T2D) or at least three of the following major risk factors: current smoking, hypertension, dyslipidaemia, overweight/obesity or family history of premature CVD. A detailed description of methods and participants has been published elsewhere [4,19,20].

The present substudy of the PREDIMED trial consists of a random subsample of 91 participants from the PREDIMED-Hospital Clinic recruitment centre (Barcelona). To evaluate the effect of plasma fatty acids (FAs) on inflammatory status, we determined circulating inflammatory biomarkers and plasma FAs at baseline and after one year of follow-up. Participants with extreme total energy intakes (>3500 or <500 kcal/day in women or >4000 or <800 kcal/day in men) were excluded from the analysis. For this reason, one man that reported > 4000 kcal/day was not considered in this analysis.

2.2. Ethics statement

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.3. Covariate assessment

Dietary intake was assessed using a validated, semi-quantitative 137-item food frequency questionnaire with the assistance of trained dietitians at baseline and yearly thereafter. Nutrient intakes were calculated from Spanish food composition tables [21].

Trained personnel took anthropometric measures by standard methods at baseline and after one year, including weight and height, from which body mass index (kg/m^2) was calculated. Physical activity (metabolic equivalent tasks per minutes per day, METs min/day) was assessed with a validated Spanish version of the Minnesota physical activity questionnaire [22].

2.4. Derivatization and analysis of plasma fatty acids

2.4.1. Sample preparation

Plasma EDTA samples were collected after an overnight fast, centrifuged, coded, and stored at − 80ºC until analysis. The FA profile was determined by fast gas chromatography after derivatization to their corresponding fatty acid methyl esters (FAMEs) [23]. Briefly, 20 μL of the internal standard tridecanoic acid (C13:0) methyl ester, purchased from Sigma-Aldrich (St. Louis, MO, USA), was added to 100 μL of plasma sample. One milliliter of sodium methylate (0.5% w/v), acquired from Sigma-Aldrich, was directly added and heated to 100 ºC for 15 min. After cooling, the samples were esterified with one milliliter of boron trifluoride-methanol reagent, purchased from Sigma-Aldrich, at 100 ºC for 15 min. Once the tubes were cooled, FAMEs were isolated by adding 500 μL of n-hexane (Sigma-Aldrich). After shaking, one milliliter of saturated sodium chloride solution purchased from Panreac Quimica SLU (Barcelona, Spain) was added. Finally, the tubes were centrifuged for 10 min at 3000 g. After drying with anhydrous sodium sulfate (Scharlab, Barcelona, Spain), the clear n-hexane top layer was transferred into an automatic injector vial equipped with a volume adapter of 300 μL.

2.4.2. Gas chromatographic conditions

Fast analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a fame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of FAMEs was carried out on a capillary column (10 m x 0.10 mm i.d.) coated with an SGE-BPX70 cross-linked stationary phase (70% cyanopropyl polysilphenylene-siloxane, 0.20 μm film thickness) from SGE (SGE Europe Ltd., United Kingdom).

Operation conditions were as follows: the split-splitness injector was used in split mode with a split ratio of 1:100. The injector volume of the sample was 1 μL. The injector and detector temperatures were kept at 250 ºC and 270 ºC, respectively. The temperature program was as follows: initial temperature 150 ºC, increased at 25 ºC/min until 250 ºC (total run time: 4 min). Helium was used as the carrier gas, with linear velocity of 59.4 cm/s (average at 150 ºC). Data acquisition and processing were performed using Shimadzu-Chemstation software for GC systems.

Methyl ester peaks were identified by comparison of their relative retention times with those of the standards Supelco 37 Component FAME mix and PUFA No. 2 (Animal Source), purchased form Merck (Darmstadt, Germany). Results were expressed as relative percentages of total FA, and their means and standard deviation (SD) at baseline and

after one year of follow-up are shown in Supplementary Table S1.

2.5. Inflammatory biomarkers

Circulating inflammatory biomarkers were analyzed as described elsewhere [24]. In summary, baseline and one-year plasma samples were determined by using commercial ELISA kits for soluble (s) intercellular cell adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), sE-Sel, sP-Sel, and IL-6 (BLK and PelkinElmer Elast Amplification System). The samples were processed by a technician blinded to group allocation. High-sensitive CRP was determined in serum by particle-enhanced immunonephelometry, as reported [4]. Means and SD of circulating inflammatory biomarkers at baseline and after one year of follow-up are shown in Supplementary Table S1.

2.6. Statistical analyses

Analyses were performed with Stata 16.0 (Stata-Corp LP, Tx. USA). Baseline characteristics of the participants are presented as means + SD for continuous variables and percentages for categorical variables.

Statistical analyses were performed for FA subtypes according to their degree of saturation and series: SFAs (C14:0, C16:0 (palmitic acid), C17:0, and C18:0), monounsaturated fatty acids (MUFAs) (C16:1 n-7, C18:1 n-9, and C18:1 n-7), omega 6 polyunsaturated fatty acids (n-6 PUFAs) (C18:2 n-6, C18:3 n-6, C20:3 n-6, and C20:4 n-6) and n-3 PUFAs (C18:3 n-3, C20:5 n-3 (EPA), and C22:6 n-3 (DHA)).

Individual baseline values of plasma FAs and inflammatory biomarkers were normalized and scaled in multiples of 1 SD with Blom inverse normal transformation [25]. Changes in plasma FAs and inflammatory biomarkers (one-year value minus the baseline value) were calculated, and the resulting difference was also normalized and scaled.

Multivariable adjusted linear regression models were used to assess associations between plasma FAs and levels of inflammatory biomarkers (baseline) and their 1-year changes per 1-SD increase. Plasma FAs were introduced as independent variables in units of SD. Therefore, the beta coefficients in ordinary least squares regression models should be interpreted as the change in the biomarker per each SD of the respective FA. Two models with adjustment of increasing complexity were applied. Model 1 was minimally adjusted for age and sex. Model 2 was additionally adjusted for physical activity, smoking habit, educational level, total energy intake and medication (insulin, other glucose lowering drugs, statins, other lipid lowering drugs, angiotensin-converting enzyme (ACE) inhibitors, other antihypertensive drugs, antiplatelet therapy, and non-steroidal anti-inflammatory drugs (NSAIDs)). Model 3 was only developed for the analysis of SFAs and palmitic acid and included an index of de novo lipogenesis (DNL) calculated as the ratio of C16:0 / C18:2 n-6 to account for endogenous synthesis of SFAs as different from nutritional sources [26]. One-year changes in inflammatory biomarkers and plasma FAs were analyzed using the same models as for the baseline values but they were further adjusted for the intervention group in the trial (MedDiet+virgin olive oil; MediDiet+-nuts; Low-fat control) for the longitudinal study. The *p* values obtained were penalized for multiple comparisons using the procedure described by Simes [27].

3. Results

3.1. General characteristics

The main characteristics of the 91 participants are described in Table 1. Among them, 52 were women and 39 were men with a mean age of 68 + 5.8 and 66 + 5.8 years, respectively. By study design, the participants had a high burden of CVD risk factors: 82.4% had been diagnosed with T2D, 57.1% with hypertension and 61.5% with hypercholesterolemia. Consequently, medications targeting inflammatory pathways, such as ACE inhibitors, statins, or insulin, were used at high rates.

Table 1
Baseline characteristics of participants.

	Total	Men	Women
n	*91*	*39*	*52*
Age, years	67 ± 5.9	66 ± 5.8	68 ± 5.8
Weight, Kg	75 ± 11.8	82 ± 9.4	70 ± 10.9
BMI, kg/m^2	29 ± 3.7	28.6 ± 3.3	29.4 ± 4.0
Type 2 diabetes, n (%)	75 (82.4)	34 (87.2)	41 (78.8)
Hypertension, n (%)	52 (57.1)	17 (43.6)	35 (67.3)
Hypercholesterolemia, n (%)	56 (61.5)	25 (64.1)	31 (59.6)
Medication use, n (%)			
ACE inhibitors	23 (25.3)	8 (20.5)	15 (28.8)
Statins	30 (33.1)	9 (23.1)	21 (40.4)
Insulin	11 (12.1)	6 (15.4)	5 (9.6)
Antiplatelet therapy	15 (16.5)	7 (17.9)	8 (15.4)
NSAIDs	9 (9.9)	1 (2.5)	8 (15.4)
Current smoker, n (%)	15 (16.5)	12 (30.8)	3 (5.8)
Leisure-time physical activity, MET·min/week	261 ± 252.8	313 ± 289.7	222 ± 215.9
Educational level, n (%)			
Low	60 (65.9)	20 (51.3)	40 (76.9)
Medium / High	31 (34.1)	19 (48.7)	12 (23.1)

BMI, body mass index; ACE, angiotensin-converting enzyme; NSAIDs, non-steroidal anti-inflammatory drugs; MET, metabolic equivalent task.
Values are percentages for categorical variables and means ± SD for continuous variables.

Up to 16.5% of the participants were current smokers, 30.8% of which were men. The mean values of physical activity revealed that men were more physically active (313 ± 289.7 MET·min/week) than women (222 ± 215.9 MET·min/week). Overall, men had a higher level of education, which was classified as high or medium in 48.7%, whereas 76.9% of women had the lowest level.

3.2. Association between fatty acids and circulatory inflammatory biomarkers at baseline

As shown in Table 2, plasma n-3 PUFAs were inversely related to circulating levels of sICAM-1 and sE-Sel per 1-SD change when the multivariable adjustment model 2 was used (−0.30 ng/mL [95% CI: −0.59; -< 0.01] per 1-SD, *p*-value = 0.047; −0.31 ng/cL [−0.57; −0.05], *p*-value = 0.019). No significant associations were found between the plasma concentration of n-6 PUFAs and any inflammatory biomarker. A direct association was observed between MUFA concentrations and sVCAM-1 (0.31 ng/mL [0.03; 0.58] per 1-SD, *p*-value = 0.028).

For the analysis of SFAs and palmitic acid, a third adjustment model was generated including a DNL index. As the concentrations of total plasma SFAs may be influenced by dietary intake and DNL, this correction was applied to subtract the possible effect of endogenous metabolism on this FA subtype. However, DNL was not taken into consideration for the analysis of unsaturated FAs, as it does not directly affect their synthesis. Total plasma SFAs exhibited a direct association with circulating levels of sE-Sel and IL-6 per 1-SD increase (0.26 ng/cL [0.02; 0.49], *p*-value = 0.033; 0.33 pg/mL [0.05; 0.61], *p*-value = 0.021), but the changes were not significant after adjustment for DNL.

Individual FAs that may interfere with the inflammatory process were also analyzed: the anti-inflammatory EPA and DHA, and palmitic acid, which activates inflammatory pathways. In the fully adjusted model, significant inverse associations were found between DHA and sICAM-1 and sE-Sel (−0.33 ng/mL [−0.60; −0.06], *p*-value = 0.019; −0.31 ng/cL [−0.56; −0.07], *p*-value = 0.014), and between EPA and sICAM-1 (−0.33 ng/mL [−0.62; −0.03], *p*-value = 0.031). Plasma palmitic acid was directly associated with circulating IL-6, whether considering DNL (0.48 pg/mL [0.03; 0.93], *p*-value = 0.037) or not (0.35 pg/mL [0.09; 0.61], *p*-value = 0.009) per 1-SD increase.

At baseline, no plasma FA was significantly associated with any

Table 2
Multivariable-adjusted associations between plasma fatty acids and circulating inflammatory biomarkers at baseline (per 1-SD increase in FA).

		sICAM-1, ng/mL *n = 68*	*p*-value	Adjusted *p*-value[d]	sVCAM-1, ng/mL *n = 78*	*p*-value	Adjusted *p*-value[d]	sE-Sel, ng/cL *n = 72*	*p*-value	Adjusted *p*-value[d]
n-3 PUFA	*B (CI) - Model 1*	-0.23 (−0.44; −0.03)	0.026	0.218	-0.18 (−0.36; 0.01)	0.066	0.302	-0.29 (−0.53; −0.04)	0.022	0.218
	B (CI) - Model 2	-0.30 (−0.59; -< 0.01)	0.047	0.219	-0.22 (−0.50; 0.06)	0.116	0.348	-0.31 (−0.57; −0.05)	0.019	0.173
n-6 PUFA	*B (CI) - Model 1*	-0.02 (−0.24; 0.20)	0.840	0.979	-0.16 (−0.35; 0.04)	0.114	0.336	-0.18 (−0.43; 0.07)	0.163	0.380
	B (CI) - Model 2	0.02 (−0.25; 0.29)	0.877	0.952	-0.22 (−0.47; 0.02)	0.074	0.259	-0.22 (−0.45; 0.01)	0.061	0.233
MUFA	*B (CI) - Model 1*	-0.02 (−0.24; 0.21)	0.875	0.979	0.22 (0.01; 0.43)	0.037	0.222	0.13 (−0.11; 0.37)	0.291	0.535
	B (CI) - Model 2	-0.05 (−0.31; 0.22)	0.728	0.913	0.31 (0.03; 0.58)	0.028	0.173	0.17 (−0.07; 0.40)	0.163	0.360
SFA	*B (CI) - Model 1*	0.14 (−0.08; 0.37)	0.209	0.418	0.11 (−0.10; 0.32)	0.293	0.535	0.22 (−0.02; 0.47)	0.072	0.302
	B (CI) - Model 2	0.11 (−0.16; 0.38)	0.404	0.738	0.17 (−0.06; 0.41)	0.148	0.355	0.26 (0.02; 0.49)	0.033	0.173
	B (CI) - Model 3	0.31 (−0.09; 0.71)	0.126	0.407	0.02 (−0.43; 0.45)	0.970	0.992	0.21 (−0.15; 0.58)	0.247	0.623
EPA	*B (CI) - Model 1*	-0.25 (−0.46; -0.04)	0.017	0.218	-0.16 (−0.37; 0.04)	0.121	0.336	-0.20 (−0.43; 0.05)	0.105	0.336
	B (CI) - Model 2	-0.33 (−0.62; −0.03)	0.031	0.173	-0.21 (−0.49; 0.08)	0.152	0.355	-0.17 (−0.41; 0.08)	0.188	0.395
DHA	*B (CI) - Model 1*	-0.25 (−0.46; −0.04)	0.020	0.218	-0.19 (−0.38; −0.01)	0.036	0.222	-0.29 (−0.53; −0.05)	0.018	0.218
	B (CI) - Model 2	-0.33 (−0.60; −0.06)	0.019	0.173	-0.25 (−0.50; 0.01)	0.060	0.233	-0.31 (−0.56; −0.07)	0.014	0.173
Palmitic acid	*B (CI) - Model 1*	0.11 (−0.11; 0.32)	0.323	0.565	0.06 (−0.15; 0.28)	0.572	0.828	0.19 (−0.05; 0.43)	0.123	0.336
	B (CI) - Model 2	0.04 (−0.23; 0.32)	0.736	0.913	0.11 (−0.14; 0.36)	0.370	0.706	0.22 (−0.3; 0.46)	0.087	0.281
	B (CI) - Model 3	0.15 (−0.29; 0.60)	0.491	0.992	-0.18 (−0.63; 0.26)	0.418	0.924	0.12 (−0.29; 0.53)	0.552	0.992
		sP-Sel, ng/cL *n = 64*	*p*-value	Adjusted *p*-value[d]	CRP, μg/mL *n = 72*	*p*-value	Adjusted *p*-value[d]	IL-6, pg/mL *n = 54*	*p*-value	Adjusted *p*-value[d]
n-3 PUFA	*B (CI) - Model 1*	0.06 (−0.18; 0.29)	0.639	0.866	< 0.01 (−0.22; 0.24)	0.956	0.979	-0.09 (−0.40; 0.22)	0.564	0.828
	B (CI) - Model 2	< 0.01 (−0.34; 0.35)	0.977	0.977	-0.01 (−0.25; 0.22)	0.905	0.952	0.19 (−0.30; 0.34)	0.907	0.952
n-6 PUFA	*B (CI) - Model 1*	0.14 (−0.08; 0.36)	0.199	0.418	-< 0.01 (−0.22; 0.22)	0.986	0.986	-0.10 (−0.42; 0.22)	0.526	0.818
	B (CI) - Model 2	0.15 (−0.05; 0.36)	0.139	0.355	- 0.06 (−0.33; 0.21)	0.657	0.913	-0.23 (−0.54; 0.09)	0.151	0.355
MUFA	*B (CI) - Model 1*	-0.17 (−0.38; 0.49)	0.128	0.336	-0.03 (−0.29; 0.24)	0.850	0.979	- < 0.01 (−0.31; 0.29)	0.949	0.979
	B (CI) - Model 2	-0.10 (−0.37; 0.17)	0.442	0.774	0.06 (−0.24; 0.36)	0.694	0.913	0.05 (−0.29; 0.39)	0.756	0.913
SFA	*B (CI) - Model 1*	-0.01 (−0.32; 0.29)	0.946	0.979	0.07 (−0.14; 0.29)	0.492	0.795	0.22 (−0.05; 0.49)	0.108	0.336
			0.693	0.913		0.542	0.876	0.33 (0.05;0.61)	0.021	0.173

(*continued on next page*)

Table 2 (*continued*)

	B (CI) - Model 2	0.07 (−0.27; 0.41)			0.07 (−0.17; 0.32)					
	B (CI) - Model 3	0.13 (−0.39; 0.64)	0.621	0.992	0.08 (−0.35; 0.51)	0.704	0.992	0.39 (−0.07; 0.86)	0.093	0.357
EPA	*B (CI) - Model 1*	0.08 (−0.14; 0.30)	0.489	0.795	-0.03 (−0.25; 0.18)	0.754	0.979	-0.27 (−0.63; 0.10)	0.153	0.378
	B (CI) - Model 2	0.06 (−0.31; 0.43)	0.761	0.913	-0.07 (−0.30; 0.16)	0.522	0.876	-0.09 (−0.53; 0.35)	0.689	0.913
DHA	*B (CI) - Model 1*	-0.1 (−0.22; 0.20)	0.923	0.979	0.01 (−0.20; 0.22)	0.956	0.979	-0.05 (−0.39; 0.30)	0.773	0.979
	B (CI) - Model 2	-0.06 (−0.36; 0.23)	0.674	0.913	-0.03 (−0.24; 0.19)	0.817	0.927	0.04 (−0.27; 0.34)	0.810	0.927
Palmitic acid	*B (CI) - Model 1*	-0.07 (−0.35; 0.21)	0.628	0.866	0.14 (−0.08; 0.35)	0.203	0.418	0.26 (−0.01; 0.53)	0.062	0.302
	B (CI) - Model 2	0.01 (−0.31; 0.33)	0.937	0.960	0.14 (−0.10; 0.39)	0.244	0.488	0.35 (0.09; 0.61)	0.009	0.173
	B (CI) - Model 3	-0.01 (−0.55; 0.52)	0.957	0.992	0.31 (−0.19; 0.81)	0.220	0.616	0.48 (0.03; 0.93)	0.037	0.259

sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; sE-Esel, soluble E-selectin; sP-Sel, soluble P-selectin; CRP, C-reactive protein; IL6, interleukine-6; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

β, standardized regression coefficient; CI, confidence interval.

Model 1: sex and age. *Model 2*: model 1 + physical activity, smoking habit, educational level, total energy intake and medication (insulin, other glucose lowering drugs, statins, other lipid lowering drugs, ACE inhibitors, other antihypertensive drugs, antiplatelet therapy and NSAIDs). *Model 3:* model 2 + de novo lipogenesis (DNL). $p < 0.05$ were considered significant.

Multiple testing adjustments were conducted by applying the method of Simes (considering 42 comparisons).

circulating inflammatory biomarker after correction for multiple testing.

3.3. Associations between one-year changes in plasma fatty acids and circulating inflammatory biomarkers

Table 3 presents the associations between one-year changes per 1-SD in plasma FAs and circulating inflammatory biomarkers. No relation was observed between changes in PUFAs, MUFAs, EPA, and DHA and changes in the pro-inflammatory molecules. Changes in plasma SFA and palmitic acid concentrations were directly associated with changes in IL-6 levels (0.59 pg/mL [0.28; 0.89], *p*-value = 0.001) and (0.50 pg/mL [0.23; 0.77, *p*-value = 0.001], respectively, per 1-SD increase. These associations were observed before and after the inclusion of DNL in the adjustment model and remained statistically significant after adjustment for multiple testing.

4. Discussion

In this substudy of the PREDIMED trial, we found that plasma SFAs and palmitic acid and their respective changes were associated with higher levels of circulating pro-inflammatory molecules, particularly IL-6. To our knowledge, this is the first study to assess the influence of plasma FAs on inflammatory biomarkers in a Mediterranean population, considering prospectively their respective changes.

It is of interest to examine the composition of plasma FAs, as some of them are involved in the molecular mechanisms of chronic diseases triggered by inflammatory processes, including diabetes and insulin resistance, obesity, CVD, metabolic syndrome, and cancer [25,28–30]. Dietary intake plays a major role in determining the plasma FA profile, and FAs are frequently used as biomarkers of specific food consumption [31]. However, certain FAs may be produced and transformed by endogenous synthesis, especially in individuals with metabolic disorders [26]. In particular, high-carbohydrate diets can stimulate DNL, in which acetyl-CoA carboxylase and FA synthetase catalyze acetyl-CoA and malonyl-CoA derived from glucose or other carbohydrates to generate palmitate [32,33]. In the present study, to subtract the effect of endogenous metabolism, a DNL index was included in the adjustment models, which allowed us to focus on the effects of dietary intake.

Our results show that DNL affected plasma SFA concentrations at baseline, as their association with IL-6 and sE-Sel was no longer significant once this endogenous metabolic pathway was included in the analysis. In contrast, the association between palmitic acid and IL-6 remained unaffected, thus highlighting the dietary origin of this FA. In the longitudinal analysis, SFAs and palmitic acid were strongly associated with IL-6, even when the effect of DNL was accounted for, indicating that endogenous metabolism may not have been involved in their increase.

SFAs have been in the spotlight for a long time due to their implication in several chronic diseases, including CVD or T2D [34–36]. Several mechanisms may explain the relationship between SFAs and inflammatory biomarkers. Previous in vitro studies have shown that SFAs can induce inflammation through the activation of toll-like receptor 4 (TLR-4) receptors and nuclear factor kB, which leads to the up-regulation of inflammatory genes [15,37,38]. Others suggested that SFAs could amplify the inflammatory response due to their processing into ceramides, which activate protein kinase C and MAP kinase [39]. Saravanan et al. also proposed that SFAs could impair the expression of genes related to inflammatory pathways, such as peroxisome proliferator-activated receptor y (PPAR-Y), which would eventually result in reduced insulin sensitivity [40]. Additionally, results in an observational study in humans showed that SFA intake was associated with elevated CRP levels [41]. It is important to consider that dietary surveys are prone to substantial measurement errors, which can be overcome by objective biomarker analyses [42]. In a study on the effect of diet on atherosclerotic disease, Kalogeropoulos et al. obtained different results according to the method used, as dietary SFAs were

Table 3
Multivariable-adjusted associations between 1-year changes in plasma fatty acids and changes in circulating inflammatory biomarkers (per 1-SD change in FA).

		sICAM-1, ng/mL *n = 68*	*p*-value	Adjusted *p*-value[d]	sVCAM-1, ng/mL *n = 78*	*p*-value	Adjusted *p*-value[d]	sE-Sel, ng/cL *n = 72*	*p*-value	Adjusted *p*-value[d]
n-3 PUFA	*B (CI) - Model 1*	< 0.01 (−0.25; 0.25)	0.999	0.999	0.14 (−0.09; 0.38)	0.231	0.999	-0.03 (−0.26; 0.21)	0.822	0.999
	B (CI) - Model 2	0.03 (−0.20; 0.27)	0.770	0.969	0.16 (−0.04; 0.35)	0.119	0.969	0.01 (−0.23; 0.25)	0.903	0.969
n-6 PUFA	*B (CI) - Model 1*	0.03 (−0.23; 0.30)	0.812	0.999	0.05 (−0.21; 0.31)	0.693	0.999	< −0.01 (−0.27; 0.27)	0.998	0.999
	B (CI) - Model 2	< 0.01 (−0.28; 0.28)	0.983	0.983	-0.04 (−0.30; 0.23)	0.795	0.969	0.04 (−0.21; 0.29)	0.740	0.969
MUFA	*B (CI) - Model 1*	-0.03 (−0.29; 0.22)	0.805	0.999	-0.12 (0.37; 0.13)	0.355	0.999	-0.09 (−0.35; 0.18)	0.524	0.999
	B (CI) - Model 2	-0.03 (−0.29; 0.24)	0.851	0.969	-0.03 (−0.29; 0.24)	0.844	0.969	-0.15 (−0.38; 0.08)	0.203	0.969
SFA	*B (CI) - Model 1*	-0.04 (−0.29; 0.22)	0.765	0.999	0.05 (−0.19; 0.29)	0.654	0.999	0.12 (−0.12; 0.34)	0.318	0.999
	B (CI) - Model 2	-0.04 (−0.35; 0.27)	0.810	0.969	0.04 (−0.18; 0.25)	0.748	0.969	0.14 (−0.08; 0.36)	0.223	0.969
	B (CI) - Model 3	0.06 (−0.25; 0.36)	0.705	0.966	0.05 (−0.21; 0.31)	0.703	0.966	0.17 (−0.09; 0.44)	0.189	0.966
EPA	*B (CI) - Model 1*	0.02 (−0.24; 0.27)	0.901	0.999	0.10 (−0.14; 0.35)	0.400	0.999	< −0.01 (−0.25; 0.25)	0.995	0.999
	B (CI) - Model 2	0.02 (−0.27; 0.31)	0.892	0.969	0.18 (−0.07; 0.43)	0.148	0.969	0.01 (−0.25; 0.28)	0.920	0.969
DHA	*B (CI) - Model 1*	-0.08 (−0.34; 0.18)	0.530	0.999	0.13 (−0.11; 0.36)	0.287	0.999	-0.01 (−0.25; 0.23)	0.943	0.999
	B (CI) - Model 2	-0.06 (−0.33; 0.22)	0.685	0.969	0.11 (−0.13; 0.34)	0.366	0.969	0.05 (−0.20; 0.30)	0.687	0.969
Palmitic acid	*B (CI) - Model 1*	-0.02 (−0.27; 0.23)	0.888	0.999	0.02 (−0.22; 0.26)	0.847	0.999	0.13 (−0.11; 0.37)	0.287	0.999
	B (CI) - Model 2	-0.01 (−0.28; 0.26)	0.946	0.969	0.01 (−0.22; 0.24)	0.935	0.969	0.13 (−0.12; 0.37)	0.309	0.969
	B (CI) - Model 3	0.01 (−0.23; 0.40)	0.603	0.966	0.01 (−0.27; 0.29)	0.933	0.966	0.16 (−0.11; 0.42)	0.232	0.966

		sP-Sel, ng/cL *n = 64*	*p*-value	Adjusted *p*-value[d]	CRP, μg/mL *n = 72*	*p*-value	Adjusted *p*-value[d]	IL-6, pg/mL *n = 54*	*p*-value	Adjusted *p*-value[d]
n-3 PUFA	*B (CI) - Model 1*	0.08 (−0.20; 0.36)	0.560	0.999	< 0.01 (−0.24; 0.24)	0.998	0.999	-0.06 (−0.34; 0.23)	0.684	0.999
	B (CI) - Model 2	0.09 (0.23; 0.42)	0.567	0.969	-0.02 (−0.34; 0.29)	0.875	0.969	-0.08 (−0.30; 0.14)	0.451	0.969
n-6 PUFA	*B (CI) - Model 1*	0.07 (−0.18; 0.33)	0.568	0.999	-0.02 (−0.27; 0.23)	0.873	0.999	-0.11 (−0.44; 0.23)	0.527	0.999
	B (CI) - Model 2	0.02 (−0.25; 0.28)	0.909	0.969	0.07 (−0.29; 0.43)	0.694	0.969	-0.15 (−0.43; 0.13)	0.280	0.969
MUFA	*B (CI) - Model 1*	-0.17 (−0.43; 0.09)	0.192	0.999	0.01 (−0.25; 0.27)	0.929	0.999	-0.12 (−0.47; 0.22)	0.478	0.999
	B (CI) - Model 2	-0.11 (−0.44; 0.21)	0.484	0.969	-0.10 (−0.53; 0.32)	0.682	0.969	-0.10 (−0.34; 0.14)	0.401	0.969
SFA	*B (CI) - Model 1*	0.06 (−0.18; 0.30)	0.623	0.999	0.02 (−0.20; 0.25)	0.842	0.999	0.41 (0.14; 0.69)	0.004	0.168
	B (CI) - Model 2	0.08 (−0.15; 0.31)	0.485	0.969	0.01 (−0.22; 0.24)	0.927	0.969	0.48 (0.22; 0.75)	0.001	0.021
	B (CI) - Model 3	0.14 (−0.11; 0.39)	0.271	0.966	0.01 (−0.24; 0.25)	0.966	0.966	0.59 (0.28; 0.89)	0.001	0.021
EPA	*B (CI) - Model 1*	0.10 (−0.19; 0.39)	0.503	0.999	0.02 (−0.22; 0.27)	0.845	0.999	-0.10 (−0.39; 0.18)	0.477	0.999
	B (CI) - Model 2	0.23 (−0.07; 0.53)	0.129	0.969	-0.04 (−0.33; 0.25)	0.785	0.969	-0.14 (−0.43; 0.15)	0.344	0.969
DHA	*B (CI) - Model 1*	-0.04 (−0.31; 0.23)	0.773	0.999	0.05 (−0.20; 0.29)	0.706	0.999	< 0.01 (−0.29; 0.30)	0.984	0.999
	B (CI) - Model 2	-0.07 (−0.35; 0.21)	0.623	0.969	0.03 (−0.23; 0.30)	0.805	0.969		0.907	0.969

(*continued on next page*)

Table 3 (*continued*)

								-0.02 (−0.31; 0.27)		
Palmitic acid	*B (CI) - Model 1*	0.04 (−0.20; 0.29)	0.713	0.999	0.07 (−0.16; 0.29)	0.555	0.999	0.38 (0.09; 0.67)	0.012	0.252
	B (CI) - Model 2	0.04 (−0.21; 0.28)	0.770	0.969	0.06 (−0.20; 0.31)	0.634	0.969	0.50 (0.23; 0.78)	0.001	0.021
	B (CI) - Model 3	0.10 (−0.15; 0.36)	0.421	0.966	0.05(−0.20; 0.29)	0.700	0.966	0.64 (0.31; 0.98)	0.001	0.021

sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; sE-Esel, soluble E-selectin; sP-Sel, soluble P-selectin; CRP, C-reactive protein; IL6, interleukine-6; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

β, standardized regression coefficient; CI, confidence interval.

Model 1: sex and age. *Model 2*: model 1 + physical activity, smoking habit, educational level, total energy intake and medication (insulin, other glucose lowering drugs, statins, other lipid lowering drugs, ACE inhibitors, other antihypertensive drugs, antiplatelet therapy and NSAIDs) and intervention group. *Model 3:* model 2 + de novo lipogenesis (DNL). $p < 0.05$ were considered significant.

Multiple testing adjustments were conducted by applying the method of Simes (considering 42 comparisons).

associated with IL-6 whereas plasma SFAs were also correlated with CRP [43].

In the present study, a significant relation was found between SFAs and IL-6, in accordance with previous results, and this pro-inflammatory effect was reflected in the association between their respective changes observed after one year of nutritional intervention. However, as shown by Santaren et al., individual SFAs have different effects on inflammatory biomarkers, palmitic acid standing out for its detrimental impact [44]. The unhealthy effects of palmitic acid have been linked to CVD, obesity, T2D and even cancer [45]. Regarding its role in inflammatory pathways, palmitic acid has been reported to induce pro-inflammatory cytokine expression by macrophages that is mediated by its binding to TLR-4 [46,47]. Our results showed that palmitic acid was consistently associated with higher concentrations of IL-6, both at baseline and when their respective changes were assessed. This consistency, together with the adjustment for potential endogenous sources, reinforces the support for a causal effect related to dietary intake. This relationship is of particular interest as IL-6 is involved in multiple pathological processes related to chronic inflammation [48]. *In vitro* studies have shown that palmitic acid stimulates IL-6 production in different biological tissues [49–51]. It increases the production of reactive species and decreases oxidative capacity, and that induces mitochondrial dysfunction that finally leads to insulin resistance [52]. The triggered inflammatory response is in part mediated by IL-6, as palmitic acid is responsible for the upregulation of IL-6 mRNA [53]. All these molecular mechanisms, isolated or combined, may explain the concomitant changes of palmitic acid and IL-6. In addition, IL-6 secretion is induced by palmitate in combination with lipopolysaccharide (LPS), a pro-inflammatory component of gram-negative bacteria [54]. In one of the few clinical studies assessing this relationship, Voon et al. reported that inflammatory biomarkers were unaltered by a high palmitic acid diet; their participants, however, were administered commercial fats without analysis of individual FAs, and therefore the FA content was not standardized [55]. In contrast, Mu et al. reported a positive association between palmitic acid (measured in red blood cell phospholipids) and IL-6 in consistency with our results [56]. Overall, studies that measured fatty acids in biological samples consistently support the hypothesis that SFAs and palmitic acid stimulate inflammatory responses mediated by IL-6.

The main strength of the current study is that we performed a prospective analysis of plasma FAs and circulating inflammatory molecules, which reflected participant status in a more reliable way than information provided by self-reported questionnaires assessing dietary intakes of these FAs. The main limitations were the small sample size and that participants were older subjects at high CVD risk, which may limit the generalization of the results. However, generalizability should be based on biological grounds and not only in "representativeness" from a statistical, survey-like point of view [57].

In conclusion, plasma SFAs, particularly palmitic acid, were directly associated with circulating IL-6 both at baseline and when assessing their changes after one year of nutritional intervention. These findings suggest that SFA intake promotes inflammatory processes mediated by IL-6.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centers. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

CRediT authorship contribution statement

Domínguez-López: Formal analysis, Writing – original draft. **C. Arancibia-Riveros**: Formal analysis, Writing – original draft. **R. Casas**: Formal analysis, Writing – review & editing. **A. Tresserra-Rimbau**: Writing – review & editing. **C. Razquin**: Writing – review & editing. **M. A. Martínez-González**: Writing – review & editing. **F.B. Hu**: Writing – review & editing. **E. Ros**: Writing – review & editing. **M. Fitó**: Writing – review & editing. **R. Estruch**: Conceptualization, Writing – review & editing. **M.C. López-Sabater**: Conceptualization, Writing – review & editing. **R.M. Lamuela-Raventós**: Supervision, Writing – review & editing.

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Conflict of interest statement

E.R. reports grants, personal fees, non-financial support and other from the California Walnut Commission while the study was carried out; grants, personal fees, non-financial support and other from Alexion; and personal fees and other from Amarin, outside the submitted work. R.M. L.-R. reports personal fees from Cerveceros de España, personal fees, and other from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. R.E. reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain), and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Alburquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain), and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113028.

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3.2.4. Publication 7

Higher circulating vitamin B12 is associated with lower levels of inflammatory markers in individuals at high cardiovascular risk and in naturally aged mice

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Supplementary Material available in Annex section.

Abstract

Aims: To investigate the relationship between circulating vitamin B12 and inflammatory markers IL-6 and CRP.

Methods: The association of peripheral levels of vitamin B12 with IL-6 and CRP was assessed in 136 human samples from a high cardiovascular risk population. To corroborate the results from the human trial, the analysis was replicated in naturally aged mice. Circulating inflammatory biomarkers IL-6 and CRP were measured by ELISA and particle-enhanced immunonephelometry, respectively. Serum vitamin B12 concentrations were measured by an automated electrochemiluminescence immunoassay system

Results: Individuals with higher serum levels of vitamin B12 showed lower concentrations of IL-6 and CRP after adjustment for potential confounders, and an inverse association was also found between serum IL-6 and vitamin B12 levels in naturally aged mice.

Conclusions: Circulating vitamin B12 was inversely associated with IL-6 and CRP in humans and with IL-6 in mice, suggesting that it may exert an anti-inflammatory effect through modulation of these pro-inflammatory molecules.

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Higher circulating vitamin B12 is associated with lower levels of inflammatory markers in individuals at high cardiovascular risk and in naturally aged mice

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Abstract

BACKGROUND: Vitamin B12 is an essential nutrient that is involved in numerous physiological processes, and its deficiency can lead to various complications, including neurological and haematological disorders. Some studies have suggested that vitamin B12 may have anti-inflammatory effects, but the mechanisms underlying this relationship are not yet fully understood. We investigated the relationship between circulating vitamin B12 and inflammatory markers interleukin (IL)-6 and C-reactive protein (CRP). The association of peripheral levels of vitamin B12 with IL-6 and CRP was assessed in 136 human samples from a high cardiovascular risk population. To corroborate the results from the human trial, the analysis was replicated in naturally aged mice.

RESULTS: Individuals with higher serum levels of vitamin B12 showed lower concentrations of IL-6 and CRP after adjustment for potential confounders, and an inverse association was also found between serum IL-6 and vitamin B12 levels in naturally aged mice.

CONCLUSION: Circulating vitamin B12 was inversely associated with IL-6 and CRP in humans and with IL-6 in mice, suggesting that it may exert an anti-inflammatory effect through modulation of these pro-inflammatory molecules.

Supporting information may be found in the online version of this article.

Keywords: micronutrients; bioactive compounds; inflammation; intereukin-6; C-reactive protein; PREDIMED trial

INTRODUCTION

Vitamin B12 or cobalamin is known for its role in essential biological processes[1] because there are two key vitamin B12-dependent enzymes: methionine synthase and methylmalonyl-CoA mutase.[2] Methionine synthase is involved in the conversion of homocysteine to methionine, an important amino acid that is required for protein

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synthesis and many other physiological processes.[3] This enzyme also generates *S*-adenosylmethionine, a vital molecule that donates methyl groups for DNA, RNA and other reactions, impacting gene expression and metabolism.[4] Methylmalonyl-CoA mutase plays a key role in the breakdown of certain amino acids, including valine, isoleucine, methionine and threonine, which otherwise could not be properly metabolized.[5]

Clinical vitamin B12 deficiency is prevalent, affecting a significant proportion of the population, with estimates ranging from 1.5% to 15%, particularly among the elderly.[6] This deficiency can lead to neurological or haematological complications. Despite its essentiality for all eukaryotes, vitamin B12 is only synthesized by some genus of bacteria and some archaea; humans obtain it almost exclusively from dietary animal-derived products, where it is stored within animal proteins.[7] Dietary insufficiency therefore is a major concern for vegan and vegetarian populations, especially when no supplementation or fortified foods are used.[8] However, vitamin B12 deficiency can also be a result of inefficient absorption and digestion because its bioavailability is highly dependent on individual metabolic characteristics.[9] The process of vitamin B12 uptake in mammals is complex and extensively regulated. It starts with salivary enzymes dissociating it from animal proteins, followed by binding with haptocorrin (also known as transcobalamin I) to prevent degradation.[10] In the stomach, digestive enzymes release B12 from haptocorrin, and intrinsic factor binds to it.[10] Intrinsic factor is necessary for uptake in the terminal ileum, where vitamin B12 is processed and bound by transcobalamin II for release into the circulation.[11] The holo-transcobalamin complex (vitamin B12 bound to transcobalamin II) is recognized and taken up by cells via CD320 receptor.[11] Mutations in genes encoding these proteins can cause B12 deficiency.[12] In addition, vitamin B12 requirements increase during pregnancy, lactation and at older ages, with the latter often as a result of decreased absorption.[13]

The Mediterranean diet is well-known for including a wide variety of products and culinary techniques, which minimize the risk of deficiencies of specific nutrients. Although it is mostly characterized by high consumption of plant-derived products, it also includes moderate consumption of both fish and dairy products, and low consumption of meat.[14] Through these animal-derived proteins, the Mediterranean diet provides enough vitamin B12 to meet the nutritional requirements of 4.0 μg day^{-1} proposed by the European Food Safety Authority.[15] The benefits of a Mediterranean diet were assessed in the PREDIMED (PREvención con Dieta MEDiterránea) trial, a multicenter study of over 7000 participants at risk for cardiovascular disease.[16,17] Adhering to a Mediterranean diet regiment supplemented with extra virgin olive oil or nuts conferred a significant decrease in heart attack, stroke or cardiovascular death.[18]

Vitamin B12 or folic acid deficit leads to increased levels of homocysteine. Homocysteine is a sulphur-containing amino acid strongly associated with inflammation because it induces the production of proinflammatory molecules and reactive oxygen species.[19] This is particularly interesting because most common chronic and autoimmune diseases, including diabetes, cancer and atherosclerosis, are closely related to inflammatory processes.[20] We hypothesized that serum vitamin B12 may be associated with lower concentrations of inflammatory biomarkers. Therefore, the present study aimed to assess serum vitamin B12 levels and to evaluate their association with the circulating inflammatory molecules interleukin (IL)-6 and C-reactive protein (CRP) in a cross-sectional subanalysis of participants in the PREDIMED trial. To extend to an experimental system the results observed in humans, the analysis was replicated in an experimental system with naturally aged mice that could be manipulated and followed-up to avoid the chance effect.

MATERIALS AND METHODS

Clinical trial

Study design

A cross-sectional analysis was carried out using baseline data from the PREDIMED trial, a large, parallel-group, multicenter, randomized, controlled, clinical trial designed to assess the effect of the Mediterranean diet on the primary prevention of cardiovascular disease (http://www.predimed.es). It included 7447 participants at high cardiovascular risk, aged between 55 to 80 years for men and 60–80 years for women, who were recruited in Spain between October 2003 and December 2010. Eligible participants had either type 2 diabetes at baseline or at least three of the following cardiovascular risk factors: current smoking, hypertension, dyslipidemia, overweight/obesity or family history of premature coronary heart disease. A detailed description of methods and participants has been reported previously.[16,17]

In the present study, 136 participants were randomly selected among the participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) from whom samples were available.

Ethics statement

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on 16 July 2002. All participants provided their written informed consent.

Covariate assessment

A validated, semi-quantitative 137-item food frequency questionnaire was used to determine food consumption within the prior year with the assistance of trained dietitians.[21] Nutrient intakes were calculated from Spanish food composition tables. Trained personnel took anthropometric measures by standard methods, including weight and height, from which body mass index (BMI) (kg m^{-2}) was calculated. Physical activity (metabolic equivalent tasks per minutes per day, METs min day^{-1}) was assessed with a validated Spanish version of the Minnesota physical activity questionnaire.[22]

Inflammatory biomarkers

Circulating inflammatory biomarkers were analysed as described elsewhere.[23] Briefly, commercial enzyme-linked immunosorbent assays (ELISA) kits were used to determine plasma IL-6 (Elast Amplification System; PelkinElmer, Waltham, MA, USA). A technician blinded to group allocation was responsible for processing the samples. As reported previously, high-sensitive CRP was determined in serum by particle-enhanced immunonephelometry.[23]

Serum vitamin B12

Serum vitamin B12 concentrations were measured by an automated electrochemiluminescence immunoassay system (Advia-Centaur; Siemens, Barcelona, Spain) in frozen aliquots kept at −80 °C.

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Statistical analysis

Baseline characteristics of the participants are presented as the mean ± SD for continuous variables and percentages for categorical variables.

We used multivariable adjusted linear regression models to assess the association between serum vitamin B12 and circulating inflammatory biomarkers. Three adjustment models of increasing complexity were used. Model 1 was minimally adjusted for sex and age. Model 2 was further adjusted for educational level, smoking habit, BMI, physical activity, diabetes, hypertension, hypercholesterolemia and aspirin use. Model 3 was additionally adjusted for energy intake, alcohol consumption and Mediterranean diet adherence.

Animal studies

Animal procedures

Animal experimentation at the IRB Barcelona was performed according to protocols approved by the Science Park of Barcelona Ethics Committee for Research and Animal Welfare. C57BL6/J mice ($n = 5$ females, $n = 13$ males, based on availability in our colony) were bred and aged in-house in a specific pathogen-free facility on a 12:12 h light/dark photocycle (lights on 20:00 h). Mice were fed commercially available diets manufactured by SAFE® Complete Care Competence (Rosenberg, Germany) (SAFE A30 during pregnancy and weaning; SAFE A40 in adulthood) containing 0.02 mg kg^{-1} vitamin B12. Mice were deeply anesthetized in a carbon dioxide chamber, and blood was collected several hours prior to the end of the light cycle, around 17:00 h, by intracardiac puncture, followed by cervical dislocation. Upon collection, blood was allowed to clot at room temperature for 10 min, and then spun at 6000 rpm for 10 min in a microcentrifuge. The supernatant containing serum was removed and snap frozen.

IL-6 determination

Serum IL-6 was analysed by ELISA using the Mouse IL-6 ELISA Kit from Sigma-Aldrich (St Louis, MO, USA). Antibody levels higher than 2 pg mL^{-1} were considered positive in accordance with the manufacturer's guidelines. It was not technically possible to measure CRP in mice because the sensitivity of the current assays for CRP is not sufficient to detect the low levels found in mice.

Vitamin B12 determination

Serum was diluted 1:20 in PBS. HoloTC was measured using an ADVIA Centaur Immunoassay System (Siemens) with ADVIA Centaur Vitamin B12 Test Packs (Ref 07847260) in accordance with the manufacturer's instructions.

Statistical analysis

Graphs were generated using Prism, version 9 (GraphPad Software Inc., San Diego, CA, USA) and statistical analysis was performed using simple linear regression. We found two mice outliers with IL-6 levels greater than 2 SD from the mean, potentially suggesting illness or infection, and one mouse with vitamin B12 levels greater than 2 SD, indicative of hematologic or hepatic disease.[24] We removed these three mice from our dataset, interpreting the subset that remained as the mice that were likely to be healthy in their natural aging. Linear regressions were used to assess the association between vitamin B12 and IL-6. We performed further analyses to describe the relationship of age with vitamin B12 and IL-6.

RESULTS

General characteristics of the participants in the clinical trial

The general characteristics of the participants in the trial with available data on IL-6 and vitamin B12 included in this substudy are shown in Table 1, according to tertiles of serum vitamin B12. The average mean concentrations of vitamin B12 in serum in each of the three groups were 0.3 ± 0.2, 0.4 ± 0.0 and 0.7 ± 0.2 ng mL^{-1}. The mean age of the participants was 68.3 ± 6.0 years and 56% were women. All the groups were well balanced in terms of age, sex and BMI. As a result of the study design, all participants were

Table 1. General characteristics of the participants by tertiles of serum vitamin B12

Characteristics	All participants ($n = 136$)	T1 ($n = 46$)	T2 ($n = 45$)	T3 ($n = 45$)	*P*-value
Age (years)	68.3 ± 6.0	69.3 ± 5.6	67.8 ± 5.5	67.6 ± 6.7	0.357
Women (%)	55.9	50.0	57.8	60.0	0.600
BMI (kg m^{-2})	29.1 ± 4.3	29.1 ± 3.4	29.2 ± 3.3	28.9 ± 3.6	0.883
Diabetes (%)	65.4	69.6	71.1	55.6	0.231
Hypertension (%)	73.5	82.6	71.1	66.7	0.205
Hypercholesterolemia (%)	72.8	58.7	77.8	82.2	0.027
Current smokers (%)	17.7	21.7	15.6	15.6	0.064
Medium and high educational level (%)	30.9	37.0	37.8	17.8	0.067
Physical activity (METS-min day^{-1})	281 ± 259	324 ± 265	195 ± 181	324 ± 305	0.022
Energy intake (kcal day^{-1})	2425 ± 556	2533 ± 599	2310 ± 593	2428 ± 451	0.161
Vitamin supplementation (%)	5.2	2.2	8.9	4.4	0.338
Mediterranean diet adherence[a]	8 ± 2	9 ± 2	8 ± 2	8 ± 2	0.784
Serum vitamin B12 (ng mL^{-1})	0.47 ± 0.20	0.29 ± 0.07	0.45 ± 0.04	0.68 ± 0.20	< 0.001

Note: Continuous variables are shown as the mean ± SD and categorical variables are shown as percentages. A *t*-test was used for continuous variables and a chi-square test was used for categorical variables.
Abbreviations: BMI, body mass index; METS, metabolic equivalents; T, tertile.
[a] Mediterranean diet adherence was assessed with a 14-item score.

Table 2. Multivariable-adjusted regression that evaluate the association between serum B12 levels and circulating IL-6 and CRP

Inflammatory molecules	Mean (pg mL^{-1})		β (95% CI)	*P*-value
IL-6	0.73	Model 1	−0.20 (−0.52 to 0.13)	0.232
		Model 2	−0.35 (−0.70 to 0.00)	0.049
		Model 3	−0.39 (−0.76 to −0.03)	0.035
	Mean (mg/dL)		**β (95% CI)**	***P*-value**
CRP	0.50	Model 1	−0.14 (−0.41 to 0.12)	0.290
		Model 2	−0.29 (−0.54 to −0.03)	0.028
		Model 3	−0.34 (−0.63 to −0.05)	0.020

Note: Model 1: adjusted for sex and age. Model 2: additionally adjusted for education level, smoking habit, body mass index, physical activity, diabetes, hypertension, hypercholesterolemia and aspirin medication. Model 3: additionally adjusted for energy intake, alcohol consumption and Mediterranean diet adherence.
Abbreviations: ß, difference between groups; CI, confidence interval; CRP, C reactive protein; IL-6, interleukin 6.

overweight or obese and harboured a high load of cardiovascular risk factors: 65.4% had type-2 diabetes, 73.5% had hypertension and 72.8% had hypercholesterolemia. A higher percentage of participants in the highest tertile of vitamin B12 had hypercholesterolemia ($P = 0.027$). The mean physical activity was 281.2 ± 258.7 METS-min day^{-1}, and participants in the second tertile were less physically active ($P = 0.022$). Total energy intake and Mediterranean diet adherence were comparable among groups.

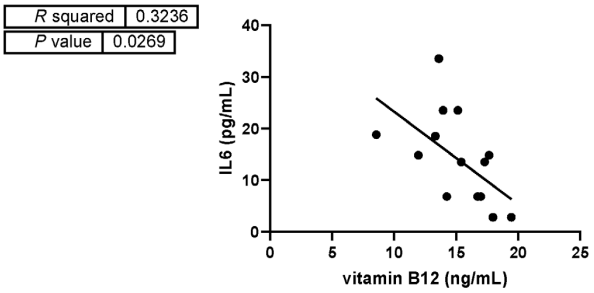

Figure 2. Association between vitamin B12 and IL-6 in mice. IL-6, interleukin-6.

The nutrient and food consumption of participants by tertiles of serum vitamin B12 are shown in the Supporting information (Table S1). The three groups of serum vitamin B12 were well-balanced in all nutrients and food groups.

Association between vitamin B12 and IL-6 and CRP in humans

The association between serum concentrations of vitamin B12 and circulating concentrations of IL-6 is presented in Table 2. In the minimally adjusted model, which accounted for sex and age, the association between IL-6 and vitamin B12 presented an inverse, albeit not statistically significant, relationship. Further adjustment for other potential confounders in the multivariable models 2 revealed a significant inverse association between IL-6 and vitamin B12 (−0.39 pg mL^{-1}; 95% confidence interval = −0.76 to

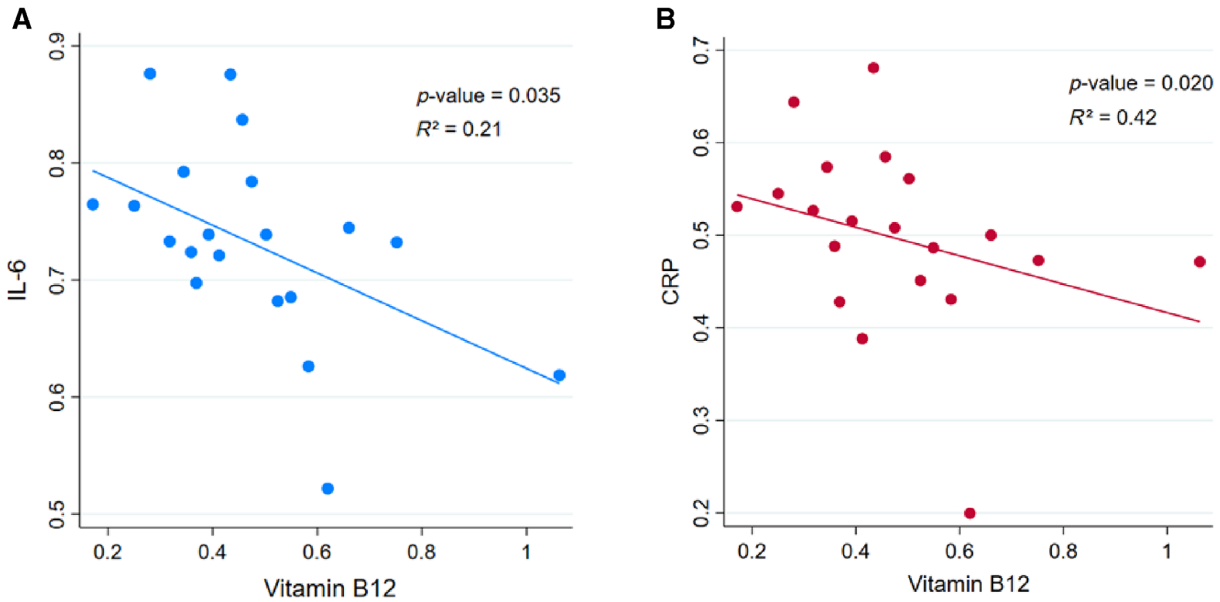

Figure 1. Binned scatterplot of the relationship between vitamin B12 and IL-6 (A) and CRP (B) in humans adjusted for sex, age, education level, smoking habit, BMI, physical activity, diabetes, hypertension, hypercholesterolemia, aspirin, medication, energy intake, alcohol consumption and Mediterranean diet adherence. IL-6, interleukin-6. CRP, C-reactive protein.

−0.03, $P = 0.031$). Similar results were obtained for CRP. In the minimally adjusted model, no significant association was found. After adjustment for models 2 and 3, a significant inverse association was found between CRP and vitamin B12 (−0.34 pg mL^{-1}; 95% confidence interval = −0.63 to −0.05, $P = 0.020$). Figure 1 illustrates that participants with higher concentrations of vitamin B12 had lower concentrations of IL-6 (A) and CRP (B) in the fully adjusted model.

Serum vitamin B12 levels are negatively correlated with IL-6 in naturally aged mice

We next wanted to extend our observation in an experimental animal model of natural aging, independent from cardiovascular disease. Although circulating IL-6 levels are generally low in healthy, unstressed laboratory mice, it has been reported that IL-6 levels increase with natural aging.[25] To assess biologically available vitamin B12 levels, we measured holotranscobalamin in the serum of mice. Following the exclusion of three outliers (defined as > 2 SD above the mean or *z*-score = 2; for details, see Materials and methods), two mice displaying IL-6 levels likely indicative of illness or infection, along with one mouse exhibiting elevated levels of vitamin B12, suggestive of hematologic or hepatic disease, there was a significantly negative correlation between serum IL-6 and vitamin B12 levels in naturally aged mice ($P = 0.027$) (Fig. 2). Taken together, our results indicate a negative association between serum IL-6 and vitamin B12 levels in healthy, naturally aged wild-type mice. When aging was considered on each variable separately, IL-6 and vitamin B12, there was no correlation between age and vitamin B12 levels, whereas IL-6 levels showed a modest but insignificant increase with age (see Supporting information, Fig. S1).

DISCUSSION

The present study aimed to characterize the relationship of circulating vitamin B12 with IL-6 and CRP. The analysis of the PREDIMED trial samples revealed that serum vitamin B12 is associated with lower levels of inflammatory molecules IL-6 and CRP. The data from naturally aged, healthy, wild-type mice provided supporting evidence by also showing an inverse relationship between serum vitamin B12 and IL-6.

Evidence suggests that vitamin B12 deficiency is associated with inflammation and metabolic complications.[26,27] Tripathi *et al.*[28] showed that dietary vitamin B12, together with folate, decreased inflammation in a mouse model of non-alcoholic steatohepatitis (NASH) through reduction of hyperhomocysteinemia. Vitamin B12 deficiency promoted NASH through activation of proinflammatory pathways with an imbalanced release of pro-inflammatory IL-1β and anti-inflammatory IL-10 in animals with colitis.[29] In humans, it was found that vitamin B12 may have a positive effect on inflammation and oxidative damage by improving the antioxidant capacity of plasma.[30] In a cross-sectional study that included middle-aged participants, a negative association was observed between tumor necrosis factor-α and serum vitamin B12.[31]

There are some potential explanations for these findings. Deficiency of vitamin B12 leads to elevated levels of homocysteine because this molecule cannot be converted into methionine via the methionine cycle because methionine synthase, which requires B12 as a cofactor, cannot function properly.[32] Hyperhomocysteinemia is a pathology characterized by homocysteine accumulation that leads to proinflammatory, cytotoxic and proatherogenic effects.[28] It has been reported that it is associated with pro-inflammatory cytokines, including IL-6, and is related to neuroinflammation.[33] Hyperhomocysteinemia also causes endothelial damage, reduces the arterial compliance and alters the process of endothelial homeostasis.[34] This condition is a potential risk for cardiovascular diseases such as atherosclerosis or coronary arterial disease.[35] Another hypothesis to explain the inverse relationship between vitamin B12 and inflammation suggests that the vitamin supresses the production of cytokines in T lymphocytes. Yamashiki *et al.*[36] demonstrated that the *in vitro* synthesis of cytokines including IL-6, interferon-γ and IL-1β was reduced when methyl B12 was added in culture. Overall, the findings suggest that vitamin B12 plays an important role in modulating inflammation.

The specific relationship between vitamin B12 and the inflammatory biomarkers IL-6 and CRP has been scarcely investigated. Scalabrino *et al.*[37] reported that vitamin B12 regulated IL-6 levels in rat cerebrospinal fluid, independently of other regulators of IL-6 production such as vasoactive intestinal peptide or somatostatin. An *in vitro* study found increased gene expression of IL-6 and other interleukins in adipocytes cultured in low vitamin B12 conditions.[38] These results are in line with the findings of the present study because we have observed that higher levels of vitamin B12 relate to lower concentrations of IL-6 in mice. Evidence from observational studies and clinical trials support these results. Ma *et al.*[39] found that supplementation of combined folate and vitamin B12 in elders with mild cognitive impairment reduced inflammatory cytokines, including IL-6, in association with lowering homocysteine levels. In patients with Alzheimer's disease, higher levels of IL-6 were detected in peripheral blood mononuclear cells when serum vitamin B12 concentrations were lower.[40] These findings are consistent with our results because we found an inverse association of vitamin B12 with inflammatory biomarkers. It is worth mentioning that the populations of these studies were similar to ours in that elderly adults were included. However, in the PREDIMED trial, participants with cognitive impairment or dementia were not included. A clinical trial with 285 patients with transient ischemic attack or stroke found that supplementation with 0.5 mg of vitamin B12, 2 mg of folic acid and 25 mg of B6 for 6 months did not reduce blood concentrations of IL-6 or CRP.[41] Nevertheless, the biological concentrations of vitamin B12 were not assessed, and thus it was not possible to determine whether the supplements were sufficient to increase blood levels of the vitamin. Previous studies have failed to establish a connection between vitamin B12 and CRP levels. Two clinical trials reported no CRP changes after an intervention with vitamin B12.[42,43] Young *et al.*[44] found a weak inverse correlation between vitamin B12 and CRP in a cross-sectional analysis. A large Finnish observational study failed to find any significant association between serum vitamin B12 and CRP levels.[45] Further studies of the clinical use of vitamin B12 as an anti-inflammatory and to reduce the risk of cardiac disease are warranted.

The inverse association of vitamin B12 and IL-6 may be relevant to inflammatory processes, as IL-6 is involved in chronic and acute inflammation.[46] In acute inflammation, IL-6 promotes the synthesis and release of most proteins involved in the acute phase response to a wide variety of stimuli. One of the acute-phase proteins is CRP,[47] which was also significantly inversely associated with vitamin B12 in our clinical study; we did not measure CRP in mouse serum because values would be below the limits of detection. IL-6 also mediates the transition from acute to chronic inflammation by recruiting monocytes to the area involved.[48] Through inflammatory processes, these molecules play an important role in the development of other diseases. Regarding

cardiovascular health, IL-6 is systematically increased in patients with obesity due to an increased release from adipocytes.[49] It is also involved in the development of insulin resistance and β-cell dysfunction that lead to type-2 diabetes. Recently, IL-6 has been proposed as a potential target for cancer treatment as a result of its involvement in the proliferation of cancerous cells.[50,51] Strong evidence suggests that CRP is a predictor of arterial thrombotic events. Two meta-analyses have confirmed that CRP is linked to a higher risk of incident cardiovascular events and can also predict future cardiovascular and all-cause mortality in individuals with type-2 diabetes.[52,53] CRP is also a potential biomarker for overall cancer because it plays a role in the occurrence of various types of cancer.[54,55]

The present study has some limitations. First, we had only a modest number of mice and humans in our study, and the human participants were older Mediterranean individuals at high cardiovascular risk; therefore, the results cannot be generalized. Second, we used a single measure of vitamin B12 and inflammatory biomarkers at baseline, which limits the potential to discern temporal and causal relationships. Our study also has important strengths. For the first time, we have assessed the relationship between circulating vitamin B12 and IL-6 both in mice and humans. Another strength is the analysis of biological samples to determine the status of the experimental animals and human participants.

CONCLUSIONS

There was an inverse association between peripheral vitamin B12 levels and IL-6 in a cross-sectional subanalysis of the PREDIMED trial, which involved a Mediterranean population at high cardiovascular risk, as well as in naturally aged mice, indicating that higher levels of vitamin B12 were linked to lower levels of IL-6. These findings support the potential role of vitamin B12 in inflammatory processes and related diseases. Further research is needed to identify the molecular mechanisms linking this vitamin to the production of IL-6 and its potential as a clinical intervention in cardiovascular and other inflammatory diseases. Importantly, extending our observation to naturally aged laboratory mice presents an attractive experimental system for future studies.

AUTHOR CONTRIBUTIONS

RE, MS and RMLR were responsible for conceptualization. IDL, MK, and RC were responsible for formal analysis. IDL and MK were responsible for writing the original draft. ET, MF, ER, RE, MS and RMLR were responsible for reviewing and editing.

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CONFLICTS OF INTEREST

ER reports grants, personal fees, non-financial support and other from the California Walnut Commission when the study was carried out; and grants, personal fees, non-financial support and other from Alexion, outside the submitted work. RML-R reports personal fees from Cerveceros de España, personal fees and others from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. RE reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain) and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Alburquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain) and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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3.2.5. Publication 8

Moderate wine consumption measured using the biomarker urinary tartaric acid concentration decreases inflammatory mediators related to atherosclerosis

Inés Domínguez-López, Camila Arancibia-Riveros, Rosa Casas, Polina Galkina, Maria Pérez, Miguel Á Martínez-González, Montserrat Fitó, Emilio Ros, Ramon Estruch, and Rosa M Lamuela-Raventós

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Abstract

Aims: To assess the anti-inflammatory properties of wine, measured as urinary tartaric acid, a new biomarker of wine consumption.

Methods: A one-year longitudinal study that included 217 participants from the PREDIMED trial was designed. Plasma inflammatory biomarkers and urinary tartaric acid were analyzed using xMAP technology and high-performance liquid chromatography, respectively. Multivariable regression analyses were performed to assess the relationship between variations over 1-year in urinary tartaric acid concentrations and 1-year changes in serum inflammatory molecules. Three categories were built according to tertiles of 1-y changes in urinary tartaric acid.

Results: Using a ROC curve, urinary tartaric acid was corroborated as a reliable biomarker of wine consumption. In the continuous analysis, participants with higher increases in tartaric acid significantly reduced their concentrations in soluble VCAM-1 after 1-year of follow-up. Moreover, tertile 2 and 3 of 1-year changes in tartaric acid presented a significant reduction in soluble ICAM-1 as compared to tertile 1. Participants in the third tertile also exhibited a reduced concentration of soluble VCAM-1 compared to those in the first tertile.

Conclusions: Our findings suggest that wine consumption is associated with lower levels of inflammation due to the anti-inflammatory properties of wine compounds.

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Original Article

Moderate wine consumption measured using the biomarker urinary tartaric acid concentration decreases inflammatory mediators related to atherosclerosis

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ABSTRACT

Objectives: Several studies suggest that moderate wine consumption, particularly red wine, may have benefits for cardiovascular health. Red wine contains a variety of bioactive compounds, including polyphenols like phenolic acids, which have demonstrated anti-inflammatory effects in experimental models. The aim of this study was to assess the anti-inflammatory properties of wine, measured as urinary tartaric acid, a new biomarker of wine consumption.
Design, settings, and participants: One-year longitudinal study that included 217 participants from the PREDIMED trial.
Measurements: Plasma inflammatory biomarkers and urinary tartaric acid were analyzed using xMAP technology and high-performance liquid chromatography, respectively. Multivariable regression analyses were performed to assess the relationship between variations over 1-year in urinary tartaric acid concentrations and 1-year changes in serum inflammatory molecules, including adhesion cell molecules, interleukine-6, tumour necrosis factor alpha, and monocyte chemotactic protein 1. Three categories were built according to tertiles of 1-y changes in urinary tartaric acid.
Results: Using a ROC curve, urinary tartaric acid was corroborated as a reliable biomarker of wine consumption (AUC = 0.818 (95% CI: 0.76; 0.87). In the continuous analysis, participants with higher increases in tartaric acid significantly reduced their concentrations in soluble vascular adhesion molecule (sVCAM-1) after 1-year of follow-up (−0.20 (−0.38; −9,93) ng/mL per 1-SD increment, *p*-value = 0.031). Moreover, tertiles 2 and 3 of 1-year changes in tartaric acid presented a significant reduction in soluble intercellular cell adhesion molecule (sICAM-1) as compared to tertile 1 (−0.31 (−0.52; −0.10) ng/mL, *p*-value = 0.014 and −0.29 (−0.52; −0.07) ng/mL, *p*-value = 0.023, respectively). Participants in the third tertile also exhibited a reduced concentration of sVCAM-1 compared to those in the first tertile (−0.31 (−0.55; −0.06) ng/mL, *p*-value = 0.035).
Conclusions: Our findings suggest that wine consumption is associated with lower levels of inflammation due to the anti-inflammatory properties of wine compounds.

Abbreviations: CVD, cardiovascular disease; CRP, C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1; IL-6, interleukin-6; MedDiet, Mediterranean diet; MCP-1, monocyte chemoattractant protein-1; NSAID, non-steroidal anti-inflammatory drug; ROC, receiver operating characteristic; T, tertile; TNF-α, tumour necrosis factor alpha; VCAM-1, soluble vascular cell adhesion molecule-1.

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1. Introduction

Inflammation and its multifaceted role in health establish a fundamental link between the body's immune response and various physiological processes [1]. While acute inflammation is a protective mechanism aimed at repairing tissue damage and combating infections, chronic inflammation can lead to a cascade of detrimental effects, including the development of arthritis, asthma, atherosclerosis, autoimmune diseases, cancer, diabetes, and obesity [2–4].

Within this framework, the Mediterranean diet (MedDiet) has garnered attention for its potential to curb inflammation. Epidemiological studies have demonstrated the effectiveness of the MedDiet in reducing circulating inflammatory molecules in a population at high cardiovascular (CVD) risk [5,6]. This plant-based dietary pattern is rich in antioxidant-rich fruits, vegetables, whole grains, healthy fats from sources like extra virgin olive oil and fatty fish and includes a moderate consumption of wine [7]. Some of its dietary components, such as polyphenols and omega-3 fatty acids, exhibit powerful anti-inflammatory properties, potentially helping to modulate inflammatory processes responsible of several chronic diseases [8–10]. In the context of the MedDiet, the moderate intake of alcohol (ethanol), mainly as wine, emerges as a complementary factor that synergizes with other heart-protective elements of the MedDiet. These components may contribute to the elevation of high-density lipoprotein (HDL)-cholesterol, the inhibition of platelet aggregation, the stimulation of antioxidant effects, and the reduction of systemic inflammation, among other effects [11].

A contentious debate persists concerning the impact of red wine consumption on health. Both epidemiological and clinical findings seem to emphasize the beneficial role of moderate red wine consumption in reducing inflammation [12]. Nonetheless, some studies did not find any noticeable impact of moderate red wine consumption on inflammatory biomarkers [13], while others even have reported proinflammatory effects [14]. However, most randomized clinical trials and observational epidemiological investigations strongly suggest that moderate drinking of wine yields positive outcomes in various inflammatory pathways linked to endothelial activation [15–18]. This connection could potentially be attributed to the presence of polyphenols, which have the capacity to alleviate mild inflammation and endothelial activation.

Consumption of wine, the main alcoholic beverage in the MedDiet, in epidemiological and clinical trials is primarily assessed using food frequency questionnaires, which might be affected by subjectivity [19]. In contrast, urinary tartaric acid concentration has emerged as an objective, reliable, selective, and sensitive biomarker for gauging moderate wine consumption [20]. The aim of the present study, nested in the PREDIMED trial, was to analyse the relationship of changes in wine consumption, measured with the reliable biomarker tartaric acid excreted in urine, with changes in plasma circulating inflammatory molecules in an older population at high CVD risk of the PREDIMED trial, using repeated measurements at baseline and after one year of follow-up.

2. Methods

2.1. Study design

A prospective cohort analysis was conducted utilizing baseline and one-year data from the PREDIMED (PREvención con Dieta MEDiterránea) study. This large, parallel-group, multicenter, randomized, controlled, five-year clinical trial aimed to assess the impact of the MedDiet enriched with olive oil or nuts on the incidence of CVD. The trial took place in Spain from October 2003 to December 2010 and involved 7447 participants at high cardiovascular risk (www.predimed.es).

Eligible participants included men (aged 55–80 years) and women (aged 60–80 years) with type-2 diabetes or exhibiting at least three of the following significant risk factors: current smoking, hypertension, dyslipidemia, overweight/obesity, or a family history of premature CVD. Detailed methodologies and participant criteria have been previously published [21,22].

The present substudy of the PREDIMED trial consisted of a subsample of 217 participants from the PREDIMED-Hospital Clinic of Barcelona and Navarra recruitment centres whose data regarding inflammatory biomarkers and urinary tartaric acid were available.

2.2. Ethics statement

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.3. Covariate assessment

Food consumption was ascertained using a validated, semi-quantitative food frequency questionnaire comprising 137 items, administered by skilled dietitians [23]. To determine nutrient intakes, Spanish food composition tables were utilized.

Anthropometric measurements, including weight and height, were taken by trained staff using established techniques, enabling the calculation of body mass index (BMI) in kg/m^2. To assess physical activity levels, a validated Spanish iteration of the Minnesota physical activity questionnaire was employed, measuring metabolic equivalent tasks per minutes per day (METs min/day) [24].

2.4. Inflammatory biomarkers

Circulating inflammatory biomarkers were analysed as described elsewhere [25]. To summarize, the concentrations of soluble adhesion molecules were measured using xMAP technology on the Luminex platform (Luminex Corporation, Austin, TX, USA), according to the manufacturer's instructions, and analysed using the Bio-Plex Manager™ Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We determined the concentration of five potential biomarkers: plasma-soluble vascular cell adhesion molecule 1 (sVCAM-1), intercellular adhesion molecule 1 (sICAM-1), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and monocyte chemotactic protein 1 (MCP-1), by using a Human Cytokine Plex assay (Bio-Rad Laboratories, Inc.).

2.5. Urinary tartaric acid

Tartaric acid concentration in first spot morning urine was measured using the methodology previously described [26]. We combined data from two substudies and corrected for batch effects using an average batch correction method [26].

2.5.1. Reagents and standards

Formic acid (approximately 98%), picric acid (98%, moistened with approximately 33% water) and sodium hydroxide (>98%) were purchased from Panreac. L-(+)-Tartaric acid and creatinine were obtained from Sigma. The labelled internal standard DL-(+)-tartaric-2,3-d2 acid was purchased from C/D/N Isotopes. Solvents were high-performance liquid chromatography grade, and all other chemicals were analytical reagent grade. Ultrapure water was obtained from a Milli-Q Gradient water purification system (Millipore).

2.5.2. Sample preparation

Tartaric acid in urine was determined following a validated stable-isotope dilution LC–ESI–MS/MS method [27]. A total of 20 μL of urine were diluted 1:50 (vol:vol) with 0.5% formic acid in water, and 10 μL of

the internal standard DL-(±)-tartaric-2,3-d2 acid (200 μg/mL) were added. The diluted sample was filtered by 0.20 μm and analyzed by LC–ESI–MS/MS, following the metod described previously by Regueiro et al. [27]. The data for urinary tartaric acid were adjusted using urine creatinine levels. The creatinine levels were determined using a modified version of the Jaffé alkaline picrate method specifically designed for Thermo microtiter 96-well plates. This approach was carried out following the methodology developed by Medina-Remón et al [28].

2.5.3. LC–ESI–MS/MS analysis

The analysis was performed using an Atlantis TE C18, 100 × 2.1 mm, 3 μm (Waters, Milford, MA, USA) reversed-phase column coupled for detection to a triple quadrupole mass spectrometer API 3000 (Applied Biosystems, Foster City, CA, USA). The mass spectrometer was operated in negative electrospray ionisation. The column was maintained at 25 °C throughout the analysis. Mobile phases A and B were, 0.5% formic acid in water and 0.5% formic acid in acetonitrile, respectively. The following linear gradient was used: held at 100%A for 3.5 min, decreased to 10%A over 2 min and held for 2 min, then returned to initial conditions for 1.5 min and re-equilibrated for 6 min. The flow rate was set at 350 μL/min and the injection volume was 10 μL. Post-column addition of acetonitrile (250 μL/min) was carried out to improve analyte ionization efficiency. The detection was accomplished in the multiple reaction monitoring (MRM) mode, and the following MS/MS transitions were used for quantification and confirmation, respectively: m/z 149/87 and m/z 149/73 for tartaric acid, and m/z 151/88 and m/z 151/74 for the deuterated isotope.

2.6. Statistical analyses

Participants were divided into 3 categories according to tertiles of 1-year changes in urinary tartaric acid concentration to assess linearity. Baseline characteristics of participants are presented as means ± standard deviation (SD) for continuous variables and percentages for categorical variables. We used one-factor ANOVA to assess differences in continuous variables, and chi-squared tests for categorical values.

Individual baseline values of inflammatory biomarkers, and tartaric acid were normalized and scaled in multiples of 1 SD with Blom inverse normal transformation due to high biological variability [29]. Changes in these variables (one-year value minus the baseline value) were calculated, and the resulting difference was also normalized and scaled.

The analysis for the receiver operating characteristic (ROC) curve was obtained through a logistic regression between wine (consumers/non-consumers) as the dependent variable and urinary tartaric acid concentration as the exposure variable, adjusted for age (continuous), sex (men/women), physical activity (continuous), smoking habit (current/past/non-smoker), educational level (low/medium & high level), waist-to-height ratio (continuous), hypertension (yes/no), hypercholesterolemia (yes/no), diabetes (yes/no), energy intake (continuous), MedDiet adherence not considering wine (continuous) and grapes and raisins consumption (continuous).

Multivariable adjusted linear regression models were used to assess associations between 1-year changes of urinary tartaric acid and concentrations (analyzed as continuous variable and using tertiles) and inflammatory biomarkers per 1-SD increment. Adjustment models of increasing complexity were applied. Model 1 was minimally adjusted for age and sex. Model 2 was further adjusted for physical activity, smoking habit, educational level, waist-to-height ratio, intervention group (MedDiet supplemented with extra virgin olive oil/MedDiet supplemented with nuts/control group) diabetes, hypercholesterolemia, hypertension, and intake of aspirin and non-steroidal anti-inflammatory drug (NSAIDs) (yes/no), energy intake, MedDiet adherence (not considering wine) and consumption of grapes and raisins. To assess the linear trend (p for trend) across tertiles of tartaric acid changes, the mean value was assigned to each tertile.

All analyses were conducted with robust estimates of the variance to correct for intracluster correlation and 2-sided significance was determined at a $p < 0.05$. Analyses were performed with Stata 16.0 (Stata-Corp LP).

3. Results

3.1. General characteristics of the participants

Baseline characteristics of the participants are presented in Table 1, categorized according to tertiles of 1-year changes of urinary tartaric acid concentrations. Then mean age was 68.8 years and 52.1% were females. All groups were well balanced in terms of sex, age, and physical activity. As expected, considering the characteristics of the participants in the PREDIMED trial, all groups had a mean BMI indicating overweight and

Table 1
General characteristics of the participants at baseline categorized for 1-year changes in urinary tartaric acid (n = 217).

	All (n = 217)	T1 (n = 69)	T2 (n = 69)	T3 (n = 68)	p-Value
Δ Tartaric acid (μg/mg creatinine)	1.3 ± 45.2	−23.2 ± 53.7	0.9 ± 1.1	26.5 ± 45.1	
Female, n (%)	113 (52.1)	37 (49.3)	40 (55.6)	36 (50.0)	0.767
Age, years	68.8 ± 5.8	68.5 ± 5.8	69.0 ± 6.0	68.8 ± 5.6	0.887
BMI, kg/m^2	29.7 ± 3.5	29.9 ± 3.7	29.4 ± 3.6	29.7 ± 3.3	0.762
Physical activity, METS-min/day	270.9 ± 222.8	280.8 ± 254.0	259.8 ± 207.0	272.0 ± 206.3	0.851
Current smoker, n (%)	31 (14.3)	12 (16.4)	7 (9.7)	12 (16.7)	0.400
Educational level, n (%)					0.089
Low	163 (75.1)	54 (74.0)	60 (83.3)	49 (68.1)	
High & medium	54 (24.9)	19 (26.0)	12 (16.7)	23 (31.9)	
Diabetes mellitus, %	119 (54.8)	45 (61.6)	37 (51.4)	37 (51.4)	0.358
Dyslipidaemia, n (%)	138 (63.6)	47 (64.4)	44 (61.1)	47 (65.3)	0.861
Hypertension, n (%)	171 (78.8)	55 (75.3)	58 (80.6)	58 (80.6)	0.674
Total energy intake, kcal/day	2369 ± 605	2332 ± 592	2391 ± 627	2385 ± 600	0.812
Mediterranean diet adherence, 14-points score	9 ± 2	8 ± 2	9 ± 2	9 ± 2	0.264
Intervention group, n (%)					0.316
MedDiet supplemented with EVOO	80 (36.9)	28 (38.4)	28 (38.9)	24 (33.3)	
MedDiet supplemented with nuts	75 (34.6)	30 (41.1)	20 (27.8)	25 (34.7)	
Low-fat control group	62 (28.6)	15 (20.1)	24 (33.3)	23 (31.9)	
Wine consumption, mL/day	82.5 ± 127.9	100.8 ± 142.0	42.6 ± 64.5	103.7 ± 149.4	0.005
Grapes & raisins consumption (g/d)	11.2 ± 19.1	11.2 ± 20.0	11.9 ± 20.7	10.5 ± 16.5	0.912

T, tertile; BMI, body mass index.
$p < 0.05$ were considered significant.
One-ANOVA factor were used for continuous variables, and a chi-square test was used for categorical variables.

Table 2
Change in dietary intake after 1 year categorized by tertiles of change in urinary tartaric acid.

	T1 (n = 73)	T2 (n = 72)	T3 (n = 72)	p-Value
Carbohydrates (g/d)	−22.21 ± 81.81	−11.24 ± 69.66	−17.43 ± 66.81	0.661
Proteins (g/d)	−3.94 ± 21.73	0.23 ± 17.45	−3.08 ± 19.83	0.409
Total fat (g/d)	8.31 ± 33.57	5.95 ± 26.80	5.74 ± 28.00	0.845
Saturated fatty acids (g/d)	−2.52 ± 10.17	−0.44 ± 8.68	−1.19 ± 7.31	0.356
Monounsaturated fatty acids (g/d)	6.34 ± 17.27	4.83 ± 15.92	4.87 ± 14.11	0.805
Polyunsaturated fatty acids (g/d)	3.23 ± 9.41	1.49 ± 6.49	1.80 ± 10.26	0.451
Alcohol (g/d)	−2.05 ± 10.72	0.68 ± 6.76	1.28 ± 13.72	0.142
EVOO (g/d)	12.76 ± 27.10	18.09 ± 27.93	18.15 ± 24.65	0.375
Vegetables (g/d)	54.72 ± 192.82	72.15 ± 161.64	10.32 ± 170.96	0.096
Fruits (g/d)	69.57 ± 261.16	27.39 ± 185.67	14.09 ± 208.19	0.289
Cereals (g/d)	−20.75 ± 101.81	−18.03 ± 77.81	−14.16 ± 98.23	0.913
Legumes (g/d)	2.12 ± 9.17	4.43 ± 10.19	−0.18 ± 37.65	0.489
Nuts (g/d)	12.38 ± 24.52	6.69 ± 18.35	7.67 ± 26.26	0.292
Fish and seafood (g/d)	7.92 ± 38.58	7.98 ± 42.79	7.80 ± 42.08	1.000
Meat and meat products (g/d)	−15.06 ± 57.33	−12.71 ± 42.33	−22.61 ± 47.03	0.455
Dairy products (g/d)	−47.90 ± 206.55	−1.24 ± 201.55	5.77 ± 166.31	0.191
Pastries (g/d)	−7.15 ± 26.29	−9.13 ± 28.72	−1.89 ± 23.91	0.237

Data are given as mean ± standard deviation (SD); statistical analyses were undertaken using 1-factor ANOVA, $p < 0.05$ indicates statistical significance. EVOO, extra virgin olive oil; T, tertiles.

showed a high prevalence of cardiovascular risk factors; 54.8% had diabetes, 63.6% had dyslipidemia, and 78.8% had hypertension. Most of the participants were either non-smokers (85.7%) or had a low educational level (75.1%), equally distributed among groups. Mediterranean diet adherence was comparable among the three groups, although it tended to be lower in T1, and wine consumption was significantly lower in T2. Baseline and one-year changes in inflammatory biomarkers across tertiles of urinary tartaric acid are presented in Supplementary Tables S1 and S2, respectively.

Changes in the dietary intake of participants after one year of follow-up according to tertiles of change of urinary excretion of tartaric acid can be found in Table 2. The consumption of all foods and nutrients was well-balanced across tertiles.

3.2. Urinary tartaric acid concentration as wine consumption biomarker

After adjustments for potential confounders (age, sex, smoking habit, educational level, BMI, physical activity, intervention group, analysis time, energy intake, and grapes and raisins consumption), we found that participants who increased their consumption of wine also excreted more tartaric acid in urine (0.39 (0.28; 0.50) μg/mg creatinine per 1-SD, p-value = <0.001). Fig. 1 shows the ROC curve analysis, where it can be observed that urinary tartaric acid can predict wine consumption with an AUC = 0.818 (0.763; 0.873).

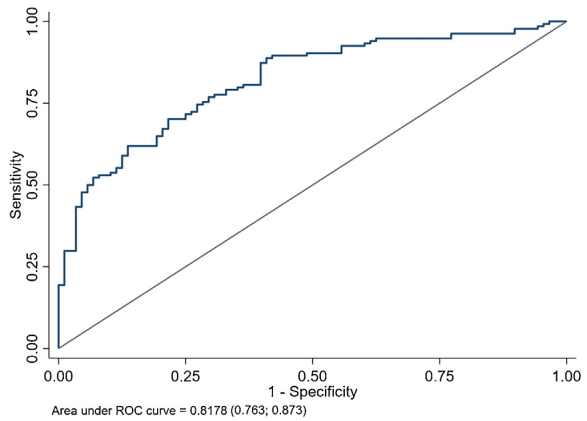

Fig. 1. Receiver operating characteristic (ROC) curves for prediction of wine consumption (yes/no) by urinary tartaric acid.

3.3. Urinary tartaric acid concentration and inflammatory molecules

As illustrated in Fig. 2, participants with higher increases in tartaric acid presented a significant reduction in sVCAM-1 concentrations after adjustment for potential confounders (−0.20 (−0.38; −0.03) ng/mL per 1-SD increase, p-value = 0.031). No other significant associations were found when changes in tartaric acid were modelled as a continuous variable.

The associations between tertiles of urinary 1-year changes in tartaric acid and 1-year changes in circulating inflammatory biomarkers are shown in Table 3. After adjustment for potential confounders in model 2, we found that an increase in urinary excretion of tartaric acid was inversely associated with changes in plasma sVCAM-1 and sICAM-1. Thus, participants in T2 and T3 had lower concentrations of sICAM-1 compared to participants of T1 (−0.31 (−0.52; −0.10) ng/mL, p-value = 0.038 and −0.29 (−0.52; −0.07) ng/mL, p-value = 0.023, respectively; p = 0.099 for trend). Similar results were found for sVCAM-1, as participants in T3 exhibited lower concentrations of the inflammatory biomarker compared to T1 (−0.31 (−0.55; −0.06) ng/mL, p-value = 0.025; p = 0.035 for trend). No other significant association was found between tertiles of change in urinary tartaric acid and changes in inflammatory biomarkers in the fully adjusted model.

4. Discussion

In this longitudinal sub-analysis within the PREDIMED trial, we assessed the anti-inflammatory potential of wine by measuring tartaric acid excretion in urine in a population at high risk for CVD. We observed an inverse association between changes in tartaric acid excretion in urine and changes in the plasma inflammatory molecules sVCAM-1 and sICAM-1.

Tartaric acid stands as the primary acid found within grapes, and during the harvest, grape juice holds between 4–8 g/L of this acid. This acid content contributes to the grapes' lower pH level and provides pleasant organoleptic characteristics to wine [30]. Intriguingly, tartaric acid is synthesized in only a limited number of plant species [31], making it a valuable compound as a biomarker for grape consumption and its derivatives, such as wine [20,32]. Our study demonstrates that urinary excretion of tartaric acid serves as a valid and reliable biomarker for assessing wine consumption, better than data obtained using validated food-frequency questionnaires. This holds substantial practical significance because assessing wine and other alcoholic beverages through food frequency questionnaires may introduce errors stemming from societal perceptions of alcohol consumption [19], and underreporting of at-risk

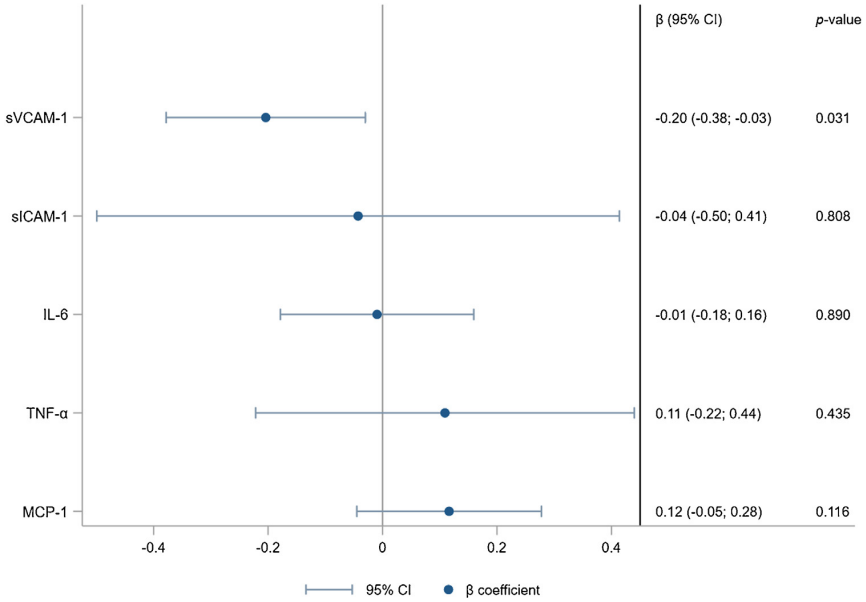

Fig. 2. Multivariable adjusted regression between 1-year changes in urinary tartaric acid and inflammatory molecules (per 1-SD increment) adjusted for age, sex, physical activity, smoking habit, waist-to-height ratio, intervention group, diabetes, hypercholesterolemia, hypertension, and intake of aspirin and NSAIDs, total energy intake, MedDiet adherence (not considering wine), grapes, and raisins. All analyses were conducted with robust estimates of the variance to correct for intracluster correlation. sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; IL6, interleukine-6; TNF-α, tumor necrosis factor- α; MCP-1, monocyte chemoattractant protein-1.

drinkers could distort the benefits of moderate drinking [33]. Apart from alcohol-related concerns, food frequency questionnaires and food records inherently entail subjectivity and the potential for inaccurate reporting, as they depend on individuals' perceptions [34]. Therefore, quantifying wine consumption through tartaric acid provides more precise and objective results.

The health effects of moderate wine consumption have been widely studied. Mendelian randomization studies suggested that the risk of CVD is decreased with a reduction of alcohol, even though the type of alcoholic beverage was not considered [35,36]. However, wine consumed during meals in the context of a MedDiet has been associated with cardioprotective effects [37], whereas these positive effects disappear with heavy episodic drinking [38]. In fact, a meta-analysis demonstrated that there is a J-shape relationship between wine consumption and vascular events and cardiovascular mortality [39]. Epidemiological studies have indicated that wine consumption is linked to a lower prevalence of metabolic syndrome and its components, including higher HDL-cholesterol, reduced lipid oxidative stress, and lower BMI [17,40,41]. In addition to its impact on the lipid profile, wine might engage other mechanisms related to inflammation that could explain its protective effects on cardiovascular health. Clinical trials provide supporting evidence for this hypothesis. Several studies have reported improvements in C-reactive protein (CRP) levels following wine consumption [42–44]. Another clinical trial involving participants with diabetes revealed a significant reduction in proinflammatory cytokines, including TNF-α, IL-6, IL-18, and CRP, after one year of intervention with moderate red wine consumption compared to the control group [43]. Other studies have documented an increase in the adipokines leptin and

Table 3
Multivariable-adjusted associations between 1-year changes in urinary tartaric acid (in tertiles) and changes in circulating inflammatory biomarkers (per 1-SD).

		T1 *vs.* T2 β (95% CI per 1SD)	*p*-Value	T1 *vs.* T3 β (95% CI)	*p*-Value	*p*-Trend
sVCAM-1 (ng/mL) n = 85	*Model 1*	−0.01 (−0.44; 0.42)	0.955	0.03 (−0.65; 0.71)	0.914	0.917
	Model 2	−0.03 (−0.46; 0.39)	0.844	−0.31 (−0.55; −0.06)	0.025	0.035
sICAM-1 (ng/mL) n = 104	*Model 1*	−0.10 (−0.59; 0.39)	0.597	−-0.05 (−0.66; 0.56)	0.825	0.861
	Model 2	−0.31 (−0.52; −0.10)	0.014	−0.29 (−0.52; −0.07)	0.023	0.099
IL-6 (pg/mL) n = 161	*Model 1*	0.07 (−0.10; 0.24)	0.311	0.04 (−0.26; 0.33)	0.755	0.761
	Model 2	0.02 (−0.16; 0.20)	0.779	−0.05 (−0.32; 0.21)	0.618	0.598
TNF-α (pg/mL) n = 110	*Model 1*	0.04 (−0.15; 0.23)	0.608	0.29 (−0.31; 0.89)	0.267	0.274
	Model 2	0.09 (−0.20; 0.37)	0.469	0.33 (−0.35; 1.01)	0.263	0.269
MCP-1 (pg/mL) n = 168	*Model 1*	−0.03 (−0.56; 0.51)	0.901	0.33 (0.01; 0.66)	0.045	0.059
	Model 2	−0.04 (−0.50; 0.43)	0.844	0.36 (−0.19; 0.92)	0.142	0.141

sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; IL6, interleukine-6; TNF-α, tumor necrosis factor- α; MCP-1, monocyte chemoattractant protein-1.
β, standardized regression coefficient; CI, confidence interval.
Model 1: sex, age. Model 2: model 1 + physical activity, smoking habit, waist-to-height ratio, intervention group, diabetes, hypercholesterolemia, hypertension, and intake of aspirin and NSAIDs, total energy intake, MedDiet adherence (not considering wine), grapes, and raisins.
All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.
$p < 0.05$ were considered significant.

adiponectin [45–47]. Concerning adhesion molecules VCAM-1 and ICAM-1, evidence suggests that they can decrease after 3 or 4 weeks of wine consumption [18,44]. These findings align with our results, as we identified an inverse association between changes in urinary tartaric acid and plasma sVCAM-1 and sICAM-1 levels after one year of follow-up. ICAM-1 and VCAM-1 function as signalling molecules that promote cell recruitment to the arterial wall, and their circulating levels reflect the degree of endothelial activation caused by CVD risk factors. Indeed, they are considered valuable predictive markers of atherosclerosis [48].

The anti-inflammatory effects of wine consumption could be partially due to its phenolic content. Red wine is a beverage rich in polyphenols such as phenolic acids, flavanols, flavonols, anthocyanins, and resveratrol [49]. *In vivo* studies have demonstrated that red wine polyphenols may have antiatherogenic properties, as they reduce the levels of the adhesion molecules ICAM-1 and VCAM-1 [15,16,50,51]. In humans, polyphenols have exhibited anti-inflammatory effects. In the PREDIMED trial, polyphenol intake was associated with decreased inflammatory biomarkers, including those related to atherosclerosis [25], and urinary polyphenols were shown to be a biomarker of anti-inflammatory diets [52,53]. Polyphenols were also linked to lower plasmatic biomarkers in healthy men, although no changes were observed in cellular adhesion molecules [53]. In addition to the non-alcoholic compounds in wine, the beneficial effects could also be attributed to ethanol, although evidence has yielded inconclusive results. A preclinical study conducted in rats observed that red wine, but not alcohol alone, reduced vascular cell adhesion molecules, oxidative stress, and improved the balance in adipocytokines [51] In humans, Chiva-Blanch et al. found that red wine, dealcoholized red wine, and gin reduced the cellular adhesion molecules, suggesting that the effects could be attributed to both the phenolic compounds and ethanol [54]. A study that combined cross-sectional data of three cohorts and included more than 40,000 participants showed that moderate alcohol intake from different alcoholic beverages was associated with better profiles of inflammatory markers and blood lipids. However, the associations were stronger when alcohol was consumed from wine [55]. On the other hand, Estruch et al. noted a decrease in fibrinogen and interleukin-1 alpha after the wine and gin interventions, but CRP and endothelial adhesion molecules were only reduced with wine [56]. Interestingly, a study comparing red and white wine—both with equal ethanol content but red wine containing higher polyphenol levels—revealed a distinct response in cellular adhesion molecules. While ICAM-1 decreased similarly with both red and white wine, VCAM-1 and E-selectin showed reductions only in response to red wine [44]. In summary, it appears that polyphenols provide wine with an additional anti-inflammatory effect that ethanol alone does not possess.

It is important to emphasize that the advantages of wine consumption are confined to moderate consumption, defined as fewer than 1–2 drinks/day [57]. This aligns with the Mediterranean dietary pattern, where red wine is predominantly enjoyed alongside main meals [22]. Furthermore, it is important to establish different recommendations for wine and alcohol consumption depending on age. There is strong evidence supporting the minimization of alcohol consumption in young individuals because the risks associated with its consumption outweigh the benefits. However, in individuals aged 40 or older, the relative risk of alcohol consumption follows a J-shaped curve, leading us to recommend moderate consumption [58]. Our study's findings are situated within this framework, as participants were older individuals and their consumption fell within these guidelines and showcased adherence to the MedDiet.

The main strength of our study is the application of a biological biomarker, tartaric acid, to evaluate wine consumption. This approach contrasts with less reliable techniques such as food-frequency questionnaires or self-reported surveys. Additionally, the study integrated the use of inflammatory biomarkers, providing a more accurate reflection of participant' status. Furthermore, the study employed a longitudinal design, enabling us to conduct a prospective analysis.

Nonetheless, our study has limitations such as a relatively small sample size and its focus on an older population with a high risk of cardiovascular disease. Furthermore, 24-h urine was not collected due to the study design, which is considered more reliable than spot urine. However, results were adjusted for creatinine levels to account for this variability. In addition, we only examined inflammatory biomarkers most closely associated with atherosclerotic plaques development and did not assess other molecules involved in inflammatory pathways.

In conclusion, our findings support the notion that urinary tartaric acid, identified as a biomarker of wine consumption measured by food-frequency questionnaires, is associated with anti-inflammatory properties related to atherosclerosis in older individuals. Further studies are needed to elucidate the molecular mechanisms behind these associations.

CRediT authorship contribution statement

I.D.L: Conceptualization, Methodology, and Writing – Original Draft. **C.A.R.:** Methodology; **R.C.:** Methodology and Writing – Review & Editing; **P.G.:** Methodology. **M.P.:** Writing – Review & Editing. **M.Á.M.G.:** Writing – Review & Editing. **M.F.:** Writing – Review & Editing; **E.R.:** Writing – Review & Editing. **R.E.:** Conceptualization and Writing – Review & Editing. **R.M.L.R.:** Conceptualization and Writing – Review & Editing.

Data sharing

There are restrictions on the availability of data for the PREDIMED trial, due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following the project purposes. Therefore, data will only be available upon request.

Data described in the manuscript, code book, and analytic code will be made available upon request pending and approval to the PREDIMED trial Steering Committee, only to external researchers for studies following the project purposes.

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Conflicts of interest

E.R. reports grants, personal fees, non-financial support and other from the California Walnut Commission while the study was carried out; grants, personal fees, non-financial support and other from Alexion; and personal fees and other from Amarin, outside the submitted work. R.M.L.-R. reports personal fees from Cerveceros de España, UNIDECO, personal fees, and other from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. R.E. reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain), and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Albuquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain), and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jnha.2023.100003.

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3.3. Cardiovascular disease and associated risk factors

Numerous studies have reported the beneficial effects of wine consumption on CVD and its associated risk factors due to its high content in phenolic compounds. However, there is a gap in knowledge regarding benefits on populations at high risk of CVD, such as postmenopausal women, which is the focus of this specific objective (Publication 9). In addition, a study was carried out in a larger cohort of men and women to identify the phenolic compounds present in urine that could serve as predictors of T2D (Publication 10). Due to the high metabolism of polyphenols by the gut microbiota, the last part of this objective focused on the analysis of MPM related to high adherence to the MedDiet and their association with cardiovascular health (Publication 11).

3.3.1. Publication 9

Urinary tartaric acid, a biomarker of wine intake, correlates with lower total and LDL cholesterol

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Nutrients. 2021. https://doi.org/10.3390/nu13082883

Abstract

Aims: To evaluate the association of urinary tartaric acid, a biomarker of wine consumption, with anthropometric (weight, waist circumference, BMI, and waist-to-height ratio), blood pressure, and biochemical variables (blood glucose and lipid profile) that may be affected during the menopausal transition.

Methods: This sub-study of the PREDIMED trial included a sample of 230 women aged 60-80 years with high CVD risk at baseline. Urine samples were diluted and filtered, and tartaric acid was analyzed by HPLC coupled to electrospray ionization tandem MS. Correlations between tartaric acid and the study variables were adjusted for age, education level, smoking status, physical activity, BMI, cholesterol-lowering, antihypertensive, and insulin treatment, total energy intake, and consumption of fruits, vegetables, and raisins.

Results: A strong association was observed between wine consumption and urinary tartaric acid. Total and LDL-c were inversely correlated with urinary tartaric acid, whereas other biochemical and anthropometric variables were unrelated.

Conclusions: The results suggest that wine consumption may have a positive effect on cardiovascular health in postmenopausal women, underpinning its nutraceutical properties.

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MDPI

Article

Urinary Tartaric Acid, a Biomarker of Wine Intake, Correlates with Lower Total and LDL Cholesterol

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Abstract: Postmenopausal women are at higher risk of developing cardiovascular diseases due to changes in lipid profile and body fat, among others. The aim of this study was to evaluate the association of urinary tartaric acid, a biomarker of wine consumption, with anthropometric (weight, waist circumference, body mass index (BMI), and waist-to-height ratio), blood pressure, and biochemical variables (blood glucose and lipid profile) that may be affected during the menopausal transition. This sub-study of the PREDIMED (Prevención con Dieta Mediterránea) trial included a sample of 230 women aged 60–80 years with high cardiovascular risk at baseline. Urine samples were diluted and filtered, and tartaric acid was analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Correlations between tartaric acid and the study variables were adjusted for age, education level, smoking status, physical activity, BMI, cholesterol-lowering, antihypertensive, and insulin treatment, total energy intake, and consumption of fruits, vegetables, and raisins. A strong association was observed between wine consumption and urinary tartaric acid (0.01 µg/mg (95% confidence interval (CI): 0.01, 0.01), p-value < 0.001). Total and low-density lipoprotein (LDL) cholesterol were inversely correlated with urinary

tartaric acid (−3.13 µg/mg (−5.54, −0.71), *p*-value = 0.016 and −3.03 µg/mg (−5.62, −0.42), *p*-value = 0.027, respectively), whereas other biochemical and anthropometric variables were unrelated. The results suggest that wine consumption may have a positive effect on cardiovascular health in postmenopausal women, underpinning its nutraceutical properties.

Keywords: PREDIMED; Mediterranean diet; lipid profile; cardiovascular risk; polyphenols; menopause; body fat; biomarkers; tartaric acid

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide in both sexes. Nevertheless, important sex-specific differences exist. According to the American Heart Association, menopause is listed as a female-specific cardiovascular risk factor (CVRF) [1]. During the menopause transition women experience adverse changes in their lipid profile, body fat distribution, metabolic syndrome risk, and vascular health [2–5]. Previous studies suggested that menopause is associated with increased total and low-density lipoprotein (LDL) cholesterol [6] and changes in body composition such as increased fat mass and loss of lean mass [7]. Changes in blood pressure (BP), waist circumference (WC), body mass index (BMI), and blood glucose and insulin have not been specifically associated with menopause and appear to reflect chronological aging [5,6,8]. Therefore, menopause-induced increases in cholesterol, body fat, and possibly other CVRFs may accelerate the risk of developing CVD.

Diet and lifestyle can also affect the incidence of CVD. Modifiable factors, such as smoking cessation, healthy diet, and regular physical activity, play a crucial role in reducing cardiovascular risk [9]. The Mediterranean diet (MedDiet) has been associated with a better control of several CVRFs [10] through improvements in BP, lipid profile, glucose metabolism, arrhythmia risk, and gut microbiome [11,12]. One of the main characteristics of the MedDiet is the abundant consumption of olive oil, vegetables, fruits, nuts, legumes, fish, and cereals, and moderate wine consumption [13,14]. Epidemiologic studies and randomized clinical trials reported that moderate consumption of wine (1 or 2 glasses/day) during meals has been consistently associated with a lower risk of CVD [15–17]. In the context of a MedDiet, moderate alcohol consumption appears to be synergistic with other cardioprotective components of the MedDiet that increase high-density lipoprotein (HDL) cholesterol, decrease platelet aggregation, promote antioxidant effects, and reduce inflammation [13].

Wine consumption is mainly determined through dietary questionnaires. A biomarker of wine intake reflects its consumption more reliably than a questionnaire, since people may not accurately report the amount of alcohol consumed due to perceived social rejection of excessive consumption [18]. Tartaric acid, the main organic acid in wine and the molecule responsible for wine acidity, is present in high amounts in wine (1.5–4.0 g/L) but is rare in most common foods [19,20]. Urinary tartaric acid has been considered as a sensitive, selective, and robust biomarker of moderate wine intake [21,22]. Therefore, determining tartaric acid stands out as a useful tool to further study the impact of moderate wine drinking on health. The aim of this study was to determine the association between urinary tartaric acid as a biomarker of wine consumption and CVRFs in postmenopausal women at risk of developing CVD.

2. Materials and Methods

2.1. Study Design

This study is a cross-sectional analysis of baseline data from a subsample of participants in the PREDIMED (PREvención con DIeta MEDiterránea) study, a large, parallel-group, multicenter, randomized, controlled, 5-year clinical trial conducted between 2003 and 2009. The objective was to assess the effect of a Mediterranean diet supplemented with

extra-virgin olive oil or mixed nuts as compared to a low-fat diet on the primary prevention of CVD in 7447 participants at high cardiovascular risk. Eligible participants were men (55–80 years old) and women (60–80 years old) who had type 2 diabetes mellitus or at least 3 of the following major CVRFs: smoking, hypertension, elevated LDL cholesterol, low HDL cholesterol, overweight or obesity, and/or family history of premature coronary heart disease [23]. All participants provided written informed consent, and the study protocol and procedures complied the ethical standards of the Declaration of Helsinki.

For the present sub-study of the PREDIMED trial, urinary tartaric acid was analyzed in a subsample of women equivalent to 5% of the total female population of the PREDIMED study. The 230 women that were randomly selected had undergone the menopausal transition and their urine samples were available at baseline. Participants who had no available data of total energy intake or reported extreme values (>3500 kcal/day) were excluded from the analysis (n = 8).

2.2. Anthropometric, Dietary, and Physical Activity Assessments

Trained personal performed the anthropometric and clinical measurements (height, weight, WC, and BP). BMI was obtained by dividing the body weight in kilograms by the square of height in cm. The waist-to-height ratio (WtHR) was calculated by dividing the WC in centimeters by height in meters. Systolic (SBP) and diastolic blood pressure (DBP) were measured in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP, Lake Forest, IL, USA). A validated semi-quantitative food frequency questionnaire (FFQ), which included 137 food items [24], and the Minnesota Leisure-Time Physical Activity Questionnaire [25] were used to assess dietary habits over the previous 12 months and physical activity (metabolic equivalent tasks per minute per day, METs min/day) of the participants.

2.3. Clinical Measurements

Medical conditions, family history of disease, and risk factors were collected though a questionnaire during the first screening visit. Biological samples (plasma and urine) were collected at baseline after 12 h overnight fast and stored at −80 °C until assay. Blood glucose, total cholesterol, triglycerides, and HDL cholesterol were determined by standard enzymatic methods, and LDL cholesterol was calculated by the Friedewald equation [26].

2.4. Tartaric Acid Determination

2.4.1. Reagents and Standards

Formic acid (approximately 98%), picric acid (98%, moistened with approximately 33% water), and sodium hydroxide (≥98%) were obtained from Panreac. L-(+)-Tartaric acid and creatinine were purchased from Sigma. The labelled internal standard DL-(±)-tartaric-2,3-d2 acid was obtained from C/D/N Isotopes. Solvents were high-performance liquid chromatography grade, and all other chemicals were analytical reagent grade. Ultrapure water was obtained from a Milli-Q Gradient water purification system (Millipore, Bedford, MA, USA).

Stock solutions of tartaric acid were prepared in water. Working standard solutions that ranged from 0.01 to 5 μg/mL were made by appropriate dilution in 0.5% formic acid in water and then stored in amber glass vials at −20 °C.

2.4.2. Sample Preparation

Determination of urinary tartaric acid was performed following a previously validated stable-isotope dilution LC-ESI-MS/MS method by our research group [27]. Briefly, urine samples (20 μL) were diluted 1:50 (v:v) with 0.5% formic acid in water, and 10 μL of a deuterated isotope standard solution in water (DL-(±)-tartaric-2,3-d2 acid, 200 μg/mL) were added. The sample dilution was passed through a 0.20 μm filter and analyzed by LC–ESI-MS/MS. Urinary tartaric acid data were corrected by urine creatinine, measured according to the adapted Jaffé alkaline picrate method for thermo microtiter 96-well plates,

according to Medina-Remón et al. [28]. Finally, urinary tartaric acid was expressed as μg of tartaric acid per mg of creatinine. According to previous data, the cut-off of 8.84 μg/mg creatinine was used to discriminate daily consumers and non-consumers of wine [21].

2.4.3. LC–ESI-MS/MS Analysis

After filtration, tartaric acid was analyzed using an Atlantis TE C18, 100 mm × 2.1 mm, 3 μm (Waters, Milford, MA, USA) reversed-phase column coupled for detection to the triple quadrupole mass spectrometer API 3000 (Applied Biosystems, Foster City, CA, USA). The mass spectrometer was operated in negative electrospray ionization mode. The column was maintained at 25 °C throughout the analysis. Mobile phases A and B were 0.5% formic acid in water and 0.5% formic acid in acetonitrile, respectively. The following linear gradient was used: holding at 100%A for 3.5 min, decrease to 10%A over 2 min and holding for 2 min, return to initial conditions for 1.5 min, and re-equilibration for 6 min. The flow rate was set at 350 μL/min and the injection volume was 10 μL. Post-column addition of acetonitrile (250 μL/min) was carried out to improve analyte ionization efficiency. The detection was accomplished in multiple reaction monitoring (MRM) mode, and the following MS/MS transitions were used for quantification and confirmation, respectively: m/z 149/87 and m/z 149/73 for tartaric acid, and m/z 151/88 and m/z 151/74 for the deuterated isotope.

2.5. Statistical Analyses

The baseline characteristics of participants are presented as means and standard deviations (SD) for continuous variables, and frequency (*n*) and percentage (%) for categorical variables.

The normality of continuous variables was assessed with the Shapiro–Wilk test. The variables without normal distribution were transformed into logarithms. Multiple adjusted linear regression models were used to assess the differences between urinary tartaric acid and wine consumption as well as anthropometric and biochemical measurements. Three different adjustment models were applied. Model 1 was minimally adjusted for age (continuous). Model 2 was additionally adjusted for educational level, smoking status, BMI (except for anthropometric criteria), physical activity, and cholesterol-lowering, antihypertensive, and insulin treatment. Model 3 was further adjusted for total energy intake and consumption of fruits, vegetables, and raisins. We used robust variance estimators to account for the recruitment center in all linear models. To illustrate the relationship between wine consumption and urinary tartaric acid, the mL per month of wine reported in the FFQ were transformed into glasses of wine (with 1 glass equivalent to 100 mL).

Values are shown as 95% confidence interval (CI) and significance for all statistical tests was based on bilateral contrast set at $p < 0.05$. All the statistical analyses were performed using Stata statistical software package version 16.0 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. Study Population

The main characteristics of the PREDIMED participants who were included in this sub study are summarized in Table 1. The mean age of the women was 66.9 + 0.4 years. Their burden of CVRFs was high: 42.1% had been diagnosed with type 2 diabetes, 87.3% with hypertension, and 76.5% with hypercholesterolemia. Among the drug treatments, statins were the most common medication, with 40.72% of them under treatment. Furthermore, 9.1% of the participants were current smokers. Finally, 82% of the participants had a low educational level.

Up to 45.7% of the participants reported wine consumption in the FFQ. The mean concentration of tartaric acid in urine was 28.34 μg/mg creatinine, and 40.4% were considered daily consumers of wine.

Table 1. Baseline characteristics of the women in the study population (n = 222).

General Characteristics	
Age, years	66.9 ± 0.4
Type 2 diabetes, *n* (%)	93 (42.08)
Hypertension, *n* (%)	193 (87.34)
Hypercholesterolemia, *n* (%)	169 (76.55)
Medication use, *n* (%)	
ACE inhibitors	64 (28.96)
Diuretics	58 (26.24)
Statins	90 (40.72)
Other lipid-lowering agents	14 (6.33)
Insulin	10 (4.52)
Oral hypoglycemic agents	53 (23.98)
Antiplatelet therapy	46 (20.81)
Current smoker, *n* (%)	20 (9.05)
Leisure-time physical activity, MET·min/week	186.5 ± 10.9
Educational level, *n* (%)	
Low	182 (82.35)
Medium	24 (10.86)
High	15 (6.79)
Wine consumption, *n* (%)	101 (45.70)
Urinary tartaric acid, µg/mg creatinine	28.47 + 4.03
Daily wine consumers, *n* (%)	81 (40.4)
Anthropometric measurements, mean + SD	
Weight, kg	72.9 ± 0.7
BMI, kg/m^2	30.3 ± 0.28
WC, cm	97.5 ± 0.7
WtHR	63.0 ± 0.5
Biochemical measurements, mean + SD	
Total cholesterol, mg/dL	221.0 ± 2.7
LDL cholesterol, mg/dL	136.5 ± 2.4
HDL cholesterol, mg/dL	57.7 ± 1.0
Triglycerides, mg/dL	134.0 ± 5.2
Glucose, mg/dL	118.1 ± 2.4
Blood pressure, mean + SD	
Systolic, mm Hg	148.7 ± 1.2
Diastolic, mm Hg	83.8 ± 0.6
Dietary intake, mean + SD	
Total energy, kcal/day	2161 ± 33
Carbohydrate, % of energy	42.0 ± 0.5
Protein, % of energy	16.8 ± 0.2
Fat, % of energy	39.8 ± 0.5

ACE: angiotensin-converting enzyme; MET: metabolic equivalent task; BMI: body mass index; WC: waist circumference; WtHR: waist-to-height ratio; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol. Data are expressed as the mean ± standard deviations (SD) for continuous variables and frequency (*n*) and percentage (%) for categorical variables.

The mean values of anthropometric measurements revealed that most participants were obese, as defined by their BMI, WC, and WtHR data [29] according to the International Diabetes Federation and the American Heart Association [30]. Regarding biochemical measurements, triglycerides and HDL cholesterol were at desirables levels, while total cholesterol, LDL cholesterol and glucose were borderline high [31,32].

The mean energy intake was 2161 kcal/day, of which carbohydrates accounted for 42.0% of the energy consumed, protein intake 16.8%, and fat intake 39.8%.

3.2. Tartaric Acid as a Biomarker of Wine Consumption

After adjustments for several covariates (age, education level, smoking status, physical activity, BMI, cholesterol-lowering, antihypertensive, and insulin treatment, total energy intake, and consumption of fruits, vegetables, and raisins), women who consumed more wine presented higher concentrations of tartaric acid in urine (0.01 μg/mg (95% CI: 0.01, 0.01), *p*-value < 0.001). Figure 1 illustrates the linear relationship between urinary tartaric acid concentrations and wine consumption expressed as glasses of wine, excluding those who reported not consuming wine.

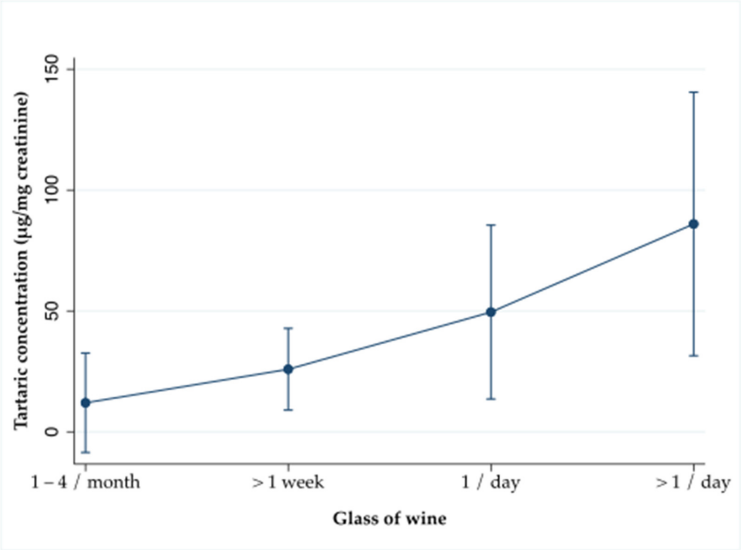

Figure 1. Relationship between urinary tartaric acid concentrations in urine and wine consumption expressed as glasses of wine.

3.3. Anthropometric Measurements and Urinary Tartaric Acid

After adjustment for several covariates, we observed no association between urinary tartaric acid and BMI, WC, weight, WtHR, and systolic or diastolic BP (Table 2).

Table 2. Association between anthropometric variables and urinary tartaric acid (μg/mg creatinine).

		β (95% IC)	*p*-Value
BMI, kg/m^2	Model 1	<−0.01 (−0.01, 0.01)	0.519
	Model 2	<−0.01 (−0.01, 0.01)	0.426
	Model 3	<−0.01 (−0.01, 0.01)	0.973
WC, cm	Model 1	0.56 (−0.25, 1.38)	0.173
	Model 2	0.61 (−0.04, 1.26)	0.064
	Model 3	0.70 (−0.12, 1.51)	0.087
Weight, kg	Model 1	<−0.01 (−0.01, 0.01)	0.770
	Model 2	<−0.01 (−0.01, 0.01)	0.766
	Model 3	<0.01 (−0.01, 0.01)	0.886
WtHR	Model 1	0.29 (−0.25, 0.82)	0.291
	Model 2	0.32 (−0.17, 0.81)	0.175
	Model 3	0.41 (−0.14, 0.96)	0.128

BMI: body mass index; WC: waist circumference; WtHR: waist-to-height ratio; CI: confidence interval. Regression coefficients (95%CI) were obtained from multivariable adjusted linear regression models. β: Non-standardized coefficient. Model 1: adjusted for age. Model 2: adjusted for age, educational level, smoking status, physical activity, and cholesterol-lowering, antihypertensive, and insulin treatment. Model 3: adjusted for age, educational level, smoking status, physical activity, cholesterol-lowering, antihypertensive, and insulin treatment, total energy intake, and consumption of fruits, vegetables, and raisins. We used robust standard errors to account for recruitment center. *p*-values < 0.05 were considered significant.

3.4. Biochemical and Clinical Measurements and Urinary Tartaric Acid

A negative association was observed between urine tartaric acid and total and LDL cholesterol after full adjustment (−3.13 µg/mg (−5.54, −0.71), *p*-value = 0.016 and −3.03 µg/mg (−5.62, −0.42), *p*-value = 0.027, respectively). By contrast, no differences were observed for HDL with different concentrations of tartaric acid. Finally, no association was found between triglycerides and glucose and tartaric acid concentrations (Table 3).

Table 3. Association between biochemical variables and blood pressure and urinary tartaric acid (µg/mg creatinine).

		β (95% CI)	*p*-Value
Total cholesterol, mg/dL	Model 1	−3.32 (−6.53, −0.10)	0.043
	Model 2	−3.24 (−5.78, −0.72)	0.017
	Model 3	−3.13 (−5.54, −0.71)	0.016
LDL cholesterol, mg/dL	Model 1	−3.44 (−6.34, −0.53)	0.021
	Model 2	−3.43 (−5.86, −1.00)	0.010
	Model 3	−3.03 (−5.62, −0.42)	0.027
HDL cholesterol, mg/dL	Model 1	<−0.01 (−0.02, 0.02)	0.689
	Model 2	−0.01 (−0.03, 0.01)	0.220
	Model 3	<−0.01 (−0.02, 0.01)	0.633
Triglycerides, mg/dL	Model 1	0.01 (−0.04, 0.05)	0.739
	Model 2	0.02 (−0.03, 0.07)	0.422
	Model 3	0.02 (−0.04, 0.07)	0.525
Glucose, mg/dL	Model 1	0.02 (<−0.01, 0.04)	0.092
	Model 2	0.03 (−0.01, 0.07)	0.119
	Model 3	0.02 (−0.01, 0.06)	0.180
Systolic BP, mmHg	Model 1	−0.34 (−1.79, 1.11)	0.647
	Model 2	−0.09 (−1.53, 1.71)	0.904
	Model 3	0.36 (−1.27, 1.99)	0.633
Diastolic BP, mmHg	Model 1	0.05 (−0.71, 0.81)	0.893
	Model 2	0.24 (−0.47, 0.95)	0.472
	Model 3	0.21 (−0.47, 0.89)	0.502

BP: blood pressure; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol; CI: confidence interval. Regression coefficients (95%CI) were obtained from multivariable adjusted linear regression models. β: Non-standardized coefficient. Model 1: adjusted for age. Model 2: adjusted for age, educational level, smoking status, physical activity, BMI, and cholesterol-lowering, antihypertensive, and insulin treatment. Model 3: adjusted for age, educational level, smoking status, physical activity, cholesterol-lowering, antihypertensive, and insulin treatment, total energy intake, and consumption of fruits, vegetables, and raisins. We used robust standard errors to account for recruitment center. *p*-values < 0.05 were considered significant.

4. Discussion

In this sub-analysis of a subset of postmenopausal women participating in the PREDIMED trial, urinary tartaric acid concentrations as an objective biomarker of wine intake were significantly associated with lower concentrations of total and LDL cholesterol. No associations with anthropometric variables or blood pressure were observed. To the best of our knowledge, the current study is the first to evaluate wine consumption based on a biomarker in a postmenopausal population at increased risk of developing CVD.

Wine consumption has been widely studied due to its beneficial effects on cardiovascular and metabolic health [10]. However, most studies have evaluated wine intake using FFQs or self-questionnaires instead of biological biomarkers, a more reliable and objective way of assessing dietary habits [33]. It has been previously demonstrated that urinary tartaric acid is a specific and sensitive biomarker, as its major sources in the diet are grapes and wine [21,34]. Accordingly, we observed a positive association between wine consumption reported in the FFQs and the concentrations of tartaric acid present in urine. Other phenolic compounds, such as resveratrol and its metabolites, have been proposed as wine biomarkers. However, the resveratrol content in wine is subject to a high

variability and its metabolism shows interindividual differences [35]. Thus, selectivity and high correlation with reported intakes make tartaric acid a reliable dietary biomarker of wine consumption.

Different studies have evaluated how alcohol intake affects different parameters of body composition. A cross-sectional study in French adults suggested an inverse association in women of wine intake 100 g/day with BMI and WtHR [36]. Tresserra-Rimbau et al. analyzed the effects of red wine consumption on the prevalence of metabolic syndrome and its components, and found a negative association between moderate red wine consumption and BMI [16]. Tolstrup et al. also described inverse associations between alcohol consumption and WC in women [37], while other studies found no relationship between alcohol consumption and body weight in women [38,39]. The mentioned literature indicates that moderate consumption of wine, an alcoholic beverage that contributes to energy intake, is not related to weight gain or detrimental changes in body composition. Our study supports this notion, as we did not observe any differences in BMI, weight, WC, and WtHR with increasing wine consumption.

Evaluating the effect of alcohol, and specifically wine, on the risk of developing CVD in women is important due to the increase in cardiovascular risk after menopause. Among CVRFs, a recent metanalysis reported that triglycerides, total cholesterol, LDL cholesterol, and the total cholesterol-to-HDL-cholesterol ratio were significantly higher in postmenopausal women compared to premenopausal women, and suggested that age was partly responsible for the differences in lipid levels [40]. We found that women with higher concentrations of tartaric acid presented lower total and LDL cholesterol. Similarly to our results, Rifler et al. reported that after 2 weeks of drinking 250 mL of red wine daily, patients post myocardial infarction presented a 5% decrease in total and LDL cholesterol [41]. Furthermore, Taborsky et al. evaluated the effect of 1 year of wine consumption, and observed a reduction in total and LDL cholesterol [42]. In another clinical trial, authors reported a similar beneficial effect on the lipid profile after consumption of red wine in asymptomatic hypercholesterolemic individuals [43]. The data are almost consistent in showing that wine consumption reduces LDL cholesterol while increasing HDL cholesterol [44,45]. Moderate consumption of alcohol has been associated with higher concentrations of HDL cholesterol and diminished lipid oxidation stress [46]. Resveratrol metabolites in urine, as biomarkers of wine consumption, were significantly associated with lower triglycerides and higher HDL-cholesterol [47]. However, we were unable to confirm that higher urinary tartaric acid as a biomarker of wine consumption was associated with raised HDL cholesterol levels. A probable reason is that the women studied had rather high baseline HDL cholesterol levels, making it more difficult to further increase these with interventions.

Many clinical studies support that light to moderate alcohol consumption, in particular of red wine, is associated with lower CVD rates and an improved lipid profile and inflammatory system [17,48]. However, it remains unknown whether this effect of wine is due to alcohol per se, the phytochemicals of wine, their combined effect, or even the time of drinking, since postprandial oxidative stress after a meal appears to be counteracted by the ingestion of red wine [49]. In this sense, it has been found that wine micro-constituents modulate inflammatory mediators and therefore may be responsible for attenuating postprandial inflammation [50]. In addition, they protect against the effect of ethanol on cytokine secretion, which are involved in inflammatory processes [51]. In support of this view, a randomized clinical trial reported that wine bioactive compounds, such as resveratrol, can decrease total cholesterol by reducing mRNA expression of hepatic 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, in addition to the increased activation of the sirtuin system in all tissues [52]. Experimental work in cell cultures and animal models has shown that the enhancement of Sirtuin 1 can lead to better metabolic profiles and anti-inflammatory activities, as well as increased reverse cholesterol transport [53]. Overall, evidence supports that wine micro-constituents play a crucial role in the protective effect of wine on cardiovascular health by exerting anti-inflammatory actions.

The main strength of this study is that it used a biological biomarker, tartaric acid, to evaluate wine consumption, instead of less reliable methods such as FFQs or self-reported questionnaires. Moreover, it involved baseline data of participants in the PREDIMED trial; therefore, the results reflect real-life conditions. The main limitations were the modest sample size and the impossibility of determining causality due to the cross-sectional design.

5. Conclusions

The findings from the current cross-sectional study support the notion that wine intake has beneficial nutraceutical effects on the cardiovascular health of postmenopausal women, as its biomarker tartaric acid was associated with lower total and LDL cholesterol concentrations. Randomized trials are needed to confirm these results and determine the impact of wine consumption on cardiovascular health in a sensitive population such as that of postmenopausal women.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centers. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent was obtained from the patients.

Data Availability Statement: There are restrictions on the availability of data for the PREDIMED trial due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following project purposes. Requestors wishing to access the PREDIMED-Plus trial data used in this study can make a request to the PREDIMED trial Steering Committee chair: restruch@clinic.cat. The request will then be passed to members of the PREDIMED Steering Committee for deliberation.

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Abbreviations

Angiotensin-converting enzyme (ACE); blood pressure (BP); body mass index (BMI); confidence interval (CI); cardiovascular disease (CVD); cardiovascular risk factors (CVRFs); food frequency questionnaire (FFQ); high-density lipoprotein (HDL); liquid chromatography with electrospray ionization and tandem mass spectrometry (LC-ESI-MS/MS); low-density lipoprotein (LDL); Mediterranean diet (MedDiet); Prevención con Dieta Mediterránea (PREDIMED); standard deviation (SD); waist circumference (WC); waist to height ratio (WtHR).

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3.3.2. Publication 10

Urinary metabolomics of phenolic compounds reveals biomarkers of type-2 diabetes within the PREDIMED trial

Inés Domínguez-López, Julián Lozano-Castellón, Anna Vallverdú-Queralt, Olga Jáuregui, Miguel Ángel Martínez-González, Frank B Hu, Montserrat Fitó, Emilio Ros, Ramon Estruch, and Rosa M Lamuela-Raventós

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Supplementary Material available in Annex section.

Abstract

Aims: We used a metabolomics approach to determine urinary phenolic metabolites associated with T2D and fasting plasma glucose.

Methods: This case-control study within the PREDIMED trial included 200 participants at high CVD risk, 102 of whom were diagnosed with T2D. A panel of urinary phenolic compounds were analysed using a novel method based on HPLC coupled to MS. Multivariate statistics and adjusted logistic regressions were applied to determine the most discriminant compounds and their association with T2D. The relationship between discriminant phenolic compounds and plasma glucose was assessed using multivariable linear regressions.

Results: A total of 41 phenolic compounds were modeled in the orthogonal projection to latent structures discriminant analysis, and after applying adjusted logistic regressions two were selected as discriminant: dihydrocaffeic acid and genistein diglucuronide. Both metabolites were associated with a lower risk of suffering from T2D, but only dihydrocaffeic acid was inversely associated with plasma glucose.

Conclusions: A novel method using a metabolomics approach was developed to analyse a panel of urinary phenolic compounds for potential associations with T2D, and two metabolites, dihydrocaffeic acid and genistein diglucuronide, were found to be associated with a lower risk of this condition.

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Urinary metabolomics of phenolic compounds reveals biomarkers of type-2 diabetes within the PREDIMED trial

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ABSTRACT

Background: Phenolic compounds have been associated with protective effects against type-2 diabetes (T2D). We used a metabolomics approach to determine urinary phenolic metabolites associated with T2D and fasting plasma glucose.

Methods: This case-control study within the PREDIMED trial included 200 participants at high cardiovascular risk, 102 of whom were diagnosed with T2D. A panel of urinary phenolic compounds were analysed using a novel method based on liquid chromatography coupled to mass spectrometry. Multivariate statistics and adjusted logistic regressions were applied to determine the most discriminant compounds and their association with T2D. The relationship between the discriminant phenolic compounds and plasma glucose was assessed using multivariable linear regressions.

Results: A total of 41 phenolic compounds were modeled in the orthogonal projection to latent structures discriminant analysis, and after applying adjusted logistic regressions two were selected as discriminant: dihydrocaffeic acid (OR = 0.22 (CI 95 %: 0.09; 0.52) per 1-SD, *p*-value = 0.021) and genistein diglucuronide (OR = 0.72 (CI 95%: 0.59; 0.88) per 1-SD, *p*-value = 0.021). Both metabolites were associated with a lower risk of suffering from T2D, but only dihydrocaffeic acid was inversely associated with plasma glucose (β = −17.12 (95 % CI: −29.92; −4.32) mg/dL per 1-SD, *p*-value = 0.009).

Conclusions: A novel method using a metabolomics approach was developed to analyse a panel of urinary phenolic compounds for potential associations with T2D, and two metabolites, dihydrocaffeic acid and genistein diglucuronide, were found to be associated with a lower risk of this condition.

Abbreviations: , AMPK1, Adenosine monophosphate-activated protein kinase; , AUC, area under the curve; , CV, cross-validation; , FDR, false discovery rate; , FC, fold change; , LTQ-Orbitrap-HRMS, linear ion trap quadrupole-Orbitrap-high-resolution mass spectrometry; , MET, metabolic equivalent for task; , OPLS-DA, orthogonal projection to latent structures discriminant analysis; , PPARy, peroxisome proliferator-activated receptor y; , ROC, receiver operator characteristic; , SIRT1, sirtuin 1; , SPE, solid-phase extraction; , T2D, type-2 diabetes; , VIP, variable importance in projection.

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1. Introduction

Polyphenols are secondary metabolites with a wide diversity of chemical structures. The intake of polyphenols can be estimated through dietary questionnaires and food-composition tables [1]. Despite recent improvements, these methods have limitations due to the variability of the phenolic content in foods, which can be modified by processing, cooking, plant variety, or season of harvest [2–4]. In addition, the bioavailability of polyphenols varies depending on the food with which they are ingested [5]. Thus, the most reliable way to measure exposure to dietary polyphenols is by analysing biological samples, especially urine, as polyphenols and their metabolites are largely excreted in urine 24–48 h after ingestion [6,7]. Individual polyphenols measured through food frequency questionnaires and analysis of biological samples have been linked with a lower risk of type-2 diabetes (T2D) [8]. The Mediterranean Diet, which is rich in plant-based foods and characterized by a high phenolic intake, has also been associated with a lower T2D incidence [9,10]. In recent years, research on T2D prevention and control has grown in response to the increasing global prevalence of the disease and associated mortality rate, with 700 million people predicted to be affected by 2045 [11].

Metabolomics has emerged as a tool for the identification of disease biomarkers, allowing earlier detection and improved monitoring, which will enable the development of personalized treatment plans for patients [12]. For example, in 2022, Larkin et al. detected biomarkers of cancer in blood samples of patients with nonspecific symptoms [13]. However, metabolomics-based research still faces challenges, such as the standardization of methods across laboratories, finding a consensus on data interpretation, and the promotion of data-sharing [14].

Metabolomics has also been applied to identify biomarkers related to T2D and its risk factors. Most of this research has focused on different lipid classes, such as ceramides or sphingolipids, bile acids, and amino acids, especially branched-chain and aromatic amino acids [15,16]. However, the relationship of polyphenols and their metabolites with T2D has been minimally explored using metabolomics. The application of this approach could shed light on polyphenols involved in the pathways leading to T2D, as well as their effect on the molecular mechanisms underlying the development of the disease. Ultimately, a greater understanding of the pathogenesis of T2D will facilitate the implementation of new prevention strategies.

Therefore, the aim of the present work was to develop a metabolomics-based method to analyse a panel of urinary phenolic compounds for potential associations with T2D in participants of the PREDIMED (PREvención con DIeta MEDiterránea) trial with and without T2D at baseline. To this end, we employed high precision analytical techniques based on linear ion trap quadrupole-Orbitrap-high-resolution mass spectrometry (LTQ-Orbitrap-HRMS), which allowed us to identify a great variety of unknown phenolic compounds. The most discriminant compounds related to T2D were then identified with multivariate statistics, which enabled us to simultaneously assess a large number of phenolic compounds. Finally, we investigated the associations of the selected individual compounds with T2D and fasting plasma glucose levels using multivariable-adjusted regressions.

2. Materials and methods

2.1. Study design

The present work is a case-control sub-study using baseline data of the PREDIMED trial, a multicentre, parallel-group, randomized, controlled trial conducted in Spain from 2003 to 2010. The methods and design of this study have been described in detail elsewhere [17,18]. Its main aim was to assess the effect of a Mediterranean diet enriched with olive oil or nuts on the primary prevention of cardiovascular disease. It included 7447 participants aged 55–80 years at high cardiovascular risk who had T2D or at least three of the following major risk factors: current smoking, hypertension, dyslipidaemia, overweight/obesity or a family history of premature cardiovascular disease. To carry out the study, 200 participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) were randomly selected, 102 of whom were diagnosed with T2D. Participants who reported extreme total energy intakes (>3500 or <500 kcal/day in women or >4000 or <800 kcal/day in men) were excluded from the analysis.

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.2. Covariate assessment

Trained dietitians completed a semi-quantitative 137-item food frequency questionnaire in interviews with participants, as well as a 14-item questionnaire to assess their adherence to the Mediterranean diet [19]. Participants were considered to suffer from hypercholesterolemia or hypertension if they had a previous diagnosis and/or were under cholesterol-lowering or antihypertensive medication, respectively. Trained personnel measured body weight, height, waist circumference, and blood pressure. Body mass index (BMI) was calculated as weight in kg divided by height in m^2. Physical activity (metabolic equivalent tasks per minutes per day, METs min/day) was assessed with a validated Spanish version of the Minnesota physical activity questionnaire [20]. Plasma glucose, total cholesterol, triglycerides, and HDL cholesterol were determined by standard enzymatic methods, and LDL cholesterol was calculated by the Friedewald equation [18].

2.3. Ascertainment of type-2 diabetes

For the present analysis, the main endpoint was the prevalence of T2D, which was defined as previous clinical diagnosis of T2D, or glycated hemoglobin (HbA1c) $\geq$ 6.5%, or use of antidiabetic medication at baseline, or fasting plasma glucose > 126 mg/dl in both the screening visit and baseline visit.

2.4. Phenolic metabolic profiling

Biological samples were collected after an overnight fast, coded, and stored at − 80 ºC until analysis. Phenolic compounds were isolated using a method previously validated by our group with minor modifications [21]. Briefly, 50 μL urine samples were diluted 1:20 (v:v) with Milli-Q Water (Bedford, MA, USA), and 100 μL of the internal standard abscisic acid-d_6 (Santa Cruz Biotechnology, Santa Cruz, CA) was added. The sample dilution was acidified with 2 μL of formic acid (Panreac Química S.A., Barcelona, Spain) and centrifuged at 15,000 g at 4 °C for 4 min. The acidified urines underwent a solid-phase extraction (SPE) in Water Oasis HLB 96-well plates 30 μm (30 mg) (Water Oasis, Milford, MA, USA). First, the 96-well plate was activated with methanol (Sigma-Aldrich, St. Louis, MO, USA) and 1.5 M formic acid, and after loading the samples, a clean-up step was performed with 1.5 M formic acid and methanol (0.5 %). The phenolic compounds were then eluted with methanol acidified with 1.5 M formic acid, evaporated to dryness with nitrogen gas and reconstituted with 100 μL formic acid (0.05 %). After 20 min of vortex mixing, the samples were filtered through 0.22 μm polytetrafluoroethylene 96-well plate filters (Millipore, Massachusetts, USA).

The analysis was performed on an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an H-ESI source working in negative mode as described elsewhere [21]. Chromatographic separation was performed on a Kinetex F5 100 Å Column (50 × 4.6 mm, 2.6 μm) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were, respectively, 0.05 % formic acid in water

and 0.05 % formic acid in acetonitrile. The following linear gradient was used: held at 98%A for 1.7 min, decreased to 92%A for 3 min, decreased to 80%A for 1.3 min, decreased to 70%A for 1.3 min, decreased to 50 % for 0.1 min, decreased to 0 % for 1.3 min, then returned to initial conditions for 1.7 min and re-equilibrated for 3 min. The flow rate was set at 0.750 μL/min and the injection volume was 5 μL.

The collected UHPLC-HRMS data (. RAW file) were converted into. abf files using the Reifycs Abf Converter and then further processed using the software MS-DIAL (version 4.24) [22]. In this regard, automatic peak finding and LOWESS normalization were performed. The mass range 100–1500 *m/z* was searched for peaks with a minimum peak height of 10,000 cps. The MS tolerance for peak centroiding was set to 0.01 Da. Retention time information was excluded from the calculation of the total score. For identification, accurate mass tolerance was 0.01 Da. The identification step was based on mass accuracy and isotopic patterns, and the annotation was carried out by manually comparing the peaks with the theoretical phenolic compounds.

Then, for annotation confirmation, a pool of representative samples was injected in the UHPLC-Orbitrap-HRMS equipment and a data-dependent scan was carried out with the use of a parent ion list, using the ions tentatively annotated previously. The data were analysed using Xcalibur software v2.0.7 (Thermo Fisher Scientific, San Jose, CA, USA) and the fragments were manually checked.

2.5. Creatinine determination

Creatinine was measured by an adapted Jaffé alkaline picrate method for 96-well plates, as described by Medina-Remón et al. [23]. As phenolic compounds were expressed as peak areas, without quantitation, it was not possible to normalize their values by creatinine concentration. Therefore, we introduced creatinine in the adjustment models to account for differences in urinary excretion.

2.6. Statistical analyses

Supervised analysis of multivariate data was carried out using SIMCA software (Umetrics) and orthogonal projection to latent structures discriminant analysis (OPLS-DA). This model was selected over a partial least squares discriminant analysis, as the orthogonal projection allows a better separation between groups when the intra-group variability is high [24]. To investigate the presence of outliers, Hotelling's T2 was applied, using a 95% limit for suspicious outliers and 99 % for strong outliers. Goodness-of-prediction (Q2 Y) and goodness-of-fit (R2 Y) were used as validation parameters, adopting a Q2 Y prediction ability of $>$ 0.5 as the acceptability threshold. ANOVA applied to cross-validated residuals (CV-ANOVA) was used for the cross-validation of the model, with a p-value $<$ 0.05 as a threshold. The p-value of the CV-ANOVA indicates the probability for an OPLS-DA model, with this F-value being the result only of chance [25]. Finally, a permutation test (200 permutations) was done to exclude overfitting. The variable importance in projection (VIP) was used to extrapolate the possible marker compounds, i.e., those with a VIP score $>$ 1. For each phenolic metabolite with a VIP score $>$ 1, the fold change comparing T2D versus T2D-free participants was calculated.

Logistic regression models were applied to assess the association of each phenolic compound (1-SD increment in transformed concentration of metabolites) with T2D, adjusting for covariates and confounders that could alter this relationship. Thus, we adjusted for age, sex, BMI, smoking habit, educational level, physical activity, total energy intake and hypercholesterolemia. Individual phenolic compounds and creatinine were natural logarithmically transformed to normalize their distributions, as were confounders that did not follow normal distribution (physical activity and total energy intake). The p-values of the logistic-adjusted associations were adjusted using the false discovery rate (FDR)-adjusted procedure to account for multiple testing [26]. Therefore, those metabolites with a VIP score $>$ 1 and a statistically significant p-value ($<$0.05) were considered as discriminant for T2D status and were used as independent variables in the following regression models. Receiver operating characteristic (ROC) curve analysis was used to assess the accuracy of the discriminant phenolic compounds.

To further explore the relationship between the discriminant phenolic compounds and T2D, multivariable regression models were used to assess their association with plasma glucose levels (mg/dL). Three adjustment models of increasing complexity were used. Model 1 was minimally adjusted for age, sex, and creatinine. Model 2 was further adjusted for smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia. As antidiabetic drugs were only used by diabetic participants with higher levels of glucose, their inclusion in the model might be an overadjustment. We therefore also applied model 3, which was additionally adjusted for antidiabetic drug usage.

Logistic and multivariable adjusted regression models were generated using Stata 16.0. P-value $<$ 0.05 was considered statistically significant.

3. Results

3.1. General characteristics

The descriptive characteristics of the 200 participants are listed in Table 1. Their mean + SD age was 66.1 + 5.3 years and approximately half were women (54.5 %). 102 participants were diagnosed with T2D and 98 were free of T2D. The two groups were well-balanced in terms of BMI, physical activity, and total energy intake. A higher percentage of participants with hypercholesterolemia was observed in the non-T2D group (93.9 %). 15 % of the total participants were current smokers, the percentage being similar in both groups. Regarding education, a higher percentage of participants with T2D had received a high or medium level of education (40.2 %) compared to those without T2D (26.5 %).

3.2. Phenolic compound identification

The HRMS-based metabolomics analysis permitted the tentative identification of 79 phenolic compounds according to their exact mass and isotopic ratio. Among the 41 marker compounds selected using the VIP method (VIP score $>$ 1), 17 were identified by their exact mass, isotopic ratio, and fragmentation pattern. The results are presented in

Table 1
Baseline characteristics of all the participants according to T2D status.

Characteristics	All (n=200)	Participants without T2D (n=102)	Participants with T2D (n=98)	p-value
Women, n(%)	109 (54.5)	56 (57.1)	53 (52.0)	0.462
Age, years	66.1 + 5.3	65.7 + 5.02	66.5 + 5.6	0.326
BMI, kg/m2	29.5 + 3.4	29.3 + 3.3	29.7 + 3.5	0.363
Hypercholesterolemia	158 (79.0)	92 (93.9)	66 (64.7)	< 0.001
Current smokers	30 (15.0)	15 (15.31)	15 (14.71)	0.958
High or medium education	67 (33.5)	26 (26.5)	41 (40.2)	0.041
Physical activity, METS-min/day	291.0 + 266.9	286.2 + 263.4	295.6 + 271.4	0.805
Total energy intake, kcal/day	2394.1 + 500.8	2419.9 + 471.3	2369.2 + 528.7	0.475

T2D, type-2 diabetes; METS, metabolic equivalents.
Continuous variables are shown as means + SDs, and categorical variables are shown as percentages.
T-test or chi-square test as appropriate.

the Appendix (Table A1). Due to the low concentrations of the urine samples and the difficulty in obtaining MS fragments, some compounds were tentatively identified according to the mass accurate measurements and the isotopic pattern, with a level 4 identification [27].

3.3. Determination of discriminant compounds

The OPLS-DA was built to model the raw data in a supervised manner and extrapolate the VIP marker discriminant compounds. The OPLS-DA analysis resulted in a model with R2 Y = 0.43 and Q2 Y = 0.101. VIP values were obtained for each variable in the OPLS-DA model and the 41 selected compounds (VIP > 1.0) were consecutively identified by product ion scan analysis (MS^2) (Table A1). The multivariate supervised statistical method allowed the identification of the discriminant compounds and provided a good separation between participants with and without T2D in the score plot (Fig. 1).

Table 2 shows the 41 phenolic compounds with a VIP score > 1 grouped according to their polyphenol class. The average fold change (FC) was calculated to compare the samples of participants with and without T2D. Among all the compounds, 27 had a negative logFC, indicating their levels were higher in participants free of T2D.

In the logistic-adjusted regression analysis, two phenolic compounds were associated with T2D after the FDR correction: dihydrocaffeic acid and genistein diglucuronide. Dihydrocaffeic acid is a colonic metabolite of caffeic and ferulic acid, although it can also be found in olives [28,29]. The aglycone of genistein diglucuronide can be found in vivo after the intake of soy, and in smaller proportions after consumption of nuts, vegetables, and fruits [30]. Both dihydrocaffeic acid and genistein diglucuronide showed an inverse association with T2D (OR = 0.22 (CI 95 %: 0.09; 0.52) per 1-SD, *p*-value = 0.021 and OR = 0.72 (CI 95 %: 0.59; 0.88) per 1-SD, *p*-value = 0.021, respectively), although the logFC of dihydrocaffeic acid was lower compared to genistein diglucuronide (−0.27 and −1.92, respectively). Dihydrocaffeic acid and genistein diglucuronide were both identified by product ion scan analysis (MS^2), comparing the fragments to those in the literature [31,32].

Fig. 2 illustrates the ROC curve of urinary dihydrocaffeic acid and genistein diglucuronide in relation to T2D adjusted by the potential confounders. The area under the curve was 0.779 and 0.783 for dihydrocaffeic acid and genistein diglucuronide, respectively, indicating that both compounds were discriminant for T2D. Dihydrocaffeic acid predicted a non-diabetic status with 70.97 % of sensitivity and 65.66 % of specificity, whereas for genistein diglucuronide, the sensitivity was 75.61 % and specificity 65.22 %.

3.4. Discriminant compounds and fasting plasma glucose

The associations between plasma glucose and the discriminant phenolic compounds according to the three adjustment models are presented in Table 3. Dihydrocaffeic acid showed a significant inverse association with plasma glucose in model 2 (β = −17.12 (95 % CI: −29.92; −4.32) mg/dL per 1-SD, *p*-value = 0.009), but in model 3, which included the use of antidiabetic drugs, the relationship was no longer significant and the β coefficient decreased. However, a strong positive correlation was found between antidiabetic drug usage and plasma glucose (data not shown), so its inclusion as a confounder could be an overadjustment. A comparison of the associations of dihydrocaffeic acid with plasma glucose using adjustment models 2 and 3 is presented in Fig. 3. Genistein diglucuronide did not show any significant association in any adjustment model.

4. Discussion

In the present case-control substudy of the PREDIMED trial, we identified urinary phenolic compounds associated with the risk of T2D in participants at high CVD risk using a metabolomics approach based on UHPLC-Orbitrap-HRMS. The metabolites dihydrocaffeic acid and genistein diglucuronide were associated with lower T2D risk, and dihydrocaffeic acid was also inversely associated with plasma glucose levels.

There is extensive evidence from epidemiological studies that dietary polyphenols reduce T2D risk [9]. This relationship could be explained by the anti-inflammatory properties of phenolic compounds, as cellular inflammation plays a key role in the development of T2D. Polyphenols can modulate the transcription of genes involved in inflammatory pathways, such as PPARγ (peroxisome proliferator-activated receptor γ), SIRT1 (Sirtuin 1), or AMPK1 (adenosine monophosphate-activated protein kinase) [33,34]. In addition, they can reduce glucose absorption by inhibiting the sodium-dependent glucose transporter 1 (SGLT1) and increase its uptake in tissues through activation of glucose transporter 4 (GLUT4) [35]. Through prebiotic effects, polyphenols can stimulate the growth of microbial species in the gut with a beneficial impact on metabolic diseases [36].

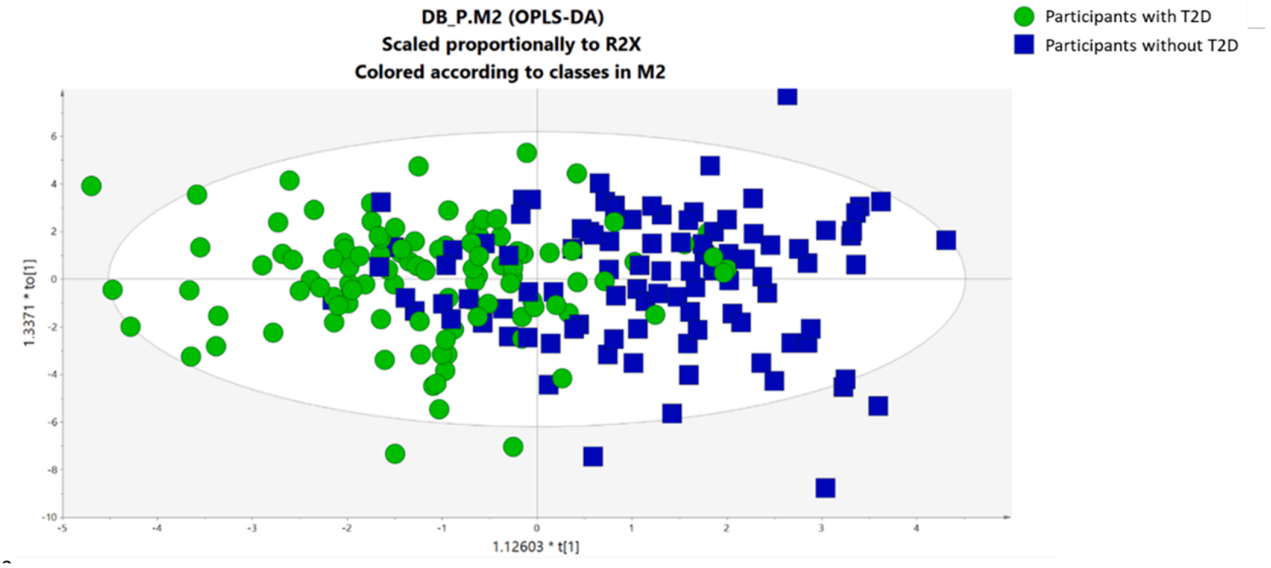

Fig. 1. OPLS-DA score plot built considering the urinary phenolic profile of the participants with and without T2D.

Table 2
Average logFC of the marker compounds (VIP > 1) of T2D and their polyphenol class. The OR (CI 95%) and *p*-value obtained for each compound in the logistic regression analysis are also shown.

Polyphenol class	Tentative metabolite identification	LogFC	OR (CI 95%) per 1-SD	*p*-value
Flavonoids	Epicatechin diglucuronide	-4.3481	0.74 (0.46; 1.17)	0.244
	Hesperetin	-2.2667	0.89 (0.76; 1.04)	0.214
	Daidzein sulfate	-2.1385	0.74 (0.60; 0.92)	0.068
	Genistein diglucuronide	-1.9158	0.72 (0.59; 0.88)	0.021
	Hesperetin diglucuronide	-1.6719	0.95 (0.84; 1.07)	0.419
	Genistein	-1.2496	0.73 (0.52; 1.03)	0.148
	Daidzein glucuronide	-1.2200	0.87 (0.71; 1.07)	0.244
	Hesperetin glucuronide	-1.0218	0.89 (0.77; 1.04)	0.214
	Daidzein	-0.4381	0.85 (0.73; 1.00)	0.128
	Naringenin diglucuronide	-0.3238	0.78 (0.62; 0.99)	0.117
	Naringenin disulfate	-0.0045	0.91 (0.68; 1.23)	0.595
	Epicatechin glucuronide	0.0821	0.94 (0.75; 1.18)	0.628
	Equol sulfate	1.1369	0.94 (0.65; 1.35)	0.740
	Hesperetin sulfate	1.7829	0.89 (0.75; 1.06)	0.244
	Equol glucuronide	2.6864	1.31 (1.07; 1.61)	0.068
Lignans	Enterolactone glucuronide	-0.5135	0.79 (0.62; 1.03)	0.148
Phenolic acids	Gallic acid diglucuronide	-4.4063	0.88 (0.72; 1.07)	0.244
	Benzoic acid diglucuronide	-2.7038	1.01 (0.78; 1.30)	0.961
	Hydroxybenzoic acid diglucuronide	-2.5572	0.71 (0.55; 0.92)	0.068
	3-Hydroxyphenylacetic acid	-1.6165	0.71 (0.56; 0.91)	0.068
	Caffeic acid diglucuronide	-0.8731	0.77 (0.61; 0.99)	0.117
	Vanillic acid disulfate	-0.7186	0.82 (0.60; 1.11)	0.244
	Vanillic acid sulfate	-0.5185	0.72 (0.48; 1.08)	0.186
	Benzoic acid glucuronide	-0.4855	0.75 (0.54; 1.03)	0.148
	Protocatechuic glucuronide	-0.3934	0.74 (0.48; 1.14)	0.244
	Protocatechuic acid	-0.3314	0.74 (0.47; 1.18)	0.244
	Dihydrocaffeic acid	-0.2748	0.22 (0.09; 0.52)	0.021
	Hippuric acid glucuronide	-0.0289	0.67 (0.47; 0.93)	0.082
	Hydroxyphenylpropionic acid sulfate	0.1788	1.19 (0.94; 1.51)	0.214
	Hydroxyphenylpropionic acid glucuronide	0.2362	1.14 (0.89; 1.46)	0.337
	Chlorogenic acid sulfate	0.2885	1.15 (0.98; 1.35)	0.148
	3-Hydroxyphenylacetic acid disulfate	0.3268	1.19 (0.91; 1.56)	0.244
	Caffeic acid sulfate	0.3724	1.31 (0.99; 1.72	0.145
	Ferulic acid sulfate	0.4121	1.41 (1.06; 1.87)	0.077
	Coumaric acid glucuronide	0.4505	1.32 (1.01; 1.73)	0.123
	Chlorogenic acid glucuronide	0.7417	1.22 (1.01; 1.47)	0.117
	Chlorogenic acid	1.0310	1.49 (1.08; 2.05)	0.077
Stilbenes	Dihydroresveratrol disulfate	0.2885	1.15 (0.98; 1.35)	0.148
Other	Urolithin A glucuronide	-0.3752	0.86 (0.77; 0.97)	0.077
	Urolithin C	-0.3273	0.80 (0.65; 0.99)	0.117
	Urolithin B diglucuronide	-0.3259	0.79 (0.61; 1.03)	0.148

FC, fold change; orthogonal projection to latent structures discriminant analysis, OPLS-DA; variable importance in projection, VIP; type-2 diabetes, T2D; OR, odds ratio; CI, confidence interval.
Log-transformation was applied to raw values of phenolic compounds.
Logistic regressions were adjusted for sex, age, smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia.
P-value was FDR-adjusted.

Most of the studies that report a negative association between polyphenols and T2D in humans are based on estimations of phenolic intake through dietary recall and food-composition tables rather than analysis of biological samples[37,38]. However, these methodologies are subject to errors due to systematic bias and subjectivity, and they do not consider other aspects such as bioavailability and the formation of new compounds through endogenous metabolism [7]. Thus, the resulting data may not reflect real exposure to biologically active compounds. In the present study, we employed liquid chromatography and HRMS, which provide highly accurate mass determinations and fragmentation patterns from multi-stage mass fragmentation and allow the structural elucidation of known and unknown compounds [39,40]. Therefore, we were able to identify a great variety of phenolic compounds in urine samples and to measure exposure to bioactive compounds objectively and accurately.

In our study, dihydrocaffeic acid was associated with a lower risk of T2D. This compound is thought to be a metabolite originated by the gut microbiota in the colon from the cinnamic acids caffeic, ferulic and chlorogenic acids [28,41,42]. These phenolic compounds can be found in different foods and beverages that form an essential part of the Mediterranean diet, such as coffee, vegetables, and fruits [29]. Previous research has reported that the beneficial effects of dihydrocaffeic acid are related to oxidative and the insulin/IGF-1 pathway [43]. However, to our knowledge, no studies until now have analysed the association of dihydrocaffeic acid with T2D or plasma glucose levels in humans.

Dihydrocaffeic acid precursors have shown that they can provide benefits against T2D through different molecular pathways, as described in Fig. 4. A study performed in rats reported that caffeic acid has a protective effect against hyperglycemia and insulin resistance, with several possible mechanisms involved. It has been suggested that caffeic acid modulates the purinergic and cholinergic pathways, thus reducing oxidative stress and inflammation [44]. It is also thought to decrease the production of proinflammatory factors, such as cytokines or leptin [45,46]. Furthermore, Un et al. found that glucokinase was down-regulated by caffeic acid, leading to an attenuation of hepatic glucose output [47]. Regarding ferulic acid, it has been shown that it reduces ß-cell dysfunction by increasing the activity of antioxidant enzymes and modulating others that are key to glucose production, as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [48]. There are numerous studies that demonstrate that chlorogenic acid can reduce blood glucose in humans. In a clinical trial, it was shown that it can enhance insulin sensibility and ameliorate insulin resistance [49]. Chlorogenic acid, a precursor of dihydrocaffeic acid through its cleavage

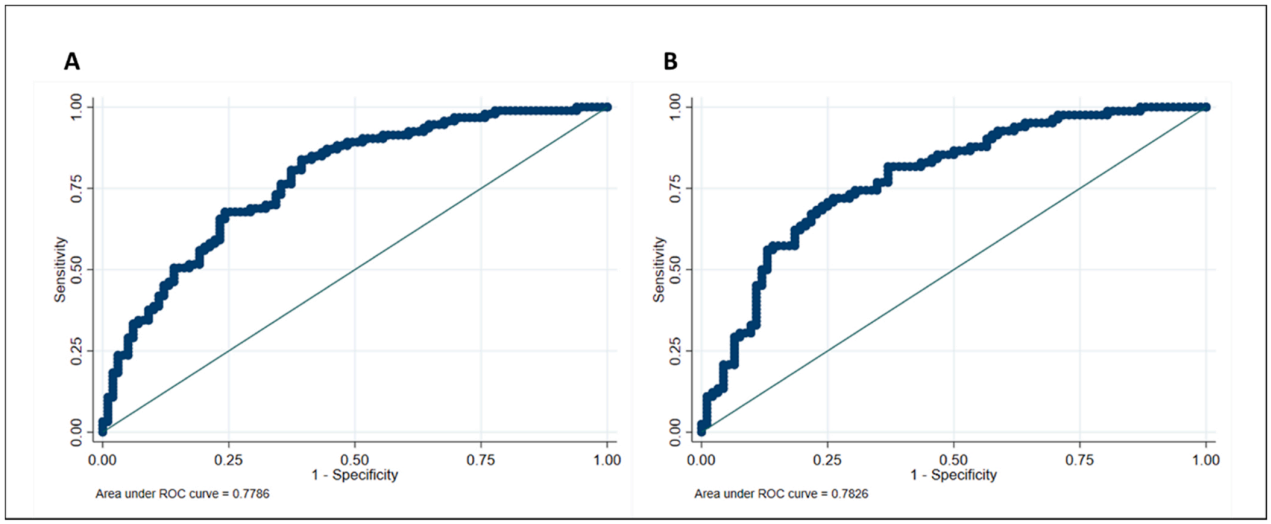

Fig. 2. Receiver operator characteristic curves for the predictive value of dihydrocaffeic acid (A) and genistein diglucuronide (B) regarding absence of type-2 diabetes.

Table 3
Multivariable linear regression between the discriminant phenolic compounds and plasma glucose.

		Dihydrocaffeic acid		Genistein diglucuronide	
		β (95% CI) per 1-SD	*p*-value	β (95% CI) per 1-SD	*p*-value
Plasma glucose (mg/dL)	*Model 1*	-12.87 (−26.61; 0.87)	0.066	-0.90 (−4.38; 2.57)	0.608
	Model 2	-17.12 (−29.92; −4.32)	0.009	-2.10 (−5.42; 1.23)	0.216
	Model 3	-10.15 (−22.46; 2.17)	0.106	0.42 (−3.48; 2.63)	0.785

ß, difference between groups; CI, confidence interval.
Log-transformation was applied to raw values of phenolic compounds.
Model 1: age and sex.
Model 2: age, sex, smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia.
Model 3: age, sex, smoking habit, educational level, BMI, physical activity, total energy intake, hypercholesterolemia, and use of antidiabetic drugs.

into caffeic and quinic acids, can modulate the activity of enzymes involved in glucose metabolism, such as α-amylase, or glucose-6-phosphatase [50,51]. It also reduces the decrease in the expression of IRS-1 and GLUT-4 typically observed after high glucose exposure [52]. In addition, chlorogenic acid can reduce the production of reactive oxygen species and protect against oxidative stress [53].

In the present study, we found that dihydrocaffeic acid was associated with lower concentrations of plasma glucose, suggesting that it exerts the same beneficial effects on glucose metabolism as its precursors, probably due to chemical structural similarity. Interestingly, when the use of antidiabetics drugs was included in the analysis this association was no longer significant. Nevertheless, this could be due to an overadjustment, as the participants using antidiabetic drugs were diabetics with high levels of plasma glucose. Overall, these findings suggest that this microbial caffeic acid metabolite has beneficial biological activity against T2D.

We found that higher levels of genistein diglucuronide were associated with a lower risk of suffering from T2D. Genistein diglucuronide is the major phase-II metabolite first to appear in plasma after genistein consumption, followed by single-conjugated metabolites [54]. The

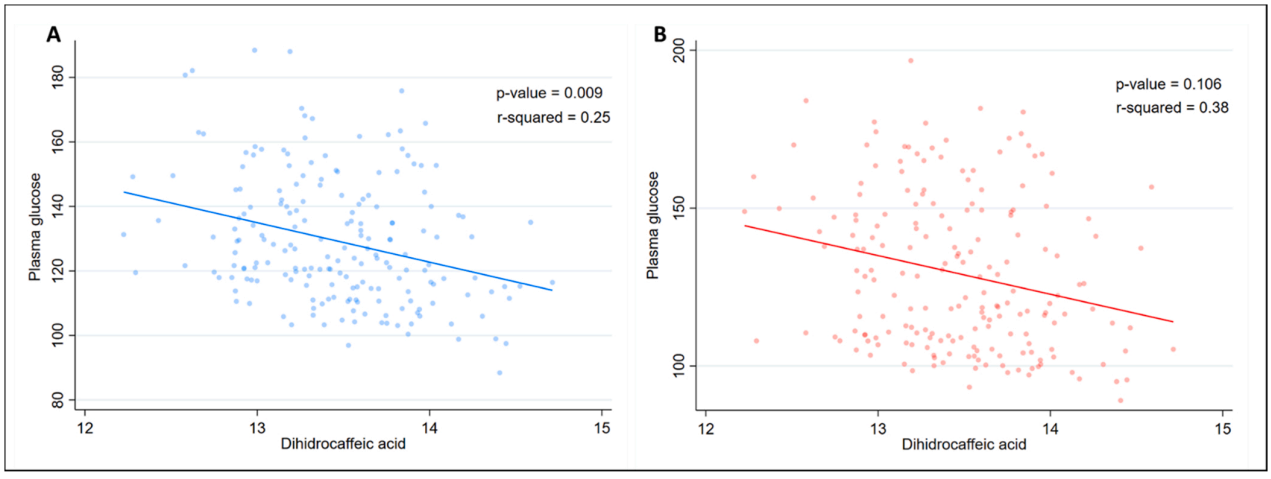

Fig. 3. Comparison of the multivariable linear regression between dihydrocaffeic acid and plasma glucose adjusted for (A): age, sex, smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia; and (B) further adjusted for the use of antidiabetic drugs.

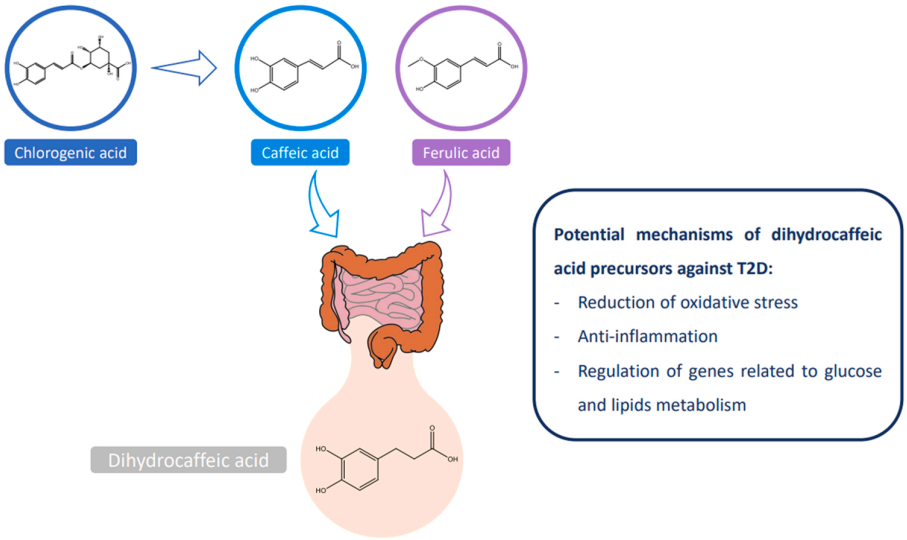

Fig. 4. Summary of dihydrocaffeic acid precursors (chlorogenic, caffeic, and ferulic acid) and their potential mechanisms against T2D. T2D, type 2 diabetes.

relationship between genistein aglycone and T2D has been widely investigated, and several studies have associated this isoflavone with a reduced risk of developing the disease, lower glucose levels, and improved insulin sensitivity [55,56]. Most of these studies were clinical trials that assessed the effects of daily genistein supplementation [57, 58]. Genistein is a phytoestrogen naturally found in soy in high concentrations, with small amounts present in other products consumed in the Mediterranean diet, such as nuts, vegetables, and fruits, [30]. Several mechanisms have been proposed to explain the protective effect of genistein against T2D. A study performed in mice suggested that it improves insulin release by inducing pancreatic β cells proliferation [59]. Mezei et al. observed a reduction of triglycerides and cholesterol through the activation of PPARγ, which is involved in glucose and lipid metabolism [60]. Therefore, genistein diglucuronide could exert a protective effect against T2D through similar mechanisms, as genistein may be released from the diglucuronide during its transport through blood or upon reaching an organ [54].

The present study has both strengths and limitations. Limitations include the relatively small sample size and the high cardiovascular risk status of the participants, which restricts the extrapolation of the results to other populations. In addition, the nature of the study precludes determination of causality. On the other hand, the main strength of our study is the use of metabolomics based on HRMS to evaluate a wide variety of phenolic compounds in biological samples. In addition, this study involved a free-living population, and the results reflect real-life conditions. Finally, the methodology here developed could be applied in other human studies to find new metabolite biomarkers of foods or disease.

5. Conclusions

A novel method using a metabolomics approach was developed to determine the association of urinary phenolic compounds with T2D revealed that two metabolites, dihydrocaffeic acid and genistein diglucuronide, were associated with a lower risk of T2D in a Mediterranean population at high cardiovascular risk. Further research is needed to explore the effects of these metabolites on the pathogenesis of T2D and their usefulness as tentative biomarker for T2D prediction.

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CRediT authorship contribution statement

Conceptualization, I.D.L. and R.M.L.M.; Formal analysis, I.D.L., J.L.C., A.V.Q., O.J.; Writing – original draft, I.D.L.; Writing – review and editing, J.L.C., A.V.Q., O.J., M.A.M.G., F.B.H., M.F., E.R., R.E., R.M.L.R.

Declaration of Competing Interest

E.R. reports grants, personal fees, non-financial support and other from the California Walnut Commission while the study was carried out; grants, personal fees, non-financial support and other from Alexion; and personal fees and other from Amarin, outside the submitted work. R.M.L.-R. reports personal fees from Cerveceros de España, personal fees, and other from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. R.E. reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain), and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Alburquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain), and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

Data availability

Data will be made available on request.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centers. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114703.

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3.3.3. Publication 11

Association of microbiota polyphenols with cardiovascular health in the context of a Mediterranean diet

Inés Domínguez-López, Camila Arancibia-Riveros, María Marhuenda-Muñoz, Anna Tresserra-Rimbau, Estefanía Toledo, Montserrat Fitó, Emilio Ros, Ramon Estruch, and Rosa M Lamuela-Raventós

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Supplementary Material available in Annex section.

Abstract

Aims: To assess the association of MPM with adherence to the MedDiet, and their relationship with ideal cardiovascular health (ICVH) and CVD risk factors.

Methods: This cross-sectional substudy within the PREDIMED trial included 200 participants of the Barcelona-Clinic recruitment center. MPM were identified and quantified using a novel method based on HPLC coupled to MS. Multivariable-adjusted regressions were used to evaluate the associations between MPM and MedDiet adherence, ICVH score, biochemical variables, and blood pressure. Additionally, an MPM score of MedDiet adherence and ICVH were calculated as the weighted sum of the variables.

Results: The MPM score was directly associated with MedDiet adherence and ICVH. We observed inverse associations of urolithin B glucuronide with LDL-c and enterodiol glucuronide with glucose, and a direct association between vanillic acid glucuronide and triglycerides and systolic blood pressure.

Conclusions: A score of urinary MPM was associated with higher adherence to the MedDiet and ICVH, and individual MPM were related to better cardiovascular health. These findings suggest that the MedDiet may affect gut microbiota, whose metabolites are linked with cardiovascular health.

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ABSTRACT

Background and aims: The Mediterranean diet (MedDiet) is rich in polyphenols, phytochemicals that are beneficial for cardiovascular health. Phenolic compounds have poor bioavailability but they are extensively metabolized by the gut microbiota. Therefore, we aimed to assess the association of microbial phenolic metabolites (MPM) with adherence to the MedDiet, and their relationship with ideal cardiovascular health (ICVH) and cardiovascular risk factors.

Methods and results: This cross-sectional substudy within the PREDIMED trial included 200 participants from the Barcelona-Clinic recruitment center. Five MPM were identified and quantified using a novel method based on liquid chromatography coupled to mass spectrometry: protocatechuic acid (PCA), enterodiol glucuronide (EDG), enterolactone glucuronide (ELG), vanillic acid glucuronide (VAG) and urolithin B glucuronide (UBG). Multivariable-adjusted regressions were used to evaluate the associations between MPM and MedDiet adherence, ICVH score, biochemical parameters, and blood pressure. Additionally, an MPM score was calculated as the weighted sum of MedDiet adherence and ICVH and found to be directly associated. Among individual polyphenols, UBG was inversely associated with LDL-cholesterol.

Conclusions: A score of urinary MPM was associated with higher adherence to the MedDiet and ICVH, and individual MPM were related to better cardiovascular health. These findings suggest that the MedDiet may affect gut microbiota, whose metabolites are linked with cardiovascular health.

1. Introduction

Cardiovascular diseases (CVD), which include various heart and circulatory system disorders, are the leading cause of premature death in Europe (Francula-Zaninovic & Nola, 2018). Individual characteristics and habits, such as smoking, hypertension, hypercholesterolemia, diabetes mellitus, obesity, and an unhealthy diet, have been identified as CVD risk factors (Joseph et al., 2017; Piepoli & Villani, 2017). In 2010, the American Heart Association (AHA) proposed an ideal cardiovascular health (ICVH) score with the purpose of improving overall health and reducing deaths from CVD (Lloyd-Jones et al., 2010). The ICVH score is based on 7 parameters of ideal health behaviors (nonsmoking, body

Abbreviations: CVD, Cardiovascular diseases; DBP, diastolic blood pressure; EDG, enterodiol glucuronide; ELG, enterolactone glucuronide; HDL-cholesterol, high-density lipoprotein-cholesterol; ICVH, ideal cardiovascular health; LTQ-Orbitrap-MS, linear ion trap quadrupole-Orbitrap-mass spectrometry; LDL-cholesterol, low-density lipoprotein-cholesterol; MedDiet, Mediterranean diet; MPM, microbial phenolic metabolites; PCA, protocatechuic acid; TG, triglycerides; SBP, systolic blood pressure; UBG, urolithin B glucuronide; VAG, vanillic acid glucuronide.

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mass index (BMI) < 25 kg/m2, appropriate physical activity, and healthy diet) and ideal health factors (total cholesterol < 200 mg/dL, blood pressure < 120/80 mmHg, and fasting blood glucose < 100 mg/dL). The benefits of meeting the 7 health metrics have been demonstrated in numerous epidemiological studies in different countries and populations, adherence being inversely associated with CVD (Ahmad et al., 2019; Aneni et al., 2017).

Diet and cardiovascular health are closely related, as certain nutrients and phytochemicals play a major role in the prevention or development of CVD (GBD, 2013). As the effects of individual nutrients depend on interactions with other components of food, it can be more useful to assess their impact within the context of a diet or dietary pattern rather than separately (Rinaldi de Alvarenga et al., 2019).

The Mediterranean diet (MedDiet) is proven to reduce the risk of developing CVD (Estruch et al., 2018). Based on the consumption of extra virgin olive oil, nuts, fruits, vegetables, whole grains, legumes, fish, and moderate quantities of wine (Bach-Faig et al., 2011), this dietary pattern is rich in healthy fats and other antioxidant bioactive molecules such as polyphenols (Tresserra-Rimbau et al., 2013). Although the intake of polyphenols has been demonstrated to improve cardiovascular health (Li et al., 2020; Tresserra-Rimbau, Rimm, Medina-Remón, Martínez-González, López-Sabater, et al., 2014), their bioavailability in the small intestine is low and 90–95 % of them reach the large intestine/colon, where they undergo enzymatic transformations catalyzed by the gut microbiota (Cardona et al., 2013; Marhuenda-Muñoz et al., 2019). After absorption, these microbial phenolic metabolites (MPM) may exert biological effects but to date, studies assessing the positive benefits of different classes of MPM on cardiovascular health are scarce.

The present study applied an innovative method to determine and quantify MPM in a subpopulation of the PREDIMED trial using a linear ion trap quadrupole-Orbitrap-mass spectrometer (LTQ-Orbitrap-MS), which provides accurate structural information to identify and quantify novel compounds. We aimed to assess the relationship between MPM and MedDiet adherence, as well as to evaluate their association with the ICVH score and cardiovascular risk factors in an elderly Mediterranean population.

2. Methods

2.1. Study design

A cross-sectional analysis was carried out using baseline data from the PREDIMED (PREvención con DIeta MEDiterránea) trial, a large, parallel-group, multicenter, randomized, controlled, five-year clinical trial that examined the effect of the traditional MedDiet on the primary prevention of CVD (https://www.predimed.es). The details of the study design can be found elsewhere (M. Á. Martínez-González et al., 2012). A total of 7,447 participants were recruited in Spain between October 2003 and December 2010, the men aged 55–80 years and women, 60–80 years. Eligible participants were free of CVD at baseline and presented type-2 diabetes or at least three of the following cardiovascular risk factors: current smoking, hypertension, dyslipidaemia, overweight/obesity or family history of premature CVD.

In the present study, 200 randomly selected participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) were included. Extreme total energy intake (>3500 or < 500 kcal/day in women or > 4000 or < 800 kcal/day in men) was an exclusion criterion for this subanalysis (Fernández-Ballart et al., 2010).

The Institutional Review Board of the Hospital Clinic (Barcelona, Spain), accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738, approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.2. Covariate assessment

Dietary intake was assessed using a validated, semi-quantitative 137-item food frequency questionnaire with the assistance of trained dietitians (Fernández-Ballart et al., 2010). To assess MedDiet adherence, a 14-item questionnaire with a value of 0 or 1 for each dietary component was used. Each item is related to a specific feature of the MedDiet, and the higher the overall score, the greater the adherence (Martínez-González, Buil-Cosiales, et al., 2019).

Trained personnel recorded the anthropometric and clinical measurements of the participants, including height, weight, waist circumference, and blood pressure. BMI was calculated by dividing the body weight in kilograms by the squared height in cm. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP, Lake Forest, IL, USA). The Minnesota Leisure-Time Physical Activity Questionnaire was used to assess physical activity (metabolic equivalent tasks per minute per day, METS min/day) of the participants (Elosua et al., 2000).

During the first screening visit, data on medical conditions, family history of disease, and risk factors were collected through a questionnaire. Biological samples (plasma and urine) were taken at baseline after a 12 h overnight fast and stored at −80 °C until analysis. Blood glucose, total cholesterol, triglycerides (TG), and high-density lipoprotein-cholesterol (HDL-cholesterol) were determined by standard enzymatic methods, and low-density lipoprotein-cholesterol (LDL-cholesterol) was calculated by the Friedewald equation (Estruch et al., 2006).

2.3. Microbial phenolic metabolite analysis

2.3.1. Standards and reagents

Protocatechuic acid (PCA), enterodiol, urolithin-A, and urolithin-B were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (+)*cis,trans*-abscisic acid d_6 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Vanillic acid, enterolactone and creatinine were obtained from Fluka (St. Louis, MO, USA). Standards were stored in powder form and protected from light.

The reagents were purchased from the following commercial suppliers: methanol of LC-MS grade and acetonitrile of HPLC grade from Sigma-Aldrich and formic acid (≥98 %) from Panreac Química S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore system (Bedford, MA, USA).

2.3.2. Sample preparation

Urinary MPM were determined following a method previously validated by our research group with minor modifications (Laveriano-santos et al., 2022). Briefly, urine samples (50 μL) diluted 1:20 (*v:v*) with Milli-Q Water and 100 μL of the internal standard abscisic acid d_6 were acidified with 2 μL of formic acid. After centrifugation at 14,000 g for 4 min at 4 °C, the acidified urines underwent solid-phase extraction in Water Oasis HLB 96-well plates 30 μm (30 mg) (Water Oasis, Milford, MA, USA) to extract the phenolic metabolites and eliminate unwanted compounds. After activating the plate with 1.5 M formic acid, the samples were loaded and cleaned with 1.5 M formic acid and 0.5 % methanol. The MPM were eluted with methanol acidified with 0.1 % formic acid, evaporated to dryness with nitrogen gas and reconstituted with 100 μL of 0.05 % formic acid. Finally, the samples were vortexed for 20 min and filtered through 0.22 μm polytetrafluoroethylene 96-well plate filters (Millipore, Massachusetts, USA).

Urine concentrations of MPM were corrected by urine creatinine, which was measured according to the adapted Jaffé alkaline picrate method for 96-well plates described by Medina-Remón et al. (2009). Urine concentrations of MPM were expressed as μmol per mg creatinine.

2.3.3. LTQ Orbitrap ESI analysis

The analysis was performed on an LTQ Orbitrap Velos mass

Table 1
General characteristics of the participants (n = 200).

Characteristics	
Women, %	54.5
Age, years	66.1 ± 5.3
Weight, kg	76.6 ± 11.7
BMI, kg/m^2	29.5 ± 3.4
Diabetes Mellitus, %	51.0
Dyslipidaemia, %	79.0
Hypertension, %	76.0
Current smoker, %	15.0
Education level, %	
Low	66.5
Medium & High	33.5
Physical activity, METS-min/day	291.0 ± 266.9
Total energy intake, kcal/day	2394.1 ± 500.8

METS, Metabolic Equivalents.
Continuous variables are shown as means ± SDs, and categorical variables are shown as percentages.

Table 2
Means and SD of urine concentrations of MPM (micromole per mg of creatinine) of the participants (n = 200).

Metabolites (Mean ± SD)	
Protocatechuic acid	0.009 ± 0.020
Vanillic acid glucuronide	0.222 ± 0.393
Enterodiol glucuronide	0.003 ± 0.004
Enterolactone glucuronide	0.020 ± 0.026
Urolithin B glucuronide	0.158 ± 0.605

MPM, microbial phenolic metabolites.

Table 3
Multivariable adjusted regression between Mediterranean diet adherence diet and MPM.

		β (95 % CI)	p-value
Protocatechuic acid	*Model 1*	0.68 (0.14; 1.22)	0.013*
	Model 2	0.62 (0.09; 1.15)	0.022
Vanillic acid glucuronide	*Model 1*	0.41 (-0.15; 0.97)	0.150
	Model 2	0.34 (-0.23; 0.91)	0.244
Enterodiol glucuronide	*Model 1*	0.36 (-0.27; 0.98)	0.260
	Model 2	0.30 (-0.31; 0.93)	0.347
Enterolactone glucuronide	*Model 1*	0.64 (-0.10; 1.18)	0.019*
	Model 2	0.55 (0.01; 1.10)	0.046
Urolithin B glucuronide	*Model 1*	0.35 (-0.17; 0.88)	0.181
	Model 2	0.42 (-0.12; 0.96)	0.125
MPM Score	*Model 1*	0.70 (0.14; 1.26)	0.021
	Model 2	0.62 (0.06; 1.18)	0.041

MPM, microbial phenolic metabolites.
β, difference between groups; CI, confidence interval.
*This difference remained statistically significant after adjusting for multiple comparisons.
Model 1: sex and age.
Model 2: sex, age, smoking habit, educational level, BMI, physical activity, and total energy intake.

spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode, as described elsewhere (Laveriano-santos et al., 2022). Chromatographic separation was performed on a Kinetex F5 100 Å (50 × 4.6 mm × 2.6 μm) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were, respectively, 0.05 % formic acid in water and 0.05 % formic acid in acetonitrile. The following linear gradient was used: held at 98 %A for 1.7 min, decreased to 92 %A for 3 min, decreased to 80 %A for 1.3 min, decreased to 70 %A for 1.3 min, decreased to 50 % for 0.1 min, decreased to 0 % for 1.3 min, then returned to the initial conditions for 1.7 min and re-equilibrated for 3 min. The flow rate was set at 0.750 mL/min and the injection volume was 5 μL.

Table 4
Frequency of participants with higher scores for overall ICVH and individual metrics.

Metrics	n (%)
Overall ICVH score	
0	1 (0.5)
1	16 (8.0)
2	67 (33.5)
3	63 (31.5)
4	43 (21.5)
5	10 (5.0)
Individual metrics	
Smoking status	164 (82.0)
Body mass index	12 (6.0)
Physical activity	167 (83.5)
Health diet score	83 (41.5)
Total cholesterol	64 (32.0)
Blood pressure	8 (4.0)
Glucose	63 (31.5)

ICVH, ideal cardiovascular health.

Table 5
Multivariable adjusted regression between overall ICVH and individual metrics and MPM score.

	β (95 % CI)	p-value
Overall ICVH score	1.29 (0.52; 2.07)	0.001
Individual Metrics		
Smoking status	0.07 (-0.18; 0.35)	0.523
Body mass index	0.02 (-0.18; 0.23)	0.839
Physical activity	0.20 (-0.11; 0.51)	0.201
Diet	0.65 (0.28; 1.01)	0.001
Blood pressure	0.09 (-0.07; (0.25)	0.275
Total cholesterol	−0.13 (-0.49; (0.22)	0.457
Glucose	0.38 (0.01; 0.75)	0.042

MPM, microbial phenolic metabolites.
β, difference between groups; CI, confidence interval.
Adjusted for sex and age.

2.3.4. Identification and quantification of MPM

Trace Finder software version 4.1 (Thermo Fisher Scientific, San Jose, CA) was used to identify and quantify urinary MPM. The glucuronidated and sulfated metabolites were quantified using their respective aglycone equivalents due to the unavailability of standards.

Metabolic differences and high inter-variability are major concerns when assessing metabolites derived from gut microbiota. Due to the wide variability of metabolites identified across the 200 participants, we focused our study on those with less than 20 % of missing values. Thus, the 5 MPM included in the statistical analysis were: PCA, enterodiol glucuronide (EDG), enterolactone glucuronide (ELG), urolithin B glucuronide (UBG), and vanillic acid glucuronide (VAG). When values for these 5 metabolites were missing, half the minimum detectable value was used.

2.4. Ideal cardiovascular health

The ICVH score was calculated based on the 7 health metrics proposed by the AHA (Lloyd-Jones et al., 2010). Thus, a value of 1 was assigned when the participants met the following criteria: never smoked or quit > 12 months ago; ≥ 9 points of adherence to the MedDiet; ≥ 150 min/week of moderate or ≥ 75 min/week of vigorous physical activity equivalent to ≥ 500 METS-min/week (Organization, 2020); BMI < 25 kg/m^2; total cholesterol < 200 mg/dL; blood pressure < 120/80 mmHg and fasting glucose < 100 mg/dL. The MedDiet was selected as a healthy dietary pattern considering the extensive and robust evidence for its beneficial effect on cardiovascular health (Martínez-González, Gea, et al., 2019). The 9 cut-point score was used to define good adherence to the MedDiet in accordance with previous studies (Hu et al., 2013).

Table 6
Multivariable adjusted regression between biochemical parameters and MPM.

	LDL, mg/dL		HDL, mg/dL		TG, mg/dL		Glucose, mg/dL	
	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value
Protocatechuic acid								
Model 1	0.09 (−3.98; 4.17)	0.964	−0.01 (−0.04; 0.03)	0.688	−0.02 (−0.08; 0.04)	0.600	−0.01 (−0.05; 0.03)	0.595
Model 2	0.93 (−2.99; 4.84)	0.641	−0.01 (−0.04; 0.02)	0.374	0.01 (−0.05; 0.07)	0.859	−0.01 (−0.02; 0.04)	0.554
Model 3	1.80 (−2.34; 5.94)	0.393	−0.01 (−0.04; 0.03)	0.688	−0.02 (−0.08; 0.04)	0.576	<0.01 (−0.03; 0.03)	0.866
Vanillic acid								
Model 1	−1.73 (−5.71; 2.26)	0.394	−0.01 (−0.04; 0.03)	0.707	0.05 (0.01; 0.11)	0.102	0.01 (−0.03; 0.05)	0.594
Model 2	−1.54 (−5.37; 2.29)	0.429	−0.01 (−0.04; 0.02)	0.574	0.06 (<0.01; 0.12)	0.039	0.01 (−0.01; 0.04)	0.338
Model 3	−1.46 (−5.55; 2.62)	0.481	−0.01 (−0.04; 0.03)	0.604	0.06 (<−0.01; 0.12)	0.054	−0.01 (−0.02; 0.04)	0.581
Enterodiol glucuronide								
Model 1	−1.01 (−5.08; 3.05)	0.624	0.01 (−0.02; 0.04)	0.604	<0.01 (−0.06; 0.06)	0.921	−0.03 (−0.07; <−0.01)	0.071
Model 2	−0.26 (−4.09; 3.56)	0.893	<0.01 (−0.03; 0.03)	0.790	0.01 (−0.05; 0.06)	0.857	−0.03 (−0.05; <0.01)	0.071
Model 3	−0.58 (−4.77; 3.61)	0.786	0.01 (−0.02; 0.04)	0.604	<0.01 (−0.06; 0.06)	0.991	−0.03 (−0.06; <0.01)	0.066
Enterolactone glucuronide								
Model 1	2.71 (−1.24; 6.66)	0.178	0.02 (−0.01; 0.05)	0.238	−0.03 (−0.09; 0.03)	0.311	−0.04 (−0.08; <−0.01)	0.041
Model 2	2.05 (−1.75; 5.86)	0.289	0.02 (−0.01; 0.05)	0.201	−0.01 (−0.07; 0.05)	0.766	<−0.01 (−0.03; 0.03)	0.958
Model 3	2.18 (−2.01; 6.37)	0.307	0.02 (−0.01; 0.05)	0.238	−0.02 (−0.08; 0.04)	0.542	<−0.01 (−0.04; 0.03)	0.773
Urolithin B glucuronide								
Model 1	−3.46 (−7.40; 0.49)	0.086	<−0.01 (−0.03; 0.03)	0.962	0.03 (−0.03; 0.09)	0.302	0.01 (−0.03; 0.05)	0.644
Model 2	−4.83 (−8.63–1.02)	0.013	<0.01 (−0.03; 0.03)	0.905	0.01 (−0.05; 0.07)	0.825	<0.01 (−0.03; 0.03)	0.940
Model 3	−5.58 (−9.66;−1.50)	0.008*	<−0.01 (−0.03; 0.03)	0.962	0.03 (−0.03; 0.10)	0.268	<0.01 (−0.03; 0.03)	0.963

MPM, microbial phenolic metabolites; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.
*This difference remained statistically significant after adjusting for multiple comparisons.
Model 1: sex and age.
Model 2: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension and medication (cholesterol-lowering, antidiabetic and antihypertensive agents).
Model 3: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension, medication (cholesterol-lowering, antidiabetic and antihypertensive agents), total energy intake and Mediterranean diet adherence.

Table 7
Multivariable adjusted regression between blood pressure and phenolic metabolites.

	DBP, mmHg		SBP, mmHg	
	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value
Protocatechuic acid glucuronide				
Model 1	0.75 (−0.68; 2.18)	0.297	0.01 (−0.01; 0.03)	0.274
Model 2	0.96 (−0.49; 2.40)	0.198	0.01 (−0.01; 0.03)	0.321
Model 3	1.01 (−0.52; 2.54)	0.193	0.01 (−0.01; 0.03)	0.233
Vanillic acid glucuronide				
Model 1	1.00 (−0.39 2.39)	0.157	0.02 (<−0.01; 0.03)	0.117
Model 2	1.26 (−0.15; 2.68)	0.079	0.02 (<−0.01; 0.03)	0.081
Model 3	1.48 (−0.02; 2.98)	0.054	0.02 (<−0.01; 0.04)	0.061
Enterodiol glucuronide				
Model 1	−0.52 (−1.93; 0.90)	0.472	<0.01 (−0.02; 0.02)	0.865
Model 2	−0.42 (−1.83; 0.99)	0.553	<0.01 (−0.02; 0.02)	0.682
Model 3	−0.61 (−2.16; 0.93	0.436	<0.01 (−0.02; 0.02)	0.900
Enterolactone glucuronide				
Model 1	−0.89 (−2.27; 0.49)	0.204	−0.01 (−0.03; 0.01)	0.221
Model 2	−0.43 (−1.84; 0.98)	0.547	<−0.01 (−0.02; 0.01)	0.682
Model 3	−0.60 (−2.15; 0.95)	0.443	<−0.01 (−0.02; 0.02)	0.711
Urolithin B glucuronide				
Model 1	1.25 (−0.11; 2.62)	0.072	0.01 (−0.01; 0.02)	0.399
Model 2	1.16 (0.26; 2.57)	0.109	0.01 (−0.01; 0.02)	0.417
Model 3	1.53 (−0.01; 3.06)	0.048	0.01 (−0.01; 0.03)	0.375

MPM, microbial phenolic metabolites; DBP, diastolic blood pressure; SBP, systolic blood pressure.
*This difference remained statistically significant after adjusting for multiple comparisons.
Model 1: sex and age.
Model 2: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension, and medication (cholesterol-lowering, antidiabetic and antihypertensive agents).
Model 3: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension, medication (cholesterol-lowering, antidiabetic and antihypertensive agents), total energy intake and Mediterranean diet adherence.

Table A1
Daily intake of foods for the total population and according to tertiles of MPM.

	All ($n = 200$)	T1 ($n = 67$)	T2 ($n = 67$)	T3 ($n = 66$)	*p*-value
Olive oil (g)	40.0 ± 13.5	40.0 ± 13.2	41.7 ± 13.3	39.3 ± 14.1	0.446
Nuts (g)	9.1 ± 11.4	10.9 ± 13.6	9.5 ± 11.3	6.9 ± 8.7	0.122
Fruits (g)	479.2 ± 232.9	451.8 ± 237.4	452.3 ± 227.0	534.3 ± 227.9	0.063
Vegetables (g)	406.6 ± 175.9	426.3 ± 219.5	397.5 ± 156.7	395.7 ± 142.4	0.531
Legumes (g)	17.8 ± 7.4	18.1 ± 7.2	18.1 ± 6.7	17.2 ± 8.4	0.732
Fish (g)	117.5 ± 44.1	119.5 ± 40.0	124.1 ± 49.1	108.8 ± 42.0	0.122
Meat or meat products (g)	148.8 ± 53.7	149.3 ± 56.7	146.7 ± 47.0	150.3 ± 57.6	0.924
Pastries (g)	21.0 ± 25.9	23.7 ± 28.5	17.84 ± 25.4	21.6 ± 23.6	0.417
Dairy products (g)	338.4 ± 207.4	364.3 ± 179.5	306.9 ± 208.0	344.1 ± 231.0	0.268
Alcohol (g)	11.7 ± 17.2	8.6 ± 12.9[a]	15.9 ± 22.3[b]	10.5 ± 14.2[a,b]	0.038
Fiber (g)	27.4 ± 7.3	26.9 ± 7.6	27.2 ± 7.6	28.2 ± 6.7	0.612
Cholesterol (g)	407.0 ± 113.1	416.0 ± 117.9	412.3 ± 110.7	392.7 ± 110.9	0.445
Sodium (mg)	2523.9 ± 779.6	2529.1 ± 856.4	2621.7 ± 847.7	2419.3 ± 604.4	0.327
Folic acid (μg)	458.7 ± 103.6	457.5 ± 108.2	456.5 ± 105.0	462.2 ± 98.7	0.946
Total polyphenols (mg)	999.8 ± 316.5	971.9 ± 300.5	991.4 ± 332.6	1036.2 ± 316.7	0.491

MPM, microbial phenolic metabolites.
p-values were calculated using the test one-way ANOVA.
Different lower-case letters indicate a significant difference among groups and were calculated analysed using Bonferroni post-hoc test.

Table A2
Multivariable adjusted regression between overall ICVH and individual metrics and MPM.

	β (95 % CI)	*p*-value
Protocatechuic acid		
Overall ICVH	0.10 (-0.06; 0.25)	0.214
Individual Metrics		
Smoking status	<0.01 (-0.06; 0.07)	0.875
Body mass index	0.01 (-0.02; 0.05)	0.379
Physical activity	−0.01 (-0.07; 0.05)	0.764
Diet	0.09 (-0.03; 0.16)	0.007
Blood pressure	<-0.01 (-0.03; 0.02)	0.803
Total cholesterol	0.01 (-0.06; 0.07)	0.863
Blood glucose	−0.01 (-0.08; 0.07)	0.842
Vanillic acid glucuronide		
Overall ICVH	−0.16 (-0.29; −0.02)	0.026
Individual Metrics		
Smoking status	−0.04 (-0.09; 0.01)	0.166
Body mass index	0.01 (-0.02; 0.04)	0.448
Physical activity	−0.04 (-0.10; 0.02)	0.227
Diet	−0.01 (-0.08; 0.06)	0.806
Blood pressure	−0.01 (-0.03; 0.01)	0.406
Total cholesterol	−0.01 (-0.08; 0.05)	0.717
Blood glucose	−0.07 (-0.13; <-0.01)	0.052
Enterodiol glucuronide		
Overall ICVH	0.03 (-0.11; 0.17)	0.689
Individual Metrics		
Smoking status	0.01 (-0.03; 0.06)	0.596
Body mass index	−0.01 (-0.04; 0.03)	0.713
Physical activity	−0.05 (-0.10; 0.01)	0.096
Diet	0.10 (0.03; 0.16)	0.003
Blood pressure	0.01 (-0.01; 0.04)	0.343
Total cholesterol	−0.02 (-0.09; 0.04)	0.504
Blood glucose	−0.02 (-0.09; 0.05)	0.623
Enterolactone glucuronide		
Overall ICVH	0.09 (-0.06; 0.24)	0.227
Individual Metrics		
Smoking status	−0.02 (-0.07; 0.02)	0.321
Body mass index	0.02 (-0.01; 0.05)	0.156
Physical activity	<0.01 (-0.06; 0.06)	0.913
Diet	0.11 (0.05; 0.18)	0.001
Blood pressure	0.02 (-0.01; 0.05)	0.148
Total cholesterol	−0.07 (-0.13; −0.01)	0.027
Blood glucose	0.03 (-0.04; 0.09)	0.452
Urolithin B glucuronide		
Overall ICVH	−0.05 (-0.8; 0.09)	0.483
Individual Metrics		
Smoking status	0.03 (-0.03; 0.08)	0.349
Body mass index	0.01 (-0.02; 0.04)	0.630
Physical activity	−0.04 (-0.09; 0.01)	0.120
Diet	−0.04 (-0.11; 0.03)	0.224
Blood pressure	<0.01 (-0.02; 0.02)	0.827
Total cholesterol	0.01 (-0.05; 0.07)	0.790
Blood glucose	−0.01 (-0.08; 0.06)	0.746

ICVH, ideal cardiovascular health; MPM, microbial phenolic metabolites.
β, difference between groups; CI, confidence interval.
Adjusted for sex and age.

Overall scores ranged from 0 to 7, with a higher score indicating a better ICVH profile.

2.5. Statistical analyses

Baseline characteristics of the participants are presented as means ± standard deviation (SD) for continuous variables and percentages for categorical values. Individual baseline values of urine metabolites were normalized and scaled in multiples of 1-SD with Blom inverse normal transformation (Blom, 1960). Multivariable adjusted linear regression was used to assess the association of urinary MPM with MedDiet adherence, ICVH scores (both overall and individual metrics), and cardiovascular risk factors. To evaluate the MPM relationship with MedDiet adherence, measured with a 14-item dietary screener, two models of increasing complexity were used. Multivariable model 1 was adjusted for age and sex, and model 2 was additionally adjusted for smoking habit, educational level, BMI, physical activity, and energy intake. Additionally, we calculated an MPM score as the weighted sum of the concentrations of the 5 quantified metabolites (PCA, VAG, EDG, ELG, and UBG). The weight for each metabolite was the regression coefficient for a 1-SD increment in urine from the multivariable adjusted regression model. The associations of MPM with ICVH scores (overall and individual metrics) were adjusted for age and sex, and an MPM score was also calculated as described. To analyse the association of MPM with biochemical parameters (LDL-cholesterol, HDL-cholesterol, TG, and glucose) and blood pressure, the normality of the outcome variables was assessed with the Shapiro-Wilk test, and those that did not follow normal distribution were transformed into logarithms (HDL-cholesterol, TG, glucose and SBP). Three models of increasing complexity were employed: model 1 was adjusted for sex and age; model 2 was further adjusted for smoking habit, educational level, BMI (except anthropometric analyses), physical activity, diabetes, hypercholesterolemia, hypertension, and medication (cholesterol-lowering, antidiabetics, and antihypertensive agents); model 3 was further adjusted for total energy intake and MedDiet adherence. We used the procedure described by Simes to correct for multiple testing of the multivariable-adjusted associations that included the 5 MPM (Simes, 1986). All statistical analyses were performed using Stata 16.0 (Stata-Corp LP, Tx. USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. General characteristics

Table 1 shows the main characteristics of all the participants. Among the 200 participants, 109 were women and 91 men, and the overall mean age was 66.1 ± 5.3 years. As expected from the inclusion criteria of the PREDIMED trial, participants were at high cardiovascular risk; 51.0 % had T2D, 79.0 % had dyslipidaemia and 76.0 % had hypertension. A total of 15.0 % of the volunteers were current smokers and most had received a low level of education. Table A.1 describes the average food consumption of the participants according to tertiles of MPM. Food nutrient and polyphenol intake was similar across the three groups, except for alcohol, which was higher in the second tertile.

The concentrations of the 5 MPM, corrected for urinary creatinine, are presented in Table 2. The predominant metabolite was VAG (0.222 ± 0.393 μmol/mg of creatinine), followed by UBG (0.158 ± 0.605 μmol/mg of creatinine), and the least abundant was EDG (0.003 ± 0.004 μmol/mg of creatinine).

3.2. MPM and MedDiet adherence

Table 3 shows the association of MedDiet adherence with individual MPM and the MPM score. In the fully adjusted model, participants with higher MPM scores also reported greater adherence to the MedDiet (β = 0.62 (0.06; 1.18), *p*-value = 0.041). Regarding individual metabolites, PCA and ELG were positively associated with the MedDiet adherence score (β = 0.62 (0.09; 1.15) per 1-SD increase, *p*-value = 0.022 and β = 0.55 (0.01; 1.10) per 1-SD increase, *p*-value = 0.046, respectively). These associations were maintained after adjusting for multiple testing in Model 1, but the significance was lost after applying Model 2.

3.3. MPM score and ICVH

The ICVH metrics and the frequency of higher ICVH scores in participants are shown in Table 4. As could be expected from the nature of the PREDIMED trial, most participants obtained low ratings for overall ICVH. The majority achieved a score of 2 (33.5 %) or 3 (31.5 %), and none responded positively for>5 health items. Most of the participants did not obtain an ideal score in the individual metrics, except for smoking status and physical activity.

The association of the MPM score with the ICVH score, both overall

and individual metrics, is presented in Table 5. A positive association was found between the MPM score and the overall ICVH score (β = 1.29 (0.52; 2.07), *p*-value = 0.001). Regarding individual metrics, ratings for diet and blood glucose were significantly higher in participants with higher MPM scores (β = 0.65 (0.28; 1.01), *p*-value = 0.001; β = 0.38 (0.01; 0.75), *p*-value = 0.042, respectively). However, no differences were observed for the other items. Results for individual MPM are presented in Table A.2.

3.4. MPM and biochemical variables and blood pressure

The associations between individual MPM with LDL-cholesterol, HDL-cholesterol, TG, and glucose are shown in Table 6. Participants with higher urinary concentrations of UBG had lower levels of LDL-cholesterol in the fully adjusted model (β = -5.58 mg/dL (-9.66; −1.50) per 1-SD increase, *p*-value = 0.008), and this association was maintained after correcting for multiple testing. In addition, EDG was associated with lower levels of blood glucose in Model 1 (β = -0.04 mg/dL (-0.08; <-0.01) per 1-SD increase, *p*-value = 0.041), but this result did not remain significant after using more complex adjustment models. No significant association was found for any metabolite with HDL-cholesterol and TG, although we observed a strong tendency towards a positive association between VAG and TG.

As shown in Table 7, SBP was not significantly associated with any urinary MPM. Unexpectedly, UBG was directly associated with DBP (β = 1.53 mmHg (-0.01; −3.06) per 1-SD increase, *p*-value = 0.048), although the relationship did not remain significant after correcting for multiple testing.

4. Discussion

In this cross-sectional substudy of the PREDIMED trial, we observed that higher urinary MPM scores were associated with greater MedDiet adherence and a better ICVH score. We also found a strong inverse association between urinary concentrations of UBG and LDL-cholesterol. These findings suggest that the MedDiet is associated with phenolic metabolites that have a positive impact on cardiovascular health. To our knowledge, this is the first study to evaluate the link between diet, urinary MPM, and cardiovascular health using a high-resolution analytical technique (LTQ-Orbitrap-MS).

In the current study, the level of adherence to the MedDiet was found to be associated with the MPM score. The MedDiet is reported to modulate the microbiome ecosystem and the microbial metabolites produced (de Filippis et al., 2016; Ghosh et al., 2020). Studies on gut microbiota have reported that greater adherence to the MedDiet is linked to higher amounts of *Bacteroidetes* (Garcia-Mantrana et al., 2018; Gutiérrez-Díaz et al., 2016), including members of the genus *Prevotella* (de Filippis et al., 2016). Several gut microbiota species are involved in polyphenol metabolism, some of which belong to the *Bacteroidetes* phylum (Achterholt et al., 2000; Clavel et al., 2006; Selma et al., 2014; Venturi et al., 1989). Altogether, these results suggest that the MedDiet promotes a favourable microbial environment for the production of MPM potentially beneficial for human health.

In our subsample of the PREDIMED trial, no participant achieved the highest ICVH rating, and therefore none met all 7 health metrics. These results are consistent with a previous study within the PREDIMED trial, in which only 0.3 % of the participants achieved scores of 6 and 7 (Díez-Espino et al., 2020). The results are not surprising due to the nature of the PREDIMED trial, which is focused on an elderly Mediterranean population at high risk of CVD. However, other studies conducted in Mediterranean countries and the United States found that approximately 20 % of the adult population met at least 5 metrics (Fernandez-Lazaro et al., 2022; Younus et al., 2016). It is well-known that polyphenols have multiple benefits on cardiovascular health, mostly due to their anti-inflammatory properties [24]. Accordingly, a high polyphenol intake is reported to reduce mortality and provide cardioprotective benefits (Salazar et al., 2022; Tresserra-Rimbau, Rimm, Medina-Remón, Martínez-González, de la Torre, et al., 2014). Furthermore, urinary polyphenols are associated with lower DBP and SBP, and higher HDL-cholesterol in individuals following a Mediterranean diet (Medina-Remón et al., 2015). However, there is a lack of research on the effect of phenolic compounds detected in biological samples on the overall ICVH. The present findings suggest that ICVH is associated with multiple MPM. Regarding individual health metrics, diet and blood glucose were the most positively linked to the MPM score, which is in accordance with the association found between the MPM score and MedDiet adherence (Table 3). These results are in accordance with a recent report that polyphenols and their metabolites are associated with a lower risk of developing T2DM and reduced insulin resistance (Chiva-Blanch et al., 2013; Marhuenda-muñoz et al., 2022). Notably, individual MPM did not seem to benefit overall ICVH, suggesting that the phenolic metabolites may have a positive impact on cardiovascular health in combination rather than individually. Interestingly, total cholesterol was not associated with the MPM score, even though a negative association was observed between UBG and LDL-cholesterol in subsequent analyses. This calls into question if total cholesterol is the most appropriate item to include in the ICVH assessment, considering that LDL-cholesterol has a negative impact on health but HDL-cholesterol does not (Ference et al., 2018).

Urolithins are microbial products synthesized from ellagitannins and ellagic acid, whose main food sources are walnuts and pomegranates. Urolithin production is particularly subject to interindividual variability, as there are 3 urolithin metabotypes in the population (Tomás-Barberán et al., 2014). In our elderly PREDIMED cohort, we mainly detected metabolites of urolithin B, the glucuronidated conjugate of urolithin B being predominant. This is consistent with Cortés-Martín et al., who reported that metabotype B, the producer of urolithin B, increases with age, whereas urolithin A decreases (Cortés-Martín et al., 2018). Interestingly, in a previous clinical trial administering a phenolic supplement, LDL-cholesterol decreased only in participants with phenotype B (González-Sarrías et al., 2010). An *in vitro* study demonstrated that urolithin B may decrease the lipid plaque deposition by modulating the expression of genes involved in reverse cholesterol pathways (Zhao et al., 2019). This mechanism could explain the inverse association observed between UBG and LDL-cholesterol.

Dietary lignans, the precursors of enterodiol and enterolactone, are mainly obtained from cereal fibre, and their effect on adiposity and biochemical parameters has been assessed by numerous studies. Two intervention trials reported that dietary lignan supplementation improved glycaemic control (Pan et al., 2007; Zhang et al., 2008). In our study, we found that glucose tended to be negatively associated with EDG but not with ELG. It should be noted that dietary intake of lignans correlates poorly with enterolignans measured in biological samples, as their bioavailability may be affected by gut microbiota composition, intestinal transit time or antibiotic use (Peterson et al., 2010).

Previous publications have reported that polyphenol intake can reduce blood pressure in a population at high cardiovascular risk (Medina-Remón et al., 2013, 2015). In contrast, our data do not support this beneficial effect, as significant associations were not observed for most MPM, and the direct association between UBG and DBP was weak. It is plausible that polyphenol effects on blood pressure are caused by those absorbed in the small intestine and not metabolized by gut microbiota.

Our study has three main limitations. As all participants were elderly Mediterranean individuals at high cardiovascular risk, the results may not be applicable to other populations. Also, the sample size is relatively small, although comparable to those in similar studies performing targeted metabolomics (Marhuenda-muñoz et al., 2022). Since fecal samples were not collected in the PREDIMED trial, we could not assess the microbiota composition of our participants. Finally, the cross-sectional nature of the study does not allow causality to be determined.

On the other hand, the strengths of the study include the analysis of

biological samples, which provides reliable information on the metabolism of participants, unavailable through intake questionnaires. Importantly, the analytical equipment used (LTQ-Orbitrap) allows a precise and accurate elucidation and quantification of novel compounds.

In conclusion, we found that urinary MPM were directly associated with MedDiet adherence and a healthier cardiovascular status. These results suggest that the MedDiet is associated with phenolic metabolites that possess beneficial properties for cardiovascular health. In addition, they show that MPM effects need to be considered in combination rather than individually when assessing their health benefits. This may be helpful in establishing dietary recommendations that favour a healthier colonic microbial environment. However, more studies are needed to confirm these potential MPM-derived benefits.

CRediT authorship contribution statement

Inés Domínguez-López: Conceptualization, Methodology, Investigation, Writing – original draft. **Camila Arancibia-Riveros:** Investigation. **María Marhuenda-Muñoz:** Methodology, Writing – review & editing. **Anna Tresserra-Rimbau:** Conceptualization, Writing – review & editing. **Estefanía Toledo:** Writing – review & editing. **Montserrat Fitó:** Writing – review & editing. **Emilio Ros:** Writing – review & editing. **Ramon Estruch:** Writing – review & editing. **Rosa M. Lamuela-Raventós:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centres. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

Appendix A

See Tables A1 and A2.

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3.4. Cognitive decline

The protective effects of dietary polyphenols against cognitive decline have been extensively studied in recent years. However, due to the low bioavailability of these compounds, it is crucial to investigate the role of phenolic compounds produced by the intestinal microbiota in the brain. The identification of MPM with a positive association with cognitive functions was achieved in two different cohorts (Publication 12 and 13). Lastly, the role of vitamin B12 in cognition was studied based on whether individuals had high or low adherence to the Mediterranean diet to assess the influence of this dietary pattern and its components on the impact that vitamin B12 may have on the brain (Publication 14).

3.4.1. Publication 12

Microbial phenolic metabolites are associated with better frontal lobe cognition

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Supplementary Material available in Annex section.

Abstract

Aims: To assess the association of MPM with cognition in an older Mediterranean population at high CVD risk.

Methods: This cross-sectional analysis was performed with 200 participants of the PREDIMED trial (Barcelona-Clinic recruitment center). A novel method based on HPLC coupled to MS was used to identify urinary MPM (protocatechuic acid, enterodiol glucuronide, enterolactone glucuronide, urolithin B glucuronide, and vanillic acid glucuronide), and cognitive function was evaluated with neuropsychological tests. Multivariable-adjusted ordinary least squares regression was used to assess the associations between cognitive function and MPM, and a score was calculated as the weighted sum of MPM.

Results: A higher MPM score was associated with better frontal lobe function. Among individual metabolites, vanillic acid glucuronide was correlated with frontal cognitive performance. Participants with higher concentrations of vanillic acid glucuronide and urolithin B glucuronide obtained better scores in the Color Trail Test part 2.

Conclusions: A higher score for urinary multi-MPM was associated with better frontal cognitive performance in an older Mediterranean population.

Microbial phenolic metabolites are associated with better frontal lobe cognition

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Abstract

With increasing life expectancy, neurodegenerative diseases have become one of the leading causes of ill-health in the elderly. Preventive strategies include following healthy diets, such as the Mediterranean diet, which is particularly rich in polyphenols, bioactive compounds with neuroprotective properties. The aim of this study was to assess the association of microbial phenolic metabolites (MPM) with cognition. This cross-sectional analysis was performed with 200 participants of the PREDIMED trial (Barcelona-Clinic recruitment center). A novel method based on liquid chromatography coupled to mass spectrometry was used to identify urinary MPM (protocatechuic acid, enterodiol glucuronide, enterolactone glucuronide, urolithin B glucuronide, and vanillic acid glucuronide), and cognitive function was evaluated with neuropsychological tests. Multivariable-adjusted ordinary least squares regression was used to assess the associations between cognitive function and MPM, and a score was calculated as the weighted sum of MPM. A higher MPM score was associated with better frontal lobe function. Among individual metabolites, vanillic acid glucuronide was correlated with frontal cognitive performance. Participants with higher concentrations of vanillic acid glucuronide and urolithin B glucuronide obtained better scores in the Color Trail Test part 2. A higher score for urinary multi-MPM was associated with better frontal cognitive performance in an older Mediterranean population.

Keywords: polyphenols, neurodegeneration, microbiota, gut-brain axis, PREDIMED.

Abbreviations: CVD, cardiovascular disease; EDG, enterodiol glucuronide; ELG, enterolactone glucuronide; LTQ-Orbitrap-MS, linear ion trap quadrupole-Orbitrap mass spectrometry; MPM, microbial phenolic metabolites; MMSE, mini-mental state examination; PCA, protocatechuic acid; RAVLT, Rey auditory verbal test learning;

UBG, urolithin B glucuronide; VAG, vanillic glucuronide; WAIS, Wechsler adult intelligence scale; WMS, Wechsler memory scale.

1. Introduction

The prevalence of neurodegenerative diseases has increased considerably in the last years due to population aging [1]. With the growth in life expectancy, dementia has become a leading cause of death in developed countries, most frequently in the form of Alzheimer's disease [2]. The neuronal loss that characterizes Alzheimer's disease particularly affects the frontal and temporal lobes; other associated changes in the brain are the formation of neurofibrillary tangles and amyloid plaques. At present, it is an incurable disease with a late diagnosis, so strategies to prevent or delay its development are urgently needed [3].

Although the precise cause of Alzheimer's disease and related dementias remains unknown, there is evidence that their development is associated with several modifiable risk factors, among them education, vascular impairment, physical activity, and dietary habits [4] . Diet in particular, is believed to play a major role in cognitive decline, [5] a pre-dementia stage. In this context, several epidemiological and clinical studies have shown that the Mediterranean diet can both delay age-related cognitive decline [6] and reduce the incidence of neurodegenerative disorders and mortality due to Alzheimer's disease [7]. The Mediterranean diet is a plant-based dietary pattern characterized by a high intake of fruits, vegetables, and seeds rich in bioactive compounds such as polyphenols, which have antioxidant and anti-inflammatory properties [8]. This may partially explain its protective effects against neurodegeneration, which is closely related to oxidative stress and inflammation [9].

Although polyphenols may have beneficial effects on cognitive function [10], they have low bioavailability. Prior to absorption, they are extensively metabolized by colonic microbiota[11], with which they have a bidirectional relationship, as polyphenols can also act as prebiotics and influence microbiota composition. The central nervous system and gut microbiota are deeply connected by biochemical signaling, forming what is known as the gut-brain axis, and polyphenols may be involved by improving cerebral blood flow or directly crossing the blood-brain barrier [12].

Despite growing interest in the gut-brain axis in recent years, to the best of our knowledge no study has evaluated the effect of microbial phenolic metabolites (MPM) detected in urine on neurocognition. Therefore, the aim of the present study was to assess the association of MPM with cognitive function in a subgroup of participants in the PREDIMED trial. A novel methodology previously developed by our group using high precision analytical techniques based on linear ion trap quadrupole-Orbitrap-mass spectrometry (LTQ-Orbitrap-MS) allowed us to identify hitherto scarcely explored compounds derived from the gut microbiota.

2. Methods

2.1 Study design

A cross-sectional analysis was conducted within the PREDIMED (PREvención con DIeta MEDiterránea) study, a large, parallel-group, multicenter, randomized, controlled clinical trial with a mean follow-up of 5 years, designed to assess the effect of the Mediterranean diet enriched in olive oil or nuts on cardiovascular disease (CVD) incidence (http://www.predimed.es) [13]. Participants were recruited in Spain from

October 2003 to December 2010 and included 7 447 men (55–80 years old) and women (60–80 years old) at high cardiovascular risk. Eligible participants had type 2 diabetes or at least three of the following major risk factors: current smoking, hypertension, dyslipidemia, overweight/obesity or a family history of premature CVD. A detailed description of methods and participants has been published elsewhere [13-14].

The present sub-study was performed in a random subsample of 200 participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) who participated in a cognitive function study [15]. Participants who reported extreme total energy intakes (> 3 500 or < 500 kcal/day in women or > 4 000 or < 800 kcal/day in men) were excluded from the analysis [16].

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.2 Covariate assessment

Participants completed a validated, semi-quantitative 137-item food frequency questionnaire with the assistance of trained dietitians, as well as a 14-item questionnaire to assess their adherence to the Mediterranean diet [17]. Trained personnel measured body weight, height, waist circumference and blood pressure. Body mass index (BMI) was calculated as weight in kg divided by height in m^2. Physical activity (metabolic equivalent tasks per minutes per day, METs min/day) was assessed with a validated Spanish version of the Minnesota physical activity questionnaire [18].

2.3 Cognitive tests

Cognitive examinations were conducted by an experienced neuropsychologist. The instruments employed for the cognitive assessment were as follows: global cognitive function was evaluated with the Mini-Mental State Examination (MMSE) [19]; intermediate and delayed episodic verbal memory were rated by the Rey Auditory Verbal Learning Test (RAVLT) [20]; episodic verbal memory was assessed with a subtest of the Wechsler Memory Scale (WMS), the verbal paired associates test [21]; semantic fluency was evaluated with an animal fluency test [22]; immediate memory and working memory were assessed with the digit span test of the Wechsler Adult Intelligence Scale (WAIS) [23]; executive function, including attention, visual-motor speed, and cognitive flexibility, were measured with the Color Trail Test (parts 1 and 2) [24]. Three composite scores of cognitive function were constructed for each participant. The frontal function composite was based on the standardized mean results of the Digit Span Backward Test and Color Trail Test (parts 1 and 2), which measured attention, cognitive flexibility and working memory. The memory composite was constructed with the standardized results from the RAVLT and WMS paired associate subtest. Finally, a global cognition composite was obtained by computing the mean standardized results of all the neuropsychological tests, including the MMSE.

2.4 Microbial phenolic metabolites

Fig. 1 shows a schematic representation of the experimental procedure for identifying and quantifying urinary MPM using a solid-phase extraction process followed by a chromatographic and spectrometric analysis in a LTQ-Orbitrap-MS.

2.4.1 Standards and reagents

Protocatechuic acid (PCA), enterodiol, urolithin-A, and urolithin-B were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (+) *cis, trans*-abscisic

acid D6 was obtained from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Vanillic acid, enterolactone and creatinine were obtained from Fluka (St. Louis, MO, USA). Standards were stored in powder form and protected from light. The reagents were purchased from the following commercial suppliers: methanol of LC-MS grade and acetonitrile of HPLC grade from Sigma-Aldrich and formic acid (≥ 98%) from Panreac Química S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore system (Bedford, MA, USA).

2.4.2 Sample preparation

Biological samples were collected after an overnight fast, coded, and stored at -80 ºC until analysis. MPM produced by the gut microbiota were determined using a method previously validated by our group with minor modifications [25]. For the present analyses, we focused only on MPM that are mostly or exclusively produced by the gut microbiota. Briefly, 50 µL of urine samples were diluted 1:20 (*V*/*V*) with Milli-Q Water and 100 µL of the internal standard abscisic acid D6 was added. The sample dilution was then acidified with 2 µL of formic acid and centrifuged at 15 000 × *g* at 4 ºC for 4 min. To isolate MPM and eliminate undesired compounds, the acidified urines underwent a solid-phase extraction in Water Oasis HLB 96-well plates 30 µm (30 mg) (Water Oasis, Milford, MA, USA). The 96-well plate was activated with methanol and 1.5 mol/L formic acid, and after loading the samples, a clean-up step was performed with 1.5 mol/L formic acid and methanol (0.5%). MPM were eluted with methanol acidified with 1.5 mol/L formic acid, evaporated to dryness with nitrogen gas and reconstituted with 100 µL formic acid (0.05%). After 20 min of vortex mixing, the samples were filtered through 0.22 µm polytetrafluoroethylene 96-well plate filters (Millipore, Massachusetts, USA).

Urinary concentrations of MPM were corrected by urine creatinine, measured according to the Jaffé alkaline picrate method adapted for Thermo microtiter 96-well plates, as described by Medina-Remón et al. [26].

2.4.3 LTQ Orbitrap ESI analysis

The analysis was performed on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode as described elsewhere [25]. Chromatographic separation was performed on a Kinetex F5 100A (50 mm × 4.6 mm × 2.6 μm) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were, respectively, 0.05% formic acid in water and 0.05% formic acid in acetonitrile. The following linear gradient was used: held at 98%A for 1.7 min, decreased to 92%A for 3 min, decreased to 80%A for 1.3 min, decreased to 70%A for 1.3 min, decreased to 50% for 0.1 min, decreased to 0% for 1.3 min, then returned to initial conditions for 1.7 min and re-equilibrated for 3 min. The flow rate was set at 0.750 μL/min and the injection volume was 5 μL.

2.4.4 Identification and quantification of MPM

The identification and quantification of MPM was performed using Trace Finder software version 4.1 (Thermo Fisher Scientific, San Jose, CA). As standards for glucuronidated and sulfated MPM were unavailable, these metabolites were quantified with their respective aglycone equivalents.

Due to high inter-individual variability in the metabolism of MPM, the identifiable metabolites were not detected in all the participants. To simplify the analysis and facilitate its comprehension, only metabolites with < 20% of missing values were included in the statistical analyses. Following this criterion, a total of 5 metabolites were

included: PCA, enterodiol glucuronide (EDG), enterolactone glucuronide (ELG), urolithin B glucuronide (UBG), and vanillic acid glucuronide (VAG). Missing values of the previously listed metabolites with less than 20% of missing values were replaced by the half of the minimum detectable value.

2.5 Statistical analysis

Baseline characteristics of the participants are described as mean ± standard deviations (SD) for quantitative variables and percentages for categorical variables. The MPM concentrations were normalized and scaled in multiples of 1-SD with Blom's inverse normal transformation to stabilize the variance [27].

The association of MPM with neuropsychological test scores and test composites was assessed with multivariable linear regression adjusted for two models of increasing complexity. Model 1 was adjusted for age and sex. Model 2 was further adjusted for smoking (former, never, current), educational level (low, medium and high), *APOE* e4 genotype, physical activity, BMI, total energy intake, Mediterranean diet adherence (continuous), hypertension, hypercholesterolemia, diabetes, statin treatment, and use of anticholinergic drugs (yes/no). A corrected *P*-value by multiple comparison following the Simes procedure was also considered. In addition, an MPM score was calculated as the weighted sum of concentrations of the target metabolites and modelled the score as a main exposure in the multivariable linear regression model. The weight for each metabolite was the regression coefficient for a 1-SD increment in the plasma concentration estimated from the multivariable linear regression model.

All statistical analyses were performed with Stata 16.0 (Stata-Corp LP, Tx. USA). Two-sided significance was set at $P < 0.05$.

3. Results

3.1 General characteristics of participants

The characteristics of all the participants are listed in Table 1 according to quartiles of total urinary MPM concentrations. All the groups were well-balanced in terms of sex, age, and educational level. By study design, all the participants were overweight or obese and had a high prevalence of conventional cardiovascular risk factors: 51% had type-2 diabetes mellitus, 79% had dyslipidemia, and 76% had hypertension. A lower percentage of participants suffered from diabetes in the third quartile of MPM, whereas the second quartile had more participants with hypertension. Only 15% of the study population were current smokers. Physical activity was comparable among groups, although participants in the first quartile tended to be more physically active. Regarding total energy intake, the first quartile displayed the highest values, whereas the last quartile had the lowest.

Table S1 presents the means and SD of urinary metabolite concentrations for the total population and according to quartiles. The predominant metabolites were VAG (0.222 $\pm$ 0.393 µmol/mg of creatinine) and UBG (0.158 $\pm$ 0.605 µmol/mg of creatinine), whereas the least abundant identified metabolite in urine was EDG (0.003 $\pm$ 0.004 µmol/mg of creatinine), followed by PCA (0.009 $\pm$ 0.020 µmol/mg of creatinine) and ELG (0.020 $\pm$ 0.026 µmol/mg of creatinine).

3.2 MPM and Cognition Composites

The associations between MPM scores and the composite scores for frontal lobe function, memory, and global cognition are presented in Table 2. In the multivariable

adjusted model that included sex, age, smoking, education, physical activity, BMI, total energy intake, hypertension, hypercholesterolemia, diabetes, *APOE* e4 genotype, statin treatment, anticholinergic drugs, and Mediterranean diet adherence, the MPM score was associated with a higher composite score for frontal lobe function (β = 0.69, 95%CI: 0.14, 1.24 per 1-SD increase, *P*-value = 0.014). On the other hand, no significant association was found with memory or global composite scores. When the MPM scores were modelled as quartiles, we observed that participants in the highest quartile had higher composite scores for frontal function and the relationship was linear (β = 0.51, 95%CI: 0.05, 0.96, *P* = 0.033 for trend).

Individual polyphenols are described in Table 3. Urine VAG was positively associated per 1-SD increment with the frontal composite score in the fully adjusted model (β = 0.17, 95%CI: 0.03, 0.31 per 1-SD, *P*-value = 0.018), but it did not remain significant after accounting for multiple testing.

3.3 MPMs and Neuropsychological Tests

Table S2 describes the associations of individual MPMs modelled continuously (per 1-SD) with neuropsychological tests. Most of the individual MPM did not show any significant association with the cognitive tests, except for UBG and VAG with the Color Trail Test part 2.

As it is shown in Fig. 2, participants with higher urinary UBG and VAG obtained better scores in the Color Trail Test part 2 (β = 0.19, 95%CI: 0.01, 0.36 per 1-SD, *P*-value = 0.035 and β = 0.19, 95%CI: 0.01, 0.38 per 1-SD, *P*-value = 0.037, respectively). However, these associations were no longer significant after adjustment for multiple comparisons.

4. Discussion

In this cross-sectional study conducted in a subsample of participants in the PREDIMED trial, we observed that a score that combined urinary MPMs was associated with better frontal lobe function in a Mediterranean population at high cardiovascular risk. Associations were found between higher composite scores for frontal function and higher concentrations of VAG, and between scores for the Color Trail Test part 2 and both VAG and UBG. Therefore, the findings from the present study suggest that MPMs may be involved in frontal cognition.

The gut microbiota has a potentially high impact on brain function due to its influence on the nervous, endocrine, and immune systems through the gut-brain axis[10]. Thus, maintaining a healthy gut microbiota has emerged as a key factor for the protection of normal brain function [28]. Polyphenols can alter the gut microbial community and, conversely, their metabolism and bioavailability depend on the microbiota composition and associated enzymatic transformations. Thus, polyphenol metabolism differs between individuals, even after the intake of similar dietary sources of phenolic compounds [12]. This would explain why in the present study only five urinary MPMs were identified and quantified as widely present among participants.

In the present cross-sectional analysis, we found associations between total MPM scores and frontal functions composite after adjusting for potential confounders, suggesting that a combination of polyphenols derived from the microbiota may have a positive impact on frontal lobe functions. Regarding dietary intakes, studies assessing the consumption of polyphenols using FFQs have demonstrated that these molecules have benefits against neurodegeneration. The two major classes of polyphenols, flavonoids

and phenolic acids, have been shown to exert neuroprotective effects in middle-aged and older adults [29,30]. While total urinary polyphenols have been associated with better cognitive performance in an older Mediterranean population [10], few studies have analyzed the relationship between individual MPMs and cognition. A recent cross-sectional study reported a neuroprotective effect of serum phenolic metabolites, some of which were derived from gut bacteria [29]. Results of animal studies are also consistent with our findings, as they support an inverse association between MPMs and cognitive impairment [30-31].

The frontal lobe of the brain is involved in processes related to executive function, attention and working memory, all of which have been associated with MPMs and are among the cognitive functions most negatively affected by aging [32-33]. Different studies have shown that polyphenols have a positive impact on cognitive functions related to the frontal lobe, which is consistent with our results. A randomized clinical trial reported an improvement in executive function after consumption of a flavonoid-rich orange juice for 8 weeks [34], and a previous study reported similar benefits 2 hours after the intake of cocoa flavonols [35]. In addition, a recent meta-analysis of randomized controlled trials concluded that short- to moderate-term interventions with polyphenols had a significant albeit small positive effect on working and episodic memory [36]. However, previous research evaluated polyphenols' intake have not considered the possible effect of microbiota metabolism. In the present study, we focused on the MPM and, therefore, showed which are the metabolites that are associated with better biological functions that impact on frontal cognition.

Several mechanisms may underlie these beneficial effects of polyphenols, including the counteraction of oxidative stress and neuroinflammation [37]. It has been shown that polyphenols inhibit ROS-forming enzymes, prevent metal deposition and neurotoxicity,

modulate transcription factors that regulate inflammatory and oxidative pathways, and have an indirect impact on brain function by improving cerebral blood flow [38]. These three processes, neuroinflammation, oxidative stress and cerebrovascular function, have been specifically linked to a decline in executive functions [39-40]. These observations provide a biological explanation for our findings of an association between urinary MPMs and frontal lobe function.

Among all the studied metabolites, VAG was the one most strongly associated with frontal cognition, and it also displayed a positive association with the Color Trail Test part 2. VAG is the glucuronidated form of vanillic acid, a phenolic acid metabolized from the anthocyanin cyanidin-3-glucoside by gut microbiota [41]. Consumption of cyanidin glycosides, present in berries, have been widely correlated with better memory response [44]. However, studies assessing the impact of the metabolite VAG or its parent compound vanillic acid on the brain are scarce, a few have evaluated the effect of the aglycone form in animal studies. In experimental models of Alzheimer's disease, vanillic acid appears to attenuate the impact of amyloid plaque accumulation [42-43]. Other studies report that this polyphenol reduced Fe^{2+} and lipopolysaccharide-induced toxicity in the brain [44-45]. Regarding UBG, associations were observed with the Color Trail Test part 2 but not with the frontal function composite score, which suggests it has a lower effect compared to VAG. Previous studies have found positive neurological effects of the parent compound of UBG, urolithin B, an ellagitannin-derived microbial metabolite [46] related to the inhibition of neuronal apoptosis, suppressed microglia activation and suppressed phosphorylation in inflammatory pathways [47-48]. Interestingly, an *in vitro* and *in silico* study demonstrated that polyphenol metabolites can cross the blood brain barrier, and VAG in particular was detected in the apical and

basolateral compartments [49]. Therefore, a higher blood brain barrier permeability could promote the neuroprotective effects of these metabolites.

Our study has limitations. First, given that the participants were older Mediterranean individuals at high cardiovascular risk, the results cannot be generalized to other populations. Second, the sample size was relatively small. Third, causality cannot be determined due to the cross-sectional design. The study also has strengths, including the use of biological samples, which provide the most accurate representation of the metabolic state of participants, and administration of a comprehensive battery of neuropsychological tests to assess cognitive function. In addition, the use of high precision equipment such as LTQ-Orbitrap allowed the identification and quantification of MPMs that until now have been scarcely studied in biological samples.

5. Conclusions

The results of the present cross-sectional study using LTQ-Orbitrap-MS suggest that higher concentrations of urinary MPMs, especially VAG, are associated with better frontal lobe function in older Mediterranean adults at high cardiovascular risk. Further studies are needed to elucidate the molecular mechanisms linking these metabolites to cognitive health.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centres. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

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Figures

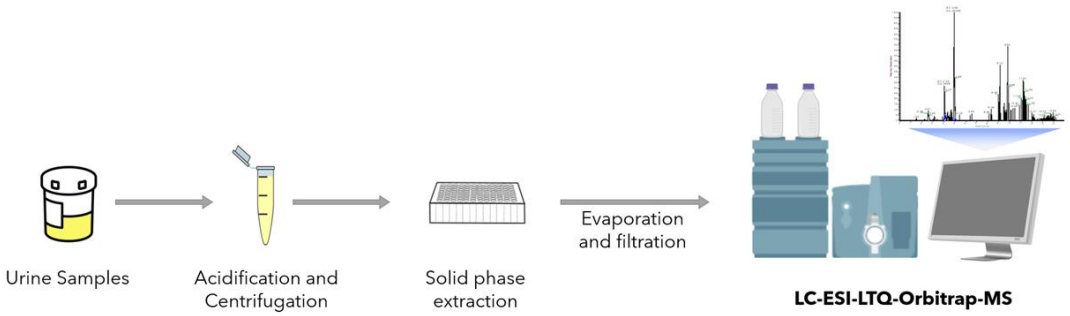

Figure 1. Schematic representation of the experimental analysis of microbial phenolic metabolites.

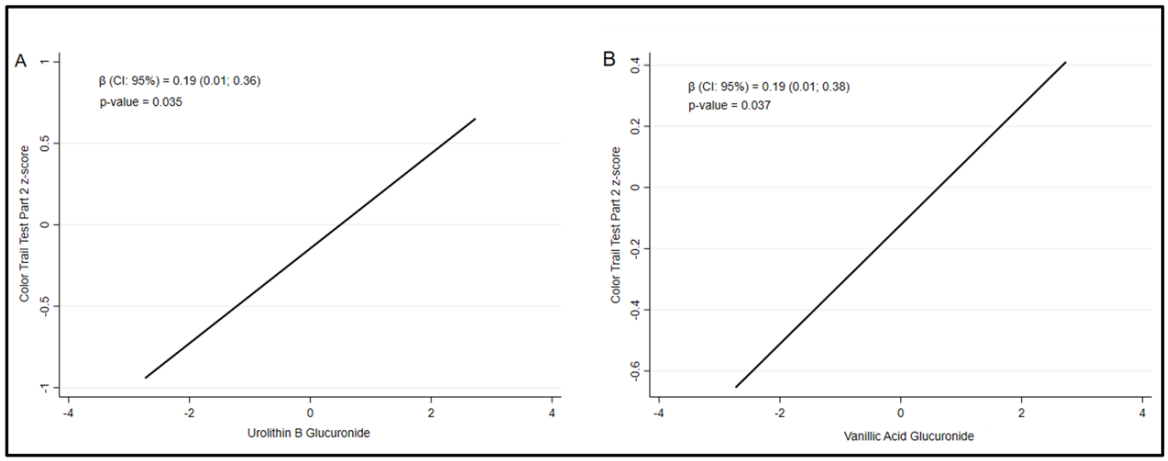

Figure 2. Multivariable adjusted regression of Color Trail Test Part 2 z-score and Urolithin B Glucuronide (A), and Vanillic Acid Glucuronide (B). Metabolites concentrations were normalized and expressed as 1-SD increment.

ß, difference between groups; CI, confidence interval.

Models adjusted for sex, age, smoking habit, educational level, physical activity, BMI, total energy intake, hypertension, hypercholesterolemia, diabetes, APO E genotype, statin treatment, anticholinergic drugs, and Mediterranean diet adherence.

Tables

Table 1. General characteristics of all participants according to quartiles of MPM[a].

Characteristics	All (n = 200)	Q1 (n = 50)	Q2 (n = 50)	Q3 (n = 50)	Q4 (n = 50)	p-value[b]
MPM	0.41 (0.02-4.53)	0.06 (0.02-0.09)	0.12 (0.09-0.15)	0.23 (0.15-0.32)	1.23 (0.34-4.53)	
Women, %	54.5	46.0	58.0	48.0	66.0	0.156
Age, years	66.1 ± 5.3	66.8 ± 5.9	65.8 ± 4.3	65.90 ± 5.4	66.0 ± 5.8	0.763
Weight, kg	76.6 ± 11.7	78.4 ± 12.0	77.6 ± 11.4	74.4 ± 11.4	76.0 ± 12.0	0.317
BMI, kg/m^2	29.5 ± 3.4	29.5 ± 2.9	29.8 ± 3.5	28.6 ± 3.6	30.2 ± 3.6	0.099
Medium & high educational level, %	33.5	28.0	40.0	28.0	38.0	0.430
Diabetes mellitus, %	51.0	52.0	70.0	38.0	44.0	0.009
Dyslipidaemia, %	79.0	80.0	70.0	86.0	80.0	0.264
Hypertension, %	76.0	74.0	82.0	78.0	70.0	0.533
Current smoker, %	15.0	12.0	16.0	20.0	12.0	0.881
Physical activity, METS-min/day	291.0 ± 266.9	332.3 ± 332.6	269.7 ± 250.6	314.0 ± 241.1	247.8 ± 230.2	0.364
Total energy intake, kcal/day	2394.1 ± 500.8	2494.5 ± 487.3	2367.4 ± 578.0	2472.2 ± 429.9	2242.1 ± 470.3	0.045

Q, Quartile; MPM, Microbial Phenolic Metabolites; METS, Metabolic Equivalents.
[a] Continuous variables are shown as means ± SDs, and categorical variables are shown as percentages.
[b] T-tests were used for continuous variables, and a chi-square test was used for categorical variables.

Table 2. Multivariable adjusted regression between phenolic metabolite scores and composite cognitive scores.

	β (CI: 95%)[a]	*p*-value	Q1	Q2	Q3	Q4	*p*-trend
Frontal Lobe Function Composite							
Model 1[b]	0.88 (0.36; 1.40)	0.001	Ref	0.43 (-0.02; 0.88)	0.30 (-0.15; 0.76)	0.62 (0.14; 1.09)	0.023
Model 2[c]	0.69 (0.14; 1.24)	0.014	Ref	0.47 (0.08; 0.86)	0.46 (0.10; 0.83)	0.51 (0.05; 0.96)	0.033
Memory Composite							
Model 1[b]	0.24 (-0.55; 1.03)	0.552	Ref	0.07 (-0.24; 0.38)	-0.07 (-0.37; 0.24)	0.13 (-0.23; 0.49)	0.658
Model 2[c]	0.22 (-0.64; 1.07)	0.620	Ref	0.10 (-0.19; 0.39)	-0.12 (-0.44; 0.19)	0.10 (-0.26; 0.45)	0.908
Global Composite							
Model 1[b]	0.73 (-0.74; 1.49)	0.512	Ref	0.06 (-0.17; 0.29)	0.03 (-0.19; 0.25)	0.16 (-0.09; 0.42)	0.198
Model 2[c]	0.37 (-0.74; 1.49)	0.512	Ref	-0.03 (-0.25; 0.20)	-0.01 (-0.25; 0.22)	0.08 (-0.16; 0.32)	0.504

Q, quartile.

[a] ß, difference between groups; CI, confidence interval.

[b] Model 1: sex and age.

[c] Model 2: sex, age, smoking habit, educational level, physical activity, BMI, total energy intake, hypertension, hypercholesterolemia, diabetes, APO E genotype, statin treatment, anticholinergic drugs, and Mediterranean diet adherence.

Table 3. Multivariable adjusted regression between individual MPM and composite cognitive test scores.

	Frontal Lobe Function Composite		**Memory Composite**		**Global Composite**	
	β (CI: 95%) [a]	*p*-value	β (CI: 95%) [a]	*p*-value	β (CI: 95%) [a]	*p*-value
Protocatechuic acid						
Model 1[b]	-0.15 (-0.29; -0.01)	0.034	0.02 (-0.09; 0.14)	0.674	-0.03 (-0.11; 0.06)	0.512
Model 2[c]	-0.06 (-0.20; 0.07)	0.343	0.05 (-0.07; 0.17)	0.411	<0.01 (-0.08; 0.09)	0.953
Enterodiol glucuronide						
Model 1[b]	0.05 (-0.14; 0.23)	0.635	<0.01 (-0.12; 0.12)	0.984	<-0.01 (-0.09; 0.08)	0.967
Model 2[c]	0.13 (-0.06; 0.32)	0.165	-0.02 (-0.14; 0.09)	0.678	<-0.01 (-0.08; 0.07)	0.905
Enterolactone glucuronide						
Model 1[b]	-0.08 (-0.25; 0.09)	0.367	-0.02 (-0.14; 0.09)	0.674	-0.04 (-0.12; 0.05)	0.386
Model 2[c]	0.01 (-0.15; 0.16)	0.941	-0.06 (-0.17; 0.06)	0.338	-0.04 (0.12; 0.03)	0.274
Urolithin B glucuronide						
Model 1[b]	0.16 (0.02; 0.30)	0.021	0.03 (-0.08; 0.15)	0.583	0.05 (-0.04; 0.12)	0.254
Model 2[c]	0.11 (-0.04; 0.26)	0.139	-0.03 (-0.15; 0.09)	0.588	-0.01 (-0.09; 0.08)	0.892
Vanillic acid glucuronide						
Model 1[b]	0.10 (-0.06; 0.26)	0.214	0.02 (-0.10; 0.14)	0.785	0.01 (-0.09; 0.10)	0.905
Model 2[c]	0.17 (0.03; 0.31)	0.018	0.01 (-0.12; 0.13)	0.889	0.01 (-0.08; 0.11)	0.793

MPM, microbial phenolic metabolites**.**

[a] ß, difference between groups; CI, confidence interval.

[b] Model 1: sex and age.

[c] Model 2: sex, age, smoking habit, educational level, physical activity, BMI, total energy intake, hypertension, hypercholesterolemia, diabetes, APO E genotype, statin treatment, anticholinergic drugs, and Mediterranean diet adherence.

Appendices

Table S1. Means and SD of urinary concentrations of metabolites (micromole per mg of creatinine) for the total population and according to quartiles of MPM[a].

	All (n = 200)	Q1 (n = 50)	Q2 (n = 50)	Q3 (n = 50)	Q4 (n = 50)
Total Microbial Phenolic Metabolites	0.412 ± 0.716	0.064 ± 0.018	0.123 ± 0.016	0.225 ± 0.053	1.234 ± 1.071
Protocatechuic acid	0.009 ± 0.020	0.004 ± 0.003	0.005 ± 0.003	0.008 ± 0.009	0.020 ± 0.037
Enterodiol glucuronide	0.003 ± 0.004	0.001 ± 0.001	0.002 ± 0.005	0.003 ± 0.004	0.004 ± 0.004
Enterolactone glucuronide	0.020 ± 0.026	0.012 ± 0.010	0.014 ± 0.016	0.023 ± 0.025	0.030 ± 0.039
Urolithin B glucuronide	0.158 ± 0.605	0.002 ± 0.005	0.005 ± 0.008	0.015 ± 0.040	0.610 ± 1.099
Vanillic acid glucuronide	0.222 ± 0.393	0.045 ± 0.016	0.097 ± 0.024	0.176 ± 0.066	0.571 ± 0.670

[a]MPM, microbial phenolic metabolites.

Table S2. Multivariable adjusted regression between individual phenolic metabolites and cognitive tests.

	MMSE		**RAVLT, total learning**		**RAVLT, delayed recall**	
	β (CI: 95%) [a]	p-value	β (CI: 95%) [a]	p-value	β (CI: 95%) [a]	p-value
Protocatechuic acid						
Model 1[b]	-0.09 (-0.21; 0.03)	0.122	0.05 (-0.09; 0.19)	0.507	0.07 (-0.06; 0.20)	0.294
Model 2[c]	-0.06 (-0.19; 0.07)	0.354	0.06 (-0.09; 0.20)	0.435	0.09 (-0.04; 0.22)	0.172
Enterodiol glucuronide						
Model 1[b]	<-0.01 (-0.15; 0.15)	0.971	-0.03 (-0.17; 0.11)	0.642	0.06 (-0.08; 0.20)	0.367
Model 2[c]	<-0.01 (-0.15; 0.15)	0.979	-0.07 (-0.20; 0.07)	0.321	0.03 (-0.11; 0.17)	0.651
Enterolactone glucuronide						
Model 1[b]	-0.01 (-0.14; 0.12)	0.962	-0.07 (-0.20; 0.07)	0.331	0.01 (-0.13; 0.14)	0.935
Model 2[c]	-0.03 (-0.16; 0.10)	0.669	-0.13 (-0.26; <0.01)	0.058	-0.04 (-0.18; 0.11)	0.611
Urolithin B glucuronide						
Model 1[b]	0.14 (0.01; 0.27)	0.039	0.02 (-0.11; 0.15)	0.811	0.05 (-0.09; 0.20)	0.446
Model 2[c]	0.12 (-0.01; 0.25)	0.073	-0.06 (-0.20; 0.08)	0.388	-0.02 (-0.17; 0.13)	0.825
Vanillic acid glucuronide						
Model 1[b]	-0.06 (-0.19; 0.08)	0.391	0.03 (-0.11; 0.17)	0.679	-0.02 (-0.16; 0.12)	0.759
Model 2[c]	-0.05 (-0.19; 0.09)	0.500	0.07 (-0.13; 0.16)	0.816	-0.03 (-0.17; 0.12)	0.731
	Paired associates		**Verbal fluency**		**Digit Span Forward**	
	β (CI: 95%) [a]	p-value	β (CI: 95%) [a]	p-value	β (CI: 95%) [a]	p-value
Protocatechuic acid						
Model 1[b]	-0.04 (-0.18; 0.09)	0.516	-0.01 (-0.20; 0.19)	0.945	-0.06 (-0.24; 0.12)	0.526
Model 2[c]	<-0.01 (-0.14; 0.14)	0.984	0.04 (-0.15; 0.22)	0.699	0.02 (-0.19; 0.23)	0.839

Enterodiol glucuronide						
Model 1[b]	-0.03 (-0.16; 0.10)	0.648	0.03 (-0.15; 0.22)	0.742	0.14 (-0.06; 0.33)	0.171
Model 2[c]	-0.04 (-0.17; 0.10)	0.602	0.03 (-0.19; 0.26)	0.769	0.22 (<0.01; 0.45)	0.054
Enterolactone glucuronide						
Model 1[b]	-0.01 (-0.13; 0.11)	0.859	0.02 (-0.25; 0.28)	0.904	-0.11 (-0.29; 0.07)	0.235
Model 2[c]	<-0.01 (-0.13; 0.13)	0.997	0.01 (-0.27; 0.29)	0.958	-0.07 (-0.31; 0.17)	0.545
Urolithin B glucuronide						
Model 1[b]	0.03 (-0.10; 0.15)	0.692	0.04 (-0.13; 0.21)	0.656	0.04 (-0.16; 0.23)	0.723
Model 2[c]	<-0.02 (-0.15; 0.11)	0.744	0.03 (-0.16; 0.22)	0.730	-0.01 (-0.21; 0.19)	0.891
Vanillic acid glucuronide						
Model 1[b]	0.04 (-0.08; 0.16)	0.510	-0.01 (-0.19; 0.17)	0.913	-0.04 (-0.25; 0.17)	0.648
Model 2[c]	0.04 (-0.10; 0.17)	0.610	0.04 (-0.16; 0.23)	0.719	<0.01 (-0.22; 0.24)	0.938

	Digit Span Backward		**Color Trail Test Part 1**		**Color Trail Test Part 2**	
	β (CI: 95%)[a]	*p*-value	β (CI: 95%)[a]	*p*-value	β (CI: 95%)[a]	*p*-value
Protocatechuic acid						
Model 1[b]	-0.06 (-0.25; 0.13)	0.522	-0.18 (-0.36; 0.01)	0.059	-0.17 (-0.34; <-0.01)	0.046
Model 2[c]	0.04 (-0.15; 0.24)	0.640	-0.12 (-0.31; 0.07)	0.201	-0.04 (-0.21; 0.13)	0.646
Enterodiol glucuronide						
Model 1[b]	0.03 (-0.19; 0.24)	0.801	0.08 (-0.19; 0.36)	0.540	0.05 (-0.17; 0.06)	0.656
Model 2[c]	0.09 (-0.10; 0.29)	0.341	0.18 (-0.12; 0.49)	0.231	0.14 (-0.10; 0.37)	0.256
Enterolactone glucuronide						
Model 1[b]	-0.02 (-0.23; 0.19)	0.886	-0.16 (-0.40; 0.09)	0.201	-0.05 (-0.25; 0.15)	0.609
Model 2[c]	0.10 (-0.10; 0.32)	0.304	-0.11 (-0.35; 0.13)	0.368	0.02 (-0.18; 0.22)	0.817
Urolithin B glucuronide						
Model 1[b]	0.05 (-0.13; 0.24)	0.551	0.19 (0.03; 0.35)	0.018	0.24 (0.08; 0.40)	0.005
Model 2[c]	-0.02 (-0.19; 0.15)	0.830	0.16 (-0.03; 0.36)	0.097	0.19 (0.01; 0.36)	0.035
Vanillic acid glucuronide						
Model 1[b]	0.08 (-0.12; 0.29)	0.413	0.08 (-0.12; 0.28)	0.419	0.11 (-0.06; 0.29)	0.208
Model 2[c]	0.15 (-0.04; 0.34)	0.127	0.14 (-0.06; 0.34)	0.164	0.19 (0.01; 0.38)	0.037

MPM, microbial phenolic metabolites; MMSE, mini-mental state scale; RAVLT, Rey auditory verbal learning test; WMS, Wechsler memory scale; WAIS, Wechsler adult intelligence scale.

[a] ß, difference between groups; CI, confidence interval.

[b] Model 1: sex and age.

[c] Model 2: sex, age, smoking habit, educational level, physical activity, BMI, total energy intake, hypertension, hypercholesterolemia, diabetes, APO E genotype, statin treatment, anticholinergic drugs, and Mediterranean diet adherence.

3.4.2. Publication 13

Microbial phenolic metabolites are associated with improved cognitive health

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Supplementary Material available in Annex section.

Abstract

Aims: To assess the relationship between MPM in urine and cognition in the context of an older population at high cardiovascular risk.

Methods: A cross-sectional analysis was conducted in 400 individuals with available measured urinary polyphenols of the PREDIMED-Plus study. Liquid chromatography coupled to mass spectrometry was used to identify urinary MPM. MedDiet adherence was estimated with a 17-item questionnaire and cognitive function was evaluated with a battery of neuropsychological tests, including the Mini-Mental State Examination and the Clock Drawing Test. Multivariable-adjusted linear regression models were fitted to assess the relationship of urinary MPM with the MedDiet and cognitive tests.

Results: Protocatechuic acid and enterolactone glucuronide were associated with higher adherence to the MedDiet. Regarding cognitive function, protocatechuic

acid, vanillic acid glucuronide, 3-hydroxybenzoic acid, enterodiol glucuronide, and enterolactone glucuronide were directly associated with a global composite score of all the cognitive tests. Furthermore, protocatechuic acid and enterolactone glucuronide were associated with higher scores in the Mini-Mental State Examination, whereas enterodiol glucuronide was associated with improved Clock Drawing Test scores. **Conclusions**: These results suggest that the MedDiet is linked to the generation of MPM associated with better cognitive performance in an older population.

RESEARCH ARTICLE

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Microbial Phenolic Metabolites Are Associated with Improved Cognitive Health

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Scope: Diets rich in polyphenols has been associated with better cognitive performance. The aim of this study is to assess the relationship between microbial phenolic metabolites (MPM) in urine and cognition in the context of an older population at high cardiovascular risk.

Methods and results: A cross-sectional analysis is conducted in 400 individuals of the PREDIMED-Plus study. Liquid chromatography coupled to mass spectrometry is used to identify urinary MPM. Mediterranean diet (MedDiet) adherence is estimated with a 17-item questionnaire and cognitive function is evaluated with a battery of neuropsychological tests. Multivariable-adjusted linear regression models are fitted to assess the relationship of urinary MPM with the MedDiet and cognitive tests. Protocatechuic acid and enterolactone glucuronide are associated with higher adherence to the MedDiet. Regarding cognitive function, protocatechuic acid, vanillic acid glucuronide, 3-hydroxybenzoic acid, enterodiol glucuronide, and enterolactone glucuronide are directly associated with a global composite score of all the cognitive tests. Furthermore, protocatechuic acid and enterolactone glucuronide are associated with higher scores in the Mini-Mental State Examination, whereas enterodiol glucuronide is associated with improved Clock Drawing Test scores.

Conclusions: These results suggest that the MedDiet is linked to MPM associated with better cognitive performance in an older population.

1. Introduction

The increasing prevalence of neurodegenerative diseases among the elderly population means there is a pressing need to develop

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early detection and prevention strategies. While an early diagnosis relies on the health care system, prevention can be addressed individually in multiple ways.[1] Modifiable risk factors include smoking, physical inactivity, sleep disturbances, social isolation, and diet.[2]

Dietary patterns and nutrients have been widely studied in relation to cognitive health.[3] Higher adherence to the Mediterranean diet (MedDiet) is reported to delay cognitive decline and reduce the risk of AD.[4,5] The cognitive benefits of the MedDiet might be related to a high intake of nutrients, micronutrients,

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and bioactive compounds involved in neurological mechanisms. This plant-based dietary pattern is characterized by a high consumption of fruits, vegetables, legumes, nuts, whole grains, and olive oil.[6] Some of these foods, such as nuts, are valuable sources of healthy fats, including vegetable omega-3 fatty acids, such as alpha-linolenic acid, which is suggested to play an important role in reducing cognitive decline.[7] Moreover, vegetables, fruits, nuts, and virgin olive oil are rich in polyphenols, which possess neuroprotective antioxidant and anti-inflammatory properties.[8]

A positive relationship between dietary polyphenols and cognition has been found in several observational studies and clinical trials.[9–11] However, these studies have not considered the extensive metabolism that polyphenols undergo before they reach the bloodstream. Most of these metabolic changes occur in the colon and are catalyzed by gut microbiota, as polyphenol absorption in the stomach and small intestine is low.[12] The resulting microbial phenolic compounds (MPM) may exert biological activities different to those of the parental polyphenols, corresponding to their altered chemical characteristics. Evidence suggests that the benefits of polyphenols on the host's health are intricately linked to their interactions with the gut microbiota.[13] The gut microbiota plays a crucial role in metabolizing polyphenols, resulting in the production of a wide array of metabolites that can exert different effects on the body, including the central nervous system.[14] Furthermore, the relationship between polyphenols and the gut microbiota is bidirectional, as dietary polyphenols are prebiotics that can influence the composition of the microbiota.[15,16]

The aim of the present study was to assess the relationship between the MedDiet and urinary phenolic metabolites derived from the gut microbiota, as well as the association between these metabolites and neurocognition in an older population at high cardiovascular risk.

2. Experimental Section

2.1. Study Design

A cross-sectional analysis was conducted using baseline data from the PREDIMED-Plus study, an ongoing multicenter, randomized, parallel-group clinical trial conducted in Spain to assess the effects of an energy-reduced MedDiet combined with physical activity and behavioral support on weight loss and cardiovascular disease morbidity and mortality. Details of the study protocol can be found at http://predimedplus.com.[17,18] A total of 6874 participants were recruited from September 2013 to December 2016 in 23 Spanish centers from universities, hospitals, and research institutes. Eligible participants were men aged 55–75 years and women aged 60–80 years who were overweight or obese (body mass index [BMI] = 27–40 kg m^{-2}) and met at least three of the metabolic syndrome criteria established by the International Diabetes Federation and the American Heart Association and National Heart, Lung, and Blood Institute.[19]

The present sub-study was performed in a random subsample of 400 participants with available urinary MPM and data from cognitive tests. Participants who reported extreme total energy intakes (>3500 or <500 kcal day^{-1} in women or >4000 or <800 kcal day^{-1} in men) were excluded from the analysis,[20] as were those with missing data on covariates (diabetes, hypercholesterolemia, or hypertension).

2.2. Ethics Statement

The study was conducted according to the ethical guidelines of the Declaration of Helsinki and all procedures were approved by

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the Institutional Review Boards of the participating centers. The clinical trial was registered with the ISRCTN (London, England), number 89898870, on July 24, 2014. Written informed consent was obtained from all participants.

2.3. Covariate Assessment

Data on covariates were collected by trained personnel in interviews using general self-report questionnaires on sociodemographics (sex, age, level of education), lifestyle (smoking habits, physical activity, alcohol consumption), and history of illness and medication use.[18] Leisure time physical activity was estimated using the validated Minnesota-REGICOR Short Physical Activity questionnaire.[21] Dietary intake was assessed using the validated, semi-quantitative 143-item PREDIMED-Plus food frequency questionnaire with the assistance of trained dietitians.[22] Adherence to the MedDiet was determined with a 17-item MEDAS questionnaire, adapted from the validated 14-item PREDIMED questionnaire.[23] Trained staff measured anthropometric variables, namely weight and height. Body mass index (BMI) was calculated as the weight in kilograms divided by height in meters^2.

2.4. Neuropsychological Assessment

Participants completed a battery of cognitive tests at baseline administered by trained personnel. The Mini-Mental State Examination (MMSE) was a commonly used brief cognitive screening test that measured five cognitive domains: serial subtraction, language, memory, orientation, and visuospatial skills; higher scores (maximum of 30) indicate an absence of cognitive decline.[24,25] The Clock Drawing Test (CDT) evaluated visuospatial and visuoconstructive capacity.[26,27]

Other neuropsychological tests used were focused on specific cognitive domains. The Verbal Fluency Test (VFT) assessed verbal ability and executive function and consisted of two parts: semantic and phonological verbal fluency tasks (VFT-a and VFT-p, respectively).[28] The Digit Span Test (DST) of the Wechsler Adult Intelligence Scale-III (WAIS-III) included forward recall (DST-f), which evaluated attention and short-term memory, and backward recall (DST-b), which evaluated working memory.[29,30] The Trail Making Test (TMT) assessed attention and processing speed (part A), and cognitive flexibility (part B).[31] The score was the time taken to complete the task, and therefore lower scores implied better performance.

Z-scores were created from raw scores for each cognitive assessment to standardize the results. The Global Cognitive Function (GCF) was calculated as a composite score that was derived from all eight assessments,[32,33] adding or subtracting each individual test value based on whether a higher score indicates higher or lower cognitive performance, respectively.

2.5. Microbial Phenolic Metabolites

2.5.1. Standards and Reagents

Protocatechuic acid (PCA), enterodiol, urolithin-A, and urolithin-B were obtained from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (+) *cis, trans*-abscisic acid D6 was purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Vanillic acid, 3-hydroxybenzoic acid (3-OHBz), enterolactone, and creatinine were purchased from Fluka (St. Louis, MO, USA). Standards were stored in powder form and protected from light. The reagents were obtained from the following commercial suppliers: methanol of LC-MS grade and acetonitrile of HPLC grade from Sigma-Aldrich and formic acid (≥98%) from Panreac Química S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore system (Bedford, MA, USA).

2.5.2. Sample Preparation

Urine samples were collected after an overnight fast, coded, and stored at −80 °C until analysis. MPM were analyzed using a method previously validated by the group with minor modifications.[34] The present study focused only on MPM that were mostly or exclusively produced by the gut microbiota. Briefly, 50 µL urine samples were diluted 1:20 (v:v) with Milli-Q Water and 100 µL of the internal standard was added. Then, 2 µL of formic acid was added to acidify the samples before centrifugating at 15 000 × *g* at 4 °C for 4 min. To eliminate undesired compounds, present in urine and to isolate the MPM, the acidified urines underwent a solid-phase extraction in Water Oasis HLB 96-well plates 30 µm (30 mg) (Water Oasis, Milford, MA, USA). The samples were loaded in an activated plate with methanol and 1.5 M formic acid, and a clean-up step was performed with 1.5 M formic acid and methanol (0.5%). The MPM were eluted with methanol acidified with 1.5 M formic acid, evaporated to dryness with nitrogen gas, and reconstituted with 100 µL formic acid (0.05%). The samples were mixed in the vortex for 20 min and filtered through 0.22 µm polytetrafluoroethylene 96-well plate filters (Millipore, MA, USA).

Urinary concentrations of MPM were corrected by urinary creatinine, measured according to the Jaffé alkaline picrate method adapted for Thermo microtiter 96-well plates, as described by Medina-Remón et al.[35]

2.5.3. HPLC-LTQ Orbitrap ESI Analysis

The high-performance liquid chromatography (HPLC) analysis was performed on an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) coupled to a linear ion trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap-MS) (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode as described.[34] Chromatographic separation was performed on a Kinetex F5 100A (50 × 4.6 mm × 2.6 µm) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were, respectively, 0.05% formic acid in water and 0.05% formic acid in acetonitrile. The following linear gradient was used: held at 98% A for 1.7 min, decreased to 92% A for 3 min, decreased to 80% A for 1.3 min, decreased to 70% A for 1.3 min, decreased to 50% for 0.1 min, decreased to 0% for 1.3 min, then returned to the initial conditions for 1.7 min and re-equilibrated for 3 min. The flow rate was set at 0.750 µL min^{-1} and the injection volume was 5 µL.

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2.5.4. Identification and Quantification of MPM

MPM identification and quantification were performed using Trace Finder software version 4.1 (Thermo Fisher Scientific, San Jose, CA, USA). The glucuronidated and sulfated MPM were quantified with their respective aglycone equivalents due to the unavailability of standards.

As microbial phenolic metabolism showed high inter-individual variability, the MPM were not detected in all the participants. For the sake of simplicity and to facilitate comprehension, only metabolites with <20% of missing values were included in the statistical analysis. Thus, a total of seven MPM were considered: PCA, vanillic acid glucuronide (VAG), vanillic acid sulfate (VAS), 3-OHBz, enterodiol glucuronide (EDG), enterolactone glucuronide (ELG), and urolithin B glucuronide (UBG). Missing values of the previously listed metabolites with less than 20% of missing values were replaced by the half of the minimum detectable value.

2.6. Statistical Analyses

Statistical analyses were performed using the PREDIMED-Plus database updated to July 2021. Baseline characteristics were described as means ± standard deviation (SD) for quantitative variables and as *n* (percentages) for qualitative variables. The study used natural logarithmic transformations to approximate a normal distribution of MPM concentrations.

Multivariable adjusted linear regression models were fitted to assess the relationship between MPM and MedDiet adherence and the neuropsychological tests adjusted by confounders. Two adjustment models of increasing complexity were used; Model 1 was minimally adjusted for age (years) and sex (men/women); Model 2 was further adjusted for smoking habits (never, former, or current), educational level (medium-high or low), BMI (obesity/overweight), physical activity (METS min week^{-1}), total energy intake (kcal day^{-1}), hypertension (yes/no), diabetes mellitus (yes/no), dyslipidemia (yes/no), and treatment with cholesterol-lowering and anticholinergic drugs (yes/no). All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.

Statistical analyses were performed using Stata 16.0 (StataCorp. LP, TX, USA) and statistical significance was set at $p < 0.05$.

3. Results

3.1. General Characteristics of Participants

The main characteristics of the 400 PREDIMED-Plus participants are summarized in **Table 1**. There were slightly more men than women, and the average age was 65 years. By study design, all participants had cardiovascular risk factors: nearly one out of four had type-2 diabetes, one-third had hypercholesterolemia, most had hypertension, and only a minority were current smokers. Actual urinary metabolite concentrations are depicted in Table S1, Supporting Information. A heat map of Spearman correlation coefficients of the urinary metabolites analyzed in the present study is presented in Figure S1, Supporting Information.

Table 1. General characteristics of the participants ($n = 400$).

Age [years]	65.3 ± 4.9
Women, *n* [%]	169 (42.5)
BMI [kg m^{-2}]	32.6 ± 3.3
Type-2 diabetes, *n* [%]	111 (27.75)
Hypertension, *n* [%]	350 (87.5)
Hypercholesterolemia, *n* [%]	275 (68.75)
Medium-high educational level, *n* [%]	222 (55.5)
Current smokers, *n* [%]	43 (10.8)
Physical activity [MET min week^{-1}]	2825.3 ± 2612.4
Total energy intake [kcal day^{-1}]	2384.5 ± 534.4
MedDiet adherence [score]	8 ± 3

Continuous variables are shown as means ± SDs, and categorical variables are shown as *n* (%). BMI, body mass index; MedDiet, Mediterranean diet; MET, metabolic equivalent of task.

3.2. Microbial Phenolic Metabolites and the Mediterranean Diet

The association between individual MPM and MedDiet adherence is described in **Figure 1**. PCA and ELG were found to be significantly associated with higher adherence to the MedDiet ($\beta = 0.14$, 95% CI: 0.01, 0.27 per 1-SD, p-value = 0.038 and $\beta = 0.12$, 95% CI: 0.02, 0.22 per 1-SD, p-value = 0.022, respectively). Results with all adjustment models can be found in Table S2, Supporting Information.

3.3. Association between Microbial Phenolic Metabolites and Cognitive Composite

Table 2 depicts the associations between individual MPM and global cognitive function (GCF). PCA and enterolactone glucuronide showed significant associations with the score ($\beta = 0.04$, 95% CI: 0.02, 0.07 per 1-SD, p-value = 0.002) and ($\beta = 0.02$, 95% CI: 0.01, 0.04 per 1-SD, p-value = 0.010), respectively. Other MPM were also positively associated with the GCF, such as VAG ($\beta = 0.06$, 95% CI: 0.01, 0.12 per 1-SD, p-value = 0.025), 3-OHBz ($\beta = 0.03$, 95% CI: 0.01, 0.06 per 1-SD, p-value = 0.017), or EDG ($\beta = 0.01$, 95% CI: <0.01, 0.02 per 1-SD, p-value = 0.039). VAS and UBG showed no association with the score.

3.4. Microbial Phenolic Metabolites and Neuropsychological Tests

The associations between individual MPM and the scores of MMSE and CDT cognitive tests are shown in **Table 3**. PCA was positively associated with the MMSE score in the fully adjusted model ($\beta = 0.06$, 95% CI: 0.03, 0.13 per 1-SD, p-value = 0.005), as was ELG ($\beta = 0.04$, 95% CI: 0.01, 0.06 per 1-SD, p-value = 0.011). A positive association was also observed between EDG and the CDT score ($\beta = 0.02$, 95% CI: <0.01, 0.04 per 1-SD, p-value = 0.047). No other significant associations were found between other metabolites and these cognitive tests. Results on specific cognitive tests can be found in Table S3, Supporting Information.

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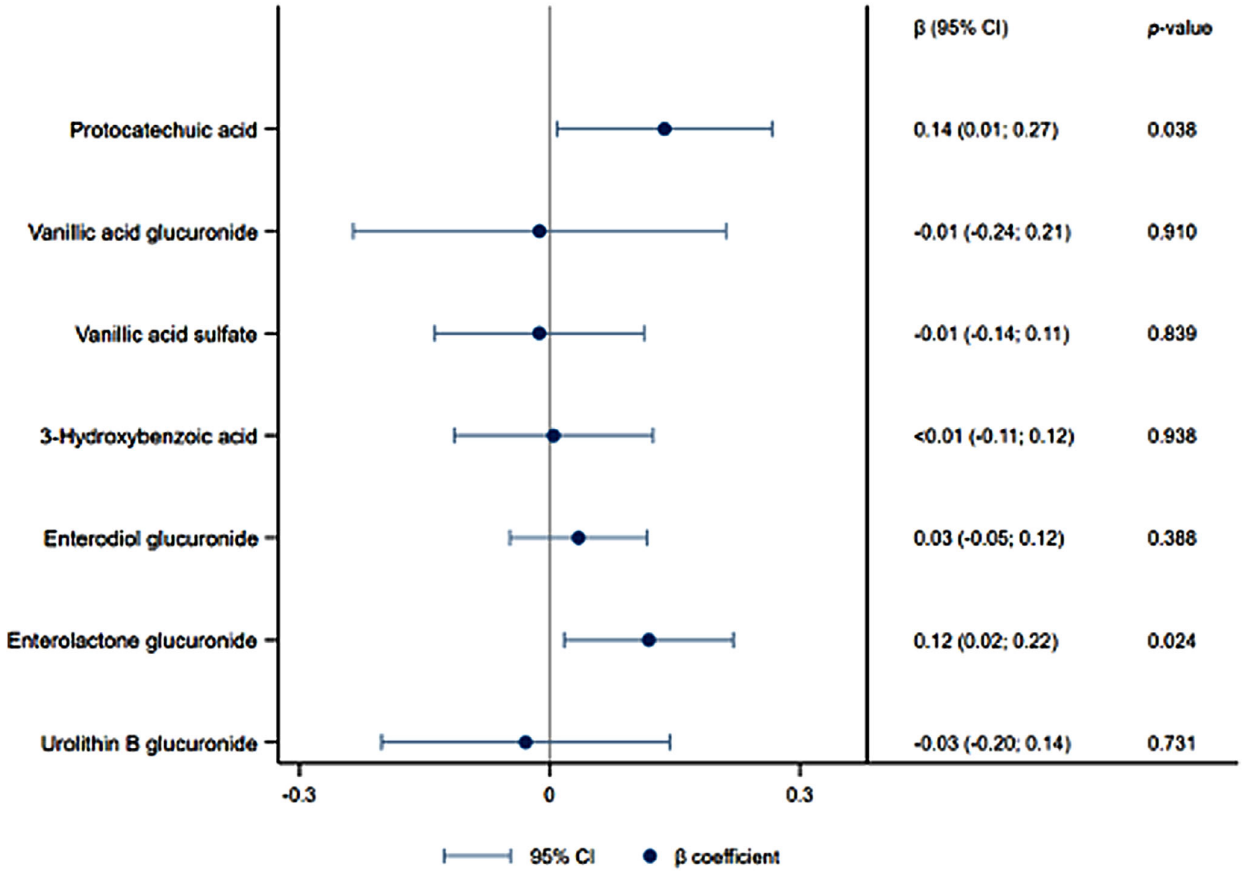

Figure 1. Association between individual MPM and MedDiet adherence (p-17 score) using multivariable adjusted linear regressions models adjusted for age, sex, smoking habit, educational level, obesity/overweight, total energy intake, diabetes, hypertension, hypercholesterolemia, and use of cholesterol-lowering drugs. All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.

4. Discussion

In this cross-sectional study of participants in the PREDIMED-Plus trial, we observed that urinary MPM were directly associated with better cognitive function in an older population at high cardiovascular risk. Moreover, the specific MPM PCA and ELG were directly associated with MedDiet adherence. These results suggest that compliance with the MedDiet is associated with the production of phenolic compounds by the gut microbiota that can contribute to better cognitive function.

In our study, individuals with higher adherence to the MedDiet presented more PCA and ELG in urine. Evidence indicates that the MedDiet induces changes in the gut microbiota and increases short-chain fatty acid production.[36] Mitsou et al. reported that higher adherence to the MedDiet was associated with greater amounts of total bacteria and lower levels of *Escherichia coli* in the gut,[37] whereas in another study this dietary pattern induced an increase of Bifidobacteria sp., which are involved in the synthesis of MPM.[38–40] Therefore, the MedDiet may influence the composition of the gut microbiota and enhances the production of specific MPM.

Several studies have demonstrated that polyphenol-enriched diets may improve cognition and reduce the risk of developing neurodegenerative diseases.[9,41] Previously, a study has shown the protective association between metabolites related to the consumption of polyphenol-rich foods and cognitive decline.[42] In the present work, PCA was one of the urinary metabolites most strongly associated with cognitive function and was also related to MedDiet adherence. PCA is present in the circulation for significantly longer periods and at higher concentrations than the parent compounds and easily crosses the blood–brain barrier.[43] The parent compounds of PCA, present in fruits, are anthocyanins and procyanidins, such as cyanidin-3-*O*-glucoside, which after ingestion are metabolized to PCA by intestinal microbiota and can be detected in blood and urine.[44–46] Experimental and clinical studies strongly support a preventative role for PCA in neurodegenerative processes, including AD and Parkinson's disease. A favorable influence of PCA on factors underlying cognitive and behavioral disorders has been described, such as the accumulation of β-amyloid plaques in brain tissues and neuroinflammation.[43] Experiments conducted by Ban et al. on cultured rat cortical neurons revealed that PCA exerts concentration-dependent protective effects against neuronal cell

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Table 2. Multivariable adjusted regression between microbial phenolic metabolites and global cognitive function.

	Global cognitive function	
	β (CI 95%) per 1-SD	*p*-value
Protocatechuic acid		
Model 1	0.04 (0.01; 0.07)	0.011
Model 2	0.04 (0.02; 0.07)	0.002
Vanillic acid glucuronide		
Model 1	0.05 (←0.01; 0.11)	0.069
Model 2	0.06 (0.01; 0.12)	0.025
Vanillic acid sulfate		
Model 1	0.01 (-0.01; 0.02)	0.499
Model 2	←0.01 (-0.02; 0.02)	0.942
3-Hydroxybenzoic acid		
Model 1	0.02 (-0.01; 0.05)	0.118
Model 2	0.03 (0.01; 0.06)	0.017
Enterodiol glucuronide		
Model 1	0.01 (←0.01; 0.03)	0.061
Model 2	0.01 (<0.01; 0.02)	0.039
Enterolactone glucuronide		
Model 1	0.03 (0.01; 0.06)	0.020
Model 2	0.02 (0.01; 0.04)	0.010
Urolithin B glucuronide		
Model 1	0.01 (-0.01; 0.02)	0.236
Model 2	0.01 (-0.01; 0.02)	0.345

A natural logarithmic transformation was applied to the raw values of individual metabolites. Model 1 was adjusted for age and sex. Model 2 was further adjusted for smoking habit, educational level, obesity/overweight, total energy intake, diabetes, hypertension, hypercholesterolemia, and use of cholesterol-lowering and anticholinergic drugs. All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.

death and prevents neurotoxicity.[47] A study in mice revealed that PCA reduced the number of β-amyloid deposits in the hippocampus and cerebral cortex, diminished inflammatory responses, and improved learning and memory performance.[48]

Another compound strongly associated with cognitive function that was detected in participants with high adherence to the MedDiet was ELG. This MPM is metabolized from dietary lignans such as pinoresinol, sesamolin, or syringaresinol, present in whole grains, nuts, and legumes.[49] A positive association with global cognition was also found for EDG, another compound of the lignan family with a similar origin to that of ELG. Even though other lignans have been associated with improvements in neurocognition,[50,51] the role of ELG and EDG has been scarcely studied. It has been suggested that the enterolactone aglycone may possess neuroprotective properties due to its capacity to inhibit the activities of carbonic anhydrase, acetylcholinesterase, and butyrylcholinesterase.[52] The few clinical and observational studies that have examined the association between dietary lignans and cognitive performance have found them to be positively related.[53–55] Altogether, these results suggest that the microbial lignans ELD and EDG may have a beneficial impact on neurocognitive health.

3-OHBz was associated with higher GCF scores and, more specifically, with better attention and short-term memory. This MPM is derived from dietary polyphenols such as epicatechin and procyanidin B, and produced after the intake of cocoa, coffee, grapes, and other plant food sources.[56] Although not previously associated with memory or cognition in humans, in vitro and animal studies have shown that 3-OHBz has a significant protective effect against AD and Parkinson's disease. Capable of penetrating the blood–brain barrier, it accumulates in the brain, and exerts neuroprotective effects.[57] An in vitro study reported that 3-OHBz potentially interferes with the self-assembly of β-amyloid peptides, which play a key role in AD neuropathogenesis.[57]

In our study, VAG was positively associated with global cognition and especially with verbal ability and executive function, as well as with short-term and working memory. A glucuronidated form of vanillic acid, VAG has been detected after the consumption of berries, being one of the main metabolites of cyanidin-3-*O*-glucoside.[58,59] Vanillic acid is reported to reduce streptozotocin-induced neurodegeneration, improve learning and memory, and exert specific anti-inflammatory and antioxidant effects that down-regulate neuroinflammatory processes.[60] Moreover, experimental studies in mice revealed that vanillic acid has a neuroprotective effect against Aβ-induced neurotoxicity, resulting in improved memory function.[61] A possible mechanism to explain the memory-enhancing effect of vanillic acid is an increased expression of nuclear factor erythroid 2-related factor 2 (Nrf2) protein, which provides neuroprotection in AD.[61] The activation of Akt/Nrf2 signaling pathways is another feasible mechanism, since their inhibition plays an important role in the development of AD.[61] Interestingly, we observed that VAS was negatively associated with attention and processing speed, a relationship not previously reported in the literature, which may indicate that glucuronidation metabolism is more beneficial than sulfation.

Our study has limitations. The sample size was relatively small, although comparable to that in similar studies[62] and given the cross-sectional nature of the study, we cannot exclude the possibility of reverse causation or residual confounding. Due to the study design, only older Mediterranean individuals at high cardiovascular risk were studied, hence the results cannot be generalized to other populations, since pre-existing conditions, age, or lifestyle factors could potentially influence the composition and function of the microbiota as well as the resulting metabolites. In the PREDIMED-trial only spot urine samples were available, however a previous study observed that both samples can be used for large scale studies.[63] Finally, since not all existing MPM have been quantified, there may be other compounds with effects on the cognitive system. The study also has strengths, such as the use of biological samples, which provide the most accurate indication of the metabolic state of participants. Furthermore, the use of high-precision equipment such as HPLC coupled with LTQ-Orbitrap MS detectors facilitated the detection, identification, and quantification of polyphenols and their metabolites with high sensitivity. Moreover, we included a comprehensive battery of neuropsychological tests to assess cognitive function.

In conclusion, our findings suggest that the MedDiet is linked to the production of MPM, especially PCA and ELG, which are associated with better cognitive functions in an older population at high cardiovascular risk. These results underscore the poten-

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Table 3. Multivariable adjusted regression between microbial phenolic metabolites and cognitive tests.

	Mini-Mental State Examination *Z*-score		Clock drawing test *Z*-score	
	β (CI 95%) per 1-SD	*p*-value	*β* (CI 95%) per 1-SD	*p*-value
Protocatechuic acid				
Model 1	0.08 (0.03; 0.13)	0.005	0.02 (−0.04; 0.08)	0.569
Model 2	0.06 (0.03; 0.13)	0.005	0.03 (−0.03; 0.09)	0.379
Vanillic acid glucuronide				
Model 1	0.03 (−0.07; 0.13)	0.550	0.05 (−0.03; 0.14)	0.191
Model 2	0.04 (−0.04; 0.13)	0.266	0.06 (−0.02; 0.13)	0.120
Vanillic acid sulfate				
Model 1	0.01 (−0.03; 0.04)	0.769	<0.01 (−0.03; 0.03)	0.951
Model 2	<0.01 (−0.03; 0.03)	0.984	<0.01 (−0.03; 0.04)	0.917
3-Hydroxybenzoic acid				
Model 1	0.04 (−0.02; 0.10)	0.214	0.03 (−0.03; 0.09)	0.280
Model 2	0.05 (−0.01; 0.11)	0.102	0.04 (−0.02; 0.09)	0.152
Enterodiol glucuronide				
Model 1	0.02 (−0.01; 0.05)	0.109	0.02 (←0.01; 0.04)	0.056
Model 2	0.02 (←0.01; 0.05)	0.067	0.02 (<0.01; 0.04)	0.047
Enterolactone glucuronide				
Model 1	0.04 (0.02; 0.07)	0.005	0.02 (−0.03; 0.06)	0.442
Model 2	0.04 (0.01; 0.06)	0.011	0.02 (−0.03; 0.06)	0.458
Urolithin B glucuronide				
Model 1	0.03 (0.01; 0.06)	0.022	−0.03 (−0.09; 0.02)	0.202
Model 2	0.03 (←0.01; 0.05)	0.055	−0.03 (−0.08; 0.03)	0.276

A natural logarithmic transformation was applied to the raw values of individual metabolites. Model 1 was adjusted for age and sex. Model 2 was further adjusted for smoking habit, educational level, obese/overweight, total energy intake, diabetes, hypertension, hypercholesterolemia, and use of cholesterol-lowering and anticholinergic drugs. All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.

tial role of the MedDiet in reducing age-related cognitive decline. Further studies are needed to investigate the molecular mechanisms linking these metabolites to cognition.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

J.S.-S. reported receiving research support from the Instituto de Salud Carlos III, Ministerio de Educación y Ciencia, Departament de Salut Pública de la Generalitat de Catalunya, the European Commission, the USA National Institutes of Health; receiving consulting fees or travel expenses from Eroski Foundation, Instituto Danone, Nestle, and Abbott Laboratories, receiving nonfinancial support from Hojiblanca, Patrimonio Comunal Olivarero, the California Walnut Commission, Almond Board of California, La Morella Nuts, Pistachio Growers and Borges S.A; serving on the board of and receiving grant support through his institution from the International Nut and Dried Foundation and the Eroski Foundation; and grants and personal fees from Instituto Danone; Serving in the Board of Danone Institute International. D.C. reported receiving grants from Instituto de Salud Carlos III. R.E. reported receiving grants from Instituto de Salud Carlos III, Fundación Dieta Mediterránea and Cerveza y Salud and olive oil for the trial from Fundación Patrimonio Comunal Olivarero and personal fees from Brewers of Europe, Fundación Cerveza y Salud, Interprofesional del Aceite de Oliva, Instituto Cervantes in Albuquerque, Milano and Tokyo, Pernod Ricard, Fundación Dieta Mediterránea (Spain), Wine and Culinary International Forum and Lilly Laboratories; non-financial support from Sociedad Española de Nutrición and Fundación Bosch y Gimpera; and grants from Uriach Laboratories. E.R. reports grants, personal fees, non-financial support and other from California Walnut Commission, during the conduct of the study; non-financial support from The International Nut Council; grants, personal fees, non-financial support and other from Alexion; grants from Amgen and Pfizer; grants, personal fees and other from Sanofi Aventis; personal fees, non-financial support and other from Ferrer International, Danone and Merck Sharp & Dohme, personal fees and other from Amarin, outside the submitted work. R.M.L.-R. reports personal fees from Cerveceros de España, personal fees and other from Adventia, other from Ecoveritas, S.A., outside the submitted work. The rest of the authors have declared that no competing interests exist. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Author Contributions

I.D.L. and R.M.L.R. conceptualized this study; I.D.L., I.P.M., and C.A.R. performed the investigation and the formal analysis. I.D.-L. and P.G. wrote the original draft. M.A.M.G, J.S.S., D.C., M.C., J.A.M., L.T.S., J.W., J.V., D.R., J.L.M., R.E., F.J.T., J.L., L.S.M., A.B.C., J.A.T., M.R.G., X.P., F.F.A., M.D.R., A.B.B., J.V., C.V., L.D., E.R., E.T., A.A., E.M.A., N.V., A.G.R., L.T.C., N.P.F., M.Z., A.C., R.C., S.M.P., J.V.L., A.M.G.P., Z.V.R., S.S., C.O.A., N.T., P.J.P.O., A.O.C., J.D.E., N.B., M.F., and R.M.L.R. reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement

There are restrictions on the availability of data for the PREDIMED-Plus trial, due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following the project purposes. Requestors wishing to access the PREDIMED-Plus trial data used in this study can make a request to the PREDIMED-Plus trial Steering Committee chair: jordi.salas@urv.cat. The request will then be passed to members of the PREDIMED-Plus Steering Committee for deliberation.

The PREDIMED-Plus study was registered at the ISRCTN of London, England: 89898870

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3.4.3. Publication 14

Serum vitamin B12 concentration is associated with improved memory in older individuals with higher adherence to the Mediterranean diet

Inés Domínguez-López, Rosa Casas, Gemma Chiva-Blanch, Miguel Ángel Martínez-González, Montserrat Fitó, Emilio Ros, Rosa M. Lamuela-Raventós, and Ramon Estruch

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Supplementary Material available in Annex section.

Abstract

Aims: To evaluate the effects of vitamin B12 on cognitive performance according to adherence to the MedDiet, and whether the MedDiet also results in increased folate or vitamin B12 levels.

Methods: This is a cohort study nested in a randomized controlled clinical trial performed in Hospital Clinic in Barcelona, Spain. A total of 170 participants of the PREDIMED trial (Barcelona – Hospital Clinic site) aged 55 to 80 years at high cardiovascular risk were included. Adherence to the Mediterranean diet was assessed using a validated 14-item questionnaire, memory function was evaluated with a battery of neuropsychological tests and serum vitamin B12 and folate were determined using an automated electrochemiluminiscence immunoassay system.

Results: In the multivariable adjusted linear regression model, serum vitamin B12 concentration presented a significant correlation with memory function in participants with high adherence to the MedDiet whereas the correlation was weak and inverse for those who presented a low adherence to the MedDiet. MedDiet adherence showed a positive association with serum folate, but not with serum vitamin B12.

Conclusions: In an older Mediterranean population at high cardiovascular risk, changes in serum vitamin B12 correlate with better memory function only in the context of a high adherence to the Mediterranean pattern, suggesting that the effects of vitamin B12 goes further than a mere nutritional requirement.

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Original article

Serum vitamin B12 concentration is associated with improved memory in older individuals with higher adherence to the Mediterranean diet

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SUMMARY

Background & aims: Vitamin B_{12} plays a crucial role in cognition, but its effect might be regulated by the presence of other micronutrients, such as folate. The aim was to evaluate the effects of vitamin B_{12} on cognitive performance according to adherence to the Mediterranean diet, and whether the Mediterranean diet also results in increased folate or vitamin B12 levels.
Methods: This is a cohort study nested in a randomized controlled clinical trial performed in Hospital Clinic in Barcelona, Spain. A total of 170 participants of the PREDIMED trial (Barcelona – Hospital Clinic site) aged 55–80 years at high cardiovascular risk were included. Adherence to the Mediterranean diet was assessed using a validated 14-item questionnaire, memory function was evaluated with a battery of neuropsychological tests and serum vitamin B_{12} and folate were determined using an automated electrochemiluminiscence immunoassay system.
Results: In the multivariable adjusted linear regression model, serum vitamin B_{12} concentration presented a significant correlation with memory function ($r^2 = 0.57$; $P = 0.028$) in participants with high adherence to the Mediterranean diet whereas the correlation was weak and inverse for those who presented a low adherence to the Mediterranean diet ($r^2 = 0.37$, $P = 0.731$). Mediterranean diet adherence showed a positive association with serum folate, but not with serum vitamin B_{12}.
Conclusions: In an older Mediterranean population at high cardiovascular risk, changes in serum vitamin B_{12} correlate with better memory function only in the context of a high adherence to the Mediterranean pattern, suggesting that the effects of vitamin B_{12} goes further than a mere nutritional requirement.
Institutional review board statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centres. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639 (https://www.isrctn.com/ISRCTN35739639).

1. Introduction

A consequence of population aging is an increased prevalence of age-related disorders, including neurodegenerative diseases.

Abbreviations: Mediterranean diet, MedDiet; recommended dietary intake, RDI; Rey Auditory Verbal Learning Test, RAVLT; Wechsler Memory Scale, WMS.

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Presently, dementia affects more than 46 million people worldwide, but this number is predicted to triple by 2050 [1]. Given that no disease-modifying pharmacologic treatments are available for dementia [2], there is a pressing public health need for effective preventive strategies. In this context, evidence suggests that certain components of the diet can play a major role in brain health [3].

Cobalamin (vitamin B_{12}) is a micronutrient most involved in cognitive functioning, since it is a water-soluble molecule that participates in the synthesis of DNA and in amino acid metabolism in the brain [4]. Dietary sources of vitamin B_{12} are limited to animal-derived products, as B_{12} is synthesized by bacteria in their gastrointestinal track and subsequently incorporated into animal tissues. It is therefore recommended that vegan and vegetarian populations use supplementation to avoid B_{12} insufficiency [5].

Evidence supports that low vitamin B_{12} status is a risk factor for cognitive decline and dementia. Insufficient dietary intake of vitamin B_{12} results in demyelization of peripheral and central neurons [6]. In addition, given that vitamin B_{12} is necessary to methylate homocysteine into methionine, B_{12} deficiency leads to elevated plasma levels of homocysteine, an amino acid that has been linked to cognitive impairment and neurodegenerative diseases [4]. The homocysteine methylation reaction also requires folate, another water-soluble vitamin mostly present in liver and vegetables (particularly dark green leafy vegetables such as spinach or asparagus) [7].

As shown in sub-studies of the PREvención con DIeta MEDiterránea (PREDIMED) trial, the Mediterranean diet (MedDiet) is beneficial for cognition in the older population at high cardiovascular risk [8,9]. This dietary pattern is characterised by high consumption of plant-derived foods and, therefore, provides a high content of dietary folate. On the other hand, the MedDiet includes moderate consumption of fish and low consumption of dairy products and meat. This dietary pattern supports sufficiency of many specific nutrients and is positively associated with micronutrient adequacy, including vitamin B_{12} [10].

We hypothesized that the adherence to a MedDiet could enhance the effect of vitamin B_{12} on cognitive functions. The MedDiet, which is rich in plant-based foods with high amounts of folate, should provide sufficient dietary amounts resulting in high body levels. Because previous evidence demonstrated that folate is essential for vitamin B_{12} to be able to exert its biological functions [11], we also hypothesized that the relationship between vitamin B_{12} and cognition is modified by folate intake and consequently by MedDiet adherence. Therefore, the aim of the present study was to assess [1]: whether vitamin B_{12} is associated with memory function and this relationship is altered by MedDiet adherence, and [2] whether the MedDiet provides higher concentrations of folate or vitamin B_{12} in serum in this aged population.

2. Materials & methods

2.1. Study design and participants

This is a longitudinal sub-study within the PREDIMED trial, a large, parallel-group, multicenter, randomized, controlled clinical trial designed to assess the effect of the MedDiet on the primary prevention of cardiovascular disease (www.predimed.es). Detailed information on the trial has been published elsewhere [12,13].

Study participants were men (55–80 y) and women (60–80 y) at high cardiovascular risk but without clinical cardiovascular disease at enrolment. Eligibility criteria were presence of type 2 diabetes or at least 3 of the following cardiovascular risk factors: smoking, hypertension, dyslipidaemia, overweight or obesity, and family history of early-onset coronary heart disease. Eligible candidates were randomly assigned to one of the following intervention groups: MedDiet supplemented with extra-virgin olive oil, MedDiet supplemented with mixed nuts (walnuts, almonds, and hazelnuts), or low-fat diet (recommendation to reduce all types of dietary fat).

For the present analysis, we used data from 170 participants consecutively recruited in the Barcelona-Clinic PREDIMED centre who had undergone sequential cognitive tests and serum folate and vitamin B_{12} determinations. Three participants who reported extreme total energy intakes (>3500 or <500 kcal/day in women or >4000 or <800 kcal/day in men) were excluded from analyses [14].

2.2. Standard protocol approvals, registrations, and patient consents

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.3. Covariates

Participants were considered to have diabetes, dyslipidaemia, or hypertension if they had a previous diagnosis of these conditions or were treated with specific drugs. Medical record and drug treatment were assessed annually. Educational level was estimated as years of formal education. Food consumption was determined using a validated, semi-quantitative 137-item food frequency questionnaire [15], and physical activity with a validated Spanish version of the Minnesota physical activity questionnaire [16]. Anthropometric and blood pressure measurements were performed by standard methods, as described [12,13]. The APOE ε4 genotype was determined with the method of Hixson and Vernier [17]. Adherence to the MedDiet was assessed using a 14-item questionnaire that gives a value of 0 or 1 for each dietary component that refers to a characteristic feature of the MedDiet [12].

2.4. Cognitive tests

Cognitive examinations were conducted by an experienced neuropsychologist who was masked to participants' group assignment. Cognitive tests were performed at baseline and repeated at the date closest to the trial's end. The instruments employed to assess memory function were as follows: intermediate and delayed episodic verbal memory were rated by the Rey Auditory Verbal Learning Test (RAVLT) [18] and episodic verbal memory was assessed with a subtest of the Wechsler Memory Scale (WMS), the verbal paired associates test [19]. The results were standardized to z-scores. Furthermore, a composite score of memory function was calculated for each individual with the standardized z-scores of the baseline results and the changes in the neuropsychological tests, as described [9]. The memory composite included the RAVLT and the WMS paired associates subtest.

2.5. Serum folate and vitamin B_{12} determinations

Serum vitamin B_{12} and folate concentrations were measured by an automated electrochemiluminiscence immunoassay system (Advia-Centaur, Siemens, Barcelona, Spain) in frozen aliquots kept at −80 °C, at baseline and the closest time point to the second cognitive evaluation. We applied logarithmic transformations to approximate a normal distribution of biological concentrations.

2.6. Statistical analyses

For statistical analyses we used Stata 16.0 (Stata-Corp LP, Tx. USA) and significance was set at $p < 0.05$. Participants were divided into two groups according to MedDiet adherence (high/low) at the closest time point to the second cognitive evaluation. This value allowed a better classification of the participants' according to their adherence after the intervention compared to baseline values or changes, which did not reflect the final status. The low MedDiet adherence group included participants with scores 0–10 points ($n = 96$), whereas the ranges for the high MedDiet adherence group were 11–14 points ($n = 71$). To obtain groups of a size as similar as possible, the cut-off was established at 11 points.

Baseline and post-intervention characteristics are described as mean ± standard deviations (SD) for quantitative variables and percentages for categorical variables for all the participants and stratified for high/low MedDiet adherence groups. Changes in the cognitive tests and serum vitamin B12 and folate were calculated as the difference between data from the last visit minus the baseline value.

Multivariable adjusted linear regression models were used to assess the relationship between changes in vitamins (B12 and folate) and changes in memory function (Memory Composite score, RAVLT delayed and intermediate, and WMS). We decided to stratify for high/low MedDiet adherence groups after testing the interaction between the MedDiet adherence and the independent variable. As the vitamins were introduced as log-transformed independent variables, the beta coefficient should be interpreted as the change in the vitamin per SD of the respective memory test. Regression models were adjusted for potential confounders using two models of increasing complexity. Model 1 was adjusted for sex, age, intervention group, and baseline MedDiet adherence, memory composite scores or subtests, and serum vitamin B_{12} or folate. Model 2 was additionally adjusted for smoking, educational level, APOE Ɛ4 genotype, follow-up time and changes from baseline in BMI, physical activity, hypertension, diabetes, hypercholesterolemia, use of anticholinergic or cholesterol-lowering drugs, and total energy intake.

The association between changes in MedDiet adherence and changes in serum vitamins (B_{12} and folate) was assessed with multivariable linear regression. Serum vitamins were introduced as log-transformed dependent variables, therefore, the beta coefficients should be interpreted as 1 unit of change in the MedDiet adherence score increases by 2 % the respective vitamin. We developed two models of increasing complexity. Model 1 was adjusted for sex, age, intervention group, and baseline levels of MedDiet adherence and serum vitamin B_{12} or folate. Model 2 was additionally adjusted for smoking, educational level, follow-up time and changes from baseline in BMI, physical activity, hypertension, diabetes, hypercholesterolemia, use of cholesterol-lowering drugs, and total energy intake.

3. Results

Baseline characteristics for the 167 participants finally included are shown in Table 1, distributed in groups according to MedDiet adherence, 71 participants in the high MedDiet adherence group, and 96 in low adherence group. At baseline, the high MedDiet adherence group included a lower percentage of women (38.0 %) compared to the low MedDiet adherence group (56.3 %). Those participants who presented a higher adherence to the MedDiet were more physically active (385.3 + 286.7 METS·min/day).

3.1. Food, energy and nutrient intake

Baseline BMI, total energy intake, serum folate and vitamin B_{12} concentrations, and memory composite scores were similar in the two groups. Postintervention data can be found in Supplementary Table S1. After the follow-up, the difference regarding physical activity was maintained between the two groups, as the high MedDiet adherence group was more physically active (368.4 + 292.5 METS·min/day). This group also disclosed higher energy intake (2140.9 + 307.1), even though that did not lead to a higher BMI. The participants allocated in the low MedDiet adherence group at baseline presented a mean score that was three points lower compared to that of the high MedDiet adherence group.

3.2. Cognitive function and vitamin B_{12}

Before analysing the relationship between changes in serum vitamins (B_{12} and folate) and the memory domain, we tested the interaction with MedDiet adherence. As the interaction was significant, we stratified the analysis for high/low MedDiet adherence groups. Table 2 displays the associations between changes in serum vitamin B_{12} and in the memory composite score and its subtests, stratified for MedDiet adherence. In the fully adjusted model, changes in vitamin B_{12} were directly associated with increases in the memory composite score in the group with high MedDiet adherence. However, no significant association was found in the low MedDiet adherence group. To ensure that these results were not confounded by the supplemented foods, olive oil, and walnuts, we checked their interaction with vitamin B_{12}, but the results were not significant (p-value = 0.739 and p-value = 0.771 for interaction, respectively). Figure 1 illustrates a strong positive correlation between changes in the memory composite and changes in vitamin B12 for participants in the high Mediet adherence group ($r^2 = 0.57$), whereas the correlation was weak and inverse for those who presented a low adherence to the MedDiet ($r^2 = 0.37$).

In the high MedDiet adherence group, similar positive associations were observed between vitamin B_{12} changes and the RAVLT delayed recall score and the WMS. Changes in vitamin B_{12} were not associated with RAVLT intermediate episodic verbal memory in any of the two groups.

3.3. Cognitive function and folate

The relationship between changes in serum folate and changes in the memory composite score and its subtests stratified for high/low MedDiet adherence is shown in Supplementary Table S2. Increases in folate concentrations were not associated with improvements in the memory composite score in any group, although we observed a tendency in the high MedDiet adherence group. No other associations were found between folate in serum and the memory tests.

Table 3 shows the multivariable adjusted regressions between changes in serum vitamins (B_{12} and folate) and changes in MedDiet adherence. In the fully-adjusted model, participants with high MedDiet adherence presented an increase in folate concentrations. No associations were detected for vitamin B_{12} in any adjustment model.

4. Discussion

In this longitudinal study conducted in an older population at high cardiovascular risk, we observed that, in participants with higher adherence to the MedDiet, changes in serum vitamin B_{12} concentration were associated with better memory function. To the best of our knowledge, this is the first study that describes the close

Table 1
General characteristics of all the participants and of groups stratified by baseline adherence to the MedDiet.

	All (167)	Low MedDiet adherence ($n = 96$)	High MedDiet adherence ($n = 71$)	p-value[a]
Women, n (%)	81 (48.5)	54 (56.3)	27 (38.0)	0.020
Age, years	66.4 ± 5.4	67.0 ± 5.4	65.7 ± 5.0	0.137
Follow-up time	3.7 ± 1.5	3.6 ± 1.5	3.8 ± 1.4	0.347
Physical activity, METS-min/day	314.1 ± 258.9	261.4 ± 223.5	385.3 ± 286.7	0.002
BMI, kg/m2	29.2 ± 3.3	29.5 ± 3.5	28.8 ± 3.0	0.159
Total energy intake, kcal/day	2463.1 ± 557.9	2418.4 ± 577.3	2523.5 ± 528.4	0.230
Medium & high educational level	66 (39.5)	38 (39.6)	28 (39.4)	0.985
Serum folate, ng/mL	9.3 ± 4.1	9.1 ± 4.2	9.5 ± 4.0	0.555
Serum vitamin B12, ng/mL	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.2	0.846
Memory Composite score	−0.1 + 0.8	−0.1 + 0.9	−0.1 + 0.7	0.800
MedDiet adherence[b]	8 ± 2	7 ± 2	9 ± 2	<0.001
Intervention group, n (%)				0.030
MedDiet supplemented with extra virgin olive oil	69 (41.3)	39 (42.6)	30 (42.3)	
MedDiet supplemented with nuts	60 (35.9)	25 (26.0)	35 (49.3)	
Low-fat diet	38 (22.8)	32 (33.3)	6 (8.6)	

MedDiet, Mediterranean diet; METS, Metabolic Equivalents.
Continuous variables are shown as means ± SDs, and categorical variables are shown n (%).
[a] T-test or chi-square test as appropriate.
[b] Based on the 14-point MedDiet screener.

Table 2
Multivariable linear regression between changes in Memory function and changes in serum vitamin B12 concentrations, stratified for MedDiet adherence.

	Low MedDiet adherence ($n = 96$)		High MedDiet adherence ($n = 71$)		
	β (95 % CI) per 1-SD	p-value	β (95 % CI) per 1-SD	p-value	p-value for interaction
Memory Composite score					
Model 1	−0.09 (-0.37; 0.19)	0.532	0.37 (-0.52; 1.26)	0.409	0.153
Model 2	−0.06 (-0.37; 0.26)	0.731	1.01 (0.11; 1.91)	0.028	0.040
RAVLT delayed episodic verbal memory					
Model 1	−0.19 (-0.67; 0.31)	0.449	0.46 (-0.60; 1.53)	0.386	0.076
Model 2	−0.24 (-0.76; 0.29)	0.378	1.27 (0.15; 2.39)	0.028	0.028
RAVLT intermediate episodic verbal memory					
Model 1	0.07 (-0.36; 0.51)	0.734	0.02 (-1.03; 1.07)	0.971	0.746
Model 2	0.16 (-0.39; 0.70)	0.562	0.81 (-0.42; 2.03)	0.190	0.346
WMS episodic memory performance					
Model 1	−0.16 (-0.53; 0.22)	0.416	0.67 (-0.54; 1.89)	0.272	0.118
Model 2	−0.03 (-0.44; 0.38)	0.881	1.40 (0.43; 2.38)	0.006	0.035

Log-transformation was applied to raw values of serum vitamin B12.
Model 1 was adjusted for sex, age, MedDiet intervention group and baseline levels of vitamin B12, Memory Composite score or subtests, and MedDiet adherence.
Model 2 was further adjusted for smoking habit, educational level, APOE ε4 genotype and changes in BMI, physical activity, hypertension, diabetes, hypercholesterolemia, use of anticholinergic and lowering-cholesterol drugs, and total energy intake.

relationship of serum vitamin B_{12} and memory function depending on the adherence to the MedDiet. Furthermore, increases in MedDiet adherence were associated with higher concentrations of folate, but not vitamin B_{12}. This observation suggests that moderate intake of animal source food, in the context of a MedDiet which is characterised by a plant-based diet pattern, is enough to cover the requirements of Vitamin B_{12} in the elderly population.

Interestingly, we observed a positive association between changes in vitamin B_{12} and memory function, as increases in serum concentrations were related to better memory composite scores, but only in those participants with higher adherence to the MedDiet. This finding is consistent with previous reports of a positive effect of vitamin B_{12} on cognition. Nalder et al. found that vitamin B_{12} deficiency was specifically associated with worst immediate and delayed memory [20], as well as poor frontal function and global cognition [21,22]. On the other hand, vitamin B_{12} intake reduces plasma homocysteine and improve cognitive impairment [23,24]. Vitamin B_{12} is also involved in the one-carbon metabolic pathway and, therefore, participates in the methylation reactions that occur in the metabolism of DNA, phospholipids, or neurotransmitters [25]. These mechanisms could explain the observed beneficial effects, as vitamin B_{12} deficiency may lead to a disruption in gene expression in the β-amyloid pathway, in phosphatidylcholine-enriched n-3 fatty acids, or in the synthesis of neurotransmitters, such as serotonin, dopamine, or noradrenaline [26]. Interestingly, we found a dose-related effect between circulating vitamin B_{12} and memory function, suggesting that as high in the serum vitamin B_{12} concentration, better memory function you achieve. However, it is important to note that the lack of significant association for specific cognitive tests, such as the RAVLT intermediate episodic verbal memory, prompts us to consider the nuanced nature of cognitive domains and their varying sensitivities to changes in vitamin B_{12} levels.

Vitamin B_{12} status was unrelated to cognition in participants with low adherence to the MedDiet, suggesting that other components of the diet, such as folate, must be present to enhance the effects of vitamin B_{12} on cognition. Circulating folate levels have been widely studied in relation to cognitive performance [20]. In the present study, we found no significant association between serum folate and memory function. Folate is another essential nutrient in one-carbon metabolism and allows the synthesis of methionine [27]. The mechanisms that may explain the implications of folate on cognition are similar to those of vitamin B_{12}, as it is also involved in endogenous methylation reactions [11]. In a randomized controlled trial Chen et al. also reported that folate supplementation could benefit Alzheimer's disease by reducing

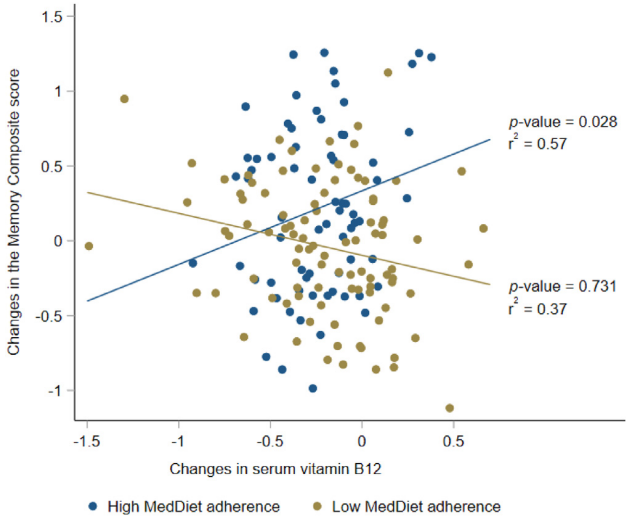

Fig. 1. Changes in the memory composite score and serum vitamin B_{12} stratified by MedDiet adherence. Log-transformation was applied to raw values of serum vitamin B_{12}. This model was adjusted for smoking habit, educational level, APOE ε4 genotype and changes in BMI, physical activity, hypertension, diabetes, hypercholesterolemia, use of anticholinergic and lowering-cholesterol drugs, and total energy intake.

Table 3
Multivariable linear regression of changes in MedDiet adherence with changes of serum vitamin B12 and folate (n = 167).

	Changes in MedDiet adherence (n = 167)	
	β (95 % CI)	p-value
Serum vitamin B12, ng/mL		
Model 1	0.01 (-0.02; 0.04)	0.511
Model 2	0.01 (-0.03; 0.04)	0.657
Serum folate, ng/mL		
Model 1	0.03 (<-0.01; 0.07)	0.088
Model 2	0.04 (<0.01; 0.08)	0.044

Log-transformation was applied to raw values of serum vitamin B12 and folate.
Model 1 was adjusted for sex, age, MedDiet intervention group and baseline levels of MedDiet adherence and vitamin B12 or folate, respectively.
Model 2 was further adjusted for smoking habit, educational level, and changes in BMI, physical activity, hypertension, diabetes, hypercholesterolemia, use of lowering-cholesterol drugs, and total energy intake.

systemic inflammation [28]. However, observational studies and other clinical trials have shown conflicting results. A large study in India following both an observational and a controlled trial design, both in children, found that neither folate intake nor circulating levels were relevant for long-term cognition [29]. Serum folate was also unrelated to cognitive function in a large Brazilian cohort [30]. Similarly, folate was not associated with cognitive functions in a cross-sectional study that included participants with different subtypes of dementia, whereas vitamin B_{12} showed a positive association with cognition in patients with Alzheimer's disease and vascular dementia [31]. By contrast, another clinical trial found that folic acid supplementation for 3 years improved cognitive function in men and women aged 50–70 years with raised homocysteine concentrations, which usually indicates folate deficiency [32]. Thus, the available evidence suggests that folate is associated with improved cognitive function only in cases of low folate levels, virtually explaining the lack of association between folate and memory function in the present study, as only 5 participants were below the cut-off of 3 ng/mL that is considered as the threshold of an adequate level [33]. According to these data, we suggest that effects of dietary vitamin B_{12} go beyond the mere requirements for an adequate metabolism (recommended dietary intake, RDI).

In our study MedDiet adherence did not increase the concentrations of serum vitamin B12, even though the effect of B_{12} on memory was enhanced by higher adherence to the MedDiet. It has been suggested that the MedDiet may not provide sufficient vitamin B12 [34], but in the present population only 11 of 167 participants had levels consistent with deficiency (<0,2 ng/mL), and 64 % of them belong to the low MedDiet adherence group. Similar to our study, Planells et al. reported that vitamin B_{12} intakes in a Spanish Mediterranean population were higher than the established RDI established 2.4 mcg [35,36].

The interaction of the MedDiet with vitamin B_{12} in memory function could be ascribable to folate, as participants with higher MedDiet adherence presented higher serum folate concentrations. The MedDiet is a plant-based dietary pattern and, therefore, promotes high intakes of folate. Observational studies conducted in Mediterranean countries have reported a correct nutritional status concerning circulating folate among their populations, while showing a link between MedDiet adherence and higher levels of folate [37,38]. Not even in vulnerable populations, as pregnant women, has folate deficiency been detected when following the MedDiet [34].

The implications of this study are multifaceted and contribute to our understanding of the intricate interplay between dietary factors, cognitive health, and the MedDiet. Furthermore, it reaffirms the potential cognitive benefits of adhering to the MedDiet, particularly among older individuals at high cardiovascular risk. Altogether, our findings suggest that vitamin B_{12} effects on memory function are potentiated in the context of a MedDiet, as it provides higher amounts of folate in serum, potentially enhancing the role of vitamin B_{12} in the one-carbon cycle. The combination of vitamin B_{12} and folate was found to be more effective than any of the two vitamins alone to improve cognitive performance [39], reinforcing the hypothesis of a synergistic effect on cognition, which is also supported by the closely related metabolism of folate and vitamin B_{12}. In this sense, a clinical trial reported that a supplement containing folate, vitamin B_{12}, and vitamin B_6 improved cognitive functions of middle-aged and elderly participants after 14 weeks [40]. However, other clinical trials did not observe any association between supplementation of these vitamins and cognitive functions in older adults [41,42]. A study conducted within older participants in the National Health and Nutrition Examination Survey (NHANES) showed that when vitamin B12 status was low, high serum folate concentrations were associated with cognitive impairment; contrarywise, when vitamin B_{12} status was within the physiological range, high serum folate was associated with a protective effect against cognitive impairment [43]. Our findings raise the intriguing possibility of synergistic effects between vitamin B_{12} and other nutrients present in the Mediterranean diet. The fact that higher adherence to the diet resulted in increased serum folate concentrations, even without a corresponding increase in serum vitamin B_{12}, hints at the interplay between nutrients synergism in influencing cognitive outcome.

Our study has limitations. First, the PREDIMED trial was not designed to examine cognition. Second, the participants were older Mediterranean individuals at high cardiovascular risk, therefore the results cannot be generalized to other populations. Third, the number of participants with available memory function tests and serum folate and vitamin B_{12} levels was relatively small. We decided to include only the memory composite and its subtests because the subsample receiving tests of frontal functions was even smaller. Our study has also strengths, such as the longitudinal design and the long duration of the intervention, the assessment of vitamins in biological samples, which reflects more accurately the status of the participants, and use of a comprehensive battery of standardized neuropsychological tests to evaluate memory function.

5. Conclusion

In an older Mediterranean population at high cardiovascular risk, changes in serum vitamin B_{12} are associated with improved memory function in the context of high adherence to the MedDiet, which also increases serum concentrations of folate but not vitamin B_{12}. Further research is needed to provide a greater insight of the role of the MedDiet in the relationship between vitamin B_{12} and cognitive performance.

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Author contributions

Conceptualization, I.D.-L., R.M.L.-R., and R.E.; methodology, I.D.-L. and R.C.; formal analysis, I.D.-L.; writing – original draft preparation, I.D.-L; writing – review and editing, G.C.-B., M.A.M.-G., M.F., E.R., R.M.L.-R., and R.E. All authors have read and agreed to the published version of the manuscript.

Data sharing

There are restrictions on the availability of data for the PREDIMED trial, due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following the project purposes. Therefore, data will only be available upon request.

Conflicts of interest

E.R. reports grants, personal fees, non-financial support and other from the California Walnut Commission while the study was carried out; grants, personal fees, non-financial support and other from Alexion; and personal fees and other from Amarin, outside the submitted work. R.M.L.-R. reports personal fees from Cerveceros de España, personal fees, and other from Adventia, Wine in Moderation, UNIDECO, and Ecoveritas S.A., outside the submitted work. R.E. reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain), and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Albuquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain), and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

Data described in the manuscript, code book, and analytic code will be made available upon request pending and approval to the PREDIMED trial Steering Committee, only to external researchers for studies following the project purposes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.10.025.

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GLOBAL DISCUSSION

4. Global Discussion

This work focuses on the influence of fatty acids, polyphenols, and vitamin B12 on chronic diseases in a population at high CVD risk. To achieve this goal, 11 substudies were conducted within the cohorts of the PREDIMED and PREDIMED-Plus trial, using biological samples of plasma, serum, and urine from the participants. Regarding metabolic and inflammatory pathways, we observed that plasma SFAs concentrations are linked to dietary intake of F&V, and changes in enzymes related to fatty acid metabolism are related to CVD risk factors. Additionally, we reported that SFAs, particularly palmitic acid, may promote circulating inflammatory biomarkers, while wine polyphenols and vitamin B12 are associated with lower levels.

Secondly, the relationship of phenolic compounds was assessed with CVD risk factors. Our results suggest that the polyphenols found in wine are associated with lower LDL-c levels in postmenopausal women. Among a population of older men and women, metabolites derived from phenolic compounds were related to a lower incidence of diabetes and better cardiovascular health.

Finally, we observed that phenolic compounds derived from the microbiota are positively linked to cognitive function. Additionally, we described the relationship between vitamin B12 and cognitive decline based on adherence to the Mediterranean diet, demonstrating that greater adherence to this dietary pattern maximizes the positive influence of vitamin B12.

4.1. Metabolic and inflammatory pathways

Plasma fatty acids are commonly used to assess the impact of diet on plasma lipids since numerous studies have shown that most plasma fatty acids mirror dietary intake[158,159]. In Publication 4 we conducted a cross-sectional analysis within the PREDIMED-Plus trial. Our findings revealed that individuals who have a higher consumption of F&V tend to exhibit lower concentrations of total fatty acids in

plasma, especially SFAs and its primary component, palmitic acid. These results indicate an inverse relationship between the consumption of F&V and SFAs, but this correlation doesn't extend to other types of fatty acids. The associations between the consumption of F&V and plasma fatty acids may be attributed to the well-known high fiber content of these foods. Fiber is recognized for its interference in nutrient absorption, potentially playing a significant role in modifying the uptake of fatty acids based on their characteristics and the quantity of fat consumed[160]. Emerging evidence has underscored the connection between SFAs and adverse health outcomes, including conditions like coronary heart disease and inflammation[161,162]. Palmitic acid, in particular, is believed to play a role in the development of obesity, diabetes, cancer, and even increased mortality risk[94,163]. However, it is not only SFAs that are linked to detrimental health effects, but also disruptions in the functions of the enzymes responsible for their metabolism. Altered desaturase enzyme activities implicated in the synthesis, elongation, or desaturation of fatty acids are associated with cardiometabolic risk factors, such as T2D, obesity and MetS[72,73]. In Publication 5, estimated desturase enzyme activities were calculated with the ratios of the fatty acids that are involved in the reaction to find that alterations were related to individual components of the MetS in a longitudinal study involving participants of the PREDIMED trial. Specifically, estimated D6D and SCD-18 activities were adversely associated with MetS, which is in line with other studies performed within the PREDIMED trial[164]. Estimated activities of D6D, SCD-16, and SCD-18 have also been positively associated with various CVD risk factors, such as BMI, blood pressure, cholesterol, or triglycerides[165–168]. This is consistent with our results, since we found that estimated D6D activity was positively associated with triglycerides and diastolic blood pressure, SCD-16 with triglycerides, and SCD-18 with triglycerides and waist circumference, and inversely associated with HDL-c. In contrast, estimated D5D activity has been favorably associated with MetS components, as it has been to related to higher HDL-c, lower blood pressure, and lower BMI[166,169]. Similarly, we observed that estimated D5D activity was associated with lower risk of MetS and also with lower levels of triglycerides, diastolic blood pressure, and waist circumference. Thus, our findings support the hypothesis that fatty acid metabolism plays a role

in metabolic health, and dysregulation of desaturases may serve as an indicator of metabolic alterations.

These enzymes play a role in metabolic health by participating in the metabolism of fatty acids with either pro-inflammatory or anti-inflammatory properties[162]. Specifically, SFAs and palmitic acid demonstrated pro-inflammatory activity after one year of follow-up in a PREDIMED subcohort in Publication 6, as they were associated with higher concentrations of the inflammatory molecule IL-6. Previous *in vitro* research has demonstrated that SFAs can trigger inflammation by activating toll-like receptor 4 (TLR-4) receptors and nuclear factor kappa B, resulting in the upregulation of inflammatory genes[170]. Others suggested that SFAs might exacerbate the inflammatory response due to their conversion into ceramides, which activate protein kinase C and mitogen-activated protein kinase[89]. Additionally, findings from human observational studies have indicated that the consumption of SFAs is associated with increased CRP levels[171]. Among SFAs, palmitic acid is particularly noteworthy for its adverse effects[172], and it stimulates the production of IL-6 in various biological tissues[173,174]. It also induces the expression of pro-inflammatory cytokines in macrophages through its interaction with TLR-4[97] and enhances the generation of reactive species, diminishes oxidative capacity, and induces mitochondrial dysfunction, ultimately leading to insulin resistance[175]. In one of the few clinical studies that examined circulating concentrations of fatty acids, Mu et al. reported a positive correlation between palmitic acid (measured in red blood cell phospholipids) and IL-6[176], consistent with our findings. Interestingly, in our analysis these associations were unaffected by *de novo* lipogenesis and endogenous metabolism, highlighting the dietary origin of these fatty acids. Surprisingly, n-3 polyunsaturated showed only a weak anti-inflammatory effect on sICAM and E-selectin.

IL-6 plays a major role in both chronic and acute inflammation[177]. In acute inflammation, it stimulates the synthesis and release of numerous proteins contributing to the acute phase response, triggered by a wide array of stimuli[177]. CRP is among the acute-phase proteins influenced by IL-6[178]. Additionally, IL-6 facilitates the transition from acute to chronic inflammation by recruiting monocytes to the affected area[179]. These molecules, through their involvement in inflammatory processes, significantly

contribute to the development of various diseases. When it comes to cardiovascular health, IL-6 levels are systematically elevated in individuals with obesity due to increased secretion from adipocytes[180]. IL-6 is also implicated in the development of insulin resistance and beta-cell dysfunction, both of which are key factors in the onset of T2D. Furthermore, recent research has suggested IL-6 as a potential target for cancer treatment, as it plays a role in the proliferation of cancer cells[181,182].

After obtaining interesting results regarding fatty acids, our research focused on investigating the relationship between IL-6 and the micronutrient vitamin B12. To achieve this, in Publication 7 we performed a cross-sectional analysis within the PREDIMED trial to assess the association between circulating vitamin B12 and IL-6. We also incorporated CRP into the analysis due to the close relationship between these two inflammatory molecules. In addition, the analysis between vitamin B12 and IL-6 was replicated in aged mice, which provided a valuable experimental model for naturally aging and free from CVD that allowed us to conduct a controlled experiment, minimizing confounding factors such as environment or genetic background. Unfortunately, CRP could not be measured in mouse serum because values would be below the limits of detection. Our results revealed that serum vitamin B12 is associated with lower levels of inflammatory molecules IL-6 and CRP in humans, and the data from naturally aged, healthy, wild-type mice provided supporting evidence by also showing an inverse relationship between serum vitamin B12 and IL-6. The available evidence suggests that vitamin B12 deficiency is linked to inflammation and metabolic complications[183] by enhancing plasma antioxidant capacity[184]. However, the specific connection between vitamin B12 and the inflammatory biomarkers IL-6 and CRP has been minimally explored. *In vitro* and animal studies showed that vitamin B12 regulated IL-6 levels independently of other regulators of IL-6 production and increased its gene expression[185,186]. Supplementation with combined folate and vitamin B12 in elders with mild cognitive impairment was found to reduce inflammatory cytokines, including IL-6, in conjunction with decreased homocysteine levels[187]. In patients with Alzheimer's disease, higher concentrations of IL-6 were detected in peripheral blood mononuclear cells when serum vitamin B12 concentrations were lower[188]. In contrast to our findings, previous studies

have not been able to establish a significant relationship between vitamin B12 and CRP levels[189–192]. The mechanisms behind this findings involve the methionine cycle, as deficits in vitamin B12 lead to the accumulation of homocysteine[152]. The pathological excess of this amino acid, hyperhomocysteinemia, has been linked to an increase in pro-inflammatory cytokines, neuroinflammation, endothelial damage, and CVD[193–195]. Another hypothesis to explain this inverse relationship is that vitamin B12 suppresses the synthesis of cytokines in T lymphocytes[196]. Overall, these findings support the potential role of vitamin B12 in inflammatory processes and related diseases.

Other circulating inflammatory molecules are considered predictive markers of atherosclerosis, such as VCAM-1 and ICAM-1. In fact, they are responsible for promoting cell recruitment to the arterial wall and reflect the degree of endothelial activation caused by CVD risk factors[197]. In Publication 8, we aimed to investigate if wine, an alcoholic beverage rich in polyphenols, was associated with lower inflammatory biomarkers related to atherosclerosis after one year of follow-up in a subcohort of the PREDIMED trial. The novelty of this study lies on the assessment of wine consumption through the measurement an objective biomarker, tartaric acid in urine[198]. In epidemiological and clinical trials it is primarily assessed using food frequency questionnaires, which might be influenced by subjectivity and negative social perceptions of alcohol intake[130]. In contrast, urinary tartaric acid provides more precise and reliable information about wine consumption. The main findings of this study supported the anti-inflammatory properties attributed to wine, as increases in urinary tartaric acid were associated with decreases in the plasma concentrations of sICAM-1 and s-VCAM-1. Previous literature aligns with our results, as clinical trials observed similar effects in adhesion molecules and other inflammatory biomarkers, such as pro-inflammatory cytokines[199–201]. These effects may, in part, be attributed to its polyphenol content, as wine is a rich source of phenolic acids, flavanols, flavonols, anthocyanins, and resveratrol[202]. Research has shown that red wine polyphenols are believed to possess antiatherogenic properties, as they lower the levels of adhesion molecules like ICAM-1 and VCAM-1[157,203,204]. Others also hypothesized that the ethanolic fraction of wine could have beneficial effects. However, most studies

comparing wine to other alcoholic beverages have failed to observe the same benefits in other drinks[199,205], suggesting that polyphenols provide wine with an additional anti-inflammatory effect that ethanol alone does not offer.

4.2. Cardiovascular disease and associated risk factors

Assessing the influence of wine on the risk of CVD in older women is crucial, considering the increased cardiovascular risk following menopause[206]. To achieve this goal, in Publication 9 we conducted a cross-sectional analysis involving postmenopausal women who participated in the PREDIMED trial to evaluate the association of wine, measured as urinary tartaric acid, with CVD risk factors. Firstly, we confirmed that tartaric acid is a sensitive biomarker in this specific population of postmenopausal women. Furthermore, our results revealed an inverse association between urinary tartaric acid and LDL-c. Previous clinical trials reported a decrease in LDL-c after interventions with wine[207,208], as well as an increase in HDL-c[209,210]. In the present study, however, we did not observe an association with HDL-C, possibly because our participants had high baseline levels of HDL-C, which made it more challenging to detect any relationship. The main hypothesis to explain these results is the presence of bioactive compounds, especially polyphenols, since, as explained before, they have anti-inflammatory activity[157]. Overall, the findings of the present study suggest that wine consumption could have beneficial effects on cardiovascular health of postmenopausal women, possibly attributable to its phenolic content.

The next step, therefore, was to assess the role of dietary polyphenols on CVD risk factors. We decided to focus initially on diabetes, a disease that affects more than 460 million people worldwide[211], and include both men and women in the study to gain a broader perspective. Publication 10 aimed to develop a metabolomics-based method to analyze a panel of urinary phenolic compounds for potential associations with T2D in participants of the PREDIMED trial with and without T2D at baseline.

A total of 41 phenolic compounds were modeled in the orthogonal projection to latent structures discriminant analysis. These compounds were initially identified through HPLC and high-resolution MS, which offer precise mass determinations and multi-stage mass fragmentation patterns, facilitating the structural elucidation of both known and unknown compounds[212]. These results are consistent with other studies that reported that dietary polyphenols reduce the risk of T2D[213]. More specifically, we found that dihydrocaffeic, a microbial metabolite of cinnamic acids, was associated with a lower risk of T2D and plasma glucose levels. Prior studies have indicated that the positive impacts of dihydrocaffeic acid are linked to oxidative stress and the insulin/IGF-1 pathway[214]. Its precursors chlorogenic, caffeic, and ferulic acid have also demonstrated benefits against T2D through reduction of oxidative stress, anti-inflammatory properties, and regulation of genes involved in glucose and lipids metabolism[215–221]. In our study, genistein diglucuronide was also identified as discriminant protective compound against T2D. This compound is a phase-II metabolite of genistein aglycone, a phytoestrogen that has been found to improve insulin release, and reduce triglycerides and cholesterol[222,223].

Based on the results obtained in the previous publication, we can deduce that the compounds with highest biological activity are metabolites derived from polyphenols present in dietary foods. Considering the extensive metabolism that polyphenols undergo in the gut, it was highly relevant to focus on studying MPM in relation to cardiovascular health. This objective was achieved in a cross-sectional study within the PREDIMED trial presented in Publication 11, in which we identified 5 MPM (protocatechuic acid, enterodiol glucuronide, enterolactone glucuronide, vanillic acid glucuronide, and urolithin B glucuronide) that were combined into a MPM score. First, we found that adherence to the MedDiet was directly associated with the MPM score, and particularly with protocatechuic acid and enterolactone glucuronide. This goes in accordance with other studies that reported that MedDiet modulates the microbial ecosystem of the gut[224], enhancing bacterial species that are involved in polyphenols metabolism[225,226]. In relation to ICVH, a positive association was observed with the MPM score, with the metrics most affected being diet and blood glucose. The relationship with glucose aligns with our prior findings (Publication 10)

and with other published research that has indicated polyphenols and their metabolites are linked to a lower risk of developing T2D and reduced insulin resistance[140,227]. Furthermore, urolithin B glucuronide was inversely associated with LDL-c in our subcohort. An *in vitro* study revealed that urolithin B could potentially decrease the deposition of lipid plaques by regulating the expression of genes associated with reverse cholesterol pathways[228]. This mechanism could offer an explanation for the observed inverse correlation between urolithin B glucuronide and LDL-c. In conclusion, these results suggest an association between the MedDiet and phenolic metabolites that have favorable implications for cardiovascular health. Moreover, they underscore the necessity of evaluating the collective effects of MPM rather than examining them individually when assessing their potential health benefits.

4.3. Cognitive decline

On the premise that MPM exert biological effects that before were attributed to dietary polyphenols, we investigated their role on cognitive health. A subcohort of participants of the PREDIMED trial recruited in the Hospital Clínic site underwent cognitive examinations to assess global, frontal, and memory functions, whose samples at baseline were analyzed in Publication 12. Similar to the methodology employed in the previous publication, a score of MPM combining the individual phenolic metabolites was developed. A higher MPM score was associated with better frontal functions composite, suggesting that the combination of MPM may have a positive influence on frontal lobe functions. The frontal lobe of the brain is responsible for processes related to executive function, attention, and working memory, which are among the cognitive faculties most negatively impacted by the aging process[229]. Consistent with our results, a few studies have reported beneficial effects of polyphenols on cognitive functions associated with the frontal lobe[230,231]. However, the effect of microbiota metabolism was not considered. Regarding individual metabolites, vanillic acid glucuronide was associated with the frontal lobe composite, whereas urolithin B glucuronide presented significant associations with only one cognitive test included

in the frontal functions composite, suggesting a weaker influence. Studies assessing the impact of these metabolites on cognition are scarce, and only *in vitro* and animal studies have specifically focused on the effect of their parent compounds, vanillic acid and urolithin B, on the brain[232–234]. It has been demonstrated that they are capable of crossing the blood-brain barrier, and the possible mechanisms of action in the brain were reviewed in Publication 3.

We replicated this study in a larger sample size of participants of the PREDIMED-Plus trial at baseline in Publication 13. Due to study design, the battery of neuropsychological tests did not correspond to those of the PREDIMED trial, and composites for global, frontal, and memory functions were not calculated. Therefore, to make the results more comprehensible and comparable, we developed a global cognitive function composite that included the results of all the neuropsychological assessments. In the present study, protocatechuic acid, vanillic acid glucuronide, 3-hydroxybenzoic acid, enterodiol glucuronide, and enterolactone glucuronide were directly associated with the global composite score. Specifically, protocatechuic acid and enterolactone glucuronide presented an association with cognition, as they were also linked to better scores on tests that assessed cognitive impairment. In Publication 3, a comprehensive summary of potential brain mechanisms of action was presented. However, the permeability of protocatechuic acid across the blood-brain barrier remains unclear, as discussed in Publication 3. The variation in cognitive health-related metabolites between the PREDIMED and PREDIMED-Plus studies could be attributed to interindividual differences among the participants. Overall, the findings from these two studies suggest that the metabolites of dietary polyphenols originated in the gut microbiota could be beneficial against cognitive decline, particularly vanillic acid glucuronide. Furthermore, the relationship of the MPM with adherence to the MedDiet was also evaluated in Publication 13. Interestingly, protocatechuic acid and enterolactone glucuronide, the metabolites significantly associated with cognition, exhibited a positive association with the MedDiet. These results support our previous findings in the PREDIMED trial (Publication 10), as the same metabolites were found to be associated with the MedDiet. This strengthens the hypothesis that the MedDiet

influences the composition of the gut microbiota and enhances the production of specific MPM with cardiovascular and cognitive health benefits.

The influence of the MedDiet goes beyond polyphenols and their microbial metabolites. This dietary pattern can also influence the biological effects of other micronutrients, including vitamin B12. Publication 14 presented a longitudinal analysis of one year of follow-up within the PREDIMED trial where the role of vitamin B12 in cognition was evaluated according to adherence to the MedDiet. We observed that only in participants with higher adherence to the MedDiet, changes in serum vitamin B12 were associated with better memory function. The positive association between vitamin B12 and memory is consistent with previous reports that found that its deficit was linked to worse immediate and delayed memory, frontal functions, and global cognition[235–237]. Due to the role that vitamin B12 plays in the one-carbon metabolic pathway, its deficit may lead to a disruption in gene expression in the β-amyloid pathway, in phosphatidylcholine-enriched n-3 fatty acids, or in the synthesis of neurotransmitters, such as serotonin, dopamine, or noradrenaline[150]. However, vitamin B12 status was not related to cognition when adherence to the MedDiet was low, suggesting that other components of the diet, such as folate, must be present to enhance the effects of vitamin B12 on cognition. In the present study, we found no significant association between serum folate and memory function, which is consistent with previous findings from other studies. Most clinical and observational studies failed to find a positive relationship between folate and cognition[238–240]. However, a clinical trial found that folic acid supplementation improved cognitive functions in participants with elevated homocysteine concentrations, which is indicative of folate deficiency[241]. Thus, current evidence indicates that folate enhances cognitive function only in situations where folate levels are low. Our research revealed that adherence to the MedDiet did not elevate serum vitamin B12 levels, even though higher adherence to the MedDiet did enhance the impact of B12 on memory. This interaction could be ascribable to folate, as participants with higher MedDiet adherence presented higher serum folate concentrations. The MedDiet is a plant-based dietary pattern and, therefore, promotes high intakes of folate[11,242]. Altogether, our results suggest that the influence of vitamin B12 on memory function is potentiated when combined with a MedDiet,

which provides increased levels of serum folate, enhancing the role of vitamin B12 in the one-carbon cycle.

The major strength of this dissertation is the assessment of nutrients, bioactive compounds, and inflammatory molecules in biological samples, which more accurately reflects the status of the participants; the determination of a biomarker for wine consumption provided more objective information than self-reported questionnaires, especially considering the negative social perception of alcohol; a novel method for the identification and quantification of phenolic compounds, whose biological effects have been scarcely studied, was used; the comprehensive dataset, including cognitive evaluations, clinical assessments, and food frequency questionnaires, available for all participants from both the PREDIMED and PREDIMED-Plus trials. This work also presents some limitations. Firstly, all research was conducted within the PREDIMED and PREDIMED-Plus trials, with participants consisting of older Mediterranean individuals at high cardiovascular risk, making it challenging to extrapolate the results to broader populations. Secondly, the number of participants in the studies was relatively small. Lastly, the imposibility of determining causality due to the design of the studies.

CONCLUSIONS

5. Conclusions

The results of this dissertation lead to postulate the following conclusions:

1. Regarding metabolic and inflammatory pathways:

    1.1. Dietary patterns with high consumption of F&V have a different influence on plasma fatty acids depending on their degree of saturation. Plasma concentrations of SFAs are negatively associated with high consumption of F&V.

    1.2. The estimated activities of the enzymes responsible for metabolizing of palmitic, oleic, α-linolenic, and linoleic acids are adversely associated with MetS and some of its components, whereas the desaturase responsible for producing arachidonic acid and EPA is positively related. Therefore, fatty acid metabolism influences metabolic health, and dysregulation of desaturases may be indicative of metabolic alterations.

    1.3. Circulating inflammatory biomarkers are related to nutritional components of the diet. SFAs, and particularly palmitic acid, are linked to inflammation through increases in IL-6. On the other hand, vitamin B12 is associated with lower levels of this molecule, as well as of CRP. Additionally, wine consumption is related to lower levels of adhesion molecules associated with atherosclerosis, potentially due to its phenolic content.

2. Regarding CVD and its risk factors:

    2.1. Wine consumption measured through the urinary biomarker tartaric acid is associated with lower levels of LDL-c in postmenopausal women.

    2.2. Among a panel of urinary phenolic compounds with potential associations with T2D that were successfully identified using a novel metabolomics approach, the metabolites dihydrocaffeic acid and genistein diglucuronide are associated with lower risk of developing this condition. Dihydrocaffeic acid also exhibits an inverse association with glucose levels.

2.3. MedDiet adherence is linked to increased production of MPM with beneficial effects on cardiovascular health. Among the metabolites, urolithin B glucuronide shows an inverse association with LDL-c. However, MPM might need to be considered collectively rather than individually, as the health benefits are mostly observed when assessing them in combination.

3. Regarding cognitive decline:

3.1. High urinary concentrations of MPM are associated with better cognitive functions. Specifically, protocatechuic acid and enterolactone glucuronide, which are positively correlated with adherence to the MedDiet, seem to help prevent cognitive impairment. Additionally, vanillic acid glucuronide is linked to higher frontal lobe functions.

3.2. Vitamin B12 correlates with better memory function, but only depending on the adherence to the MedDiet. When adherence to this dietary pattern is high, vitamin B12 is associated with an improvement in memory, whereas when the adherence is low there is no association. This suggests that the MedDiet enhances the benefits of vitamin B12 on cognition.

Overall, this dissertation provides valuable insights into the potential benefits of fatty acids, phenolic compounds, and vitamin B12 in the context of an older Spanish population at high risk of CVD. The findings emphasize the influence of these dietary and metabolic factors on inflammation, with SFAs, particularly palmitic acid, associated with inflammation, while vitamin B12 and wine consumption show potential for mitigating these inflammatory processes. Furthermore, it uncovers the potential cardioprotective effects of phenolic compounds and their microbial metabolites. Lastly, the importance of these compounds in cognitive health was uncovered, as well as the influence of the MedDiet in the role of the micronutrient vitamin B12. These results underscore the importance of dietary choices in promoting healthy aging and preventing chronic diseases. Further studies are needed to identify the molecular mechanisms that explain these effects, as well as larger and more diverse randomized clinical trials to confirm these associations in other populations.

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6. References

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ANNEX

7. Annex

7.1. Other publications

1. D. Murcia-Lesmes, **I. Domínguez-López**, E.P. Laveriano-Santos, et al. Association between tomato consumption and blood pressure in an older population at high cardiovascular risk: observational analysis of PREDIMED trial. *European Journal of Preventive Cardiology*. 2023 Nov.

2. J. Lozano-Castellón, A. Olmo-Cunillera, E. Casadei, E. Valli, **I. Domínguez-López**, et al. A targeted foodomic approach to assess differences in extra virgin olive oils: Effects of storage, agronomic and technological factors. *Food Chemistry*. 2024 Mar 1;435:137539.

3. I. Parilli-Moser, R. López-Solís,**I. Domínguez-López**, A. Vallverdú-Queralt, S. Hurtado-Barroso, R. M. Lamuela-Raventós. Consumption of peanut products enhances the production of microbial phenolic metabolites related with memory and stress response: Results from the ARISTOTLE trial. *Journal of Functional Foods*. 2021 Nov;40(11):5556-5567.

4. A. López-Yerena, **I. Domínguez-López**, M.M. Abuhabib, R. M. Lamuela-Raventós, A. Vallverdú-Queralt, M. Pérez. Tomato wastes and by-products: upcoming sources of polyphenols and carotenoids for food, nutraceutical, and pharma applications. *Critical Reviews in Food Science and Nutrition*. 2023 Jun 23:1-18.

5. I. Parilli-Moser, **I. Domínguez-López**, A. Vallverdú-Queralt, et al. Urinary Phenolic Metabolites Associated with Peanut Consumption May Have a Beneficial Impact on Vascular Health Biomarkers. *Antioxidants*. 2023 Mar;10.3390/antiox12030698

6. C. Arancibia-Riveros, **I. Domínguez-López**, A. Tresserra-Rimbau, et al. Total urinary polyphenol excretion: a biomarker of an anti-inflammatory diet in females associated with improved metabolic syndrome status. Prospective study

within a cohort of PREDIMED participants. *The American Journal of Clinical Nutrition*. 2023 Apr;117(4):814-822.

7. A. Olmo-Cunillera, E. Casadei, E. Valli, J. Lozano-Castellón, E. Miliarakis, **I. Domínguez-López**, et al. Aromatic, Sensory, and Fatty Acid Profiles of Arbequina Extra Virgin Olive Oils Produced Using Different Malaxation Conditions. *Foods*. 2022 Oct 30;11(21):3446.

8. A. Gázquez, M. Sabater-Molina, **I. Domínguez-López**, et al. Milk fat globule membrane plus milk fat increase docosahexaenoic acid availability in infant formulas. *European Journal of Nutrition*. 2022 Oct 25.

9. M. Marhuenda-Muñoz, **I. Domínguez-López**, K. Langohr, et al. Circulating carotenoids are associated with favorable lipid and fatty acid profiles in an older population at high cardiovascular risk. *Frontiers in Nutrition*. 2022 Sep 29;9:967967.

10. M. Marhuenda-Muñoz, **I. Domínguez-López**, E.P. Laveriano-Santos, et al. One-Year Changes in Urinary Microbial Phenolic Metabolites and the Risk of Type 2 Diabetes-A Case-Control Study. *Antioxidants*. 2022 Aug 8;11(8):1540.

11. J. Lozano-Castellón, A. López-Yerena, **I. Domínguez-López**, et al. Extra virgin olive oil: A comprehensive review of efforts to ensure its authenticity, traceability, and safety. *Comprehensive Reviews in Food Science and Food Safety*. 2022 May;21(3):2639-2664.

12. I. Parilli-Moser, **I. Domínguez-López**, C. Arancibia-Riveros, et al. Effect of Crushing Peanuts on Fatty Acid and Phenolic Bioaccessibility: A Long-Term Study. *Antioxidants*. 2022 Feb 19;11(2):423.

13. **I. Domínguez López**, M. Pérez, A. López-Yerena, et al. Chapter 9: Human Health and the Consumption of Fat-associated Compounds: Tyrosol, Hydroxytyrosol, Oleuropein, Oleacein, and Oleocanthal. *Royal Society of Chemistry*. 2022 January.

14. I. Parilli-Moser, **I. Domínguez-López**, M. Trius-Soler, et al. Consumption of peanut products improves memory and stress response in healthy adults from the ARISTOTLE study: A 6-month randomized controlled trial. *Clinical Nutrition*. 2021 Nov;40(11):5556-5567.

15. M. Perez, **I. Domínguez-López**, A. López-Yerena, et al. Current strategies to guarantee the authenticity of coffee. *Critical Reviews in Food Science and Nutrition*. 2021 Jul;19:1-16.

16. A. López-Yerena, **I. Domínguez-López**, A. Vallverdú-Queralt, et al. Metabolomics Technologies for the Identification and Quantification of Dietary Phenolic Compound Metabolites: An Overview. *Antioxidants*. 2021 May 25;10(6):846.

17. **I. Domínguez-López**, M. Yago-Aragón, A. Salas-Huetos, et al. Effects of Dietary Phytoestrogens on Hormones throughout a Human Lifespan: A Review. *Nutrients*. 2020 Aug 15;12(8):2456.

7.2. Communications

1. **I. Domínguez-López**, A. Tresserra-Rimbau, I. Parilli-Moser, et al. Microbial phenolic metabolites are associated with better adiposity parameters. Poster in XIV Symposium Ciber Fisiopatología de la Obesidad. May 2023. Santiago de Compostela, Spain.

2. I. Parilli-Moser, **I. Domínguez-López**, C. Arancibia-Riveros, et al. Prebiotic effect of peanut consumption on fecal short chain fatty acids seems to improve lipid profile. Poster in International World of Microbiome Conference 2022. May 2022. Viena, Austria.

3. **I. Domínguez-López**, I. Parilli-Moser, C. Arancibia-Riveros,et al. Bioavailability of bioactive fatty acids increases with peanut crushing in peanut butter. Poster in XII Symposium Ciber Fisiopatología de la Obesidad y Nutrición. October 2021. Madrid, Spain.

4. M. Marhuenda-Muñoz, E. P. Laveriano-Santos, I. Parilli-Moser, **I. Domínguez-López** and R. M. Lamuela-Raventós. "Biomarkers of polyphenols intake." Oral communication in the 5th International Symposium on Phytochemicals in Medicine and Food. August 25-31, 2021. Nanchang, China.

5. R. M. Lamuela-Raventós, **I. Domínguez-López**, I. Parilli-Moser, and R. Estruch. Moderate consumption and weight management. Oral Communication in 2nd Science & Wine World Congress. July 2021. Porto, Portugal.

7.3. Supplementary material

Supplementary Material of Publication 4

Table 1. Nutrient and food consumption according to F&V consumption and fat intake.

	Low F&V		High F&V		*p*-value
	Low-moderate fat	Very high fat	Low-moderate fat	Very high fat	
No. of participants	57	58	60	53	
Mediterranean diet adherence score	7.3 ± 2.3 [a]	6. 7 ± 2.6 [a]	10.0 ± 2.5 [b]	9.8 ± 2.8 [b]	< 0.001
Total energy (Kcal/day)	1707 ± 519 [a]	2803 ± 420 [b]	2026 ± 373 [c]	3161 ± 356 [d]	< 0.001
Nutrient intake					
Carbohydrates (g/day)	181 ± 78.7 [a]	256 ± 74.8 [b]	249 ± 66.7 [b]	324 ± 67.9 [c]	< 0.001
Fiber (g/day)	15.4 ± 6.2 [a]	19.2 ± 4.8 [b]	35.3 ± 6.4 [c]	42.5 ± 8.9 [d]	< 0.001
Protein (g/day)	71.1 ± 18.4 [a]	99.1 ± 19.6 [b]	89.8 ± 19.5 [b]	127 ± 21.7 [c]	< 0.001
Total fat (g/day)	64.9 ± 15.6 [a]	140 ± 14.6 [b]	69.7 ± 12.6 [a]	142 ± 16.9 [b]	< 0.001
Cholesterol (mg/day)	295 ± 105 [a]	437 ± 130 [b]	317 ± 97.1 [a]	459 ± 134 [b]	< 0.001
Alcohol (g/day)	16.6 ± 21.2 [a]	17.3 ± 18.1 [a]	6.08 ± 11.1 [b]	11.6 ± 13.4 [a,b]	< 0.001
Food consumption (g/day)					
F&V	277 ± 69.1 [a]	300 ± 52.7 [a]	1265 ± 299 [b]	1328 ± 270 [b]	< 0.001
Legumes	14.0 ± 8.0 [a]	18.3 ± 7.9 [a,b]	24.3 ± 16.4 [b,c]	30.4 ± 17.2 [c]	< 0.001
Cereals	139 ± 89.1 [a,b]	170 ± 85.3 [a,b]	129 ± 78.7 [a]	177 ± 82.4 [b]	0.005
Dairy	240 ± 106 [a]	360 ± 226 [b]	297 ± 213 [a,b]	380 ± 216 [b]	0.001
Meat	118 ± 44.8 [a]	151 ± 58.4 [b]	120 ± 48.0 [a]	171 ± 67.9 [b]	< 0.001
Fish	65.2 ± 38.9 [a]	92.6 ± 53.8 [b]	100 ± 48.0 [b]	136 ± 56.5 [c]	< 0.001
Nuts	5.0 ± 6.4 [a]	20.6 ± 21.4 [b]	10.5 ± 10.5 [a]	39.1 ± 30.7 [c]	< 0.001
Olive oil	23.1 ± 11.3 [a]	53.4 ± 15.5 [b]	24.0 ± 10.6 [a]	48.1 ± 16.4 [b]	< 0.001
Sunflower oil	1.4 ± 3.8 [a]	2.0 ± 5.7 [a]	0.9 ± 3.5 [a]	2.3 ± 7.2 [a]	0.464
Butter	0.8 ± 2.3 [a]	1.9 ± 4.8 [a]	0.6 ± 2.18 [a]	0.9 ± 2.1 [a]	0.098
Margarine	0.7 ± 1.7 [a]	1.4 ± 3.4 [a]	0.4 ± 1.0 [a]	1.3 ± 3.1 [a]	0.071
Pastries	18.3 ± 20.5 [a,b]	40.9 ± 43.6 [c]	14.6 ± 18.8 [b]	31.8 ± 32.1 [a,c]	< 0.001

a) F&V, fruit and vegetable.

b) Values are expressed as mean ± SD.

c) P-values were calculated by analysis of variance–one factor, $p < 0.05$

d) Different lower-case letters indicate a significant difference among groups.

Table 2. Association between individual plasma fatty acids (mg/cL) with F&V consumption and fat intake groups.

		High F&V vs. Low F&V	*p*-value	High F&V vs. Low F&V (low-moderate fat)	*p*-value	High F&V vs. Low F&V (high fat)	*p*-value
C14:0	Mean (mg/cL)	0.29 vs. 0.34		0.29 vs. 0.31		0.29 vs. 0.36	
	ß [CI]-model 1	-0.04 [-0.09; <0.01]	0.076	-0.02 [-0.09; 0.05]	0.583	-0.07 [-0.13; <-0.01]	0.041
	ß [CI]-model 2	-0.04 [-0.10; 0.01]	0.081	-0.02 [-0.10; 0.05]	0.565	-0.07 [-0.14; <-0.01]	0.043
	ß [CI]-model 3	-0.05 [-0.10; <0.01]	0.054	-0.05 [-0.13; 0.03]	0.231	-0.09 [-0.17; -0.01]	0.026
Palmitic acid (C16:0)	Mean (mg/cL)	9.36 vs. 10.26		9.18 vs. 9.79		9.56 vs. 10.73	
	ß [CI]-model 1	-0.88 [-1.64; -0.12]	0.024	-0.57 [-1.66; 0.53]	0.307	-1.20 [-2.28; -0.12]	0.029
	ß [CI]-model 2	-0.90 [-1.69; -0.11]	0.026	-0.76 [-1.89; 0.38]	0.191	-1.14 [-2.24; -0.04]	0.043
	ß [CI]-model 3	-0.92 [-1.71; -0.13]	0.023	-0.87 [-2.25; 0.51]	0.215	-1.29 [-2.59; 0.01]	0.052
C16:1 n-9	Mean (mg/cL)	0.25 vs. 0.25		0.24 vs. 0.25		0.25 vs. 0.26	
	ß [CI]-model 1	-0.01 [-0.03; 0.01]	0.477	<-0.01 [-0.04; 0.03]	0.765	-0.01 [-0.04; 0.02]	0.509
	ß [CI]-model 2	-0.01 [-0.03; 0.02]	0.597	<-0.01 [-0.04; 0.03]	0.769	-0.01 [-0.04; 0.02]	0.612
	ß [CI]-model 3	-0.01 [-0.03; 0.01]	0.456	<-0.01 [-0.04; 0.03]	0.905	-0.03 [-0.07; 0.01]	0.141
C16:1 n-7	Mean (mg/cL)	0.75 vs. 0.96		0.75 vs. 0.99		0.76 vs. 0.92	
	ß [CI]-model 1	-0.20 [-0.34; -0.06]	0.004	-0.24 [-0.46; -0.01]	0.041	-0.17 [-0.33; -0.01]	0.038
	ß [CI]-model 2	-0.21 [-0.35; -0.06]	0.005	-0.28 [-0.53; -0.03]	0.026	-0.17 [-0.34; <-0.01]	0.045
	ß [CI]-model 3	-0.17 [-0.30; -0.04]	0.013	-0.27 [-0.57; 0.02]	0.071	-0.20 [-0.41; 0.02]	0.071
C18:0	Mean (mg/cL)	2.96 vs. 3.19		2.89 vs. 3.10		3.04 vs. 3.27	
	ß [CI]-model 1	-0.22 [-0.43; -0.02]	0.030	-0.22 [-0.51; 0.07]	0.138	-0.23 [-0.52; 0.05]	0.109
	ß [CI]-model 2	-0.22 [-0.43; -0.02]	0.035	-0.27 [-0.56; 0.02]	0.071	-0.20 [-0.49; 0.09]	0.174
	ß [CI]-model 3	-0.23 [-0.44; -0.03]	0.028	-0.20 [-0.55; 0.15]	0.260	-0.35 [-0.69; -0.02]	0.039
Oleic acid (C18:1 n-9)	Mean (mg/cL)	12.43 vs. 13.30		12.01 vs. 12.87		12.90 vs. 13.72	
	ß [CI]-model 1	-0.82 [-1.95; 0.31]	0.156	-0.76 [-2.35; 0.82]	0.341	-0.88 [-2.55; 0.78]	0.295
	ß [CI]-model 2	-0.82 [-1.97; 0.32]	0.158	-0.96 [-2.56; 0.63]	0.234	-0.79 [-2.43; 0.85]	0.341
	ß [CI]-model 3	-0.78 [-1.93; 0.38]	0.186	-0.90 [-2.82; 1.02]	0.355	-0.85 [-2.78; 1.08]	0.384
Linoleic acid (C18:2 n-6)	Mean (mg/cL)	11.94 vs. 12.20		11.49 vs. 11.41		12.44 vs. 12.97	
	ß [CI]-model 1	-0.27 [-1.05; 0.51]	0.500	-0.02 [-1.11; 1.08]	0.978	-0.53 [-1.59; 0.53]	0.325
	ß [CI]-model 2	-0.23 [-1.02; 0.56]	0.567	-0.09 [-1.11; 0.94]	0.867	-0.37 [-1.45; 0.72]	0.505
	ß [CI]-model 3	-0.54 [-1.38; 0.31]	0.210	0.20 [-0.95; 1.35]	0.729	-0.99 [-2.31; 0.32]	0.137
Gamma-linolenic acid (C18:3 n-6)	Mean (mg/cL)	0.20 vs. 0.23		0.20 vs. 0.23		0.20 vs. 0.24	
	ß [CI]-model 1	-0.03 [-0.06; <-0.01]	0.026	-0.03 [-0.07; 0.01]	0.147	-0.04 [-0.08; 0.01]	0.096
	ß [CI]-model 2	-0.03 [-0.06; <-0.01]	0.029	-0.03 [-0.06; 0.01]	0.184	-0.03 [-0.08; 0.01]	0.115
	ß [CI]-model 3	-0.03 [-0.06; <-0.01]	0.035	-0.03 [-0.07; 0.02]	0.269	-0.05 [-0.10; <-0.01]	0.033
	Mean (mg/cL)	0.14 vs. 0.13		0.13 vs. 0.13		0.16 vs. 0.15	

α-linoleic acid (C18:3 n-3)	ß [CI]-model 1	0.01 [-0.02; 0.03]	0.531	<0.01 [-0.03; 0.04]	0.864	0.01 [-0.03; 0.05]	0.522
	ß [CI]-model 2	0.01 [-0.01; 0.03]	0.484	<-0.01 [-0.03; 0.03]	0.803	0.02 [-0.02; 0.06]	0.310
	ß [CI]-model 3	0.01 [-0.02; 0.03]	0.654	-0.01 [-0.05; 0.02]	0.401	0.03 [-0.02; 0.06]	0.210
C20:1 n-9	Mean (mg/cL)	0.15 vs. 0.19		0.21 vs. 0.31		0.07 vs. 0.07	
	ß [CI]-model 1	-0.05 [-0.19; 0.10]	0.525	-0.12 [-0.41; 0.16]	0.391	<0.01 [-0.01; 0.02]	0.786
	ß [CI]-model 2	-0.03 [-0.15; 0.10]	0.681	-0.10 [-0.35; 0.15]	0.431	<0.01 [-0.01; 0.02]	0.823
	ß [CI]-model 3	0.02 [-0.11; 0.15]	0.797	-0.05 [-0.36; 0.27]	0.772	<0.01 [-0.02; 0.02]	0.865
C20:2 n-6	Mean (mg/cL)	0.07 vs. 0.08		0.07 vs. 0.08		0.07 vs. 0.08	
	ß [CI]-model 1	-0.01 [-0.01; <0.01]	0.148	<-0.01 [-0.02; 0.01]	0.407	-0.01 [-0.02; <0.01]	0.180
	ß [CI]-model 2	-0.01 [-0.02; <0.01]	0.110	-0.01 [-0.02; 0.01]	0.339	-0.01 [-0.02; <0.01]	0.163
	ß [CI]-model 3	-0.01 [-0.02; <0.01]	0.133	-0.01 [-0.02; 0.01]	0.448	-0.01 [-0.03; <0.01]	0.082
C20:3 n-6	Mean (mg/cL)	0.69 vs. 0.69		0.69 vs. 0.68		0.69 vs. 0.70	
	ß [CI]-model 1	-0.01 [-0.06; 0.05]	0.862	<0.01 [-0.09; 0.09]	0.996	-0.01 [-0.09; 0.07]	0.794
	ß [CI]-model 2	-0.01 [-0.07; 0.05]	0.680	-0.02 [-0.11; 0.08]	0.699	-0.02 [-0.10; 0.07]	0.721
	ß [CI]-model 3	-0.02 [-0.08; 0.05]	0.619	-0.03 [-0.13; 0.07]	0.541	-0.06 [-0.16; 0.04]	0.241
Arachidonic acid (C20:4 n-6)	Mean (mg/cL)	3.09 vs. 3.43		3.13 vs. 3.37		3.04 vs. 3.49	
	ß [CI]-model 1	-0.35 [-0.61; -0.09]	0.009	-0.25 [-0.59; 0.08]	0.139	-0.45 [-0.86; -0.04]	0.032
	ß [CI]-model 2	-0.32 [-0.58; -0.06]	0.017	-0.24 [-0.60; 0.11]	0.173	-0.38 [-0.80; 0.03]	0.069
	ß [CI]-model 3	-0.32 [-0.61; -0.03]	0.029	-0.09 [-0.48; 0.30]	0.653	-0.66 [-1.12; -0.20]	0.005
C20:5 n-3	Mean (mg/cL)	0.34 vs. 0.30		0.34 vs. 0.29		0.35 vs. 0.31	
	ß [CI]-model 1	0.05 [-0.01; 0.10]	0.108	0.04 [-0.04; 0.12]	0.309	0.05 [-0.04; 0.14]	0.263
	ß [CI]-model 2	0.04 [-0.01; 0.10]	0.102	0.03 [-0.04; 0.10]	0.441	0.05 [-0.04; 0.15]	0.279
	ß [CI]-model 3	0.05 [<0.01; 0.10]	0.036	0.04 [-0.04; 0.11]	0.326	0.05 [-0.06; 0.17]	0.337
C22:4 n-6	Mean (mg/cL)	0.07 vs. 0.08		0.07 vs. 0.08		0.07 vs. 0.08	
	ß [CI]-model 1	-0.02 [-0.02; -0.01]	0.001	-0.01 [-0.03; <-0.01]	0.020	-0.02 [-0.03; <-0.01]	0.016
	ß [CI]-model 2	-0.02 [-0.03; -0.01]	<0.001	-0.02 [-0.03; -0.01]	0.005	-0.02 [-0.03; <-0.01]	0.020
	ß [CI]-model 3	-0.02 [-0.03; -0.01]	<0.001	-0.02 [-0.03;<-0.01]	0.018	-0.02 [-0.04; <-0.01]	0.021
C24:1 n-9	Mean (mg/cL)	0.04 vs. 0.03		0.04 vs. 0.04		0.03 vs. 0.03	
	ß [CI]-model 1	<0.01 [<-0.01; 0.01]	0.563	<0.01 [-0.01; 0.01]	0.767	<0.01 [-0.01; 0.01]	0.712
	ß [CI]-model 2	<0.01 [<-0.01; 0.01]	0.483	<0.01 [-0.01; 0.01]	0.862	<0.01 [-0.01; 0.01]	0.559
	ß [CI]-model 3	<0.01 [<-0.01; 0.01]	0.183	<0.01 [-0.01; 0.01]	0.769	0.01 [<-0.01; 0.01]	0.210
C22:5 n-6	Mean (mg/cL)	0.05 vs. 0.06		0.05 vs. 0.06		0.05 vs.0.06	
	ß [CI]-model 1	-0.01 [-0.02; <-0.01]	0.002	-0.01 [-0.02; <-0.01]	0.018	-0.01 [-0.02; <-0.01]	0.046
	ß [CI]-model 2	-0.01 [-0.02; <-0.01]	0.004	-0.01 [-0.02; <-0.01]	0.022	-0.01 [-0.02; <0.01]	0.085
	ß [CI]-model 3	-0.01 [-0.02; <-0.01]	0.010	-0.01 [-0.02; <0.01]	0.099	-0.01 [-0.02; <0.01]	0.066
	Mean (mg/cL)	0.14 vs 0.15		0.14 vs. 0.15		0.14 vs. 0.15	
	ß [CI]-model 1	-0.01 [-0.02; 0.01]	0.164	-0.01 [-0.03; 0.01]	0.318	-0.01 [-0.03; 0.01]	0.335

C22:5 n-3	ß [CI]-model 2	-0.01 [-0.03; <0.01]	0.106	-0.02 [-0.03; <0.01]	0.105	-0.01 [-0.03; 0.01]	0.392
	ß [CI]-model 3	-0.01 [-0.03; <0.01]	0.115	-0.02 [-0.04; <0.01]	0.111	-0.01 [-0.04; 0.01]	0.304
C22:6 n-3	Mean (mg/cL)	1.02 vs. 0.97		0.99 vs. 0.95		1.06 vs. 0.99	
	ß [CI]-model 1	0.05 [-0.04; 0.14]	0.282	0.04 [-0.08; 0.16]	0.499	0.07 [-0.08; 0.21]	0.388
	ß [CI]-model 2	0.04 [-0.05; 0.14]	0.360	0.02 [-0.09; 0.14]	0.717	0.06 [-0.10; 0.23]	0.455
	ß [CI]-model 3	0.04 [-0.05; 0.14]	0.361	0.04 [-0.09; 0.16]	0.552	0.04 [-0.16; 0.24]	0.682

a) F&V, fruit and vegetable.

b) ß, difference between groups; CI, confidence interval.

c) Model 1—adjusted for age and sex. Model 2—adjusted for age, sex, physical activity, and BMI. Model 3—adjusted for age, sex, physical activity, BMI, total energy intake and alcohol consumption. P-values < 0.05 were considered significant.

Supplementary Material of Publication 5

Table S1. Plasma fatty acid levels at baseline and 1-year changes according to MetS status

		Mean ± SD	*p*-value[1]	Mean ± SD	*p*-value[1]	*p*-value[2]
C14:0	Baseline	0.57 ± 0.18		0.77 ± 0.28		
	Change	-0.01 ± 0.19	0.748	-0.02 ± 0.247	0.469	<0.001
C16:0	Baseline	21.19 ± 1.54		23.01 ± 2.26		
	Change	0.04 ± 1.38	0.842	-0.08 ± 2.00	0.705	<0.001
C16:1 n-7	Baseline	1.21 ± 0.41		1.49 ± 0.61		
	Change	-0.05 ± 0.34	0.284	-0.06 ± 0.35	0.073	0.004
C18:0	Baseline	6.54 ± 0.61		6.38 ± 0.61		
	Change	0.08 ± -0.48	0.237	0.03 ± 0.56	0.552	0.150
C18:1 n-9	Baseline	26.47 ± 5.05		28.40 ± 4.35		
	Change	0.07 ± 4.03	0.904	-0.29 ± 3.83	0.442	0.019
C18:2 n-6	Baseline	31.01 ± 5.00		28.13 ± 5.45		
	Change	0.17 ± 4.12	0.773	-0.09 ± 4.22	0.823	0.003
C18:3 n-6	Baseline	0.38 ± 0.17		0.42 ± 0.18		
	Change	0.02 ± 0.11	0.234	0.01 ± 0.12	0.429	0.263
C18:3 n-3	Baseline	0.29 ± 0.12		0.36 ± 0.20		
	Change	0.07 ± 0.16	0.008	<0.01 ± 0.25	0.869	0.031
C20:3 n-6	Baseline	1.40 ± 0.31		1.49 ± 0.36		
	Change	0.01 ± 0.23	0.800	0.01 ± 0.27	0.761	0.174
C20:4 n-6	Baseline	7.06 ± 1.35		6.21 ± 1.66		
	Change	-0.31 ± 0.97	0.033	0.21 ± 1.07	0.051	0.003
C20:5 n-3	Baseline	0.94 ± 0.70		0.75 ± 0.46		
	Change	0.01 ± 0.52	0.883	0.12 ± 0.54	0.030	0.058
C22:6 n-3	Baseline	2.75 ± 0.84		2.39 ± 0.68		
	Change	-0.11 ± 0.65	0.266	0.16 ± 0.66	0.016	0.006

MetS, Metabolic Syndrome.

Values are expressed as geometric mean (% of total fatty acids) ± SD.

p-value[1] for within-group differences from baseline by paired T-test.

p-value[2] for between-group differences from baseline by paired T-test.

Supplementary Material of Publication 6

Table S1. Mean and SD of plasma concentrations of fatty acids and circulating inflammatory molecules in the total population.

	Baseline	1 year	*p*-value
Plasma fatty acids, %			
n-3 PUFA	3.62 (1.20)	3.75 (1.26)	0.204
n-6 PUFA	38 (5.22)	37.64 (5.02)	0.375
MUFA	28.72 (4.57)	28.70 (4.11)	0.942
SFA	29.60 (2.22)	29.91 (2.53)	0.138
EPA	0.81 (0.58)	0.86 (0.62)	0.240
DHA	2.46 (0.75)	2.51 (0.73)	0.423
Palmitic acid	22.27 (2.11)	22.52 (2.35)	0.726
Inflammatory biomarkers			
sICAM-1, ng/mL	375.96 (156.86)	369.57 (128.29)	0.726
sVCAM-1, ng/mL	875.71 (305.47)	739.17 (307.00)	0.003
sE-Sel, ng/cL	28.21 (12.42)	26.31 (12.16)	0.123
sP-Sel, ng/cL	78.19 (70.71)	70.54 (58.15)	0.183
CRP, µg/mL	4.34 (1.98)	2.77 (2.10)	<0.001
IL-6, pg/mL	0.81 (1.99)	0.58 (0.34)	0.001

a) PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; sE-Esel, soluble E-selectin; sP-Sel, soluble P-selectin; CRP, C-reactive protein; IL6, interleukine-6.

b) Differences were calculated by T-tests.

Appendix

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Supplementary Material of Publication 7

Table S1. Nutrient and food consumption of all participants and according to tertiles of serum vitamin B12.

Nutrients	All *n* = 136	T1 *n* = 46	T2 *n* = 45	T3 *n* = 45	*p*-value
Carbohydrates (g/day)	260.9 ± 77.3	277.9 ± 85.9	245.5 ± 77.0	259.1 ± 65.5	0.131
Fiber (g/day)	29.1 ± 7.9	30.1 ± 8.5	28.2 ± 7.3	28.8 ± 7.7	0.534
Protein (g/day)	101.2 ± 21.3	102.1 ± 23.1	97.9 ± 21.0	103.6 ± 7.7	0.435
Total fat (g/day)	100.1 ± 27.5	104.5 ± 28.4	96.1 ± 30.3	99.7 ± 23.1	0.348
Cholesterol (mg/day)	387.0 ± 111.1	391.3 ± 113.4	382.0 ± 127.7	287.7 ± 91.3	0.923
Alcohol (g/day)	10.7 ± 14.8	10.4 ± 14.2	10.2 ± 15.9	11.5 ± 14.4	0.915
Vitamin B12 (µg/day)	10.4 ± 4.1	10.2 ± 4.3[a,b]	9.0 ± 3.5[a]	12.0 ± 4.1[b]	0.002
Foods (g/day)					
Vegetables	390.2 ± 158.2	420.9 ± 179.0	368.0 ± 140.0	380.9 ± 151.3	0.252
Fruits	513.2 ± 247.1	551.6 ± 252.4	503.7 ± 248.7	483.4 ± 240.3	0.404
Legumes	19.8 ± 9.0	20.1 ± 8.8	19.2 ± 6.7	19.9 ± 11.0	0.877
Cereals	161.2 ± 91.7	176.3 ± 105.8	147.7 ± 79.2	159.2 ± 87.6	0.329
Dairy	346.5 ± 202.6	353.2 ± 204.5	357.7 ± 211.1	328.6 ± 195.1	0.767
Meat	151.3 ± 55.4	148.9 ± 57.5	144.1 ± 55.4	160.9 ± 52.9	0.338
Fish	110.4 ± 43.5	110.2 ± 40.8	104.1 ± 46.9	117.1 ± 42.7	0.369
Nuts	14.1 ± 6.4	15.2 ± 16.3	12.9 ± 13.9	14.1 ± 16.2	0.784
Olive oil	35.0 ± 13.9	37.1 ± 14.6	34.3 ± 13.6	33.5 ± 13.7	0.434
Pastries	17.2 ± 24.2	16.4 ± 19.2	18.0 ± 29.5	17.3 ± 23.4	0.956

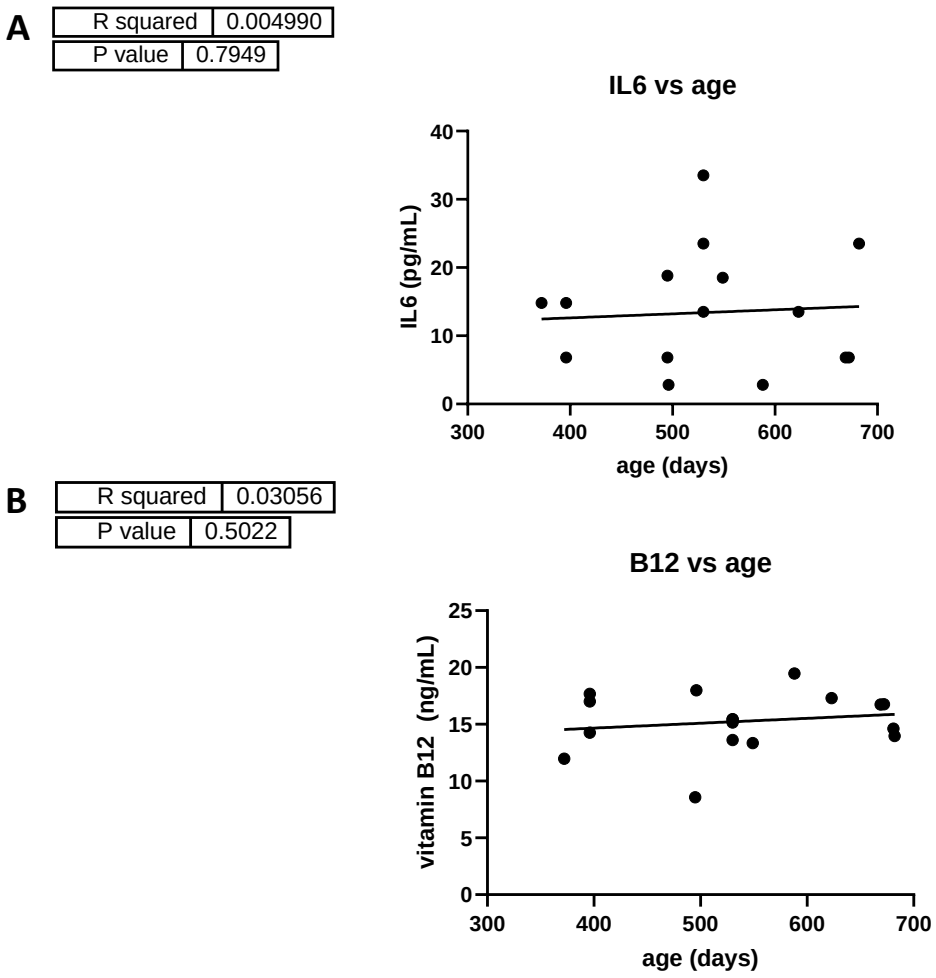


Figure S1. Association of age with vitamin B12 (A) and IL-6 (B) in mice. IL-6, interleukin-6.

Supplementary Material of Publication 8

Table S1. Inflammatory biomarkers categorized by tertiles of baseline urinary tartaric acid.

	T1 (*n* = 74)	T2 (*n* = 74)	T3 (*n* = 74)	*p*-value
sVCAM-1 (ng/mL)	172.06 ± 43.59	198.85 ± 54.08	177.65 ± 45.73	0.077
sICAM-1 (ng/mL)	146.14 ± 53.11	199.46 ± 87.67	152.30 ± 57.41	0.002
IL-6 (pg/mL)	5.60 ± 3.98	6.53 ± 3.97	5.65 ± 3.68	0.406
TNF-a (pg/mL)	9.66 ± 9.62	13.42 ± 12.28	12.25 ± 10.13	0.256
MCP1 (pg/mL)	35.70 ± 15.72	42.38 ± 30.20	36.28 ± 19.28	0.231

Data are given as mean ± standard deviation (SD); statistical analyses were undertaken using 1-factor ANOVA, $p < 0.05$ indicates statistical significance.
T, tertiles, sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; IL6, interleukine-6; TNF-α, tumor necrosis factor- α; MCP-1, monocyte chemoattractant protein-1.

Table S2. Changes in inflammatory biomarkers categorized by tertiles of 1-year changes in urinary tartaric acid.

	T1 (*n* = 69)	T2 (*n* = 69)	T3 (*n* = 68)
sVCAM-1 (ng/mL)	-7.61 ± 56.98	-4.11 ± 28.46	-3.34 ± 34.62
sICAM-1 (ng/mL)	0.08 ± 31.45	-1.89 ± 25.75	-2.38 ± 37.32
IL-6 (pg/mL)	-0.77 ± 2.98	-0.82 ± 4.18	-0.67 ± 4.54
TNF-a (pg/mL)	-2.06 ± 8.66	-1.81 ± 11.12	0.09 ± 16.22
MCP-1 (pg/mL)	-6.55 ± 18.32	-6.49 ± 19.27	-0.28 ± 16.26

Data are given as mean ± standard deviation (SD).
T, tertiles, sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; IL6, interleukine-6; TNF-α, tumor necrosis factor- α; MCP-1, monocyte chemoattractant protein-1.

Supplementary Material of Publication 10

Table A1. Marker compounds identified in the OPLS-DA.

Retention Time	Tentative Identification	Exact Mass [M-H]	VIP	MS1 isotopic spectrum *m/z* (intensity)	ID level	Fragmentation
3.333	Hydroxyphenylacetic acid	151.0401	2.76136	151 (6917952) 152 (584483 153 (26790)	MS^2, isotopic ratio, accurate mass	107/93/132
3.162	Chlorogenic acid	353.0878	2.39544	353 (404209) 354 (74465) 355 (23219)	MS^2, isotopic ratio, accurate mass	191/316
2.91	Chlorogenic acid glucuronide	401.1606	2.06082	529 (172168) 530 (46985) 531 (6079)	Isotopic ratio, accurate mass	
4.879	Epicatechin diglucuronide	641.1359	2.03178	641 (34291 642 (16296) 643 (2695)	Isotopic ratio, accurate mass	
6.967	Gallic acid diglucuronide	521.0784	2.01575	521 (210396) 522 (68018) 523 (16898)	Isotopic ratio, accurate mass	
2.89	Dihydrocaffeic acid	181.0506	2.01233	181 (612509) 182 (62292) 183 (118859)	MS^2, isotopic ratio, accurate mass	137/122
5.488	Caffeic acid diglucuronide	531.0992	1.89014	531 (20264) 532 (5566) 533 (0)	Isotopic ratio, accurate mass	
2.765	Coumaric acid glucuronide	339.0722	1.87224	339 (372120) 340 (60125) 341 (203558)	Isotopic ratio, accurate mass	
2.984	Ferulic acid sulfate	273.0074	1.85452	273 (4333605) 274 (518722) 275 (1264532)	MS^2, isotopic ratio, accurate mass	193/229
6.659	Hydroxybenzoic acid diglucuronide	489.0886	1.78092	489 (154037) 490 (43874) 491 (7765)	Isotopic ratio, accurate mass	
5.529	Enterolactone glucuronide	473.1453	1.69943	473 (8602714) 474 (2694708) 475 (456734)	MS^2, Isotopic ratio, accurate mass	297/175
0.856	Protocatechuic acid glucuronide	329.0514	1.62486	329 (314188) 330 (68290) 331 (10467)	Isotopic ratio, accurate mass	
5.7	Hesperetin sulfate	381.0286	1.53965	381 (2196506) 382 (412041) 383 (121080)	MS^2, isotopic ratio, accurate mass	301
1.763	Hippuric acid glucuronide	354.0831	1.52866	354 (1549020) 355 (279158) 356 (35365)	Isotopic ratio, accurate mass	
1.255	Protocatechuic acid	153.0193	1.50853	153 (606883) 154 (43983) 155 (4782)	Standard	
1.301	Dihydroresveratrol disulfate	389.0006	1.48895	388 (34413) 389 (12932) 391 (0)	Isotopic ratio, accurate mass	
6.508	Equol glucuronide	417.1191	1.47749	417 (360086) 418 (84760) 419 (13486)	Isotopic ratio, accurate mass	
1.43	Chlorogenic acid sulfate	417.0497	1.46752	433 (24256) 434 (4301) 435 (1340)	Isotopic ratio, accurate mass	
0.875	Naringenin diglucuronide	623.1254	1.4613	623 (114944) 624 (58440) 625 (18289)	MS^2, isotopic ratio, accurate mass	-
5.587	Hesperetin glucuronide	477.1038	1.39966	477 (5450046) 478 (1570311) 479 (278363)	MS^2, isotopic ratio, accurate mass	301/459
6.374	Urolithin c	243.0299	1.37759	243 (117137) 244 (15832) 245 (0)	MS^2, isotopic ratio, accurate mass	-
2.18	Caffeic acid sulfate	256.9761	1.33992	258 (458478) 259 (42322) 260 (115839)	Isotopic ratio, accurate mass	
4.701	Hesperetin diglucuronide	653.1359	1.29545	653 (2167040) 654 (1011129) 655 (279961)	Isotopic ratio, accurate mass	
2.15	Benzoic acid glucuronide	297.0616	1.28875	297 (476543) 298 (60814) 299 (5823)	Isotopic ratio, accurate mass	

1.904	Hydroxyphenylpropionic acid sulfate	245.0125	1.23838	245 (7377760) 246 (750487) 247 (348916)	MS^2, isotopic ratio, accurate mass	165/121
0.791	Hydroxyphenylacetic disulfate	310.9537	1.20412	310 (17318) 311 (2985) 312 (8521)	Isotopic ratio, accurate mass	
3.519	Epicatechin glucuronide	465.1038	1.18906	465 (606839) 466 (185407) 467 (32721)	MS^2, isotopic ratio, accurate mass	289/327
7.68	Benzoic acid diglucuronide	473.0937	1.15755	473 (52014) 474 (18981) 475 (3259)	Isotopic ratio, accurate mass	
3.779	Daidzein sulfate	333.0074	1.14893	333 (641601) 334 (98026) 335 (36736)	Isotopic ratio, accurate mass	
1.142	Vanillic acid sulfate	246.9918	1.10915	246 (4877512) 247 (437393) 248 (268946)	MS^2, Isotopic ratio, accurate mass	203/123/167
5.828	Daidzein	253.0506	1.08223	253 (790928) 254 (126690) 255 (7720)	Isotopic ratio, accurate mass	
3.885	Genistein	269.0455	1.08015	269 (138935) 270 (24106) 271 (4377)	Isotopic ratio, accurate mass	
2.458	Hydroxyphenylpropionic acid glucuronide	341.0878	1.07598	341 (2958146) 342 (490589) 343 (58779)	MS^2, isotopic ratio, accurate mass	113/165/175/323
6.009	Hesperetin	301.0718	1.0752	301 (361025) 302 (64834) 303 (4142)	MS^2, isotopic ratio, accurate mass	283
1.097	Vanillic acid disulfate	326.9486	1.07222	326 (19481) 327 (0) 328 (2639)	Isotopic ratio, accurate mass	
3.864	Genistein diglucuronide	621.1097	1.05636	621 (1231562) 622 (509789) 623 (106345)	MS^2, isotopic ratio, accurate mass	445
4.374	Urolithin A glucuronide	403.0671	1.03142	403 (16104702) 404 (3539286) 405 (551757)	MS^2, isotopic ratio, accurate mass	227/341/385
3.874	Naringenin disulfate	430.9748	1.02372	430 (35091) 431 (5758) 432 (40505)	Isotopic ratio, accurate mass	
5.71	Equol sulfate	320.036	1.00884	321 (454955) 322 (39855) 323 (65314)	Isotopic ratio, accurate mass	
4.142	Daidzein glucuronide	429.0827	1.00835	429 (5123791) 430 (1500309) 431 (220632)	MS^2, isotopic ratio, accurate mass	253/385
4.368	Urolithin B diglucuronide	563.1042	1.00621	563 (16766) 564 (6185) 565 (0)	Isotopic ratio, accurate mass	
1.516	Oleacein sulfate	399.0755	0.982853	399 (16961) 400 (2635) 401 (538)		
4.637	Naringenin	271.0612	0.980981	271 (413015) 272 (65433) 273 (13735)		
0.937	Hydroxytyrosol glucuronide sulfate	409.0446	0.967151	409 (41671) 410 (208410) 411 (27236)		
3.296	Hydroxyphenylpropionic acid	165.0557	0.956291	165 (4090766) 166 (404230) 167 (19279)		
1.792	Hydroxyphenylpropionic acid disulfate	324.9693	0.933361	324 (33395) 325 (0) 326 (2423)		
1.4	Hydroxybenzoic acid sulfate	216.9812	0.887876	216 (1082870) 217 (72910) 218 (2503285)		
1.527	Vanillic acid glucuronide	343.0671	0.883415	343 (3479812) 344 (649208) 345 (95924)		
2.914	Oleocanthal diglucuronide	655.188	0.845394	655 (72807) 656 (37288) 657 (11887)		
3.988	Catechol diglucuronide	461.0937	0.840377	461 (38631) 462 (8846) 463 (2197)		
1.336	Hippuric acid sulfate	258.0078	0.813007	258 (770822) 259 (62849) 260 (40704)		
3.652	Urolithin c diglucuronide	595.0941	0.790573	595 (55887) 596 (26772) 597 (7938)		

1.665	Noradrenalina	168.0666	0.783844	168 (132888) 169 (11433) 170 (2129)
6.746	Dihydroresveratrol glucuronide	405.1191	0.772716	405 (35730864) 406 (8869813) 407 (1337700)
3.5	Caffeic acid	179.035	0.751008	179 (262442) 180 (26226) 181 (21457)
0.947	Hippuric acid diglucuronide	530.1151	0.733755	530 (24342) 531 (5943) 532 (1103)
4.744	Enterodiol glucuronide	477.1766	0.729934	477 (3331297) 478 (1116390) 479 (175938)
5.291	Naringenin glucuronide	447.0933	0.727083	447 (5587213) 448 (1418777) 449 (1442592)
3.259	Daidzein diglucuronide	605.1148	0.724998	605 (294730) 606 (146625) 607 (31416)
6.712	Vanillic acid diglucuronide	519.0992	0.713891	519 (88263) 520 (24440) 521 (10971)
3.946	Urolithin d diglucuronide	611.089	0.697244	611 (37478) 612 (12780) 613 (3088)
3.503	Ferulic acid glucuronide	369.0827	0.681933	369 (11524215) 370 (2250158) 371 (750362)
1.118	Protocatechuic acid sulfate	232.9761	0.674623	232 (604720) 233 (36761) 234 (37709)
1.255	Hydroxytyrosol glucuronide	329.0878	0.668752	329 (1100428) 330 (223623) 331 (30568)
1.307	Hydroxyphenylacetic glucuronide	327.0705	0.648311	327 (701503) 328 (132864) 329 (16817)
2.71	Syringic acid glucuronide	373.0776	0.634087	373 (350442) 374 (59852) 375 (8255)
1.396	Benzoic acid	121.0295	0.633353	121 (11412496) 122 (753689) 123 (60133)
7.268	Enterodiol	301.1445	0.621114	301 (46083) 302 (7412) 303 (0
7.094	Urolithin B	211.0401	0.556842	211 (2078438) 212 (348793) 213 (11778)
3.971	Equol diglucuronide	593.1512	0.555531	593 (115951) 594 (54179) 595 (11893)
3.447	Enterolactone diglucuronide	649.1774	0.544151	649 (163773) 650 (75056) 651 (21921)
2.654	Syringic acid sulfate	277.0024	0.539111	277 (168127) 278 (11679) 279 (9697)
1.359	Hydroxybenzoic acid glucuronide	313.0565	0.536635	313 (6277628) 314 (939152) 315 (121747)
1.496	Caffeic acid glucuronide	355.0671	0.492937	355 (113660) 356 (29333) 357 (118416)
3.747	Dihydroresveratrol diglucuronide	581.1512	0.48588	581 (17044) 582 (6955) 583 (887)
0.673	Urolithin A sulfate	306.9918	0.485108	306 (77881) 307 (8118) 308 (66930)
5.467	Genistein glucuronide	445.0776	0.47837	445 (4925684) 446 (1358308) 447 (308113)
4.735	Oleocanthal glucuronide	479.1559	0.474214	479 (516328) 480 (142366) 481 (26387)
2.715	Ferulic acid diglucuronide	545.1148	0.454398	545 (10567) 546 (3147) 547 (0)

Supplementary Material of Publication 11

Table A.1. Daily intake of foods for the total population and according to tertiles of MPM.

	All (*n* = 200)	**T1 (*n* = 67)**	**T2 (*n* = 67)**	**T3 (*n* = 66)**	***p*-value**
Olive oil (g)	40.0 ± 13.5	40.0 ± 13.2	41.7 ± 13.3	39.3 ± 14.1	0.446
Nuts (g)	9.1 ± 11.4	10.9 ± 13.6	9.5 ± 11.3	6.9 ± 8.7	0.122
Fruits (g)	479.2 ± 232.9	451.8 ± 237.4	452.3 ± 227.0	534.3 ± 227.9	0.063
Vegetables (g)	406.6 ± 175.9	426.3 ± 219.5	397.5 ± 156.7	395.7 ± 142.4	0.531
Legumes (g)	17.8 ± 7.4	18.1 ± 7.2	18.1 ± 6.7	17.2 ± 8.4	0.732
Fish (g)	117.5 ± 44.1	119.5 ± 40.0	124.1 ± 49.1	108.8 ± 42.0	0.122
Meat or meat products (g)	148.8 ± 53.7	149.3 ± 56.7	146.7 ± 47.0	150.3 ± 57.6	0.924
Pastries (g)	21.0 ± 25.9	23.7 ± 28.5	17.84 ± 25.4	21.6 ± 23.6	0.417
Dairy products (g)	338.4 ± 207.4	364.3 ± 179.5	306.9 ± 208.0	344.1 ± 231.0	0.268
Alcohol (g)	11.7 ± 17.2	8.6 ± 12.9[a]	15.9 ± 22.3[b]	10.5 ± 14.2[a,b]	0.038
Fiber (g)	27.4 ± 7.3	26.9 ± 7.6	27.2 ± 7.6	28.2 ± 6.7	0.612
Cholesterol (g)	407.0 ± 113.1	416.0 ± 117.9	412.3 ± 110.7	392.7 ± 110.9	0.445
Sodium (mg)	2523.9 ± 779.6	2529.1 ± 856.4	2621.7 ± 847.7	2419.3 ± 604.4	0.327
Folic acid (µg)	458.7 ± 103.6	457.5 ± 108.2	456.5 ± 105.0	462.2 ± 98.7	0.946
Total polyphenols (mg)	999.8 ± 316.5	971.9 ± 300.5	991.4 ± 332.6	1036.2 ± 316.7	0.491

MPM, microbial phenolic metabolites.

p-values were calculated using the test one-way ANOVA.

Different lower-case letters indicate a significant difference among groups and were calculated analysed using Bonferroni post-hoc test.

Table A.2. Multivariable adjusted regression between overall ICVH and individual metrics and MPM.

	β (95% CI)	***p*-value**
Protocatechuic acid		
Overall ICVH	0.10 (-0.06; 0.25)	0.214
Individual Metrics		
Smoking status	<0.01 (-0.06; 0.07)	0.875
Body mass index	0.01 (-0.02; 0.05)	0.379
Physical activity	-0.01 (-0.07; 0.05)	0.764
Diet	0.09 (-0.03; 0.16)	0.007
Blood pressure	<-0.01 (-0.03; 0.02)	0.803
Total cholesterol	0.01 (-0.06; 0.07)	0.863
Blood glucose	-0.01 (-0.08; 0.07)	0.842
Vanillic acid glucuronide		
Overall ICVH	-0.16 (-0.29; -0.02)	0.026
Individual Metrics		
Smoking status	-0.04 (-0.09; 0.01)	0.166
Body mass index	0.01 (-0.02; 0.04)	0.448
Physical activity	-0.04 (-0.10; 0.02)	0.227
Diet	-0.01 (-0.08; 0.06)	0.806
Blood pressure	-0.01 (-0.03; 0.01)	0.406
Total cholesterol	-0.01 (-0.08; 0.05)	0.717
Blood glucose	-0.07 (-0.13; <-0.01)	0.052
Enterodiol glucuronide		
Overall ICVH	0.03 (-0.11; 0.17)	0.689
Individual Metrics		
Smoking status	0.01 (-0.03; 0.06)	0.596
Body mass index	-0.01 (-0.04; 0.03)	0.713
Physical activity	-0.05 (-0.10; 0.01)	0.096
Diet	0.10 (0.03; 0.16)	0.003
Blood pressure	0.01 (-0.01; 0.04)	0.343
Total cholesterol	-0.02 (-0.09; 0.04)	0.504
Blood glucose	-0.02 (-0.09; 0.05)	0.623
Enterolactone glucuronide		
Overall ICVH	0.09 (-0.06; 0.24)	0.227
Individual Metrics		
Smoking status	-0.02 (-0.07; 0.02)	0.321
Body mass index	0.02 (-0.01; 0.05)	0.156
Physical activity	<0.01 (-0.06; 0.06)	0.913

Diet	0.11 (0.05; 0.18)	0.001
Blood pressure	0.02 (-0.01; 0.05)	0.148
Total cholesterol	-0.07 (-0.13; -0.01)	0.027
Blood glucose	0.03 (-0.04; 0.09)	0.452
Urolithin B glucuronide		
Overall ICVH	-0.05 (-0.8; 0.09)	0.483
Individual Metrics		
Smoking status	0.03 (-0.03; 0.08)	0.349
Body mass index	0.01 (-0.02; 0.04)	0.630
Physical activity	-0.04 (-0.09; 0.01)	0.120
Diet	-0.04 (-0.11; 0.03)	0.224
Blood pressure	<0.01 (-0.02; 0.02)	0.827
Total cholesterol	0.01 (-0.05; 0.07)	0.790
Blood glucose	-0.01 (-0.08; 0.06)	0.746

ICVH, ideal cardiovascular health; MPM, microbial phenolic metabolites.

ß, difference between groups; CI, confidence interval.

Adjusted for sex and age.

Supplementary Material of Publication 13

Table S1. Means and SD of urinary concentrations of metabolites of the 400 participants expressed as micromole per mg of creatinine.

Protocatechuic acid	0.015 ± 0.033
Vanillic acid glucuronide	0.643 ± 1.354
Vanillic acid sulfate	0.968 ± 0.002
3-Hydroxybenzoic acid	0.082 ± 0.197
Enterodiol glucuronide	0.006 ± 0.015
Enterolactone glucuronide	0.027 ± 0.043
Urolithin B glucuronide	0.097 ± 0.451

Table S2. Multivariable adjusted regression between microbial phenolic metabolites and Mediterranean diet adherence (p17-score).

	β (CI 95%) per 1-SD	*p*-value
Protocatechuic acid		
Model 1	0.14 (<-0.01; 0.28)	0.054
Model 2	0.14 (0.01; 0.27)	0.038
Vanillic acid glucuronide		
Model 1	<0.01 (-0.23; 0.23)	0.968
Model 2	-0.01 (-0.24; 0.21)	0.907
Vanillic acid sulfate		
Model 1	-0.04 (-0.15; 0.07)	0.483
Model 2	-0.01 (-0.14; 0.11)	0.829
3-Hydroxybenzoic acid		
Model 1	<0.01 (-0.12; 0.13)	0.938
Model 2	<0.01 (-0.11; 0.12)	0.932
Enterodiol glucuronide		
Model 1	0.04 (-0.05; 0.13)	0.398
Model 2	0.03 (-0.05; 0.12)	0.398
Enterolactone glucuronide		
Model 1	0.12 (0.01; 0.23)	0.041
Model 2	0.12 (0.02; 0.22)	0.022
Urolithin B glucuronide		
Model 1	-0.02 (-0.20; 0.15)	0.779
Model 2	-0.03 (-0.20; 0.15)	0.747

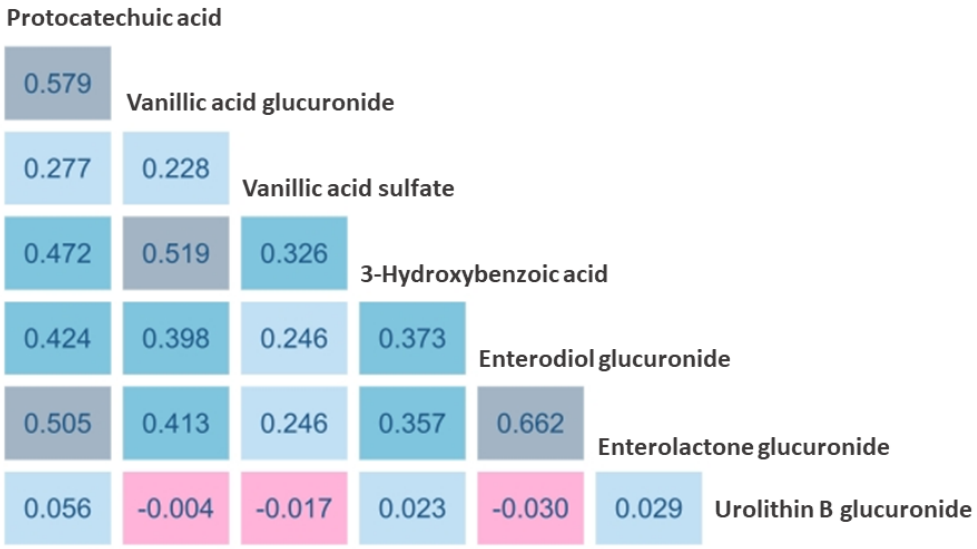
A natural logarithmic transformation was applied to the raw values of individual metabolites.
Model 1 was adjusted for age and sex.
Model 2 was further adjusted for smoking habit, educational level, obese/overweight, total energy intake, diabetes, hypertension, hypercholesterolemia and use of lowering-cholesterol drugs.
All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.

Table S3. Multivariable adjusted regression between microbial phenolic metabolites and cognitive tests.

	Semantic verbal fluency tasks		Phonological verbal fluency tasks		Digit Span Test forward		Digit Span Test backward		Trail Making Test Part A		Trail Making Test Part B	
	β (CI 95%) per 1-SD	*p*-value	β (CI 95%) per 1-SD	*p*-value	β (CI 95%) per 1-SD	*p*-value	β (CI 95%) per 1-SD	*p*-value	β (CI 95%) per 1-SD	*p*-value	β (CI 95%) per 1-SD	*p*-value
Protocatechuic acid												
Model 1	0.02 (-0.03; 0.07)	0.422	0.04 (-0.02; 0.11)	0.192	0.06 (<-0.01; 0.13)	0.055	0.01 (-0.06; 0.08)	0.705	-0.06 (-0.14; 0.01)	0.077	-0.03 (-0.08; 0.02)	0.275
Model 2	0.02 (-0.03; 0.07)	0.441	0.04 (-0.02; 0.09)	0.178	0.07 (0.02; 0.13)	0.014	0.03 (-0.03; 0.09)	0.292	-0.06 (-0.12; 0.01)	0.072	-0.02 (-0.06; 0.02)	0.257
Vanillic acid glucuronide												
Model 1	-0.02 (-0.09; 0.05)	0.640	0.06 (-0.03; 0.14)	0.111	0.09 (-0.01; 0.20)	0.070	0.08 (0.01; 0.15)	0.031	-0.07 (-0.18; 0.03)	0.152	-0.06 (-0.14; 0.02)	0.130
Model 2	<-0.01 (-0.07; 0.07)	0.916	0.07 (<0.01; 0.14)	0.046	0.10 (<0.01; 0.21)	0.044	0.09 (0.01; 0.17)	0.024	-0.08 (-0.20; 0.03)	0.144	-0.07 (-0.15; 0.02)	0.123
Vanillic acid sulfate												
Model 1	<-0.01 (-0.03; 0.03)	0.967	0.03 (<0.01; 0.06)	0.023	0.04 (<0.01; 0.07)	0.042	0.03 (-0.03; 0.08)	0.310	0.02 (-0.01; 0.05)	0.174	0.02 (-0.02; 0.06)	0.344
Model 2	-0.01 (-0.04; 0.02)	0.516	0.02 (<-0.01; 0.05)	0.071	0.03 (<-0.01; 0.07)	0.063	0.03 (-0.03; 0.08)	0.367	0.03 (<0.01; 0.06)	0.039	0.03 (-0.01; 0.07)	0.177
3-Hydroxybenzoic acid												
Model 1	<-0.01 (-0.05; 0.05)	0.845	0.03 (-0.02; 0.08)	0.282	0.06 (<0.01; 0.11)	0.035	0.03 (-0.04; 0.09)	0.303	-0.01 (-0.06; 0.04)	0.716	0.01 (-0.03; 0.05)	0.632
Model 2	0.01 (-0.04; 0.05)	0.770	0.04 (-0.02; 0.09)	0.175	0.06 (0.01; 0.11)	0.028	0.04 (-0.02; 0.10)	0.194	-0.02 (-0.07; 0.03)	0.473	<-0.01 (-0.05; 0.05)	0.977
Enterodiol glucuronide												
Model 1	<-0.01 (-0.04; 0.03)	0.805	0.02 (-0.01; 0.04)	0.207	0.03 (<-0.01; 0.06)	0.093	0.02 (<-0.01; 0.05)	0.078	-0.01 (-0.04; 0.02)	0.652	<-0.01 (-0.02; 0.01)	0.621
Model 2	<-0.01 (-0.04; 0.03)	0.791	0.02 (-0.01; 0.04)	0.238	0.03 (<-0.01; 0.05)	0.062	0.03 (<-0.01; 0.06)	0.063	<-0.01 (-0.03; 0.03)	0.886	<-0.01 (-0.02; 0.02)	0.854
Enterolactone glucuronide												
Model 1	0.02 (-0.04; 0.08)	0.423	0.04 (<0.01; 0.07)	0.037	0.05 (0.01; 0.08)	0.011	0.04 (0.01; 0.08)	0.021	-0.01 (-0.05; 0.04)	0.764	-0.03 (-0.06; 0.01)	0.091
Model 2	0.02 (-0.04; 0.07)	0.516	0.03 (<0.01: 0.06)	0.043	0.05 (0.02; 0.08)	0.003	0.05 (0.01; 0.09)	0.013	<0.01 (-0.03; 0.04)	0.959	-0.02 (-0.05; 0.01)	0.266
Urolithin B glucuronide												
Model 1	0.04 (<-0.01; 0.08)	0.051	0.04 (-0.02; 0.09)	0.153	-0.02 (-0.05; 0.02)	0.282	-0.01 (-0.03; 0.02)	0.585	0.02 (-0.03; 0.06)	0.395	-0.02 (-0.04; 0.01)	0.161
Model 2	0.04 (<-0.01; 0.08)	0.051	0.04 (-0.02; 0.09)	0.166	-0.01 (-0.05; 0.03)	0.603	<0.01 (-0.02; 0.03)	0.823	0.02 (-0.03; 0.06)	0.403	-0.01 (-0.03; 0.01)	0.389

A natural logarithmic transformation was applied to the raw values of individual metabolites.
Model 1 was adjusted for age and sex.
Model 2 was further adjusted for smoking habit, educational level, obese/overweight, total energy intake, diabetes, hypertension, hypercholesterolemia, and use of cholesterol-lowering and anticholinergic drugs.
All analyses were conducted with robust estimates of the variance to correct for intracluster correlation.

Figure S1. Correlation matrix for urinary metabolites. Spearman Correlations coefficients of microbial phenolic metabolites.



Supplementary Material of Publication 14

Supplementary Table S1. General characteristics of all the participants and of groups stratified by adherence to the MedDiet after 1-year of follow-up.

	All (167)	Low MedDiet adherence (*n*=96)	High MedDiet adherence (*n*=71)	*p*-value[a]
Age, years	70.7 ± 5.5	71.2 ± 5.6	69.9 ± 5.3	0.139
Physical activity, METS-min/day	314.4 ± 261.9	274.5 ± 230.3	368.4 ± 292.5	0.022
BMI, kg/m2	28.7 ± 3.5	29.2 ± 3.8	28.2 ± 3.0	0.077
Total energy intake, kcal/day	2022.8 ± 370.3	1936.7 ± 389.7	2140.9 ± 307.1	<0.001
Serum folate, ng/mL	7.9 ± 3.7	7.6 ± 3.7	8.3 ± 3.7	0.236
Serum vitamin B12, ng/mL	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.2	0.428
MedDiet adherence[b]	10 ± 2	9 ± 1	12 ± 1	<0.001

MedDiet, Mediterranean diet; METS, Metabolic Equivalents.
Continuous variables are shown as means ± SDs, and categorical variables are shown *n* (%).
[a]T-test or chi-square test as appropriate.
[b]Based on the 14-point MedDiet screener.

Supplementary Table S2. Multivariable linear regression between changes in Memory function and changes in serum folate concentration, stratified by MedDiet adherence group.

	Low MedDiet adherence (*n*=96)		High MedDiet adherence (*n*=71)	
	β (95% CI) per 1-SD	*p*-value	β (95% CI) per 1-SD	*p*-value
Memory Composite				
Model 1	-0.02 (-0.39; 0.35)	0.907	0.49 (-0.01; 0.99)	0.054
Model 2	0.08 (-0.29; 0.44)	0.668	0.48 (-0.07; 1.02)	0.085
RAVLT delayed episodic verbal memory				
Model 1	0.13 (-0.37; 0.63)	0.615	0.41 (-0.22; 1.04)	0.196
Model 2	0.13 (-0.46; 0.72)	0.667	0.25 (-0.44; 0.94)	0.469
RAVLT intermediate episodic verbal memory				
Model 1	0.05 (-0.44; 0.055)	0.826	0.23 (-0.53; 0.98)	0.550
Model 2	0.06 (-0.44; 0.56)	0.819	0.27 (-0.51; 1.05)	0.491
WMS episodic memory performance				
Model 1	-0.28 (-0.81; -0.26)	0.304	0.61 (-0.17; 1.38)	0.121
Model 2	0.01 (-0.50; 0.52)	0.971	0.47 (-0.33; 1.27)	0.242

Log-transformation was applied to raw values of serum folate.
Model 1 was adjusted for sex, age, MedDiet intervention group and baseline levels of folate, memory composite score or subtests, and MedDiet adherence.
Model 2 was further adjusted for smoking habit, educational level, APOE Ɛ4 genotype and changes in BMI, physical activity, hypertension, diabetes, hypercholesterolemia, use of anticholinergic and lowering-cholesterol drugs, and total energy intake.