UNIVERSITAT DE BARCELONA

Effects of Total Polyphenol Intakes on Cardiovascular Disease Risk Factors in an Elderly Population at High Cardiovascular Risk

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Abbreviations

ADA	American Diabetes Association
AGE	Advanced glycation end product
BMI	Body mass index
BP	Blood pressure
CHD	Coronary heart disease
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
FFQ	Food frequency questionnaire
FPG	Fasting plasma glucose
GAE	Gallic acid equivalent
HDL-C	High-density lipoprotein
HR	Heart rate
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
LDL-C	Low-density lipoprotein cholesterol
NO	Nitric oxide
PAD	Peripheral artery disease
PG	Two-hour plasma glucose
PREDIMED	Prevención con Dieta Mediterránea/ prevention with Mediterranean diet
ROS	Oxygen species
SBP	Systolic blood pressure
SPE	Solid phase extraction
T2D	type 2 diabetes
TG	Triglycerides
TPE	Urinary total polyphenol excretion
WC	Waist circumference
WHO	World health organization
WHtR	Waist to height ratio

Index

Chapter 1

Abstract

1 Abstract

There is a consensus that CVD has been the leading cause of death worldwide in recent decades, and it is predicted that will raise from 17.5 million in 2012 to 22.2 million in 2030 [1]. Besides, CVD is a heavy economic burden on the health care system at both global and national scales. For the primary prevention, prediction models based on established risk factors are useful tools in the prevention of CVD. In this study, the cardiovascular risk factors among the elderly population have been assessed, which used to set up associations between total polyphenol intakes from a Mediterranean diet and prevention of CVD.

The Mediterranean diet is a nutritional recommendation that has recently shown beneficial effects on human health[2]. Numerous studies have demonstrated there is a negative association between consumption of the Mediterranean diet and the prevalence of CVD. The evidence concerning the potential mechanisms of action which underlie the cardio-protective effects may be attributed to a high amount of dietary fiber, vitamins, folic acid, natural antioxidants, monounsaturated fat; moderate amounts of animal protein, moderate amount of alcohol mainly in the form of wine; and low amount of saturated and trans fats [3]. However, only limited studies have focused on the observed protection from the most abundant antioxidants in nature, polyphenol. Therefore, in this study, we hypothesized that a high dietary polyphenol intakes, recorded by urinary polyphenol excretion, could be associated with low CVD risk parameters, diabetes, and obesity in an elderly population with high cardiovascular risk.

Traditional methods of obtaining information on polyphenol intakes, such as from dietary recalls, FFQs, and databases on the polyphenol content of foods, are not accurate enough to reflect polyphenol concentration after metabolism. To solve this problem, we used excretion of urine as a reliable and effective biomarker to track polyphenol after digestion.

High glucose levels, TG concentration, DBP are classic cardiovascular risk factors for developing of CVD. In this thesis, we found significant inverse correlations between changes in TPE and plasma TG concentration, glucose concentration, and DBP after adjustment for potential confounders after a 5-year of intervention.

Overweight and obesity are also important risk factors for developing of CVD. Inverse correlations were observed between TPE at 5 years of follow-up and BW, BMI, WC and WHtR after adjustment for potential confounders, indicating higher polyphenol intakes improve body weight managements.

Prevalence of T2D is positively associated with incidence of CVD. We found a high intake of total polyphenols, calculated by FFQs and the Phenol-Explorer database, was associated with a reduced risk of diabetes in elderly people at high risk of CVD.

To conclude, we suggest that a high consumption of polyphenol-rich foods in the frame of a Mediterranean diet could potentially help to reduce multiple risk factors of CVD.

Chapter 2

Hypothesis and aims

2 Hypothesis and aims

2.1 Hypothesis

Epidemiological studies have linked polyphenol-rich food intake with reduced risk of cardiovascular diseases (CVDs). The Mediterranean diet is considered as a polyphenol-rich dietary pattern because it is characterized by a high consumption of legumes, fruit and vegetables, grains and olive oil, a moderate consumption of wine and dairy products and a low consumption of red and processed meat, cream and pastries. Urinary polyphenol excretion is a reliable biomarker of total polyphenol intake. Therefore, in this study, we hypothesized that a high dietary polyphenol intake, recorded by urinary polyphenol excretion, could be associated to low CVD risk in an elderly population with high cardiovascular risk.

2.2 Aims

The general objective of this study was to evaluate the cardiovascular protective role of dietary polyphenols, using total polyphenol excretion (TPE) in urine as a reliable biomarker in the PREDIMED population.

Specific aims:

To evaluate dietary polyphenol intake expressed by TPE in urine in an elderly Spanish population at high cardiovascular risk after a 5-year intervention with the Mediterranean diet (supplemented with nuts or extra virgin olive oil) or a low-fat diet.

To review the available epidemiological evidence on long-term polyphenol-rich diet consumption and classical cardiovascular risk factor, including blood pressure, glucose, triglycerides, total cholesterol, HDL, LDL and heart rate.

To evaluate the anti-obesity effects of polyphenol intake, studying the association between total polyphenol intake, expressed by TPE in urine, and anthropometric indexes, including body weight, body mass index, waist circumference, and waist to height ratio.

To compare the prevalence of obesity among various levels of total polyphenol intake after 5 years of intervention.

To analyze the inverse association between total polyphenols and some classes intake and diabetes among elderly people at high cardiovascular risk.

Chapter 3

Introduction

3.1 Polyphenols and cardiovascular disease (CVD) risk factors

3.1.1 Overview

Polyphenols in the nature

Polyphenols are the most abundant antioxidants in daily diet, widely spread around plant-derived food such as fruits, vegetables, cereals, legumes, and beverages like coffee, tea and wine [1]. Polyphenols represent a group of chemical substances common in plants, structurally characterized by the presence of one or more phenol units [4]. These compounds may be classified into five main groups depending on the number of phenolic rings that they contain and the structural elements that bind these rings to one another: phenolic acids, flavonoids (flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols), stilbenes, lignans and others [5] (Figure 1). Of them, phenolic acids account for about one third while flavonoids account for two thirds of human intake [6].

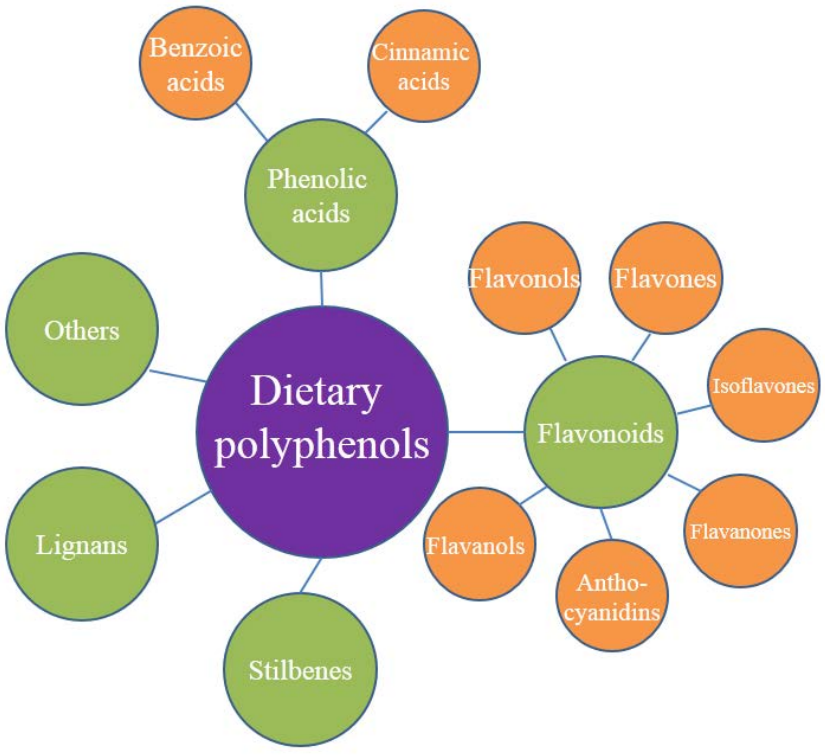


Fig.1 Classification of dietary polyphenols

- Phenolic acids are aromatic secondary plant metabolites, widely spread throughout the plant kingdom, which can be further divided into two main types, benzoic acids and cinnamic acids [7]. The benzoic acids content of edible plants is generally very low, while cinnamic acids are more common. Common examples of cinnamic acids are p-coumaric, caffeic, ferulic, and sinapic acids [5]. Phenolic acids are mainly provided by coffee, red fruits, cereal grains.
- Flavonoids represent a large class of more than 6,000 phenolic compounds in plant-derived foods and beverages. All flavonoids share constituted by two benzene rings linked through a heterocyclic pyran ring, and can be divided into several sub-families according to the degree of oxidation of the oxygen heterocycle and the substitution patterns, such as flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols [8]. Among them, flavanols is the highest intakes and mainly provided by green tea [9].
- Stilbenes are characterized by two benzene rings linked via an isopropylene moiety that form a compact ring structure separated by a double bond. Stilbenes include *trans*-resveratrol and its natural glycoside, *trans*-piceid [10]. The presence in diet is very low and usually comes from wine.
- Lignans are formed of 2 phenylpropane units, widely present in seeds, berries, grains, fruits, vegetables, and other fiber-rich foods [11].

1.1.2 Daily intakes of polyphenols

Estimated total intakes of polyphenols is approximately 1000 mg/day, however, due to the differences in regions, dietary habits, sources of food, population characteristics and methods of information collection, results of polyphenols intakes were variable as have been shown by previous publications. Table 1 summarizes polyphenol intakes from different countries and regions.

Table 1. Daily polyphenol intakes in different countries/regions (mg/day).

Country/Region	Population	Number	Database	Daily polyphenol intakes	Study	Reference
European	Adults	36,037	Phenol-Explorer	1,186	EPIC	[12]
Mediterranean	Elderly	304	Phenol-Explorer	312	-	[13]
Italy	Elderly	811	Own database	594	InCHIANTI	[14]
Polish	Adults	10,728	Phenol-Explorer	1,756	HAPIEE	[15]
Finland	Adults	2007	USDA	863	FINDIET2002	[16]
Brazil	Adults	1,103	Phenol-Explorer	377,5	Health Survey-São Paulo	[17]
Spain	Elderly	7,200	Phenol-Explorer	820	PREDIMED	[18]

Polyphenol intakes in the PREDIMED population

The Mediterranean diet recommended in the PREDIMED study could be considered as a polyphenol rich diet because it is characterized by: a) a high consumption of cereals, legumes, nuts, vegetables, and fruits; b) a relatively high-fat consumption, mostly provided by olive oil; c) moderate to high fish consumption; d) poultry and dairy products consumed in moderate to small amounts; e) low consumption of red meats, and meat products; and f) moderate alcohol intake, usually in the form of red wine [19]. Among them, fruits, vegetables, nuts, olive oil and red wine are important polyphenol sources. However, polyphenol intakes from the PREDIMED study was not very high compared to other studied cohorts.

In the frame of the PREDIMED study, the mean total polyphenol intakes were 820±323 mg/day at baseline. Of them, 443±218 mg/day was from flavonoid and 304±156 mg/day was from phenolic acids. Specifically, hydroxycinnamic acids were the phenolic group with the highest intake and 5-caffeoylquinic acid was the most abundantly ingested individual polyphenol. Considering individual foods, coffee was the main source of total dietary polyphenols (18%), followed by two fruits: oranges (16%) and apples (12%). Olives and olive oil were the fourth source, together providing

11% of total polyphenol intakes followed by red wine, which contributed 6%. Details of polyphenol intakes divided into main polyphenol subclasses, as well as flavonoid and phenolic acid intake from the different food groups are shown in figures 2 and 3[18].

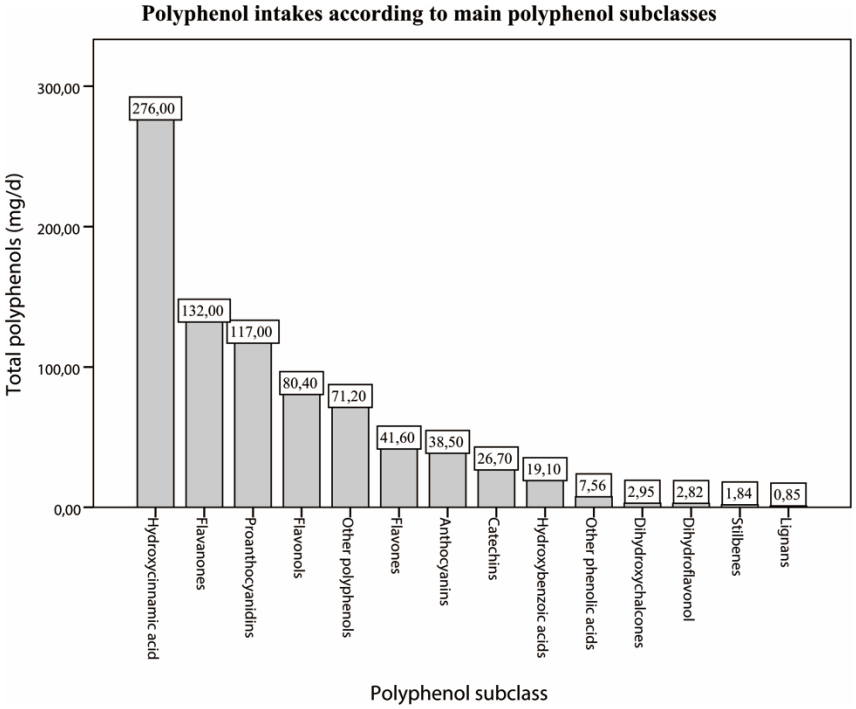


Fig 2. Polyphenol intakes according to main polyphenol subclasses in the PREDIMED study

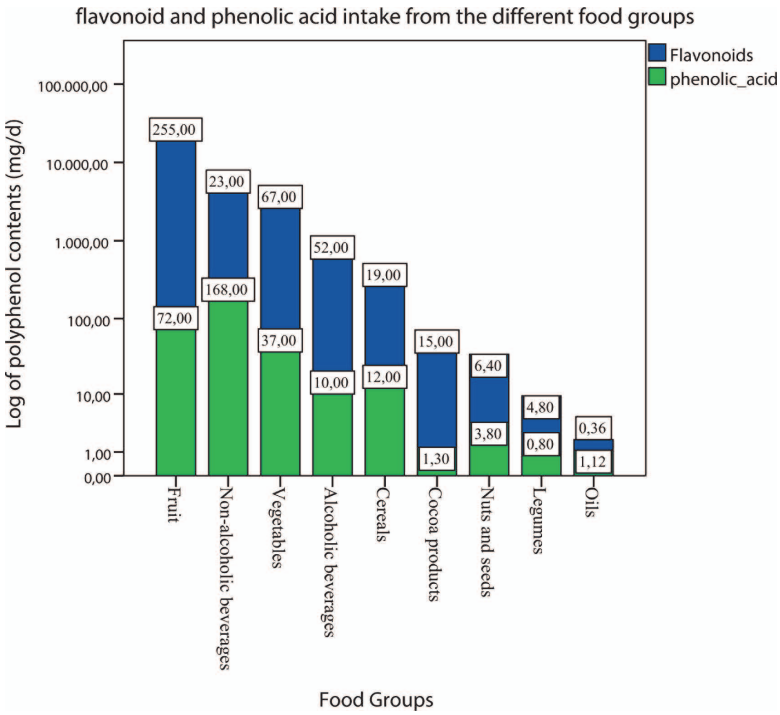


Fig 3. Flavonoids and phenolic acids intakes from the different food groups in the PREDIMED cohort

Bioavailability and metabolism of polyphenols

Bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized so that is available at the site of action [20]. To know in which proportion polyphenols are available and reach target tissues is more important than only the value of polyphenol intakes.

The chemical structure of polyphenols determines their absorption and the structure of the metabolites circulating in plasma. Absorption of polyphenols also depends on fat intake, food matrix, dose, intestinal transit, and other factors [20]. In addition, emerging findings suggest that microbiota also plays a crucial role in the metabolism of polyphenols.

Briefly, the metabolism of polyphenols takes place as follows: some polyphenols, aglycones and anthocyanins, are directly absorbed in the stomach and small intestine after ingestion. Polyphenols that are not absorbed in the small intestine reach the colon where they undergo substantial structural modifications. In fact, the colonic microbiota hydrolyzes glycosides into aglycones and degrades them to simple phenolic acids. Prior passing into the bloodstream, polyphenols undergo other structural modifications due to conjugation processes, mainly in the liver [21]. Then polyphenol metabolites enter into the bloodstream by the portal vein to the liver, where they may be subjected to more conjugations. After that, metabolites travel through the bloodstream again attached to carriers such as albumin.

There are two ways of excretion that depends on the molecular weight. The heavier compounds are usually eliminated as bile components. Back in the intestine (enterohepatic circulation), some of them are deconjugated and regenerated by gut microbial enzymes and are reabsorbed. The unabsorbed ones are eliminated via feces. The lighter polyphenols are excreted through the urine via the kidney [22,23].

Because of bioavailability greatly differs from one polyphenol to another, although polyphenol intakes from the PREDIMED study is not the highest compared with other

studies, it is not necessary the most abundant polyphenols in the diet lead to the highest concentrations of active metabolites in target tissues [24].

Properties of polyphenols and their protective effects

Epidemiological studies and meta-analyses strongly suggest that long term consumption of diets rich in plant polyphenols offer protection against the development of cancers, cardiovascular diseases (CVDs), diabetes, osteoporosis and neurodegenerative diseases [25]. Those protective effects may be attributed to several biochemical properties of polyphenol, including anti-oxidant, anti-inflammatory, anti-platelet, anti-atherogenic, anti-proliferative, and anti-angiogenic properties [4,25–29]. Figure 4 shows the associations between properties of polyphenols and their protective effects.

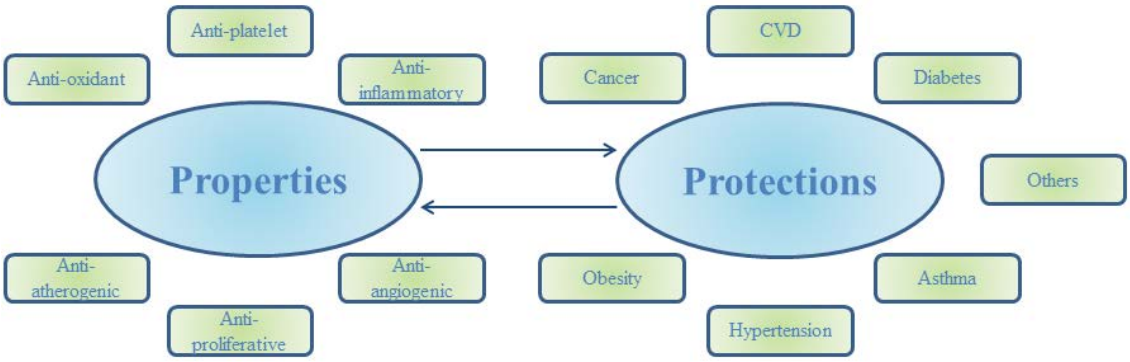


Fig 4. Biochemical properties of dietary polyphenol and their protection against chronic diseases

Prevention of CVD

CVDs are a cluster of various diseases that involve the heart or blood vessels, and mainly include coronary artery disease, carotid artery disease and peripheral artery disease [30]. There is a consensus that CVDs have been the leading cause of death worldwide in recent decades, and it is predicted that will raise from 17.5 million in 2012 to 22.2 million in 2030 [1]. Also, CVDs are a heavy economic burden on the health care system at both global and national scales. In the United States, currently, CVDs constitute 17% of overall national health expenditures, and the medical costs of CVDs have grown at an average annual rate of 6% and have accounted for 15% of the increase

in medical spending [31]. Moreover, unfortunately, these deadly killers no longer just affect privileged individuals and nations, because more than 80% of deaths related to CVD worldwide now occur in low- and middle- income countries [32].

Prevention of CVD could be achieved by different guidelines among people with or without clinically manifest CVD (primary or secondary prevention, respectively) [33]. For those people with established coronary heart disease (CHD), cerebrovascular disease or peripheral vascular disease, intensive lifestyle interventions and appropriate drug therapy are needed; while for the primary prevention, prediction models based on established risk factors are available. However, such numerous risk prediction tools can only estimate an individual's short-term risk of CVD, but do not capture the true cumulative burden of CVD [34]. Therefore, a lifestyle-based prediction, including age, smoking, body mass index (BMI), exercise, alcohol, and a healthy diet, might provide more precise guidelines.

Polyphenol and CVD

Diet is a key modifiable risk factor in the prevention and risk reduction of CVD. Over the last two decades, literature on polyphenols has grown exponentially following the recognition of their properties and more evidence of their potential beneficial effects upon health has been accumulated. For instance, numerous epidemiological and human intervention studies have suggested that regular consumption of polyphenol-rich foods, such as fruits, vegetables, olive oil and wine, may exert cardio-protective effects in humans [35–37]. Prospective and observational studies have indicated a correlation between the intakes of flavonols, resveratrol, anthocyanin and a reduced risk of CVD [26,38,39]. Furthermore, systematic reviews have indicated that the consumption of food rich in polyphenol plays an important role in the prevention of CVD [40–42].

Mechanisms used to explain such observed evidence are so far inconclusive. Protective effect on endothelial and platelet function by polyphenol intake may partly explain the observed benefits [43]. Modulation of oxidative stress due to the antioxidant properties of polyphenols has been widely accepted as a potential explanation [44]. Also, more

consistent effects have been observed on endothelial function and homeostasis and support a reduction of risk by polyphenol intakes [45]. In addition, evidence shows polyphenols from diet and red wine increase endothelial NO production, leading to endothelium-dependent relaxation in conditions such as hypertension, stroke or the metabolic syndrome [46,47]

3.1.2 Polyphenols and blood pressure

Blood pressure and CVD risk

High blood pressure is a continuous, graded cardiovascular risk factor by several epidemiologic studies [48–55]. Such risk found in both genders, all ages (young, middle-age, and elderly), health status (healthy, low cardiovascular risk, high cardiovascular risk, cardiovascular patients), different regions and countries [51,54–56].

According to the 7th report of the Joint National Committee on prevention, detection, evaluation, and treatment of high BP, definition of different categories of BP are shown in figure 5 [57]. Although prehypertension is not an independent disease category, a 10-year cumulative incidence shows high-normal blood pressure is associated with an increased risk of CVD [58].

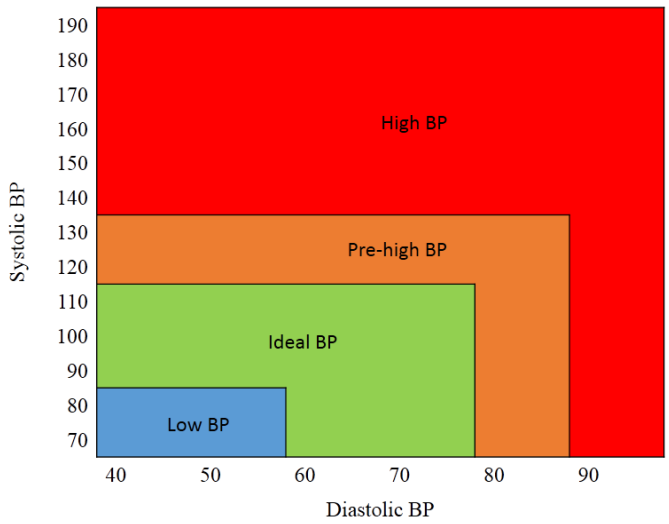


Fig 5. BP categories

DBP and SBP, which is the major determinant of cardiovascular risk, depend on the age

and gender of the subjects. In the 1970s, publications suggested DBP was the main determinant of CVD, while recent attention were paid on SBP, particularly in the elderly population [59]. In middle-aged subjects, classification of cardiovascular risk, according to DBP levels should take into account gender, especially when SBP levels are elevated. In men with systolic hypertension, a U-shaped curve relationship between cardiovascular mortality and DBP was observed, while in women, DBP was positively correlated with cardiovascular mortality [60].

The fact that BP reduction provides cardiovascular protection has been demonstrated by several epidemiological studies; however, the quantitative association between BP lowing and cardiovascular risk was different. In prospective observational studies, a long-term difference of 5-6 mm Hg in usual DBP is associated with about 35-40% less stroke and 20-25% less CHD [61]. Ten mm Hg reduction in SBP leads to approximated 22% decrements in a CHD population and 41% in stroke in large-scale placebo-controlled randomized trials [62]. Furthermore, with intensive BP reduction, risk decreased 11% for major cardiovascular events, 13% for myocardial infarction, and 24% for stroke, respectively [63]. In conclusion, the preventive effect of lowering BP is substantial and capable of reducing the incidence of CVD.

Polyphenol intakes and BP

Numerous observational and short or long term intervention studies have demonstrated that consumption of polyphenol-rich food, such as fruits, vegetables, wine, coffee, tea, dark chocolate, nuts, and legumes, was negatively associated with BP among various populations [64–74]. A polyphenol-rich diet may have a beneficial effect on BP, helping to lower high BP and prevent it from increasing.

The anti-hypertensive effects from polyphenol intakes are mainly attributed to regulation of nitric oxide (NO) bioavailability. Potential mechanisms of anti-hypertensive effects of polyphenol are shown in figure 6. Considerable evidence suggests that oxidative stress, which results in an excessive generation of reactive oxygen species (ROS), plays a key role in the pathogenesis of hypertension. This

phenomenon leads to endothelial dysfunction, an imbalance between endothelium-derived relaxing factors, such as nitric oxide and contracting factors, like endothelin (ET)-1 [75]. The beneficial role of polyphenols in the prevention and therapy of hypertension is done by acting as free radical scavengers, metal chelators, and in enzyme modulation and expression. Specifically, polyphenols activate and enhance endothelial nitric oxide synthase (eNOS) expression by several signaling pathways, increase glutathione (GSH), and inhibit ROS-producing enzymes such as NADPH and xanthine oxidases. These pathways lead to improved endothelial function, subsequent normalization of vascular tone, and finally leading to an overall antihypertensive effect.

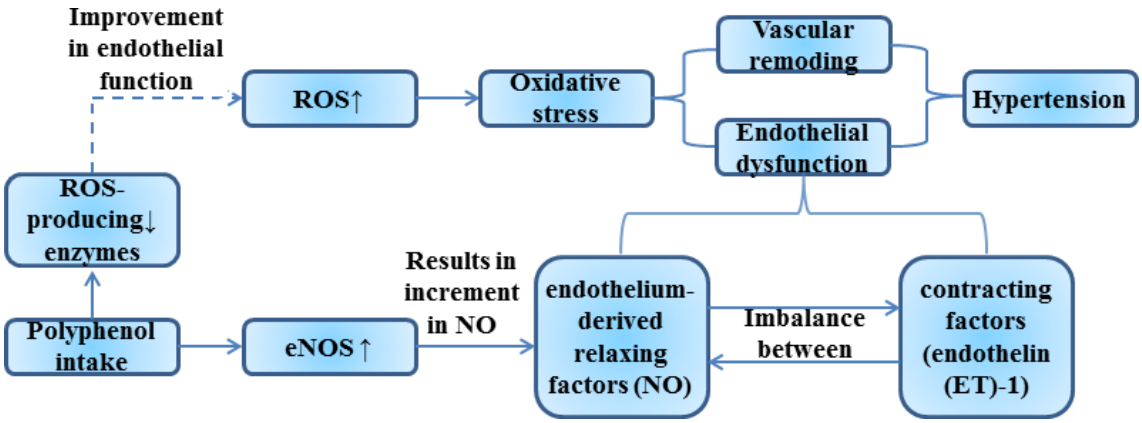


Fig 6. Potential mechanisms of anti-hypertensive effects from polyphenol intakes

Studies aimed to assess the association between polyphenols and BP have some limitations.

- Firstly, most of them focus on a single source of polyphenol or food item. The whole impact of polyphenol-rich dietary patterns, such as the Mediterranean diet, need more evidence.
- Secondly, the protection against hypertension from polyphenols not only depends on the profiles themselves, such as their concentration and chemical structure, but also on their bioavailability and metabolism among different subjects [76–78]. Thus, the degrees of observed benefits were mixed and inconclusive. To solve this problem, one option is to use a reliable and effective biomarker to track polyphenol after digestion from body tissues, such as excretion of urine or plasma.

- Thirdly, aging is an important factor for the occurrence of CVD. In older population is that other defined risk factors co-vary in number or severity with increasing age [79]. Most of them focus on mid-age subjects [69,80], only limited studies have evaluated the effects of dietary polyphenol on elderly population [47,81].
- Fourthly, compared with the daily consumption of plant-derived foods, most intervention studies used higher doses than those in real life. Hence, closer to real life may provide more reliable results.

In conclusion, to avoid the mentioned limitations above, it is necessary to focus on a dietary pattern, instead of single polyphenol-rich food resource, to track long-term effects from polyphenol intakes.

3.1.3 Polyphenols and body weight

Overweight and obesity and CVD risk

Prevalence of overweight and obesity has been increasing in recent years. In 2010, overweight and obesity were estimated to cause 3.4 million deaths, 4 % of years of life lost [82]. According to World Health Organization (WHO), in 2014, more than 1.9 billion adults were overweight and over 600 million were obese [83]. Therefore, overweight and obesity posed a grave threat to the public health worldwide. Besides, substantial economic cost on prevention and treatments of obesity has also become a huge burden nowadays and annual spending on such an area are projected to reach $861 billion in 2030 [84].

Obesity is not only a risk factor, but also a predictor of cardiovascular diseases [85–87]. High body mass index (BMI) is associated with the development of cardiovascular risk factors such as hypertension, dyslipidemia, insulin resistance, and diabetes mellitus, leading to CVDs such as CHD and ischemic stroke. Moreover, the development of these comorbidities is proportionate to the BMI and obesity is considered as an independent risk factor for CVD [88].

According to WHO, overweight and obesity are defined as "abnormal or excessive fat

accumulation that may impair health". BMI is a commonly used index to classify overweight and obesity. Overweight categorized as BMI ≥25 to <30 kg/m² and obesity as BMI ≥30 kg/m².

However, BMI cannot make the distinction between an elevated body weight due to high levels of lean versus fat body mass. Besides, recent studies have shown that abdominal anthropometric measurements, waist-to-height-ratio (WHtR) and waist circumference (WC), could be better predictors of CVD than BMI because of the closer relationship between central obesity and CVDs [89,90].

Genetic, social and environmental factors may contribute to the pathogenesis of overweight and obesity, such as polymorphism of some genes, diet, body metabolism, physical activity, intestinal microbiota, as well as social status [91]. Energy balance is important for maintaining a healthy weight. Body weight can change only when energy intake is not equal to energy expenditure over a given period of time. Thereby, reducing obesity requires modifying both energy intake and energy expenditure [92]. To increase physical activity might be helpful in rising energy expenditure and dietary and lifestyle modification could help to decrease energy intake.

Polyphenol intakes and weight control

Numerous investigations have demonstrated the role of oxidative stress in the pathophysiology of obesity. Therefore, antioxidants are considered protective agents to decrease the oxidative-inflammatory status associated with body weight gain and to be used for the treatment of the different diseases induced by obesity [91].

There are several studies that evaluated the anti-obesity effects of polyphenol intake based on animal models and in some human studies [93–96]. However, there is not sufficient data to support recommending long-term, safe usage of dietary polyphenols for prevention and treatment of obesity and there are still some limitations for those studies.

- Firstly, regarding *in vivo* approaches, most of the studies have been performed in

rodents. Extrapolation of these results to humans is a matter of concern.

- Secondly, most previous human studies have focused on certain polyphenols and effect of total polyphenol intakes and their synergistic actions were never evaluated.
- Thirdly, due to the fact that polyphenols have a low level of bioavailability in humans and research animals, intervention studies always gave high doses of polyphenols, therefore could not assess effects based on real life consumption. Moreover, it is important to note that high doses of these polyphenols in supplement form may have adverse effects.

Evidences indicate that dietary polyphenol having anti-obesity effects can be classified into several categories based on their distinct mechanisms, shown in figure 7, including: suppression of fat absorption from the gut; uptake of glucose by skeletal muscles; suppression of anabolic pathways; stimulation of catabolic pathways in adipose tissues, liver and other tissues; inhibition of angiogenesis in adipose tissues; inhibition of differentiation of pre-adipocytes to adipocytes; stimulation of apoptosis of mature adipocytes; and reduction of chronic inflammation associated with adiposity [93,97,98].

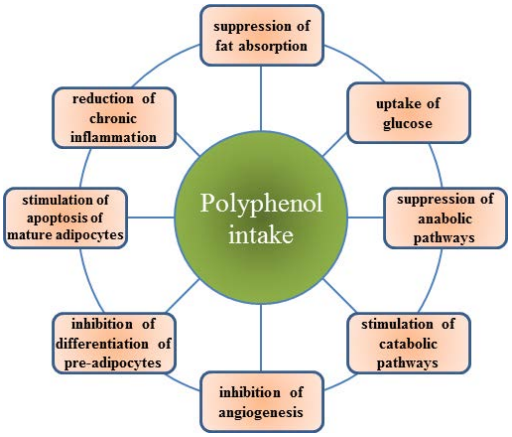


Fig. 7 Potential mechanism of anti-obesity of polyphenol intakes

In conclusion, excessive accumulation of fat leads to a chronic low-grade inflammatory state associated with high circulating levels of inflammatory markers. Obesity is a complex and multifactorial condition of chronic inflammation and oxidative stress. In this respect, several polyphenols have been shown to possess anti-inflammatory effects and might be exploited in the adjunct therapy of obesity.

3.1.4 Polyphenols and glucose

Glucose and CVD risk

There is a substantial amount of epidemiological evidences showing the relationship between glucose levels in plasma and CVD, particularly with diabetic patients. Publications have demonstrated the risk of CVD in type-2 diabetic population is about 2-4 folds higher than in subjects without diabetes [99–102]. Thereby, glucose control appears to be associated with a reduction in the incidence of major cardiovascular events, such as CHD and stroke. A systemic study conducted in Asia Pacific region found that, overall, each 1 nmol/l lower of usual fasting glucose was associated with a 21% lower risk of total stroke and a 23% lower risk of total ischemic heart disease [103].

According to the American Diabetes Association (ADA), criteria for the diagnosis of diabetes and prediabetes are shown in table 2.

Table 2. Categories of different glucose level

	PG	FPG
Normal	< 140 mg/dL (7.8mmol/L)	70-99 mg/dL (3.9-5.5 mmol/L)
Prediabetes	140-199 mg/dL (7.8-11.0 mmol/L)	100–125 mg/dL(5.6–6.9 mmol/L)
Diabetes	>200 mg/dL (11.1 mmol/L)	>126 mg/dL (7.0 mmol/L)

PG: Two-hour plasma glucose; FPG: fasting plasma glucose.

The role of hyperglycemia as an independent risk factor for the development of CVD is strongly suggested by data from large epidemiologic studies as well as by numerous clinical trials. These studies support the view that there is a graded relation between glucose above the normal range and extending into the diabetes range (ie, dysglycaemia) and subsequent cardiovascular outcomes:

- CVD is more common in people with diabetes than in subjects without the disease [104]. Moreover, hypertension and dyslipidemia, among other risk of CVD, are common in subjects with diabetes. Therefore, they can explain most but not all of the excess of risk of CVD in patients affected by diabetes.

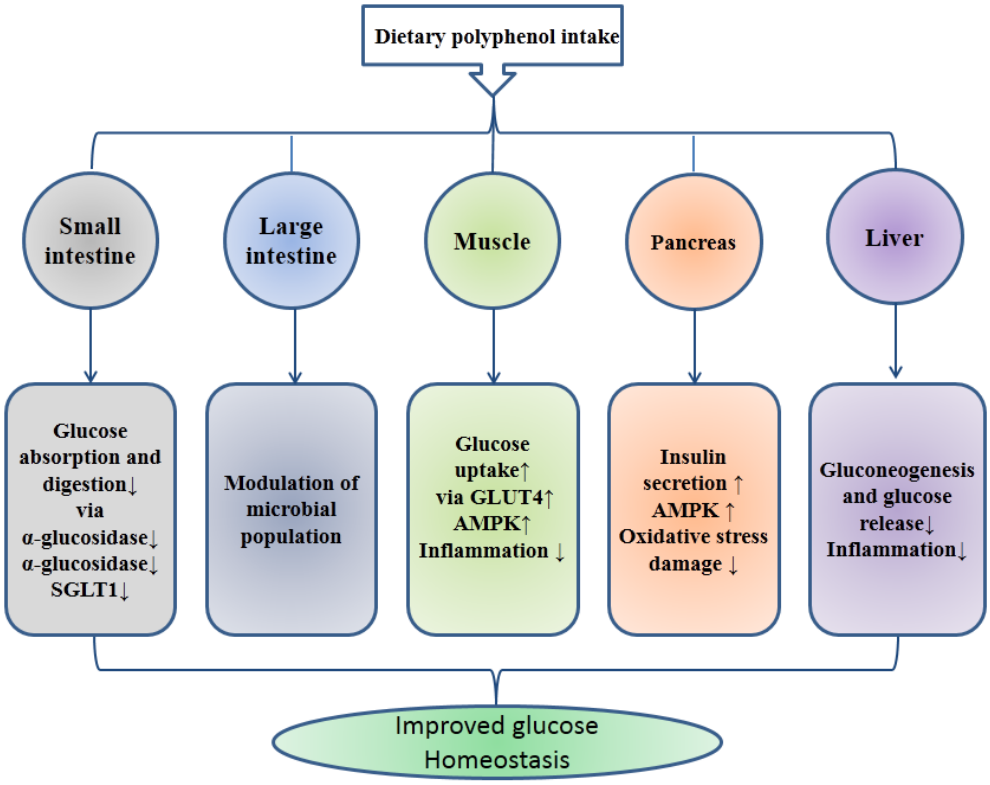
- Individuals with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) are at high risk, not only to develop diabetes mellitus, but also to experience an adverse CVD event later in life [105].

To conclude, diabetes confers about a 2-4 fold excess risk for a wide range of vascular diseases, independently from other conventional risk factors [106]. However, the rising trend in the prevalence of diabetes complications suggests that current medical treatments for the management of diabetes are not sufficient and use of supplementary treatments, including functional foods and their nutraceuticals, could increase the effectiveness of diabetes management.

Polyphenol intakes and glucose control

Growing evidence from animal studies supports the anti-diabetic properties of some dietary polyphenols, suggesting that dietary polyphenols could be one dietary therapy for the prevention and management of Type-2 diabetes (T2D) [107–113]. However, human' studies are limited and have shown inconsistent results [107]. A meta-analysis of nine interventions showed that Mediterranean diet reduced a 0.3% of hemoglobin A1c, an index used to identify the three month average plasma glucose concentration, and a reduction in fasting plasma glucose by 0.72 mmol/L [114].

Potential mechanisms of action of dietary polyphenols in the regulation of glucose homeostasis and insulin sensitivity based on *in vitro* and *in vivo* studies were summarized as follows: dietary polyphenolic compounds may be related to inhibition of carbohydrate digestion; inhibition of glucose absorption; stimulation of insulin secretion; protection of pancreatic β-cells; suppression of glucose release; inhibition of advanced glycation end product (AGE) formations. Specific mechanisms are shown in figure 8.



SGLT1: sodium-glucose linked transporter 1; GLUT4: Glucose transporter type 4; AMPK: activated protein kinase

Fig.8 Potential mechanisms of anti-diabetic effects from polyphenol intakes [107]

3.1.5 Polyphenols and triglycerides

A long-standing association exists between elevated triglycerides (TG) levels and CVD. Observational studies have shown that TG levels reflect the presence of remnant lipoproteins that may promote atherosclerosis and are thus significant predictors of CVD [115]. A study indicated that TG, with concentrations of 2–10 mmol/L conferred increased risk of CVD, and concentrations greater than 10 mmol/L conferred increased risk of acute pancreatitis and possibly CVD [116]. Moreover, having high TG is a risk factor for the development of T2D in individuals with normal or IFG [117]. Therefore, raised TG could be potentially seen as an important CVD risk factor.

To data, randomized trials showing cardiovascular benefit of TG reduction are scarce. However, there is increasing evidence that raised concentrations of remnant cholesterol, marked by raising triglycerides, are an additional causal risk factor for CVD and all-

cause mortality[116]. Treatments of dyslipidemia mainly include drug therapies and modification of lifestyle. Particularly, the most important lifestyle modification is to lose weight through dietary control and enhance physical activity [118].

Numerous mechanisms and targeted molecular processes about polyphenols and hypertriglyceridemia reduction have been explored in animal models and/or cell lines, but only limited human clinical trials provide evidence of their efficacy [119]. Most of previous studies have been focusing on a single polyphenol-rich food, but never paid attention on polyphenol rich dietary patterns such as the Mediterranean diet [64,120,121]. Synergistic effects from total polyphenol intakes, as well as potential mechanisms used to explain the anti-hypertriglyceridemia effects have been never explored. In addition, large, long-term intervention studies are asked for further research.

3.1.6 Polyphenols and others cardiovascular risk factors

Cholesterol is a fatty substance that circulates in the blood and is an important structural component of all human cells. High levels of total cholesterol and low levels of high-density lipoprotein cholesterol (HDL-C) are risk factors for CHD [122,123]. In a meta-analysis of individual data from 61 prospective studies, 0.33 mmol/L increased HDL-C was associated with approximately a 30% lower CHD mortality [124]. A recent study also indicated a positive association between total cholesterol and total and ischemic stroke risks in men and an inverse association between total cholesterol and intra hemorrhagic stroke risk in women [125]. During 2011–2014, 12.1% of adults had high total cholesterol and 18.5% had low HDL-C in United States[122].

Low-density lipoprotein cholesterol (LDL-C) is also widely recognized as an established cardiovascular risk marker according to results from numerous clinical trials that demonstrate the ability of LDL-C to independently predict development and progression of CHD [126,127]. All currently available guidelines state that LDL-C levels should be used as the primary target to initiate and titrate lipid-lowering therapy

[128]. A randomized trial showed that a reduction in LDL-C of 1.6 mmol/L halves the risk of ischemic heart disease. Another study indicated that LDL-C is a strong independent predictor of CHD in individuals with diabetes and a 10-mg/dL increase in LDL-C was associated with a 12% increase in CVD risk [129].

Cell studies, animal models and limited human studies have pointed out that there is an association between polyphenol intake and improvements in lipid profile, including increment in HDL-C, and decrement in total cholesterol and LDL-C. However, results are not conclusive.

Most of the studies explored the effects of polyphenols contained in individual foods, such as cocoa, green tea, or red wine [130–132]; but the effects of diets containing different natural sources of polyphenols were not investigated [133].

Various mechanisms are used to explain the observed beneficial effects, mainly including inhibition of cholesterol synthesis; increment in LDL receptor activity; reduction of intestinal cholesterol absorption; and ability to interfere with bile metabolism [134].

3.2 Biomarker of total polyphenol intakes

3.2.1 The need for a biomarker

Biomarkers are some measurable characteristics of an organism that reflect a particular physiological state. In clinical studies, biomarker refers to a broad subcategory of medical signs, which used to identify medical state observed from outside the patient and could be measured accurately and reproducibly [135].

In the studies of the associations between dietary exposure and health outcomes, the use of robust biomarkers of food exposure has been proposed as an accurate measurement to estimate real intake.

There are several reasons for the need of a biomarker of total polyphenol intakes, including [24,136,137]:

- There is a great diversity of polyphenol content between foods.
- There is limited data regarding the polyphenol content of specific foods within the commonly-used food composition databases.
- There are challenges in characterizing and quantifying habitual food intake.
- The health effects of polyphenols depend on their bioavailability, which varies greatly from one molecule to another and among individuals.
- After dietary intake, polyphenols are deglycosylated and conjugated by reactions such as methylation, sulphation and glucuronidation in the mouth, stomach, upper intestinal epithelial cells and liver.

Strengths and limitations of biomarker

Compared with traditional methods of obtaining information on polyphenol intakes, such as from dietary recalls, FFQs, and databases on the polyphenol content of foods, there are numerous strengths for the use of biomarkers. For instance, biomarkers are objective measures that significantly limit biases and errors associated with dietary assessment and inaccuracies in food-composition data [138]; biomarkers may better reflect tissue exposure to polyphenols more directly linked to their health effects than

intakes [139]; and, when important dietary sources of polyphenols are not included in dietary records, biomarkers may be useful.

Also, there are some limitations. First, it is clear that the choice of biomarker for the estimation of polyphenol intakes is complex with different classes of polyphenols and possibly even individual polyphenols requiring different approaches. Thus, it may difficult to get a general biomarker for total polyphenol intakes. Second, the quantification of a number of metabolites in 24h urine samples may be a suitable approach in small-scale human intervention trials but may not be a realistic possibility in large-scale epidemiological studies due to problems in organizing the collection of 24 h urine samples from a large study population [136].

Biomarker is an improvement of dietary reports

Even though polyphenol biomarkers could provide more accurate data than traditional methods, such as self-reporting of dietary patterns by the study participants, and food composition databases, but they cannot completely replace them. Indeed, introduction of biomarkers to calibrate the measurement error in dietary reports, and as additional measures of exposure, is a significant development in the effort to improve estimates of the magnitude of the contribution of diet in affecting individual disease risk within populations [138].

Factors affecting choice of potential biomarkers of polyphenols intakes

Due to the fact that the relationship between dietary intake and resulting concentrations of biomarkers in body fluids is highly complex, several factors may affect the choice of the biomarker of polyphenol intake, including [136,140]:

- Metabolism of polyphenols in human.
- The time–response relationship between polyphenol intakes and the appearance of the biomarker in biological fluid.
- The extent to which certain physiological and environmental factors affect the rate of polyphenol metabolism in human subjects.

- The biomarker should be specific to the dietary component of interest.

3.2.2 Urinary total polyphenol excretion (TPE) as a reliable biomarker of total polyphenol intake

The F–C assay

The F-C assay has been used to measure total phenolics in natural products for many years [141]. The F–C reagents (phosphomolybdic-phosphotungstic acid reagents) reduce polyphenols in alkaline medium. A series of molybdic and tungstic oxides are formed in this redox reaction, in alkaline conditions, showing a blue coloration proportional to the concentration of polyphenols [142]. However, The F-C assay is affected by several interfering reductant substances, such as sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, and Fe (II), as well as other reducing substances that react with the F-C reagent [143]. Therefore, it is necessary to remove these substances to avoid the interference.

Solid phase extraction, clean-up procedure

Sample preparation is a key procedure in modern chemical analysis. SPE is one of the simplest, yet most effective and versatile, method of sample preparation. Sample components of interest are separated from other species by applying the sample mixture to an appropriately chosen solid sorbent and selectively eluting the desired components [144]. After SPE procedure applied on the Oasis® MAX 96-well plate, interference water-soluble compounds were removed from urine samples, thus avoiding the unpleased reaction with the F–C reagent [145].

Correlations between TPE and total polyphenol intakes

Several publications have reported that urinary total polyphenol excretion, expressed as mg gallic acid equivalent (GAE) per g creatinine, and analyzed by a Folin-Ciocalteu (F–C) assay, after a solid phase extraction (SPE) clean-up with Oasis MAX 96-well plate cartridges could be considered as an accurate biomarker of polyphenol-rich food intake [14,66,145–148].

One of previous studies has evaluated the specific correlation between total polyphenol in urine and polyphenol intakes, showing a positive significant correlation between the TPE in spot urine samples and total polyphenol intakes ($r = 0.257$, $p = 0.04$), and the total fruit and vegetable intake ($r = 0.339$, $p = 0.008$), therefore demonstrated TPE could be considered as a marker of total dietary polyphenol intakes [145,146].

3.3 The PREDIMED study

3.3.1 Overview

The PREDIMED Study (PREvención con DIeta MEDiterránea, ISRCTN35739639, www.predimed.es), was a large, parallel-group, randomized, multicenter, controlled, clinical trial [149]. Participants were recruited from 2004 to 2009. After a median of 4.8 years of follow-up, the study was stopped by an external scientific committee. The study was funded by the *Instituto de Salud Carlos III*, from the *Ministerio de Economia y Competitividad*

Aims

- To assess the effects of two Mediterranean diets on a composite endpoint of cardiovascular death, myocardial infarction and stroke (primary endpoint) in comparison with a low-fat control diet.
- To assess the effects of two Mediterranean diets on the death of any cause, incidence of heart failure, diabetes mellitus, dementia or other neurodegenerative disorders and major cancers (colorectal, breast, lung, stomach and prostate) [149].

Intervention

Participants were randomly assigned, in a 1:1:1 ratio, to one of three dietary intervention groups: a Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with nuts, or a control diet (low-fat diet according to the American Heart Association recommendations).

Participants

High-risk participants (7447) were randomly selected from 8 different Spanish regions. Participants were men (55–80 years old) or women (60–80 years old) who were free of CVD but at high risk at baseline. Both inclusion criteria and exclusion criteria are shown in table 4 [150]:

Table 4. inclusion and exclusion criteria of participants in PREDIMED study.

Inclusion criteria	Exclusion criteria

• having either type 2 diabetes or more than 3 major cardiovascular risk factors:
  • current smoking (>1 cig/day during the last month)
  • hypertension (SBP ≥ 140mm Hg or DBP ≥ 90mm Hg or antihypertensive medication)
  • hypercholesterolemia (LDL cholesterol ≥ 160 mg/dL or lipid-lowering therapy; HDL cholesterol ≤ 40 mg/dL in men or ≤ 50 mg/dL in women)
  • BMI ≥ 25 kg/m^2
  • family history of premature CHD
	• previous history of CVD.
	• any severe chronic illness.
	• immunodeficiency or human immunodeficiency virus (HIV) positive status
	• illegal drug or alcohol misuse
	• history of allergy to olive oil or nuts
	• unlikelihood of changing dietary habits

Measurements

Selected participants were invited to complete some questionnaires:

- Inclusion questionnaire;
- A general questionnaire used to collect demographic and sociological data;
- A validated 137-item food frequency questionnaire (FFQ) used to assess dietary habits [151];
- A 47-item general questionnaire aimed to summarize information about lifestyle, health condition, education, history of illnesses and medication use;
- A 14-point questionnaire to evaluate the degree of adherence to the Mediterranean diet [152];
- A validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire used to record physical activity [153].

All questionnaires except inclusion questionnaire and general questionnaire were administered and repeated annually during the follow-up by trained staff in face-to-face interviews.

Group visits were repeated every 3 months with the same frequency of contacts mentioned above. Blood and urine samples were collected at baseline and years 1, 3, 5 and 6 (or final visit).

3.2.2 Latest evidence from the PREDIMED study

On the basis of the results of an interim analysis, the trial was stopped after a median follow-up of 4.8 years. Several evidences were found to sustain the cardiovascular protective effects of the Mediterranean diets.

- A primary endpoint event, including stroke, myocardial infarction, and death from cardiovascular causes, occurred in 288 participants. Specifically, the multivariable-adjusted hazard ratios were 0.70 (95% confidence interval [CI], 0.54 to 0.92) and 0.72 (95% CI, 0.54 to 0.96) for the group assigned to a Mediterranean diet with extra-virgin olive oil (96 events) and the group assigned to a Mediterranean diet with nuts (83 events). Also, compared with control group, a 30% reduction in incidence of cardiovascular events was observed when comparing the two Mediterranean diet groups with control group [150]. So, compared with a low-fat diet, Mediterranean diets supplemented with olive oil or nuts were associated with a reduction in the risk of major cardiovascular events among high-risk persons. The results support the benefits of the Mediterranean diet for the primary prevention of cardiovascular disease.
- Respective hazard ratios for incident diabetes (273 cases) among 3541 non-diabetic participants were 0.60 (0.43-0.85) and 0.82 (0.61-1.10) for olive oil group and nuts group, respectively versus control [154].
- After 1-y follow-up, participants in the Mediterranean diet with nuts group showed a significant 13.7% reduction in the prevalence of metabolic syndrome compared with reductions of 6.7% and 2.0% in the Mediterranean diet with olive oil group and control groups, respectively [154]. However, after 4.8 years of follow-up, Mediterranean diet supplemented with either extra virgin olive oil or nuts was not associated with the onset of metabolic syndrome [155].

- Mediterranean diets reduced the risk of peripheral artery disease (PAD). Both Mediterranean diet interventions were associated with a lower risk of PAD compared with the control group. Specifically, the hazard ratio (HR) was 0.34 (95%CI, 0.20-0.58) for participants in the Mediterranean diet plus extra-virgin olive oil group and 0.50 (95%CI, 0.30-0.81) for the Mediterranean diet plus nuts group versus control group after adjustments of classical atherosclerotic risk factors [156].
- Significant improvements in classical and emerging CVD risk factors were also supported. Both Mediterranean diets showed favorable effects on blood pressure, insulin sensitivity, lipid profiles, lipoprotein particles, inflammation, oxidative stress, and carotid atherosclerosis [157].

3.3.3 Strengths and limitations of the PREDIMED study

The strengths of the study are:

- The randomized design, blinded assessment and adjudication of events, and adjustment for a large number of potential confounders minimize the threat of biases in this study.
- The large sample size and the long follow-up period allowed us to obtain relatively precise estimates.
- The community-dwelling participants with unrestricted energy intake, reveals a real lifestyle.

Some limitations of our study should be acknowledged:

- Whether the results can be generalized to persons at lower risk or to other settings is still unknown.
- Lost participants, mainly in the control groups, which might cause a bias toward a benefit in the two Mediterranean-diet groups.
- The FFQ relies on self-reported information, which may account for bias.

Chapter 4

Material and methods

4 Material and methods

4.1 Subjects

The PREDIMED study is a parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the MD on the primary prevention of cardiovascular disease (CVD) (http://www.predimed.es) [149]. Recruitment took place between October 2003 and January 2009, and the 7447 participants were randomly assigned to one of three interventions (two Mediterranean diets enriched with extra virgin olive oil (EVOO) or mixed nuts, and a control low-fat diet). The design, methodology and eligibility criteria for the PREDIMED study. have been previously described [158].

Briefly, we recruited men aged 55 to 80 years and women aged 60 to 80 years with no previously documented CVD. They were eligible if they had type 2 diabetes, or 3 or more major cardiovascular risk factors (hypertension, high plasma LDL-cholesterol, low plasma HDL cholesterol, overweight or obesity, current smoking, or a family history of premature coronary heart disease). The trial was stopped after a median follow-up of 4.8 years due to the benefits of the Mediterranean diet with respect to major cardiovascular events: myocardial infarction, stroke or death from cardiovascular causes (analysis performed by the Drug and Safety Monitoring Board of the trial), compared with a control low-fat group [159].

For the study of the effects of polyphenol on cardiovascular risk factors after a long-term follow-up in the PREDIMED study and the study of effects of polyphenol on body weight and obesity after 5 years of follow-up, 612 participants randomly selected for the present study among the participants from the Hospital Clinic of Barcelona and University of Valencia who collected urinary samples after 5 years of follow-up. However, 39 were excluded because they had extreme TPE values; hence a total of 573 participants were finally included. Period of recruitment is from 2003 to 2009, average follow-up is 5.9 years.

For the study of effects of intake of total polyphenols and some classes of polyphenols on diabetes in elderly people at high cardiovascular disease risk, we excluded 3614 who reported diabetes (including types 1 and 2) at baseline, we also excluded 371 participants who did not complete the FFQs at baseline, and 27 who had an extreme energy intake from 7447 participants. Hence, 3430 subjects were finally selected.

4.2 Assessment of diet, and lifestyle

At baseline and yearly, participants filled out the following validated questionnaires: a 137-item semi-quantitative FFQ [151], a 14-point score questionnaire on adherence to the traditional Med Diet [152], and the Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire [153]. In addition, participants filled out a general questionnaire to provide data on lifestyle habits, concurrent diseases, and medication use. Total energy and nutrient intake were calculated on the basis of Spanish food composition tables.

4.3 Assessment of total polyphenol intake and urinary polyphenol excretion

Individual polyphenol intake was calculated by multiplying the content of each polyphenol in a particular food item (milligrams per gram) by the consumption of this food item (grams per day) and then summing the product across all food items. We obtained the polyphenol content of foods with the use of the Phenol-Explorer database (www.phenol-explorer.eu).

Urine samples were collected and coded, and then immediately shipped to a central laboratory, to be stored at -80ºC until analyzed. The Folin-Ciocalteu method was applied to determine the content of TPE, using a clean-up procedure with solid phase extraction (SPE) performed in 96-well plate cartridges (Oasis MAX), which helped to remove urinary interferences. Finally, TPE was expressed as milligrams gallic acid equivalent (GAE)/g of creatinine [145].

4.4 Measurements

4.4.1 Clinical Measurements.

Weight and height were measured with light clothing and no shoes with a calibrated balance and a wall-mounted calibrated stadiometer, respectively. BMI was calculated as weight in kilograms divided by the square of height in meters. For the measurement of blood pressure (BP), a validated semiautomatic sphygmomanometer (Omron HEM-705CP) was used by trained nurses. Measurements were taken at 5-minute intervals with participants in a seated position. Data were collected as an average of 2 measurements in each arm, repeated twice [160]. Plasma glucose, total cholesterol, and triglyceride concentrations were measured using standard enzymatic auto-mated methods. Levels of HDL-cholesterol were measured by an enzymatic procedure after precipitation, and LDL-cholesterol was estimated by the Friedewald formula [161].

4.4.2 Anthropometric measurements

BMI was calculated as weight in kilograms divided by the square of height in meters. WC was measured midway between the lowest rib and the iliac crest. WHtR was calculated as the waist in centimeters divided by the height in meters. Obesity is defined as BMI more than 30 kg/m^2.

4.4.3 Assessment of diabetes

For the present analysis, the main endpoint was incidence of type 2 diabetes diagnosed according to the American Diabetes Association criteria, namely, fasting plasma glucose concentrations of ≥7.0 mmol/L (≥126.1 mg/dL) or 2-h plasma glucose concentrations of ≥11.1 mmol/L (≥200.0 mg/dL) after an oral dose of 75 g glucose[162]. A review of all medical records of participants was completed yearly in each center by physician investigators who were blinded to the intervention. When new-onset diabetes cases were identified on the basis of a medical diagnosis reported in the medical charts or by a glucose test during routine biochemical analyses (done≥1 time/y), these reports were sent to the PREDIMED Clinical Events Ascertainment Committee, whose

members also were blinded to treatment allocation. Only when a second test that used the same criteria and repeated within the next 3 mo was available and confirmed the new diabetes case was the endpoint definitively confirmed by the adjudication committee [163].

4.5 Statistical analysis

Results were expressed as mean ± SD for continuous variables or percentages for categorical variables. Kolmogorov tests were applied to examine the normality distribution and skewness.

- For the study of the effects of polyphenol on cardiovascular risk factors after a long-term follow-up in the PREDIMED study, all participants were divided into three categories according to changes in TPE during the follow-up ($\Delta TP < -11.4$mg gallic acid/g creatinine, $-11.4 \leq \Delta TP \leq 24.6$mg gallic acid/g creatinine, and $\Delta TP > 24.6$mg gallic acid/g creatinine).

  Changes in nutrient and key food consumption during the follow-up were assessed with ANOVA for repeated measurements analysis. Bonferroni post hoc test and paired t-test were used to compare each variable within and between groups.

  Multivariate linear regression models were used to assess the relationship between serum glucose, total cholesterol, HDL, LDL, triglyceride concentrations, SBP, DBP, and heart rate, and tertiles of changes in TPE during the follow-up period, adjusted for potential confounders (sex, age, intervention groups, BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, medication use, and 14-unit Mediterranean diet score at baseline). Sensitivity analyses were used to further assess the relationship between specific cardiovascular risk factors and subcategories.

  General Linear Model (GLM) approach to ANCOVA was used to determine differences between tertiles of changes in TPE aſter5-year of follow-up, adjusted for potential confounders as did in multivariate linear regression models.

- For the study of effects of polyphenol on body weight and obesity after 5 years of follow-up, all participants were divided into five categories (roughly quintiles) according to the TPE at 5 years of follow-up.

  Changes in nutrient intakes and key food consumption according to the FFQs were assessed with yearly repeated-measures analysis during the follow-up period. A Bonferroni post-hoc test and paired T-test were used to compare each variable within and between groups.

  Multivariate linear regression models were used to assess the relationship between anthropometric parameters (BW, BMI, WC, and WHtR) and quintiles of TPE at 5 years, adjusted for potential confounders, including sex, age, intervention groups, smoking status (never, current, former), family history of coronary heart disease (CHD), physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) and recruitment centers, 14-unit Mediterranean diet score and energy intake at baseline.

  Multiple logistic regression analyses were used to calculate the odds ratio (OR) for quintiles of TPE and obesity (BMI>30 kg/m^2). Models were adjusted for potential confounders as in linear regression analyses.

  Sensitivity analyses were used to further assess the relationship between polyphenol urinary excretion and weight in subcategories (gender and age).

Fort both study, all analyses were performed using SPSS software V21.0 (Chicago, USA). All models were tested for the detection of outliers, multicollinearity, homoscedasticity, and normality and independence of errors. All statistical tests were two- tailed, and the significance level was $p< 0.05$.

- For the study of effects of intake of total polyphenols and some classes of polyphenols on diabetes in elderly people at high cardiovascular disease risk, all

participants were divided into three categories according to total polyphenol intake at baseline adjusted for energy intake.

We calculated person-years of follow-up for each individual from the date of inclusion to the date of diagnosis of type 2 diabetes, death, or end of the follow-up, whichever came first.

We used time-dependent Cox proportional hazards regression models with updated diet and covariate information to estimate the HRs for polyphenol intake in relation to type 2 diabetes risk, while using the lowest tertiles of intake as the reference group.

Tertiles were used to avoid assumptions about linearity and also to reduce the effect of potential outliers. Then, the median intake value and the 25th and 75th percentiles, the number of cases, and the median years of follow-up (with the 25th and 75th percentiles) were assigned to each tertile. A test for linear trend was performed with the use of the resulting variable as a continuous one.

Total polyphenols and subclasses were previously adjusted for total energy intake with the use of the residual method. To assess long-term polyphenol intake and reduce within-person variation, we also calculated the weighted cumulative mean of polyphenol intake at each yearly visit. Nondietary covariates such as smoking, BMI, physical activity, and medication use, as well as dietary covariates, were updated yearly.

In multivariable models, we adjusted for age (<60, 60–64.9, 65–69.9, 70–74.9, and ≥75 y), BMI (continuous), smoking status (never, current, or former), physical activity (continuous), education (primary education, secondary education, or academic/graduate), fasting blood glucose concentrations at baseline (continuous), prevalence of dyslipidemia (yes/no) and hypertension (yes/no), alcohol consumption (continuous in grams per day, adding a quadratic term), energy intake (continuous), and adherence to the traditional MedDiet (14-point score).We also stratified for sex, recruitment center, and intervention group in all models.

Stratification allows for the assessment of modifying effects, as well as controlling for confounding factors. The stratums were then pooled by the software (SAS) to give an overall estimate of the RR adjusted for other potential confounders.

We conducted additional stratified analyses for sex, age, alcohol intake, smoking, physical activity, intervention group, and fasting glucose concentrations at baseline to evaluate potential effect modification. We present the HRs and 95% CIs for each risk factor category, comparing the third tertile with the first tertile and using the fully- adjusted model, taking out the risk factor that we were evaluating. We also included the number of cases and median years of follow-up for each category.

To test for linearity, we used the median intake in each tertile as a continuous variable. To test for statistical interactions, we also added to the model interaction terms between total polyphenol intake and each of these factors.

All statistical analyses were conducted with the use of SAS software, version 9. All t tests were 2-sided and P values below 0.05 were considered to be significant.

Chapter 5

Results

5 Results

5.1 Effects of polyphenol intakes on cardiovascular risk factors after 5-year of follow up.

5.1.1 Baseline characteristics of participants

After 5 years of follow-up of 612 participants randomly selected for this sub study of the PREDIMED trial, 39 were excluded because of extreme TPE values, hence a total of 573 participants were included in the present study.

Table1 shows the baseline characteristics of participants.The average age was 67.3 ± 5.9 years with a BMI of 29.2 ± 3.3 kg/m^2.Most of the participants gathered a high number of cardiovascular risk factors: 41.5% had diabetes; 80.5% had hypertension; 66.8% had dyslipidemia; 16.9% were current smokers, and 37.5% had a family history of CHD.

Table 1. Baseline characteristics of participants according to tertiles of changes in TPE

	TPE (mg GAE/ g creatinine)						
	Q1 (ΔTP < -11.4)		Q2 (-11.4≤ ΔTP≤24.6)		Q3 (ΔTP > 24.6)		P
No. of subjects	191		191		191		
Women, n (%)	101	(52.9)	83	(43.5)	112	(58.6)	0.011
Age (y), mean (SD)	66.7	(5.9)	67.3	(5.8)	68.00	(6.0)	0.113
Weight (kg), mean (SD)	73.9	(10.6)	77.1	(11.6)	74.5	(10.7)	0.01
BMI (kg/m^2), mean (SD)	28.9	(3.1)	29.6	(3.5)	29.2	(3.2)	0.103
Systolic BP (mm Hg), mean (SD)	149.8	(17.9)	151.6	(16.9)	152.8	(18.6)	0.238
Diastolic BP (mm Hg), mean (SD)	84.3	(9.8)	85.9	(10.0)	85.5	(10.4)	0.269
Hypertension, n (%)	151	(79.1)	152	(79.6)	158	(82.7)	0.621
Diabetes, n (%)	78	(40.8)	85	(44.5)	75	(39.3)	0.567
Dyslipidemia, n (%)	136	(72.3)	117	(61.3)	128	(67)	0.074
Smoking status							0.641
Current, n (%)	35	(18.3)	34	(17.8)	28	(14.7)	0.586
Former, n (%)	36	(18.8)	43	(22.5)	47	(24.6)	0.388
Never, n (%)	120	(62.8)	114	(59.7)	116	(60.7)	0.814
Family history of CHD, n (%)	65	(35.3)	75	(40.3)	75	(41.2)	0.460
Medication							
Aspirin, n (%)	33	(32.0)	35	(34.0)	35	(34.0)	0.949
Antihypertensive drugs, n (%)	131	(68.6)	142	(74.3)	141	(73.8)	0.381
Hypolipidemic drugs, n (%)	91	(47.6)	70	(36.6)	78	(40.8)	0.089
Insulin, n (%)	10	(5.2)	9	(4.7)	8	(4.2)	0.890
Oral hypoglycemic drugs, n (%)	40	(20.9)	46	(24.1)	45	(23.6)	0.736
Vitamin or minerals, n (%)	18	(9.5)	16	(8.5)	13	(6.9)	0.644
Educational level							
Primary school, n (%)	140	(74.1)	139	(73.5)	146	(76.8)	
High school, n (%)	32	(16.9)	28	(14.8)	28	(14.7)	0.793
University, n (%)	17	(9.2)	22	(11.6)	16	(8.4)	
Physical activity at leisure time, (MET-min/d)	275	(212)	287	(204)	269	(183)	0.696
Polyphenol intake (mg/d)	853.4	(239.8)	831.2	(248.9)	882.7	(247.8)	0.135

BMI: body mass index; CHD: coronary heart disease; GAE: gallic acid equivalent; TPE: total polyphenol excretion. Data are given as means (SD) for continuous variables and percentages for categorical variables; P < 0.05 indicates statistical significance.

*P-values calculated by analysis of variance or $\chi 2$ tests.

5.1.2 Changes in daily intake of key foods and nutrition after 5 years with energy adjustment categorized by tertile of changes in TPE.

Table 2 and 3 shows changes in daily intake of key foods and nutrition categorized by tertile of changes in TPE and quintile of TPE at 5-year of follow-up, respectively.

Table 2. Changes in key food intake and nutrients according to the FFQs after energy adjustment categorized by tertile of changes in TPE[a]

		TPE (mg GAE/ g creatinine)								
		Q1 (ΔTP < -11.4)		Q2 (-11.4≤ ΔTP≤24.6)		Q3 (ΔTP > 24.6)		P [b]		
		mean	SD	mean	SD	mean	SD	TIME [c]	GROUP [d]	TIME*GROUP [e]
Vegetables (g/d)	baseline	302.1	117.5	293.9	109.0	289.6	118.4	<0.001	0.195	0.369
	5-years	366.0**	122.9	340.7**	115.8	354.3**	120.9			
Fruits (g/d)	baseline	346.3	176.7	354.7	169.3	385.4	183.6	<0.001	0.477	0.103
	5-years	459.4**	181.4	456.4**	172.6	454.0**	158.8			
Legumes (g/d)	baseline	18.7	7.2	19.2	7.2	19.69	8.8	0.445	0.149	0.736
	5-years	18.7	8.3	19.1	7.7	19.9	8.1			
Cereals (g/d)	baseline	240.0	73.2	242.9	79.2	238.9	70.7	<0.001	0.867	0.712
	5-years	221.1**	63.4	216.4**	68.5	216.1**	63.0			
Milk (g/d)	baseline	368.8	201.3	345.4	193.9	393.2	233.2	0.005	0.300	0.166
	5-years	402.2*	223.4	386.4**	204.3	395.6	196.6			
Meat (g/d)	baseline	140.9	49.1	140.1	48.8	138.0	45.3	<0.001	0.992	0.431
	5-years	126.6**	41.9	126.9**	43.3	130.1	44.1			
Fish (g/d)	baseline	94.7	37.2	90.4	39.1	91.7	39.2	<0.001	0.521	0.882
	5-years	101.4	45.6	98.8**	43.2	97.8*	36.2			
Pastries (g/d)	baseline	26.1	26.1	25.7	27.1	26.6	25.1	0.001	0.846	0.485
	5-years	20.1	24.2	23.1	28.4	21.5	27.3			
EVOO (g/d)	baseline	24.1	24.2	21.8	23.8	21.3	22.9	<0.001	0.848	0.346
	5-years	48.2**	22.8	48.1**	25.0	49.7**	23.1			
Nuts (g/d)	baseline	11.0	12.1	9.8	13.1	10.6	13.1	<0.001	0.794	0.656
	5-years	16.0**	12.5	16.1**	13.1	16.7**	12.2			
Wine (g/d)	baseline	98.3	140.1	105.2	157.2	96.8	136.1	0.002	0.781	0.979
	5-years	80.1**	130.5	89.0	130.8	80.7	123.4			

Folic acid (µg/d)	baseline	376.7	83.8	379.4	81.1	381.9	87.6	<0.001	0.939	0.322
	5-years	432.6**	75.9	425.3**	87.3	424.5**	72.4			
Coffee (ml/d)	baseline	38.4	57.0	33.2	44.2	33.9	47.2	0.004	0.902	0.258
	5-years	27.4*	48.0	28.6	46.4	30.7	48.8			
Chocolate (g/d)	baseline	2.9	5.7	2.5	4.7	3.1	5.9	0.940	0.422	0.203
	5-years	2.2	4.2	3.1*	6.1	3.4	7.9			
Total Carbohydrates (g/d)	baseline	235.6	36.5	238.4	43.2	239.9	35.9	0.736	0.964	0.41
	5-years	239.7	63.1	235.0	68.9	235.6	61.0			
Protein (g/d)	baseline	88.4	36.5	91.2	43.2	92.7	35.9	0.12	0.649	0.498
	5-years	94.5	18.6	92.7	19.5	94.4	17.7			
Total Fat (g/d)	baseline	102.5	12.8	100.8	12.6	102.7	13.6	<0.001	0.528	0.331
	5-years	110.7**	23.1	112.9**	25.6	113.4**	24.4			
MUFA (g/d)	baseline	53.5	13.6	52.3	17.4	51.6	15.2	<0.001	0.97	0.19
	5-years	58.3**	12.7	59.6**	13.5	59.8**	13.5			
SFA (g/d)	baseline	25.5	9.0	24.6	10.1	24.0	9.7	0.949	0.887	0.114
	5-years	24.2	6.4	24.8	7.4	25.1	7.3			
PUFA (g/d)	baseline	15.7	4.7	15.7	6.1	15.6	5.4	<0.001	0.716	0.675
	5-years	19.0**	5.9	19.0**	5.6	19.6**	5.5			
Alcohol (g/d)	baseline	14.1	5.2	13.3	5.4	13.4	4.8	0.039	0.979	0.75
	5-years	11.9	14.6	12.4	15.2	12.2	14.7			
Fibre (g/d)	baseline	24.2	6.0	24.6	5.6	25.2	6.4	<0.001	0.564	0.204
	5-years	26.6**	7.5	25.8	7.4	26.4	7.0			
Cholesterol (mg/d)	baseline	352.4	84.6	353.1	94.6	350.5	94.0	0.2	0.946	0.975
	5-years	359.9	90.9	358.0	98.7	356.8	92.7			
Na (mg/d)	baseline	2322.4	479.6	2273.1	528.7	2263.7	479.9	0.736	0.963	0.41
	5-years	2229.8	644.5	2230.8	728.0	2253.7	652.0			
K (mg/d)	baseline	4230.9	723.7	4164.3	682.7	4300.6	796.1	<0.001	0.234	0.542

Mg (mg/d)	5-years	4654.5**	826.8	4546.0**	963.7	4614.7**	805.9	<0.001	0.432	0.365
	baseline	359.5	62.7	358.4	58.1	367.1	61.8			
	5-years	398.5**	82.1	388.1**	86.4	394.3**	80.8			

a. Data are given as means (SD); P < 0.05 indicates statistical significance. EVOO: extra virgin olive oil. GAE: gallic acid equivalent; TPE: total polyphenol excretion. Values with asterisks are statistically different from baseline values by the Paired-samples T test (*P < 0.05; **P < 0.01).

b. Data analysed by repeated-measures 2-factor ANOVA.

c. Comparison between before and after intervention.

d. Comparison between tertiles of TPE changes.

e. Comparison between measurements obtained before and after intervention and between tertiles of TPE changes.

Table 3. Changes in key food intake and nutrients according to the FFQs after energy adjustment categorized by quintile of TPE at 5 years [a]

		TPE (mg GAE/ g creatinine)													
		Q1 (<79.02)		Q2 (79.03-99.50)		Q3 (99.51-124.53)		Q4 (124.54-160.06)		Q5 (>160.07)		P[b]	P[c]		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	ANOVA	TIME	GROUP	INTERACTION
Vegetables (g/d)	baseline	302.6	126.5	295.2	122.6	283.3	114.1	312.9	142.4	297.0	107.0	0.484	<0.001	0.916	0.440
	changes	47.7 **	131.5	53.1 **	130.2	76.3 **	150.4	46.0 **	162.3	59.0 **	131.6	0.514			
Fruits (g/d)	baseline	328.9	184.8	361.6	179.7	358.6	182.3	389.5	162.5	394.5	189.3	0.064	<0.001	0.051	0.530
	changes	89.1 **	220.1	82.9 **	222.7	111.0 **	220.6	80.6 **	208.4	67.9 **	194.8	0.658			
Legumes (g/d)	baseline	18.5	8.4	19.3	7.4	20.0	9.2	19.2	7.2	19.0	6.6	0.634	0.446	0.251	0.045
	changes	0.3	10.7	1.7	11.3	-3.2	29.1	-1.0	8.5	1.0	8.3	0.160			
Cereals (g/d)	baseline	246.8	83.8	246.3	81.2	232.8	74.2	242.9	71.9	237.7	61.9	0.636	<0.001	0.530	0.386
	changes	-22.2 *	91.1	-21.7 **	85.1	-14.5	91.5	-31.4 **	80.1	-25.6 **	77.5	0.673			
Milk (g/d)	baseline	322.8	191.3	370.1	202.2	365.8	210.0	354.5	203.7	422.5	231.6	0.010	0.005	0.009	0.728
	changes	38.3	207.4	21.4	200.9	10.3	198.6	40.2 *	188.3	20.6	208.9	0.776			
Meat (g/d)	baseline	138.1	50.4	136.2	43.9	145.6	56.5	139.3	41.7	141.6	47.1	0.613	<0.001	0.198	0.983

	changes	-9.9	52.1	-13.9 **	44.6	-12.1 *	52.5	-13.8 **	51.5	-11.7 *	47.7	0.975			
Fish (g/d)	baseline	92.0	39.6	92.5	36.9	90.4	38.0	97.0	41.3	93.0	39.0	0.787	0.005	0.970	0.481
	changes	8.2 *	40.4	6.2	42.2	11.5**	44.2	4.2	39.4	7.3	43.3	0.764			
Pastries (g/d)	baseline	29.6	32.1	23.6	22.4	24.1	24.7	23.9	22.4	26.6	26.5	0.379	0.006	0.291	0.920
	changes	-4.8	32.4	-3.0	29.4	-4.3	27.2	-3.2	24.3	-3.9	29.2	0.991			
EVOO (g/d)	baseline	22.7	25.6	20.5	22.4	22.6	23.6	22.9	23.7	22.8	23.3	0.960	<0.001	0.961	0.626
	changes	24.9 **	29.6	27.6 **	27.6	25.4**	28.2	26.4 **	30.1	26.9 **	25.6	0.955			
Olive oil (g/d)	baseline	45.4	17.6	46.3	14.2	45.3	13.5	46.3	14.5	44.2	15.3	0.791	<0.001	0.575	0.161
	changes	7.8 **	18.2	10.5 **	17.5	9.8 **	16.8	9.3 **	18.7	11.2**	16.8	0.654			
Nuts (g/d)	baseline	10.7	14.2	11.2	12.6	10.0	12.7	10.0	13.6	10.9	11.5	0.904	<0.001	0.634	0.192
	changes	3.1	16.4	5.3 **	17.1	5.5 **	15.8	9.5 **	17.3	4.2 **	14.0	0.039			
Wine (g/d)	baseline	104.9	144.8	97.0	138.3	103.9	171.5	98.2	136.2	81.2	125.6	0.739	0.013	0.647	0.984
	changes	-8.4	124.2	-13.3	129.4	-17.1	111.3	-18.1 *	95.3	-14.5	91.9	0.971			
Tea (ml)	baseline	4.8	14.5	4.6	15.1	6.4	17.0	5.2	12.5	7.6	21.1	0.605	0.401	0.479	0.172
	changes	0.1	16.6	-1.9	14.5	-1.8	16.7	3.2	24.9	-2.0	22.4	0.204			
Coffee (ml)	baseline	39.1	58.4	36.7	52.4	30.7	43.0	35.0	47.1	30.9	43.0	0.717	0.002	0.546	0.098
	changes	-11.5 *	50.1	-1.8	50.2	-7.3 *	36.8	-13.1 **	47.0	3.7	51.3	0.048			
Total carbohydrates (g/d)	baseline	237.5	43.2	242.1	38.0	237.0	41.7	235.8	36.3	239.7	34.8	0.682	0.769	0.114	0.025
	changes	4.5	78.6	-10.3	71.6	5.7	72.4	-13.2 *	68.1	-0.4	64.6	0.166			
Protein (g/d)	baseline	90.3	43.2	94.9	38.0	89.7	41.7	88.5	36.3	92.5	34.8	0.682	0.274	0.307	0.474
	changes	2.3	47.6	-3.0	41.4	6.1	46.9	2.5	40.9	4.6	39.6	0.592			
Total Fat (g/d)	baseline	100.4	13.6	100.9	12.7	102.8	13.7	102.7	12.9	104.5	13.6	0.132	<0.001	0.235	0.981
	changes	10.0 **	30.8	9.2 **	28.5	10.9 **	28.0	8.5 **	26.9	9.4 **	31.5	0.980			
Fiber (g/d)	baseline	24.2	6.0	24.5	6.5	24.2	6.5	25.8	5.8	25.5	5.6	0.256	0.006	0.632	0.013
	changes	1.1	8.2	1.4	9.7	3.2 **	8.4	0.2	8.2	1.0	7.8	0.107			
Alcohol (g/d)	baseline	13.5	5.6	13.6	5.2	13.7	5.3	13.3	4.7	14.2	4.9	0.780	0.032	0.961	0.765
	changes	-0.2	17.8	-1.6	15.0	-1.2	16.7	-1.9	14.4	-3.2 *	14.2	0.707			

SFA (g/d)	baseline	24.9	11.2	24.2	9.7	24.5	8.3	25.7	9.1	23.9	9.9	0.663	0.541	0.772	0.266
	changes	-0.5	12.9	-0.1	10.6	0.7	9.8	-1.5	11.1	1.6	11.3	0.301			
MUFA (g/d)	baseline	52.1	16.8	51.3	15.4	52.4	16.0	53.6	14.5	52.4	15.0	0.828	<0.001	0.941	0.694
	changes	6.0 **	20.3	7.5 **	19.2	7.4 **	17.5	4.8 **	18.1	7.1 **	19.0	0.798			
PUFA (g/d)	baseline	15.6	5.2	15.4	5.7	15.5	5.8	16.0	5.2	15.5	5.5	0.887	<0.001	0.575	0.670
	changes	3.0 **	7.7	3.1 **	7.8	3.4 **	7.1	3.4 **	8.1	4.2 **	8.9	0.800			
Folic acid (μg/d)	baseline	379.3	89.5	373.3	90.5	369.8	76.5	394.4	98.8	394.2	85.7	0.152	<0.001	0.447	0.086
	changes	42.7 **	91.3	46.5 **	99.7	65.7 **	92.6	39.2 **	91.6	38.7**	100.4	0.196			
Cholesterol (mg/d)	baseline	354.9	92.4	340.0	92.2	351.4	93.3	353.5	81.6	359.1	95.5	0.505	0.234	0.406	0.329
	changes	0.2	115.0	11.9	101.3	11.4	109.2	-3.5	105.5	15.2	112.6	0.644			
Na (mg/d)	baseline	2331.2	570.5	2254.1	499.7	2272.1	480.3	2286.1	491.3	2298.5	447.1	0.815	0.257	0.258	0.319
	changes	-64.0	941.6	-83.1	728.6	10.6	730.0	-184.2 **	713.4	10.8	752.6	0.306			
K (mg/d)	baseline	4130.5	769.6	4218.7	756.2	4208.9	696.0	4312.6	808.1	4410.2	748.0	0.069	<0.001	0.246	0.067
	changes	379.8 **	1070.7	286.0**	1042.2	497.3 **	1007.2	249.1 *	1116.2	268.1 *	1100.4	0.389			
Mg (mg/d)	baseline	355.0	64.0	361.1	68.6	356.9	56.6	368.6	58.2	374.8	61.9	0.147	<0.001	0.474	0.056
	changes	30.7 **	97.9	26.8 **	99.2	42.9 **	87.9	22.0 **	87.3	23.9 *	95.9	0.481			
P-14 score	baseline	8.9	1.8	9.0	1.8	9.2	1.9	8.8	1.9	9.1	1.8	0.441	<0.001	0.370	0.571
	changes	1.6 **	2.5	1.8**	1.9	1.7 **	2.1	1.9**	2.0	1.6 **	2.1	0.626			
Total Energy intake (Kcal/d)	baseline	2508.8	582.3	2285.5	578.5	2338.5	463.0	2262.2	510.7	2191.7	470.7	<0.001	0.034	<0.001	0.234
	changes	-25.9	627.6	32.0	497.4	100.2*	503.9	8.9	564.0	157.8**	520.1	0.089			

a. Data are given as means (SD); P < 0.05 indicates statistical significance. Values with asterisks are statistically different from baseline by paired-samples T test (*P < 0.05; **P < 0.01).

b. Data analysed by one-way ANOVA.

c. Data analysed by repeated-measures 2-factor ANOVA.

As shown in **table 2**, most key foods changed considerably after the long-term intervention, with the exception of legumes and chocolate. Also, we observed a significant increase in total fat, fibre, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), K, and Mg, while other items such as total carbohydrates, protein, saturated fatty acids (SFA), Na, and cholesterol remained unchanged. This may be due to dietary changes based on recommendations to adhere to a Mediterranean diet, which is characterized by a high consumption of vegetables, fruits, olive oil, wine, and nuts and a low consumption of red meat, high-fat dairy products, and sweets. However, there were no significant changes when comparing tertiles and their interaction. In addition, we found that significant changes in TPE did not significantly affect the intake of nutritional elements among groups.

As shown in **table 3**, at the end of the intervention, the consumption of most of the items belonging to a Mediterranean dietary pattern had increased significantly, including vegetables, fruits, fish, milk, extra virgin olive oil, olive oil, nuts, and coffee. However, intake of wine decreased significantly, as well as intakes of cereals, meat, and pastries. Significant increments were observed in the consumption of total fat, fiber, polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), folic acid, potassium (K) and magnesium (Mg), while total carbohydrates, protein, cholesterol, sodium (Na) and saturated fatty acids (SFA) remained similar throughout.

5.1.3 Associations between polyphenol intake and cardiovascular risk factors

a. Long-term polyphenol intake reduce CVD risk factors such as DBP, glucose and triglycerides.

Table 4 shows multivariate linear regression analyses with changes in cardiovascular risk factors as dependent variables and tertiles of changes in TPE, adjusted for potential confounders. Significant inverse associations were found between tertiles of changes in TPE and glucose ($\beta = -4.372$; $p = 0.026$), triglycerides ($\beta = -8.572$; $p = 0.006$), and DBP ($\beta = -1.156$; $p = 0.031$) after adjustment for potential confounders. However, other parameters did not show significant associations. The standardized coefficients (Beta) in the model were used to measure degrees of contribution to different risk factors. Results indicate that, among the CVD risk factors, triglyceride levels showed the highest beneficial effects of dietary polyphenol intake (Beta = −0.126; $p = 0.031$).

Table 4. Multivariate linear regression analyses with changes in cardiovascular risk factors as dependent variables, and tertiles of changes in TPE in spot urine samples (mg GAE/g creatinine) as exposure variables, adjusted for potential confounders.

		β	SE	Beta	sig.	95% CI	
Change in GLU (mg/dL)	Model 1	-4.164	1.979	-0.095	0.036	-8.053	-0.275
	Model 2	-4.316	1.981	-0.098	0.030	-8.208	-0.424
	Model 3	-4.355	1.949	-0.099	0.026	-8.186	-0.525
	Model 4	-4.372	1.953	-0.099	0.026	-8.209	-0.534
Change in COL (mg/dL)	Model 1	-2.51	2.001	-0.057	0.210	-6.442	1.421
	Model 2	-2.236	2.011	-0.050	0.267	-6.187	1.715
	Model 3	-1.845	2.013	-0.042	0.360	-5.800	2.109
	Model 4	-1.802	2.015	-0.041	0.372	-5.762	2.157
Change in HDL (mg/dL)	Model 1	0.102	0.448	0.010	0.820	-0.778	0.982
	Model 2	0.135	0.448	0.014	0.763	-0.744	1.015
	Model 3	0.133	0.456	0.014	0.771	-0.764	1.030
	Model 4	0.174	0.454	0.018	0.701	-0.718	1.067
Change in LDL (mg/dL)	Model 1	-0.205	1.775	-0.005	0.908	-3.693	3.283
	Model 2	-0.039	1.784	-0.001	0.983	-3.545	3.467
	Model 3	0.448	1.783	0.012	0.802	-3.056	3.952
	Model 4	0.469	1.786	0.012	0.793	-3.041	3.979
Change in TG (mg/dL)	Model 1	-8.356	3.06	-0.123	0.007	-14.369	-2.344
	Model 2	-8.563	3.058	-0.126	0.005	-14.572	-2.554
	Model 3	-8.627	3.094	-0.127	0.006	-14.708	-2.546
	Model 4	-8.572	3.099	-0.126	0.006	-14.662	-2.483
Change in SBP (mm Hg)	Model 1	-1.367	0.994	-0.058	0.169	-3.319	0.585
	Model 2	-1.222	1.001	-0.052	0.222	-3.188	0.744
	Model 3	-1.127	1.003	-0.048	0.262	-3.098	0.843
	Model 4	-1.098	1.005	-0.046	0.275	-3.071	0.876
Change in DBP (mm Hg)	Model 1	-1.316	0.531	-0.104	0.013	-2.359	-0.273
	Model 2	-1.254	0.532	-0.099	0.019	-2.298	-0.209
	Model 3	-1.153	0.532	-0.091	0.031	-2.198	-0.108
	Model 4	-1.156	0.533	-0.091	0.031	-2.203	-0.109
Change in HR	Model 1	-0.002	0.555	0.000	0.997	-1.091	1.087
	Model 2	0.043	0.559	0.003	0.938	-1.055	1.142
	Model 3	-0.011	0.567	-0.001	0.985	-1.125	1.103
	Model 4	-0.074	0.565	-0.006	0.895	-1.184	1.035

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance

Model 1. unadjusted; Model 2 was adjusted for sex, age and intervention groups; Model 3 adjusted as in Model 2 plus BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidaemia, medication use: antihypertensive drugs, vitamins, insulin, oral hypoglycaemic drugs, aspirin or other antiplatelet drug; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score.

b. Long-term polyphenol intake leads to reduction in body weight.

Figure 1 shows comparison of total urinary polyphenol excretion between baseline and 5-year of follow up by quintiles of TPE at 5 years. For the first two quintiles, TPE at baseline was significantly higher than at 5-year. By contrast, TPE at top two categories were higher than at baseline.

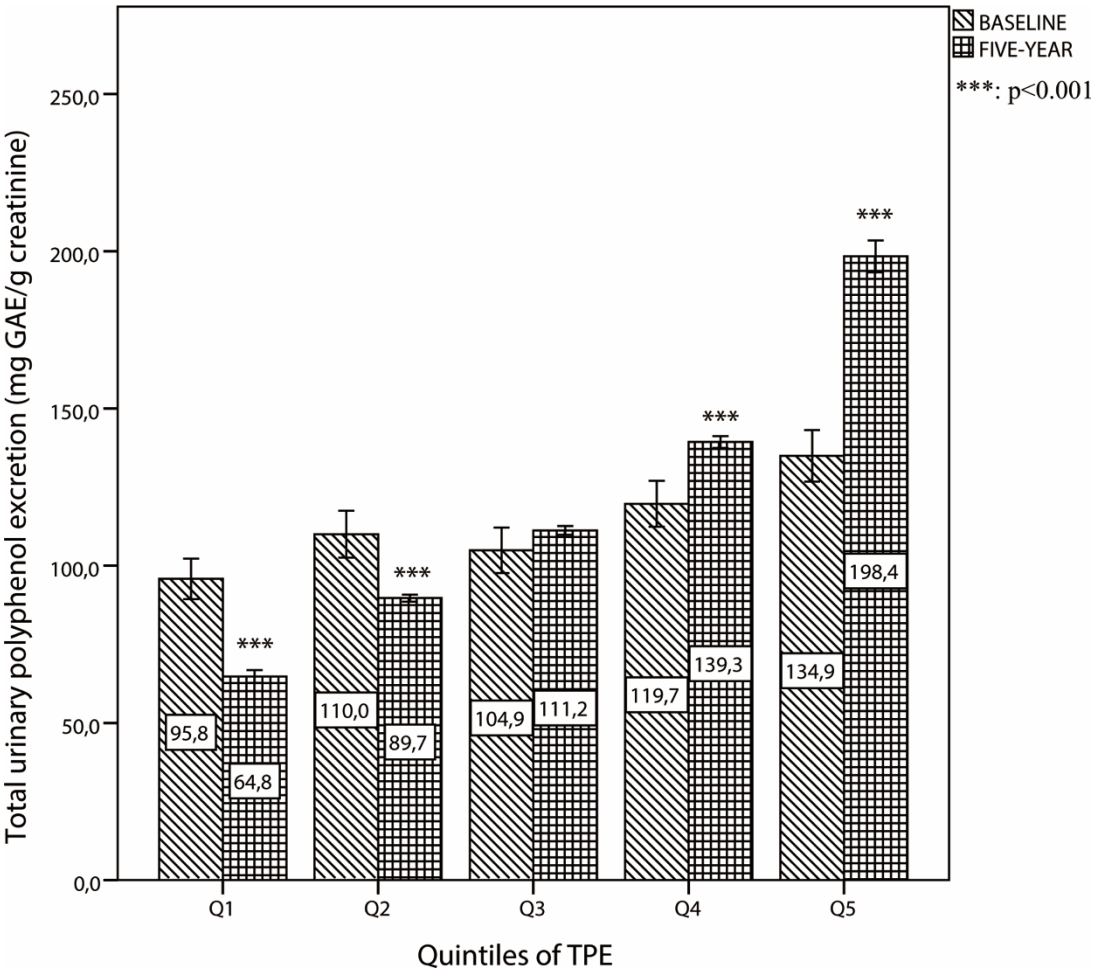


Fig 1. Total polyphenol excretion at baseline and at 5-years of follow-up by quintiles of TPE (at the 5th year).

Table 5 shows changes of obesity parameters during follow-up categorized by quintile of TPE at 5-year. Subjects in the highest TPE category had the lowest BW (70.29±10.25 Kg) and BMI (28.40±3.75 Kg/m2) after the intervention. Inversely, those participants in the first quintile of TPE had significantly higher WC (101.41±9.35 cm) and WHtR (61.80±5.15) compared with baseline values.

Table 5. Comparisons of obesity parameters during follow-up categorized by quintile of TPE (mg GAE/ g creatinine) at 5 years [a]

		Q1 (<79.02)		Q2 (79.03-99.50)		Q3 (99.51-124.53)		Q4 (124.54-160.06)		Q5 (>160.07)		P[b]	P[c]	P[d]
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Q1VsQ5	ANOVA	
BW (kg)	baseline	79.98	11.52	75.24	10.07	76.42	11.55	72.50	10.44	71.52	9.50	<0.001	<0.001	
	5-year	80.50	11.13	75.07	11.10	76.04	11.72	71.92	11.29	70.29**	10.25	<0.001	<0.001	0.101
	changes	0.72	5.06	-0.17	5.06	-0.39	5.04	-0.57	4.23	-1.23**	4.57	0.024	0.045	
BMI (Kg/m^2)	baseline	29.53	2.92	29.07	3.07	29.44	3.41	29.09	3.43	28.90	3.44	1.000	0.549	
	5-year	29.81	3.04	28.98	3.31	29.30	3.63	28.85	3.76	28.40**	3.75	0.027	0.039	0.092
	changes	0.30	1.88	-0.09	2.10	-0.13	1.86	-0.24	1.73	-0.50**	1.87	0.015	0.031	
WC (cm)	baseline	99.96	9.64	96.44	8.88	98.03	10.14	95.18	9.83	94.15	8.60	<0.001	<0.001	
	5-year	101.41**	9.35	97.13	9.90	98.78	9.78	95.89	10.97	94.50	9.50	<0.001	<0.001	<0.001
	changes	0.72**	5.06	-0.17	5.06	-0.39	5.04	-0.57	4.23	-1.23	4.57	0.024	0.045	
WHtR (cm/m)	baseline	60.84	5.09	60.07	5.80	60.95	5.83	60.37	5.98	59.92	5.74	1.000	0.572	
	5-year	61.80**	5.15	60.46	5.94	61.37	5.71	60.80	6.61	60.12	6.36	0.360	0.216	0.001
	changes	1.02**	3.83	0.38	3.89	0.45	3.53	0.51	4.35	0.25	3.60	1.000	0.618	

a. Data are given as means (SD); $P < 0.05$ indicates statistical significance. Values with asterisks are statistically different from baseline by paired-samples T-test ($P < 0.05$): *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

b. Data analyzed by Bonferroni post-hoc comparisons.

c. Data analyzed by ANOVA.

d. Data analyzed by paired-samples T-test.

Table 6 shows associations between associations between polyphenol intake and obesity parameters. Significant inverse associations were found between quintiles of TPE at 5 years and BW (β=-1.004; P=0.002), BMI (β=-0.320; P=0.005), WC (β=-0.742; P=0.013) and WHtR (β=-0.408; P=0.036) after adjustment for potential confounders.

Table 6. Multivariate linear regression analyses with obesity indexes and quintiles of TPE at 5-year

		β	SE	Beta	P	95%CI	
BW (Kg)	Model 1	-2.350	0.331	-0.285	<0.001	-3.000	-1.700
	Model 2	-1.070	0.315	-0.130	0.001	-1.689	-0.451
	Model 3	-1.148	0.323	-0.139	<0.001	-1.783	-0.513
	Model 4	-1.004	0.320	-0.124	0.002	-1.634	-0.375
BMI (Kg/m^2)	Model 1	-0.295	0.104	-0.118	0.005	-0.499	-0.090
	Model 2	-0.328	0.110	-0.131	0.003	-0.544	-0.111
	Model 3	-0.358	0.113	-0.143	0.002	-0.580	-0.136
	Model 4	-0.320	0.113	-0.129	0.005	-0.541	-0.098
WC (cm)	Model 1	-1.500	0.296	-0.208	<0.001	-2.082	-0.918
	Model 2	-0.721	0.293	-0.100	0.014	-1.296	-0.147
	Model 3	-0.877	0.302	-0.122	0.004	-1.471	-0.283
	Model 4	-0.742	0.297	-0.104	0.013	-1.326	-0.158
WHtR(cm/m)	Model 1	-0.298	0.178	-0.070	0.094	-0.648	0.051
	Model 2	-0.367	0.189	-0.087	0.052	-0.739	0.004
	Model 3	-0.474	0.195	-0.112	0.016	-0.857	-0.090
	Model 4	-0.408	0.194	-0.097	0.036	-0.788	-0.028

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance.

Model 1. unadjusted; Model 2 was adjusted for sex, age and intervention groups; Model 3 adjusted as in Model 2 plus smoking status (never, current, former), family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) and recruitment centers; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score and energy intake at baseline

Table 7 shows the OR (95% confidence interval) for obesity according to the quintile of TPE at 5 years. In fully-adjusted models, participants in the category of highest TPE had a lower prevalence of obesity (odds ratio (OR) = 0.346, 95% confidence interval (CI) 0.176 to 0.178; P-trend, 0.039) than those in the lowest category.

Table 7 Multivariate adjusted odds ratios (95% CI) for prevalent obesity (213 cases) after 5-year follow-up.

	Q1	Q2	95% CI		Q3	95% CI		Q4	95% CI		Q5	95% CI		P-trend
Model 1	1 (ref.)	0.639	0.375	1.089	0.769	0.454	1.302	0.664	0.390	1.129	0.450	0.259	0.782	0.073
Model 2	1 (ref.)	0.597	0.344	1.035	0.691	0.400	1.192	0.618	0.350	1.091	0.383	0.211	0.694	0.036
Model 3	1 (ref.)	0.559	0.314	0.995	0.649	0.367	1.147	0.543	0.296	0.996	0.318	0.166	0.606	0.015
Model 4	1 (ref.)	0.604	0.332	1.100	0.720	0.399	1.300	0.560	0.298	1.054	0.346	0.176	0.678	0.039

Obesity was defined as BMI>30 kg/m^2.

Model 1. unadjusted; Model 2 was adjusted for sex, age and intervention groups; Model 3 adjusted as in Model 2 plus smoking status (never, current, former), family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) and recruitment centers; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score and energy intake at baseline.

Table 8 shows sensitivity analyses to account for differences in sex and age. In fully-adjusted models, only males showed significant inverse associations with BW (β=-1.004; P=0.031) and BMI (β=-0.298; P=0.036). For age categories (<67 years and ≥67 years), all adiposity parameters [BW (β=-1.358; P=0.002), BMI (β=-0.466; P=0.003), WC (β=-1.061; P=0.012), WHtR (β=-0.623; P=0.023))] were lower in the older group (age≥67).

Table 8. Sensitivity analyses of obesity indexes with linear regression analyses.

		BW						BMI						WC						WHtR					
		β	SE	Beta	sig.	95%CI		β	SE	Beta	sig.	95%CI		β	SE	Beta	sig.	95%CI		β	SE	Beta	sig.	95%CI	
Sex	Male	-1.004	0.462	-0.133	0.031	-1.915	-0.094	-0.298	0.142	-0.130	0.036	-0.577	-0.020	-0.502	0.379	-0.082	0.187	-1.249	0.246	-0.230	0.230	-0.063	0.319	-0.684	0.224
	Female	-0.747	0.458	-0.101	0.104	-1.649	0.155	-0.265	0.176	-0.094	0.134	-0.612	0.082	-0.766	0.463	-0.105	0.099	-1.677	0.145	-0.483	0.314	-0.097	0.125	-1.100	0.135
Age (year)	<67	-0.638	0.495	-0.081	0.198	-1.614	0.337	-0.174	0.167	-0.073	0.299	-0.502	0.155	-0.351	0.432	-0.053	0.417	-1.203	0.500	-0.152	0.280	-0.039	0.588	-0.704	0.400
	≥67	-1.358	0.430	-0.164	0.002	-2.204	-0.512	-0.466	0.157	-0.176	0.003	-0.774	-0.158	-1.061	0.418	-0.137	0.012	-1.883	-0.239	-0.623	0.272	-0.136	0.023	-1.157	-0.088

BW: body weight; BMI: body mass index; WC: waist circumference; WHtR: waist to height ratio.

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance

Model was adjusted for sex, age, intervention groups, smoking status (never, current, former), family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) recruitment centers, 14-unit Mediterranean diet score and energy intake at baseline.

5.2 Effects of intake of total polyphenols and some classes of polyphenols on diabetes in elderly people at high cardiovascular disease risk.

5.2.1 Baseline characteristics of participants

Table 9 show the basic information of the participants at baseline. The present study was conducted on 3430 subjects: 1314 men aged 65.2 ± 6.3 y and 2116 women aged 67.5 ± 5.6 y. At baseline, participants in the third tertile were more likely to be men (P < 0.001), younger (P < 0.001), and current (P < 0.001) or former smokers (P = 0.03); have a lower BMI (in kg/m^2; P = 0.02); and be more physically active (P < 0.001). They also had a higher adherence to the traditional MedDiet (P < 0.001) and tended to consume foods with a high polyphenol content, such as fruits and vegetables, nuts, coffee, and wine (P < 0.001). Those with a lower intake of polyphenols had a lower education level (P < 0.001), were more hypertensive (P = 0.003), and had a higher waist-to-height ratio (P < 0.001).

Table 9. Baseline characteristics of the PREDIMED study cohort according to tertiles of calorie-adjusted total polyphenol intake at baseline[1]

Characteristics	T1 (*n* = 1143)	T2 (*n* = 1144)	T3 (*n* = 1143)	P[2]
Polyphenol intake (cutoff values), mg/d	554 ± 103 (<701)	805 ± 63 (701–914)	1131 ± 203 (>914)	
Female	722 (63.2)[b]	778 (68.0)[b]	616 (53.9)[a]	<0.001
Age, y	66.9 ± 6.1[a]	67.1 ± 5.9[a]	66.0 ± 5.9[b]	<0.001
BMI, kg/m^2	30.2 ± 3.5[a]	29.8 ± 3.4[b]	29.8 ± 3.5[b]	0.02
Leisure-time physical activity, MET-min/d	211 ± 214[b]	223 ± 205[b]	263 ± 248[a]	<0.001
Smoking				<0.001
Never	746 (65.3)[b]	774 (67.7)[b]	613 (53.6)[a]	
Current	196 (17.1)[b]	174 (15.2)[b]	283 (24.8)[a]	
Former	201 (17.6)[b]	196 (17.1)[b]	247 (21.6)[a]	
Education				<0.001
Primary	906 (79.3)[b]	885 (77.3)[b]	802 (70.2)[a]	
Secondary	174 (15.2)	179 (15.7)	201 (17.6)	
Academic/graduate	63 (5.5)[b]	80 (7.0)[b]	104 (12.2)[a]	
Intervention group				<0.001
MedDiet–EVOO	384 (33.6)	380 (33.2)	364 (31.9)	
MedDiet–nuts	374 (32.7)[a]	387 (33.8)[a,b]	437 (38.2)[b]	
Low-fat diet (control group)	385 (33.7)	377 (33.0)	342 (29.9)	
Drug use				
Hypolipidemic	539 (47.2)[a]	591 (51.7)[b]	579 (50.9)[a,b]	0.07

Antihypertensive	907 (79.5)[a]	878 (76.8)[a,b]	863 (75.8)[b]	0.09
Aspirin	174 (15.3)	211 (18.4)	201 (17.7)	0.11
Multivitamins	152 (13.3)	165 (14.5)	142 (12.5)	0.36
Mean intake				
Total energy intake, kcal/d	2372 ± 621[a]	2196 ± 513[b]	2310 ± 558[c]	<0.001
Carbohydrates, g/d	258 ± 87[a]	235 ± 71[b]	248 ± 75[c]	<0.001
Protein, g/d	94.5 ± 22.4[a]	89.3 ± 19.0[b]	91.0 ± 20.7[c]	<0.001
SFAs, g/d	26.6 ± 9.7[a]	23.9 ± 7.7[b]	23.9 ± 8.7[b]	<0.001
MUFAs, g/d	49.9 ± 15.4[a]	46.6 ± 13.6[b]	47.3 ± 14.4[b]	<0.001
PUFAs, g/d	16.2 ± 6.7[a]	14.9 ± 6.0[b]	15.3 ± 6.3[b]	<0.001
Fiber, g/d	23.2 ± 7.6[a]	24.7 ± 7.7[b]	28.7 ± 9.7[c]	<0.001
Total cholesterol, mg/d	378 ± 135[a]	357 ± 117[b]	352 ± 112[b]	<0.001
Alcohol, g/d	6.2 ± 10.9[a]	7.4 ± 12.2[b]	14.4 ± 19.3[c]	<0.001
Vegetables, g/d	303 ± 125[a]	322 ± 132[b]	359 ± 152[c]	<0.001
Fruits, g/d	279 ± 152[a]	359 ± 170[b]	480 ± 227[c]	<0.001
Legumes, g/d	20.8 ± 15.7	20.3 ± 11.3	20.5 ± 12.3	0.63
Cereals, g/d	256 ± 117[a]	220 ± 95[b]	217 ± 94[b]	<0.001
Dairy products, g/d	389 ± 228[b]	371 ± 212[b]	350 ± 220[a]	<0.001
Meat or meat products, g/d	139 ± 61[a]	129 ± 51[b]	129 ± 52[b]	<0.001
Fish, g/d	96.0 ± 44.5[b]	96.3 ± 45.3[b]	101 ± 45.5[a]	0.01
Sugar-sweetened soft drinks, g/d	28.9 ± 88.9[a]	21.1 ± 69.4[b]	17.9 ± 60.1[b]	0.001
Nuts, g/d	9.7 ± 13.1[b]	8.7 ± 1.9[b]	11.4 ± 14.0[a]	0.003
Coffee, mL/d	45.2 ± 42.6[a]	65.8 ± 44.9[b]	90.8 ± 58.8[c]	<0.001
Tea, mL/d	4.9 ± 20.1	5.2 ± 18.0	6.5 ± 25.2	0.17
Wine, mL/d	36.5 ± 74.0[a]	52.3 ± 94.2[b]	110.3 ± 154.0[c]	<0.001
14-point MedDiet score	8.24 ± 1.89[a]	8.67 ± 1.93[b]	9.08 ± 1.84[c]	<0.001
Clinical variables				
Hypertension	1076 (94.1)[a]	1049 (91.7)[b]	1034 (90.5)[b]	<0.001
Hypercholesterolemia	925 (80.9)[a]	999 (87.3)[b]	991 (86.7)[b]	<0.001
Waist-to-height ratio	0.63 ± 0.06[a]	0.62 ± 0.06[b]	0.62 ± 0.06[b]	<0.001
Systolic BP, mm Hg	149 ± 19[a]	148 ± 19[b]	148 ± 18[b]	0.02
Diastolic BP, mm Hg	84 ± 10	84 ± 10	84 ± 10	0.41
Glucose,[3] mg/dL	98 ± 15	98 ± 16	99 ± 16	0.57
Total cholesterol,[3] mg/dL	210 ± 37	214 ± 39	214 ± 38	0.09
HDL cholesterol,[3] mg/dL	52 ± 12	53 ± 11	53 ± 11	0.05
LDL cholesterol,[3] mg/dL	139 ± 34	140 ± 33	140 ± 34	0.83
TGs,[3] mg/dL	128 ± 73	129 ± 71	129 ± 63	0.94

1 Values are frequencies (percentages) for categorical variables or means 6 SDs for continuous variables; n = 3430. Values in a row without a common superscript letter are significantly different, P< 0.05. BP, blood pressure; MedDiet–EVOO, Mediterranean diet supplemented with extra-virgin olive oil; MedDiet–nuts, Mediterranean diet supplemented with nuts; MET, metabolic equivalent task; PREDIMED, Prevención con Dieta Mediterránea, T tertile.

2 Calculated by ANOVA or x^2 tests.

3 Measured in plasma.

5.2.2 Cox proportional HRs for new-onset diabetes

Table 10 shows the Cox proportional HRs for type 2 diabetes according to tertiles of cumulative intake of total polyphenols (adjusted for calories) and the main polyphenol groups. During a median of 5.5±2.0 y of follow-up (18,900 person-years), a total of 314 incident cases of diabetes were diagnosed (9.1%).After adjustment for anthropometric, sociodemographic, lifestyle, and dietary variables (fully adjusted model) and stratifying by sex, recruitment center, and intervention group, significant and linear inverse associations were found for total polyphenols (HR: 0.72; 95% CI: 0.52, 0.99; P-trend = 0.05), total flavonoids (HR: 0.67; 95% CI: 0.48, 0.93; P-trend = 0.02), and stilbenes (HR: 0.57; 95% CI: 0.38, 0.84; P-trend = 0.003), whereas nonsignificant results were found for other polyphenol groups.

Table 10. Cox proportional HRs for new-onset diabetes in the PREDIMED cohort by cumulative intake of polyphenols, adjusted for energy intake and divided into tertiles[1]

	T1	T2	T3	P-trend[2]	P[3]
Total polyphenols, mg/d	600 (518, 653)	781 (739, 825)	1002 (929, 1119)		
Cases, *n*	117	103	94		
Person-years, *n*	5910	6785	6205		
Follow-up, y	5.6 (4.0, 7.2)	5.4 (4.0, 7.2)	5.3 (3.9, 7.2)		0.7
Incidence, %	10.9	8.4	8.3		0.06
Model 1	1.00 (ref)	0.82 (0.62, 1.07)	0.81 (0.61, 1.08)	0.15	
Model 2	1.00 (ref)	0.78 (0.59, 1.04)	0.74 (0.55, 0.99)	0.04	
Model 3	1.00 (ref)	0.74 (0.54, 1.00)	0.72 (0.52, 0.99)	0.05	
Flavonoids, mg/d	291 (236, 334)	425 (392, 462)	596 (533, 698)		
Cases, *n*	133	91	90		
Person-years, *n*	5659	6685	6556		
Follow-up, y	5.0 (3.9, 7.1)	5.9 (4.0, 7.3)	5.8 (4.0, 7.3)		<0.0001
Incidence, %	12.4	7.7	7.7		<0.0001
Model 1	1.00 (ref)	0.66 (0.50, 0.87)	0.69 (0.52, 0.92)	0.01	
Model 2	1.00 (ref)	0.64 (0.48, 0.85)	0.69 (0.51, 0.93)	0.02	
Model 3	1.00 (ref)	0.60 (0.44, 0.82)	0.67 (0.48, 0.93)	0.02	
Phenolic acids, mg/d	164 (130, 192)	256 (234, 279)	381 (342, 442)		
Cases, *n*	101	109	104		
Person-years, *n*	6577	6555	6767		
Follow-up, y	6.0 (4.1, 7.3)	5.8 (4.0, 7.2)	4.9 (3.8, 7.1)		<0.0001
Incidence, %	8.7	9.3	9.5		0.83
Model 1	1.00 (ref)	1.03 (0.78, 1.36)	1.03 (0.78, 1.37)	0.84	
Model 2	1.00 (ref)	1.06 (0.80, 1.41)	0.96 (0.71, 1.29)	0.73	
Model 3	1.00 (ref)	0.89 (0.65, 1.21)	0.85 (0.62, 1.17)	0.34	
Stilbenes, mg/d	0.04 (0, 0.17)	1.01 (0.73, 1.35)	3.89 (2.77, 6.95)		
Cases, *n*	102	115	97		

Person-years, *n*	3141	6519	6238		
Follow-up, y	5.4 (4.0, 7.3)	5.1 (4.0, 7.1)	6.0 (4.0, 7.3)		0.003
Incidence, %	9.2	9.5	8.8		0.85
Model 1	1.00 (ref)	1.08 (0.82, 1.42)	0.81 (0.60, 1.09)	0.09	
Model 2	1.00 (ref)	1.01 (0.76, 1.34)	0.84 (0.62, 1.14)	0.23	
Model 3	1.00 (ref)	0.90 (0.64, 1.27)	0.57 (0.38, 0.84)	0.003	
Lignans, mg/d	0.42 (0.35, 0.47)	0.59 (0.56, 0.63)	0.78 (0.73, 0.88)		
Cases, *n*	111	96	107		
Person-years, *n*	5401	6345	7153		
Follow-up, y	4.9 (3.6, 7.0)	5.4 (4.0, 7.1)	6.1 (4.4, 7.4)		<0.0001
Incidence, %	10.5	8.3	8.8		0.17
Model 1	1.00 (ref)	0.73 (0.55, 0.97)	0.78 (0.58, 1.05)	0.12	
Model 2	1.00 (ref)	0.68 (0.51, 0.92)	0.75 (0.55, 1.01)	0.08	
Model 3	1.00 (ref)	0.68 (0.48, 0.94)	0.82 (0.58, 1.15)	0.35	
Others,[4] mg/d	41.3 (32.8, 47.3)	63.3 (58.1, 69.5)	96.5 (85.3, 115.0)		
Cases, *n*	90	113	111		
Person-years, *n*	5701	7143	6055		
Follow-up, y	5.1 (3.9, 7.2)	5.9 (4.0, 7.3)	5.4 (4.0, 7.2)		0.004
Incidence, %	8.5	8.9	10.1		0.43
Model 1	1.00 (ref)	0.97 (0.72, 1.29)	1.08 (0.81, 1.45)	0.51	
Model 2	1.00 (ref)	0.95 (0.71, 1.28)	1.06 (0.79, 1.44)	0.6	
Model 3	1.00 (ref)	0.98 (0.71, 1.36)	0.97 (0.70, 1.36)	0.89	

1 Values are HRs (95% CIs), unless otherwise indicated. Polyphenol intake and follow-up values are medians (25th, 75th percentiles). Model 1 is adjusted for age and stratified by sex, recruitment center, and intervention group. Model 2 is adjusted for factors in model 1, in addition to smoking, BMI, physical activity, dyslipidemia, hypertension, and education level. Model 3 is adjusted for factors in model 2, in addition to total energy intake, alcohol intake, adherence to the Mediterranean diet, and fasting glucose concentrations at baseline. PREDIMED, Prevención con Dieta Mediterránea; ref, reference; T, tertile.

2 Based on tests for trend across tertiles of polyphenol intake by assigning the median value of each tertile.

3 Calculated by ANOVA (continuous variables) or $\chi 2$ tests (categorical variables).

4 Includes alkylmethoxyphenols, alkylphenols, curcuminoids, furanocoumarins, hydroxybenzaldehydes, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxycoumarins, hydroxyphenylpropenes, methoxyphenols, naphtoquinones, phenolic terpenes, and tyrosols.

5.2.3 The HRs for new-onset type 2 diabetes

Table 11 shows The HRs for new-onset type 2 diabetes and tertiles of cumulative flavonoid class intake. Dihydroflavonols and flavanones were significantly associated with theriskoftype2diabetes in thefully adjusted model when comparing the third with the first tertile (HR: 0.59; 95% CI: 0.40, 0.88; P-trend = 0.003; and HR: 0.69; 95% CI: 0.49, 0.97; P-trend = 0.03, respectively). Nevertheless, it is worth mentioning that, for catechins, the middle tertile was significantly associated with the risk of type 2 diabetes compared with the first tertile, even in the fully adjusted model (HR: 0.61; 95% CI: 0.44, 0.85). This

association was not observed for the group with the highest intake (HR: 0.84; 95% CI: 0.60, 1.17; P-trend = 0.45). There were substantial changes from model 2 to model 3; for instance, HRs for stilbenes changed from 0.84 to 0.57, and for dihydroflavonols, from 0.87 to 0.59, when comparing the third to the first tertile, and flavonols changed from 0.93 to 0.77 when comparing the second to the first tertile. This was due to the inclusion of both alcohol and fasting glucose concentrations at baseline in the model.

Table 11. Cox proportional HRs for new-onset diabetes in the PREDIMED cohort by cumulative intake of flavonoid classes, adjusted for energy intake and divided into tertiles1

	T1	T2	T3	P-trend[2]	P[3]
Anthocyanidins, mg/d	14.9 (8.6, 19.6)	30.9 (26.9, 35.3)	58.9 (48.3, 77.1)		
Cases, n	104	97	113		
Person-years, n	6642	6485	5772		
Follow-up, y	5.2 (3.9, 7.2)	5.9 (4.0, 7.3)	5.6 (4.0, 7.2)		0.02
Incidence, %	8.5	8.4	10.7		0.11
Model 1	1.00 (ref)	0.84 (0.63, 1.12)	0.99 (0.75, 1.30)	0.89	
Model 2	1.00 (ref)	0.84 (0.63, 1.13)	0.96 (0.72, 1.27)	0.89	
Model 3	1.00 (ref)	0.82 (0.59, 1.13)	0.88 (0.62, 1.24)	0.54	
Catechins, mg/d	13.8 (10.5, 16.4)	23.2 (20.8, 26.2)	39.4 (33.7, 48.2)		
Cases, n	125	83	106		
Person-years, n	6080	6593	6227		
Follow-up, y	5.4 (4.0, 7.3)	5.5 (4.0, 7.2)	5.4 (4.0, 7.2)		0.35
Incidence, %	11.3	7	9.3		0.002
Model 1	1.00 (ref)	0.64 (0.48, 0.85)	0.85 (0.64, 1.11)	0.37	
Model 2	1.00 (ref)	0.64 (0.47, 0.85)	0.84 (0.63, 1.11)	0.35	
Model 3	1.00 (ref)	0.61 (0.44, 0.85)	0.84 (0.60, 1.17)	0.45	
Dihydrochalcones, mg/d	0.99 (0.41, 1.38)	2.40 (2.00, 2.77)	3.96 (3.48, 6.18)		
Cases, n	117	97	100		
Person-years, n	5477	6906	6516		
Follow-up, y	5.1 (3.9, 7.1)	6.0 (4.1, 7.4)	5.3 (3.9, 7.2)		<0.0001
Incidence, %	11.3	8.1	8.4		0.22
Model 1	1.00 (ref)	0.77 (0.58, 1.01)	1.00 (0.75, 1.01)	0.99	
Model 2	1.00 (ref)	0.79 (0.60, 1.06)	1.00 (0.74, 1.35)	0.92	
Model 3	1.00 (ref)	0.88 (0.64, 1.19)	1.15 (0.83, 1.61)	0.44	
Dihydroflavonols, mg/d	0 (0, 0.16)	1.48 (1.03, 2.03)	6.09 (4.31, 11.0)		
Cases, n	100	117	97		
Person-years, n	6132	6546	6220		
Follow-up, y	5.6 (4.0, 7.3)	5.1 (3.9, 7.1)	6.0 (4.0, 7.3)		0.002
Incidence, %	9	9.6	8.8		0.8
Model 1	1.00 (ref)	1.13 (0.86, 1.49)	0.83 (0.62, 1.12)	0.12	
Model 2	1.00 (ref)	1.05 (0.79, 1.39)	0.87 (0.64, 1.17)	0.27	
Model 3	1.00 (ref)	0.99 (0.70, 1.38)	0.59 (0.40, 0.88)	0.003	
Proanthocyanidins, mg/d	74.5 (54.3, 87.4)	122 (111, 134)	187 (164, 228)		

Cases, n	122	102	90		
Person-years, n	5778	6831	6290		
Follow-up, y	5.1 (3.9, 7.2)	5.8 (4.0, 7.3)	5.6 (4.0, 7.2)		0.003
Incidence, %	11.3	8.4	7.9		0.01
Model 1	1.00 (ref)	0.78 (0.59, 1.02)	0.73 (0.55, 0.97)	0.04	
Model 2	1.00 (ref)	0.80 (0.61, 1.06)	0.70 (0.52, 0.95)	0.02	
Model 3	1.00 (ref)	0.75 (0.55, 1.02)	0.75 (0.54, 1.04)	0.09	
Flavanones, mg/d	43.4 (15.9, 63.8)	114 (96.4, 132)	197 (166, 292)		
Cases, n	121	105	88		
Person-years, n	5206	6670	7024		
Follow-up, y	5.1 (3.9, 7.1)	6.0 (4.1, 7.3)	5.6 (3.9, 7.3)		<0.0001
Incidence, %	12.3	8.9	7		<0.0001
Model 1	1.00 (ref)	0.77 (0.59, 1.01)	0.73 (0.55, 0.98)	0.04	
Model 2	1.00 (ref)	0.81 (0.61, 1.07)	0.74 (0.54, 1.00)	0.05	
Model 3	1.00 (ref)	0.87 (0.65, 1.17)	0.69 (0.49, 0.97)	0.03	
Flavones, mg/d	21.5 (16.6, 25.1)	34.7 (31.5, 38.0)	56.8 (48.4, 71.2)		
Cases, n	116	94	104		
Person-years, n	5328	6474	7098		
Follow-up, y	5.1 (3.9, 7.1)	5.9 (4.0, 7.2)	5.8 (4.0, 7.3)		<0.0001
Incidence, %	11.5	8.1	8.3		0.009
Model 1	1.00 (ref)	0.78 (0.59, 1.03)	0.87 (0.66, 1.15)	0.46	
Model 2	1.00 (ref)	0.79 (0.49, 1.06)	0.87 (0.65, 1.17)	0.46	
Model 3	1.00 (ref)	0.78 (0.56, 1.06)	0.98 (0.71, 1.35)	0.92	
Flavonols, mg/d	57.5 (46.8, 64.5)	82.9 (76.9, 87.9)	106 (99.1, 122)		
Cases, n	114	100	100		
Person-years, n	6242	6279	6378		
Follow-up, y	5.0 (3.9, 7.0)	5.7 (4.0, 7.3)	6.0 (4.2, 7.4)		<0.0001
Incidence, %	9.6	8.9	9		0.84
Model 1	1.00 (ref)	0.94 (0.71, 1.25)	1.03 (0.75, 1.42)	0.88	
Model 2	1.00 (ref)	0.93 (0.69, 1.24)	1.04 (0.75, 1.45)	0.96	
Model 3	1.00 (ref)	0.77 (0.56, 1.06)	0.97 (0.68, 1.39)	0.77	

1Values are HRs (95% CIs), unless otherwise indicated. Polyphenol intake and follow-up values are medians (25th, 75th percentiles). Model 1 is adjusted for age and stratified by sex, recruitment center, and intervention group. Model 2 is adjusted for factors in model 1, in addition to smoking, BMI, physical activity, dyslipidemia, hypertension, and education level. Model 3 is adjusted for factors in model 2, in addition to total energy intake, alcohol intake, adherence to the Mediterranean diet, and fasting glucose concentrations at baseline. PREDIMED,

Prevenci´on con Dieta Mediterr´anea; ref, reference; T, tertile.

2 Based on tests for trend across tertiles of polyphenol intake by assigning the median value of each tertile.

3 Calculated by ANOVA (continuous variables) or x^2 tests (categorical variables)

Figure 2 shows the HRs and 95% CIs of diabetes risk, comparing the highest with the lowest tertile of intake of total polyphenols and subclasses after adjustment for all potential confounders.

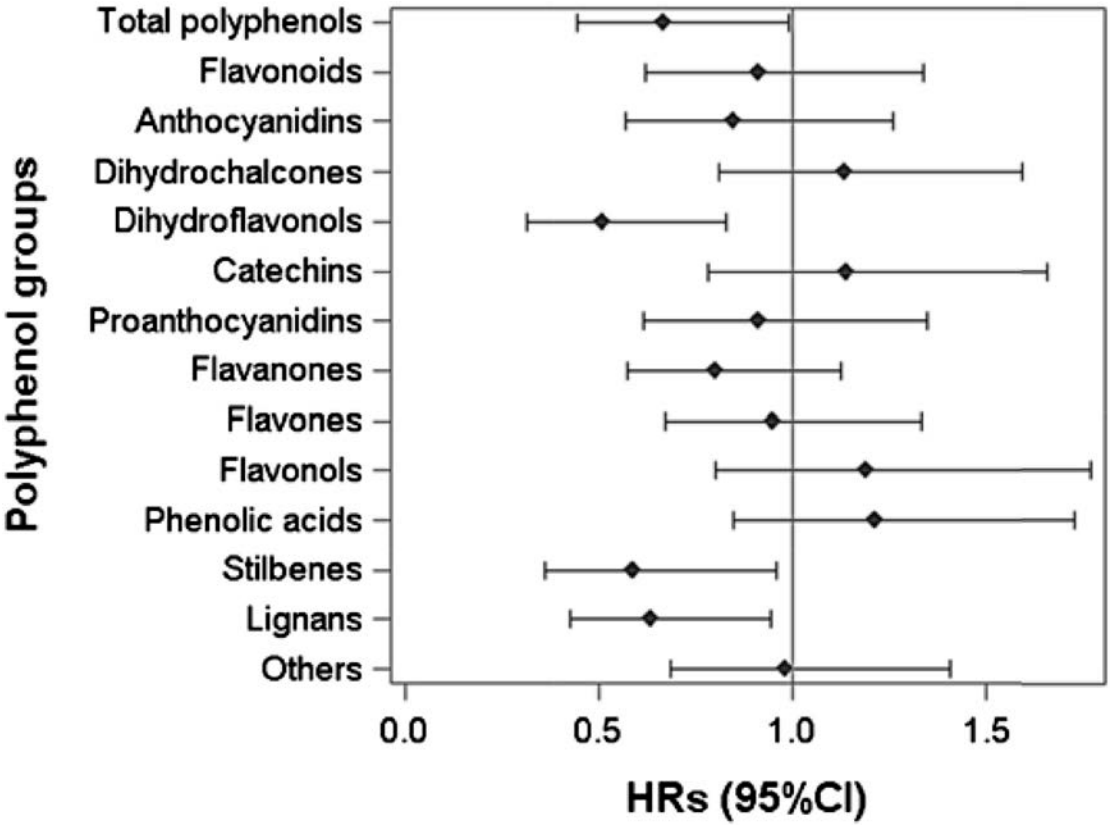


Figure 2. HRs (95% CIs) of diabetes incidence for the highest compared with the lowest tertile of polyphenol intake (fully adjusted model) in the PREvención con Dieta MEDiterránea study cohort (n = 3430).

We also conducted stratified analyses by different predictors of diabetes and total polyphenol intake. None of the stratified results had a significant interaction term; therefore, we cannot draw conclusions.

Chapter 6

Discussion

6 Discussion

The Mediterranean diet has shown beneficial effects on human health [3,159,164–166]. This dietary pattern promotes a high consumption of fruits, vegetables, olive oil, legumes, and unrefined cereals; relatively low consumption of meat; and moderate consumption of red wine. To data, the evidence concerning the potential mechanisms of action which underlie the cardio-protective effects may be attributed to a high amount of dietary fiber, vitamins, folic acid, natural antioxidants, monounsaturated fat; moderate amounts of animal protein, moderate amount of alcohol mainly in the form of wine; and low amount of saturated and trans fats [3].

Numerous epidemiological and human intervention studies have suggested that regular consumption of polyphenol-rich foods, such as fruits, vegetables, olive oil and wine, may exert cardio-protective effects in humans [35–37].

Compared with current studies, one of potential limitation is the estimation of polyphenol intake [38,66,167,168]. Most of previous studies, data on the polyphenol content in foods were obtained from the USDA or Phenol-Explorer database and the correspondence between food items in the FFQ and the database, therefore, the absence of information about some foods could lead to an underestimation of the intake. Moreover, the study did not consider the bioavailability of the polyphenol intakes.

6.1 Urinary polyphenol excretion

6.1.1 Folin-Ciocalteu method and urinary polyphenol excretion

We used the Folin-Ciocalteu method with solid phase extraction to determine TPE in urine samples., Folin-Ciocalteu method could be applied for determining total polyphenols. Previous studies concerned the determination of total polyphenol are mostly focus on nature extraction [169], only limited studies have been applied on biological samples in clinical studies [145,170,171]. To avoid the presence of reducing

interferants in the biological samples, we used of solid phase extraction (SPE) cartridges to separate interferants from phenolics. TPE among 573 participants, randomly selected from the 7447 participants of the PREDIMED study, were 113.1±41.8 mg GAE/g creatinine at baseline and 120.6±48.1 mg GAE/g creatinine at 5 years. A former study assessed the normal distribution of TPE among healthy male children and adolescents on a typical Egyptian diet, showing an averaged 89.5±8.4 mg GAE/g creatinine [172]. The major difference may be raised from variation in the sources and amount of dietary polyphenol, as well as the age of participants. Another human study showed a mean TPE of 67.82±1.83 mg GAE/g creatinine after 1-year of intervention in healthy adults without any major CVD risk factors from Spain [173]. Both of their results are lower than ours, which may be explained by the observed positive relationship between TPE and ages that our participants were older (67.3±5.9) than theirs (44.6±15.8 for women and 42.3 ±16.4 for men) [173].

6.1.2 Urinary polyphenol excretion in subgroup analysis

Regarding TPE variation due to gender, TPE was higher in women (133.3±48.7 mg GAE/g creatinine) than in men (107.1±43.8 mg GAE/g creatinine) after five years of intervention. Similar trends were observed for participants at baseline. Assuming that the amount of polyphenols excreted in urine corresponds to the intake, our finding is in agreement with a previous study concerning total polyphenol intakes that 869.6±343.3 mg/d among women and 840.1±317.9 mg/d among men after energy-adjustment [15]. However, in the SU.VI.MAX study, results were different from ours as mean total polyphenol intake was higher in men [174]. This might be because their results were not adjusted for energy intake; besides, diversity of country-specific food resource is another plausible reason for the difference. Comparing different age groups, younger participants (<67 years old) showed lower TPE (110.9±46.5mg GAE/g creatinine) than those over 67 years (130.1±47.9 mg GAE/g creatinine) at 5-year. According to WHO recommendations, it is the fact that elderly adults are the highest consumers of fruits and vegetables, thus possibly explained a higher intake of polyphenols among elderly

participants [17]. However, compared with previous studies, results are inconsistent. A study conducted in Japan indicated that polyphenol intake was higher in participants with higher age, while the HAPIEE and EPIC studies showed inverse associations [12,15,175]. Aging affects dietary habits and it is an important factor related to other cardiovascular risk factors

Regarding the smoking status, former smoker (122.3±50.6 mg GAE/g creatinine) and none smoker groups (124.1±47.1 mg GAE/g creatinine) showed higher TPE than smokers (105.9±46.3 mg GAE/g creatinine) at 5 years of intervention And same trends were found at baseline that former smoker (110.4±39.9 mg GAE/g creatinine) and none smoker groups (118.1±43.0 mg GAE/g creatinine) showed higher TPE than smokers (98.5±35.7 mg GAE/g creatinine). To data, few studies have analyzed the association between polyphenol excretion and smoking habits. One sub-study from PREDIMED agreed with our findings, showing the same negative trends regarding TPE among smokers and non-smokers [66]. Oppositely, one study showed higher polyphenol intakes among current smokers instead of former smokers and non-smokers, which may be possible explained by the fact that smokers were more likely to drink coffee, which was the major contributor to polyphenol intake in this study. [17]. It has been observed that dietary patterns are different between smokers and nonsmokers [176].

According to original study design that participants were categorized into three dietary groups supplemented with olive oil, nut, and low-fat, respectively, changes of TPE during 5-year of intervention did not show any significant difference (p=0.189). This is because the concentration of TPE is corresponded to polyphenol intake and the changes of total polyphenol intake did not show significant difference neither (p=0.365).

6.2 Association between polyphenol intake and cardiovascular risk factors

6.2.1 Polyphenol excretion and blood pressure

Hypertension is a critical risk factor for CVD. In our study, significant inverse associations were found between tertiles of changes in TPE after 5 years' intervention and DBP (β=-1.156; P=0.031). Our results are in line with other studies within the PREDIMED study and other intervention studies [47,66,158].

Comparing with other large clinical trials, for instance, the EPIC study, the SUN study, and the Dietary-Approaches-to-Stop-Hypertension (DASH), also support our finding that consumption of foods rich in polyphenol such as fruits and vegetables are associated with lowering BP [177–179].

There are also some latest short period clinical trials support current finding. A study conducted in the similar aged population (50–70 years) but with high normal range BP (130/85–139/89mmHg) and stage 1-2 hypertension (140/90–179/109mmHg), suggested polyphenol-rich berry juice may contribute to a BP and BP variability lowering effect, being more pronounced in hypertensive than in normotensive subjects [180]. In another trail conducted in overweight-to-obese patients with pre-hypertension and stage 1 hypertension, finally found supplementation with 162 mg/d quercetin from onion skin extract lowers ambulatory blood pressure in patients with hypertension, suggesting a cardio protective effect of quercetin [181].

Mainly contribution of BP-lowing effects from polyphenol intake is the role of antioxidants. Consumption is associated with an improvement in endothelial function via vascular eNOS (endothelial nitric oxide synthase) and Akt (protein kinase B) activation [182].

6.2.2 Polyphenol excretion and T2D

T2D is one of the most common chronic diseases worldwide, and the increasing

prevalence in recent years is in parallel to obesity [183]. We found an inverse association between TPE and glucose concentration (β= –4.164; P=0.036). Related to T2D, results from two cohorts of US women, the Nurses's Health Study (NHS) and NHSII, suggested that specific flavonoid subclasses, including flavanones and flavonols, as well as caffeic acid, are associated with a lower T2D risk in relatively short-term follow-up but not during longer follow-up [184]. One of the limitations is that urinary polyphenol excretions, collected from their subjects, are less likely to represent long-term intakes because of the substantial within-person variability. Our results provide more general evidence because we focused on total polyphenols, not only some flavonoid subclasses.

We also evaluated the relationship between all polyphenol subclasses and the incidence of T2D in a longitudinal and observational study within the PREDIMED. We found a high intake of total polyphenols, total flavonoids (specifically flavanones and dihydroflavonols), and stilbenes was associated with a reduced risk of diabetes. In our study, catechins were significantly associated with a decreased risk of type 2 diabetes when comparing the second to the first tertile. However, proanthocyanidins, which are polymers of the flavan-3-ols found in grapes, red wine, apples, berries, chocolate, seeds, and legumes, were only inversely associated with diabetes risk when glucose concentrations were taken out of the model. Besides, similar results were found for anthocyanidins, although their bioavailability seems to be low compared with other flavonoid. Furthermore, our results suggest an inverse association between lignan intake and diabetes incidence, but only when glucose concentrations at baseline were not added to the model. In addition, we also found a strong inverse association between dihydroflavonols and diabetes, which has been previously demonstrated in animal and in vitro models.

Compared with other research, numerous evidence support are in line with our finding. A clinical study from Framingham Heart Study Offspring cohort supported evidence of a possible beneficial relationship between increased flavonol intake and risk of T2D,

indicating that each 2.5-fold increase in flavonol intake was associated with a 26% lower incidence of T2D [185]. In a prospective study conducted in 2 cohorts of US women, urinary excretion of hesperetin, another flavanone, was associated with a decreased risk of type 2 diabetes [186]. Results from human and animal trials have also shown that anthocyanidins improve glucose homeostasis through different mechanisms [112,187].

There is a lack of consensus on the antidiabetic properties of flavonols, the most consumed flavonoids, and flavones. We did not find any relation between flavonols or flavones and diabetes in our study, and neither did Kataja-Tuomola et al [188].

Several mechanisms have been invoked to explain the inverse associations between polyphenol consumption and diabetes incidence. Indeed, classic cases of hormonal disruption are insulin resistance, which causes hyperinsulinemia, or the β cell burnout in type 2 diabetes that results in chronic hyperglycemia [189]. The anti-diabetic effects of polyphenols may be attributed to the inhibition of oxidative stress, which seems to contribute to the development of insulin resistance and β-cell dysfunction, the two key events in the clinical development of T2D [190,191].

Consideration of sub classes of polyphenol, some polyphenols can inhibit cellular inflammation through the activation of PPARg and AMPK (Adenosine Monophosphate-activated Protein Kinase), an upstream activator of the anti-inflammatory gene transcription factors SIRT1 (Sirtuin 1) and FOX (Forkhead box) [189,192]. Two flavanones, naringin and hesperidin, showed antidiabetic properties partially mediated through the regulation of PPARg [193].

Previous results from animal and cell-cultured studies have shown that flavan-3-ols, especially epigallocatechin gallate, which belongs to the family of catechins, have antidiabetic effects. According to these studies, epigallocatechin gallate acts through multiple signaling pathways, leading to improvements in insulin secretion, glucose uptake, insulin resistance, glucose tolerance, oxidative stress, inflammation, and

mitochondrial function [193].

Overall, thinking that current medical treatments for the management of diabetes are tedious, the rising trend in the prevalence of diabetes complications asks for supplementary treatments, such as dietary polyphenol, which could increase the effectiveness of diabetes management.

6.2.3 Polyphenol excretion and hyperlipidemia

Triglycerides are considered the highest source of energy, and inhibition of triglyceride absorption also plays a role in the prevention of CVDs [194]. Treating hyperlipidemia is multifaceted, including lifestyle changes, risk factor modifications, and drug therapy [195]. From our study, we observed an inverse correlation between changes in TPE and plasma triglyceride concentration ($\beta = -8.563$; $P = 0.007$).

Only limited studies agreed with the hypothesis that polyphenol intake help to reduce triglyceride concentration because lacking clinical evidence. A similar study aimed to assess the relationship between dietary polyphenol intake and metabolic syndrome in Polish adults indicated that TG were significantly lower among individuals in the higher quartiles of polyphenol intake [196]. However, they did not show a linear association and such association only appeared in women. Another supporting from animal model report that plasma triglycerides was 39% lower, respectively in guinea pigs fed the grape diet compared with controls ($P < 0.05$), indicating a protective effects[197].

Plausible mechanisms to explain the improvement in triglyceride from polyphenol intake are various due to the consideration that the Mediterranean diet is a constellation of several polyphenol-rich foods. Sugiyama et al. investigated the inhibitory effect of oligomeric procyanidins from apples on triglyceride absorption, explained by the inhibition of pancreatic lipase activity *in vivo* and in animal models [198]. Data from animal models indicated that such lowering effects could be attributed to the very low-density lipoprotein (VLDL) secretion rates and a decrease in apolipoprotein B secretion [197]. In addition, a study of haemodialysis patients fed with polyphenol-rich pomegranate juice also reported improvements in triglyceride levels, but this was

explained by an inhibition of intestinal absorption and clearance of plasma triglycerides *in vivo* [121].

It is worth noting that a number of metabolic conditions are frequently associated with high TG levels. For instance, obesity is the most frequently metabolic stressor, and poorly controlled T2D is also very common [115]. Therefore, a well control of triglycerides level help to reduce other cardiovascular risk factors.

6.2.4 Polyphenol excretion and obesity

Overweight and obesity have been steadily increasing in recent years and currently represent a serious threat to public health. Few human studies have investigated the relationship between polyphenol intake and body weight, even though obesity is considered as a major independent risk factor for various chronic diseases. Reduction in energy intake and increment in energy expenditure may lead to a reduction in prevalence of obesity and overweight. From our study, we observed an inverse association between polyphenol intake and several anthropometric parameters, including BW (β=-1.004; 95% CI: -1.634 to -0.375, P=0.002), BMI (β=-0.320; 95% CI: -0.541 to -0.098, P=0.005), WC (β=-0.742; 95% CI: -1.326 to -0.158, P=0.013) and WHtR (β=-0.408; 95% CI: -0.788 to -0.028, P=0.036) after adjustment for potential confounders.

The present findings are consistent with previous reports on the inverse associations between polyphenol intake and weight parameters. A 16-year longitudinal study from the Netherlands associated a higher intake of total flavonols/flavones and catechins with a lower increase in BMI [199]. Other supporting evidence showed a significant decrease of 1.9 cm in WC and 1.2 Kg in BW after supplementation of catechin-rich green tea for 90 days, although at a much higher dose than habitual intakes [200]. Two 12-week intervention studies also demonstrated anti-obesity effects of green tea intake, finding a considerable reduction in BW, BMI, WC and total abdominal fat area [201,202]. A prospective, randomized, double-blind clinical study among obese subjects found an inverse relationship between green tea consumption and BMI, WC, GLU, TG [203].

However, their results were based on a three-month intervention, and larger scales with longer time of observation are needed. Another study showed moderate wine consumption might lower incidence rates of abdominal obesity [132]. However, it did not give a causal relationship because of the nature of cross-sectional study.

Beyond BW and BMI, abdominal obesity, including WC and WHtR, may show closer inverse association with CVD risk [89]. A cross sectional study has demonstrated an inverse association between the adherence to the Mediterranean diet, expressed by the p14-item score and abdominal obesity indexes in a population of adults at high cardiovascular risk [204]. However, a higher p14-item score is not directly correlated with higher polyphenol intake. In our study, we found TPE was significant higher among subjects with a p14-item score >10 than participants with a lower score. From other sub studies within the PREDIMED study, we also conform that the Mediterranean diet was negatively associated with WC and WHtR [205–207].

Knowledge of the anti-obesity effects of polyphenols is limited and only a few specific compounds have been analyzed in this context, for instance:

- Resveratrol, widely present in red grapes and red wine, exerts an anti-obesity action by reducing adipogenesis and increasing apoptosis in mature adipocytes, and inhibiting fat accumulation processes and stimulating lipolytic and oxidative pathways in *in vivo* studies [108,208].
- Anthocyanins, water-soluble plant pigments in blue, purple, and red fruits, have also been found to significantly reduce body weight. This effect may be due to suppression of lipid synthesis, up-regulation of adiponectin, which enhances insulin sensitivity, and reduction in of serum triglycerides and leptin levels [98,209].
- Flavonoids, which are a large group of polyphenols found in a wide range of Mediterranean diet foods [18,210], have been mainly attributed to improvement in adipocyte functionality and fatty oxidation[211]. Also playing a key role in weight control is the down-regulation of a variety of pro-inflammatory adipocytokines, particularly tumor necrosis factor alpha (TNF-α) [212].

Furthermore, overweight and obesity are associated with numerous comorbidities, including hypertension, T2D, dyslipidemia and CVDs [213]. Hence, on one hand, dietary polyphenol intakes helps to reduce cardiovascular risk directly; on the other hand, anti-obesity effect of dietary polyphenol intake leads to improvements in modifiable risk factors.

6.2.5 Limitation in comparisons with other evidence

Even though numerous studies have investigated the relationship between polyphenol-rich diets and classical cardiovascular risk factors, supporting our finding in several ways, comparisons between their results and ours are still difficult. Potential reasons are as following:

- Lake of common or reliable biomarker of dietary polyphenols. Most of the studies analyzed associations between polyphenol intakes and cardiovascular outcomes, ignoring affections of digestion and metabolism after ingestion.
- Differences in the profiles of participants, including health status, age, region.
- Difference in types of studies (cross-sectional study or longitudinal study) and lengths.
- Variation among dietary control. For instance, some of them focused on a single polyphenol-rich food source (tea, coffee, cocoa, wine and others); some focus on different food patterns such as Mediterranean diet and western diet. Even within Mediterranean countries, the definition of Mediterranean diet varies with geography, historical period, and the nationality of the authors [214].

6.3 strength and limitation of current study

6.3.1 Strength of current study

The main strengths of the current study are the following:

- The prospective design, relatively long-term follow-up and comprehensive data on risk factors and confounders.

- The evaluation of overall effects of polyphenol intakes on specific cardiovascular risk factors, which provide clinical evidence from the viewpoint of the protective role of polyphenol intake against CVD, within the framework of the PREDIMED trial.
- The use of a reliable biomarker of total polyphenol intakes. Compared with self-reported information in FFQ or polyphenol database such as Phenol-Explorer, the use of biomarker could be considered as a supplement and provides a more precise data.
- The Folin-Ciocalteu assay is a rapid, cheap, and environmentally friendly measurement that can be applied in large intervention studies.

6.3.2 Limitation of current study

Also, there are some limitations that need to be noted:

- As an intrinsic limitation of the PREDIMED study, our sub-study was conducted only among elderly subjects at high cardiovascular risk; therefore, it is difficult to extrapolate the results to the general population.
- Residual confounding could still exist even though we adjusted for potential confounders related to living habits, profiles of participants, family history of CVD, and eating habits.
- Possible synergistic effects between different types of polyphenols or polyphenols and other dietary components have not been measured.
- Regarding the analysis of the association between polyphenol intakes and diabetes, the nature of observational study within an intervention trial makes it impossible to establish causality.
- For the sub-study of the association between polyphenol intake and weight management, groups were categorized by TPE at 5-year instead of changes of TPE during the intervention.
- Intrinsic limitations of biomarkers: measurement error from the analysis and variability between individuals.

6.4 Summarize

This thesis provides detailed associations between total polyphenol intakes, measured by both FFQs and biomarker, and specific CVD risk factors, including BP, glucose concentration, lipid profile (TG, total cholesterol, HDL, LDL), heart rates, anthropometric parameters (BW, BMI, WC and WHtR), prevalence of diabetes and obesity in an elderly population at high cardiovascular risk. Compared with previous studies relating beneficial effects on CVD from dietary polyphenol consumption, our results give systematic and detailed associations. Therefore, for the establishment of dietary recommendations for public health, we suggest that a high consumption of polyphenol-rich foods in the frame of a Mediterranean diet could potentially help to reduce multiple risk factors of CVD.

Absorption, metabolism and elimination vary widely among polyphenols so future intervention studies should include a detailed assessment of polyphenols bioavailability. Therefore, we suggest that more studies with polyphenols are also needed to establish their role in the prevention of CVD. Besides, the dose–response relationship between total polyphenol and risk factors are also recommended.

Chapter 7

Conclusion

7 Conclusions

1. Urinary polyphenol excretion, significantly increased after 5-year of follow-up in an elderly population at high cardiovascular risk. TPE in urine samples, with solid phase extraction, were 113.1±41.8 mg GAE/g creatinine at baseline and 120.6±48.1 mg GAE/g creatinine at 5-year.
2. Total polyphenol intake, estimated by TPE analyzed by the Folin-Ciocateu colorimetric analysis, was inversely associated with several cardiovascular risk factors among participants in the PREDIMED study after 5-year of intervention.
3. A high intake of total polyphenols, calculated by FFQs and the Phenol-Explorer database, was associated with a reduced risk of diabetes in elderly people at high risk of cardiovascular disease.
4. Regarding socio-demographic and lifestyle factors, higher TPE levels are observed among women compared with men, none smokers and former smokers compared with current smokers, elder population (elder than 67 years old) compared with younger population at both of the beginning and end of the intervention, respectively.
5. After a 5-year follow-up, significant inverse correlations were observed between changes in TPE and plasma TG concentration, glucose concentration, and DBP after adjustment for potential confounders. For other biochemical cardiovascular risk factors, including total cholesterol, HDL, LDL, SBP, and heart rate, we did not find any significant improvements.
6. Inverse correlations were observed between TPE at 5 years of follow-up and BW, BMI, WC and WHtR after adjustment for potential confounders.
7. After 5-year of follow-up, participants in the category of highest TPE had a lower prevalence of obesity (odds ratio (OR) = 0.346, 95% confidence interval (CI) 0.176 to 0.178; P-trend, 0.039) than those in the lowest category.
8. We observed a 28% reduction in new-onset diabetes in the highest tertile compared with the lowest tertile of total polyphenol intake. For subclasses of polyphenols intake, we found a 33% reduction from total flavonoids; a 43% reduction from stilbenes; a 41% reduction from dihydroflavonols in new-onset diabetes, respectively.

Chapter 8

Reference

Reference

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Chapter 9

Annex

Publication 1: Effects of Polyphenol, Measured by a Biomarker of Total Polyphenols in Urine, on Cardiovascular Risk Factors after a Long-Term Follow-Up in the PREDIMED Study

Xiaohui Guo, Anna Tresserra-Rimbau, Ramón Estruch, Miguel A. Martínez-González, Alexander Medina-Remón, Olga Castañer,6 Dolores Corella, Jordi Salas-Salvadó, and Rosa M. Lamuela-Raventós. *Oxid Med Cell Longev. 2016; 2016:2572606. doi: 10.1155/2016/2572606.*

Abstract: Several epidemiological studies have shown an inverse association between the consumption of polyphenol-rich foods and risk of cardiovascular diseases. However, accuracy and reliability of these studies may be increased using urinary total polyphenol excretion (TPE) as a biomarker for total polyphenol intake. Our aim was to assess if antioxidant activity, measured by a Folin-Ciocalteu assay in urine, is correlated with an improvement in cardiovascular risk factors (blood pressure and serum glucose, cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride concentrations) in an elderly population at high risk. A longitudinal study was performed with 573 participants (aged 67.3 ± 5.9) from the PREDIMED study (ISRCTN35739639). We used Folin-Ciocalteu method to determine TPE in urine samples, assisting with solid phase extraction. Participants were categorized into three groups according to changes in TPE. Multiple linear regression models were used to assess relationships between TPE and clinical cardiovascular risk factors, adjusting for potential confounders. After a 5-year follow-up, significant inverse correlations were observed between changes in TPE and plasma triglyceride concentration (β = -8.563; P = 0.007), glucose concentration (β = -4.164; P = 0.036), and diastolic blood pressure (β = -1.316; P = 0.013). Our results suggest that the consumption of more polyphenols, measured as TPE in urine, could exert a protective effect against some cardiovascular risk factors.

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Research Article

Effects of Polyphenol, Measured by a Biomarker of Total Polyphenols in Urine, on Cardiovascular Risk Factors After a Long-Term Follow-Up in the PREDIMED Study

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Several epidemiological studies have shown an inverse association between the consumption of polyphenol-rich foods and risk of cardiovascular diseases. However, accuracy and reliability of these studies may be increased using urinary total polyphenol excretion (TPE) as a biomarker for total polyphenol intake. Our aim was to assess if antioxidant activity, measured by a Folin-Ciocalteu assay in urine, is correlated with an improvement in cardiovascular risk factors (blood pressure and serum glucose, cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride concentrations) in an elderly population at high risk. A longitudinal study was performed with 573 participants (aged 67.3 ± 5.9) from the PREDIMED study (ISRCTN35739639). We used Folin-Ciocalteu method to determine TPE in urine samples, assisting with solid phase extraction. Participants were categorized into three groups according to changes in TPE. Multiple linear regression models were used to assess relationships between TPE and clinical cardiovascular risk factors, adjusting for potential confounders. After a 5-year follow-up, significant inverse correlations were observed between changes in TPE and plasma triglyceride concentration ($\beta = -8.563$; $P = 0.007$), glucose concentration ($\beta = -4.164$; $P = 0.036$), and diastolic blood pressure ($\beta = -1.316$; $P = 0.013$). Our results suggest that the consumption of more polyphenols, measured as TPE in urine, could exert a protective effect against some cardiovascular risk factors.

1. Introduction

Cardiovascular diseases (CVDs) are considered to be the leading global cause of death, accounting for 17.3 million deaths per year, which is predicted to rise to more than 23.6 million by 2030 [1]. The main causes of CVDs involve nonmodifiable risk factors, such as age, sex, and family history of coronary heart disease (CHD), and modifiable risk factors, such as an unhealthy diet, lack of physical activity, smoking, and excessive alcohol intake [2, 3]. Therefore, an improvement of dietary habits could help to prevent CVDs.

Several studies have described protective roles of polyphenols in the cardiovascular system. The cardiovascular protection by polyphenol consumption can be explained by various mechanisms, including their anti-inflammatory properties, antioxidant capacity, improvement in endothelial function, inhibition of platelet aggregation and antithrombotic properties, and mechanisms that are not mutually exclusive [4–8]. Hence, further exploration of polyphenol consumption will help to discern its beneficial effects on human health. Prior information on polyphenol intake has often been collected through food frequency questionnaires (FFQs) or dietary recalls, whose bias can result in data not so accurate [9]. Therefore, in order to analyse associations between polyphenol intake and main cardiovascular risk factors, there is a need for biomarkers that can accurately reflect polyphenol intakein human studies.

The Folin-Ciocalteu method, an antioxidant assay based on electron transfer that measures the reductive capacity of an antioxidant, has been widely applied for measuring total polyphenol content in plant-derived food and recently in biological samples for clinical studies [10, 11]. Briefly, polyphenols from urine samples react with the Folin-Ciocalteu reagent to form a blue complex in alkaline medium, measured in spectrophotometry at 765 nm [12]. A solid phase extraction method is used to clean up the sample from possible interferences. This measurement of total urinary polyphenol excretion (TPE) has been considered as reliable biomarker of total polyphenol intake in recent years [8, 13, 14].

Several studies have addressed the relationship between polyphenol intake and cardiovascular risk factors; however, the results have led to mixed and inconsistent conclusions. Two studies conducted in healthy participants observed that improvement in cardiovascular health was due to higher HDL levels after intake of polyphenol-rich foods [15, 16]. Different results were obtained in other two studies in overweight subjects: one showed cardioprotective effects due to a reduction in body weight and an improvement in total cholesterol and LDL concentration after ingestion of a polyphenol extract from *Ecklonia cava*, while the other study observed a reduction in fasting glucose concentration when supplied with polyphenol-rich dark chocolate [17, 18]. Additionally, reduction in systolic blood pressure was observed in hemodialysis patients after the consumption of a polyphenol-rich beverage for one year [19]. Moreover, in the frame of the PREDIMED study, we found that specific categories of polyphenols, calculated through yearly FFQs and the Phenol-Explorer database, were significantly associated with decreased CVD risk [20].

Most of the aforementioned studies were conducted in small populations or over short periods of time. The association between polyphenol intake and cardiovascular risk factors has also been evaluated in large, long-term epidemiological trials, but with the limitations associated with using FFQs [21–24]. Therefore, the aim of the present study was to apply the reliable and validated antioxidant activity test, the Folin-Ciocalteu method, in urine samples as a biomarker of total polyphenol intake, to analyse the association between polyphenol intake and cardiovascular risk factors in an elderly population at high cardiovascular risk after a long-term follow-up (median: 4.8 years).

2. Methods

The present study was conducted within the frame of the PREDIMED study, which aimed to assess effects of the Mediterranean diet on the primary prevention of CVDs in Spain. The protocol and recruitment methods have been reported in detail elsewhere [25]. Eligible participants were men aged 55–80 and women aged 60–80 years without any history of cardiovascular disease but fulfilling at least one of the following two criteria: type-2 diabetes or three or more cardiovascular risk factors (family history of early-onset CVDs, hypertension, current smoking, low HDL-cholesterol, high LDL-cholesterol, and overweight or obesity). Exclusion criteria included any severe chronic illness, previous history of CVDs, alcohol or drug abuse, body mass index (BMI) of more than 40 kg/m^2, and history of allergy or intolerance to olive oil or nuts. The trial was stopped after a median follow-up of 4.8 years due to the benefit of the Mediterranean diet with respect to major cardiovascular events: myocardial infarction, stroke, or death from cardiovascular causes (analysis performed by the Drug and Safety Monitoring Board of the trial), compared to a control low-fat diet [26].

The present longitudinal analysis included 612 volunteers, randomly selected from two recruitment centers in Spain. All participants provided written informed consent, and the protocol was approved by the Institutional Review Boards of the participating centers and registered.

2.1. Nutritional Assessments. Dietary habits of participants were assessed through a validated 137-item FFQ [27]. Nutrient intake was adjusted by calories using the residuals' method. Information about lifestyle, health condition, education, history of illnesses, and medication use was collected by a 47-item general questionnaire. The degree of adherence to the Mediterranean diet was assessed by a 14-point questionnaire [28]. Physical activity was assessed using the validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire [29]. All questionnaires were administered and repeated annually during the follow-up by trained staff in face-to-face interviews.

Information on polyphenol intake was obtained using the FFQ and the Phenol-Explorer database. The relationship between food items in the FFQ and the database has been described previously [30]. The content of total polyphenol intake equals the sum of all the individual polyphenol from each food item.

2.2. TPE Measurements. Urine samples were collected and coded and then immediately shipped to a central laboratory, to be stored at −80°C until analysed. The Folin-Ciocalteu method was applied to determine the content of TPE, using a clean-up procedure with solid phase extraction (SPE) performed in 96-well plate cartridges (Oasis MAX), which helped to remove urinary interferences. Finally, TPE was expressed as mg gallic acid equivalent (GAE)/g of creatinine.

All details have been previously described by Medina-Remón et al. [14].

2.3. Clinical Measurements. Weight and height were measured with light clothing and no shoes with a calibrated balance and a wall-mounted calibrated stadiometer, respectively. BMI was calculated as weight in kilograms divided by the square of height in meters. For the measurement of blood pressure (BP), a validated semiautomatic sphygmomanometer (Omron HEM-705CP) was used by trained nurses. Measurements were taken at 5-minute intervals with participants in a seated position. Data were collected as an average of 2 measurements in each arm, repeated twice [31].

Plasma glucose, total cholesterol, and triglyceride concentrations were measured using standard enzymatic automated methods. Levels of HDL-cholesterol were measured by an enzymatic procedure after precipitation, and LDL-cholesterol was estimated by the Friedewald formula [32].

2.4. Statistical Analysis. Results were expressed as mean ± SD for continuous variables or percentages for categorical variables. Kolmogorov tests were applied to examine the normality distribution and skewness. All participants were divided into three categories according to changes in TPE during the follow-up ($\Delta TP < -11.4$ mg gallic acid/g creatinine, $-11.4 \leq \Delta TP \leq 24.6$ mg gallic acid/g creatinine, and $\Delta TP > 24.6$ mg gallic acid/g creatinine). Changes in nutrient and key food consumption during the follow-up were assessed with ANOVA for repeated measurements analysis. Bonferroni *post hoc* test and paired *t*-test were used to compare each variable within and between groups.

Multivariate linear regression models were used to assess the relationship between serum glucose, total cholesterol, HDL, LDL, triglyceride concentrations, systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate, and tertiles of changes in TPE during the follow-up period, adjusted for potential confounders (sex, age, intervention groups, BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, medication use, and 14-unit Mediterranean diet score at baseline). Sensitivity analyses were used to further assess the relationship between specific cardiovascular risk factors and subcategories.

General Linear Model (GLM) approach to ANCOVA was used to determine differences between tertiles of changes in TPE after 5-year follow-up, adjusted for potential confounders as did in multivariate linear regression models.

All analyses were performed using SPSS software V21.0 (Chicago, USA). All models were tested for the detection of outliers, multicollinearity, homoscedasticity, and normality and independence of errors. All statistical tests were two-tailed, and the significance level was $P < 0.05$.

3. Results

After 5 years of follow-up of 612 participants randomly selected for this substudy of the PREDIMED trial, 39 were excluded because of extreme TPE values, hence a total of 573 participants were included in the present study. Baseline characteristics of participants grouped by tertiles of changes in TPE during the follow-up are shown in Table 1. According to the study design, the average age was 67.3 ± 5.9 years with a BMI of 29.2 ± 3.3 kg/m^2. Most of the participants gathered a high number of cardiovascular risk factors: 41.5% had diabetes; 80.5% had hypertension; 66.8% had dyslipidemia; 16.9% were current smokers, and 37.5% had a family history of CHD. In the second tertile, individuals were less likely to be women and had a higher body weight.

Table 2 shows changes in key food consumption during the follow-up. Most key foods changed considerably after the long-term intervention, with the exception of legumes and chocolate. Table 3 summarizes information on nutrient intake at baseline and 5 years according to changes in TPE during the follow-up. Comparing nutrient intake at 5 years versus baseline, we observed a significant increase in total fat, fibre, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), K, and Mg, while other items such as total carbohydrates, protein, saturated fatty acids (SFA), Na, and cholesterol remained unchanged. This may be due to dietary changes based on recommendations to adhere to a Mediterranean diet, which is characterized by a high consumption of vegetables, fruits, olive oil, wine, and nuts and a low consumption of red meat, high-fat dairy products, and sweets. However, there were no significant changes when comparing tertiles and their interaction. In addition, we found that significant changes in TPE did not significantly affect the intake of nutritional elements among groups.

Several antioxidant substances such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acid, Fe(II), and nonphenolic organic substances might affect total polyphenol when applying Folin-Ciocalteu assay; however, after a solid phase extraction (SPE), the aforementioned interfering substances were eliminated through the cleaning-up process [14].

Linear regression analyses were conducted to assess the relationship between TPE ($Q_1 = -48.70 \pm 30.11$ mg GAE/g creatinine; $Q_2 = 6.95 \pm 10.55$ mg GAE/g creatinine; $Q_3 = 64.48 \pm 31.61$ mg GAE/g creatinine) and clinical possible cardiovascular risk factors (plasma glucose, triglyceride, cholesterol, HDL-c, and LDL-c concentrations, and SBP, DBP, and heart rate). Results are shown in Table 4. Significant inverse associations were found between tertiles of changes in TPE and glucose ($\beta = -4.372$; $P = 0.026$), triglycerides ($\beta = -8.572$; $P = 0.006$), and DBP ($\beta = -1.156$; $P = 0.031$) after adjustment for potential confounders. However, other parameters did not show significant associations. The standardized coefficients (Beta) in the model were used to measure degrees of contribution to different risk factors. Results indicate that, among the CVD risk factors, triglyceride levels showed the highest beneficial effects of dietary polyphenol intake (Beta = −0.126; $P = 0.031$).

We also conducted sensitivity analyses to ascertain whether significant changes were related to specific variables. As shown in Table 5, men were more likely to improve their plasma triglyceride concentration than women, according to tertiles of changes in TPE. In contrast, the lowering effects

Table 1: Baseline characteristics of participants according to tertiles of changes in TPE.

	TPE (mg GAE/g creatinine)						
	Q_1 (ΔTP < −11.4)		Q_2 (−11.4 ≤ ΔTP ≤ 24.6)		Q_3 (ΔTP > 24.6)		P
Number of subjects	191		191		191		
Women, *n* (%)	101	(52.9)	83	(43.5)	112	(58.6)	0.011
Age (y), mean (SD)	66.7	(5.9)	67.3	(5.8)	68.00	(6.0)	0.113
Weight (kg), mean (SD)	73.9	(10.6)	77.1	(11.6)	74.5	(10.7)	0.01
BMI (kg/m^2), mean (SD)	28.9	(3.1)	29.6	(3.5)	29.2	(3.2)	0.103
Systolic BP (mm Hg), mean (SD)	149.8	(17.9)	151.6	(16.9)	152.8	(18.6)	0.238
Diastolic BP (mm Hg), mean (SD)	84.3	(9.8)	85.9	(10.0)	85.5	(10.4)	0.269
Hypertension, *n* (%)	151	(79.1)	152	(79.6)	158	(82.7)	0.621
Diabetes, *n* (%)	78	(40.8)	85	(44.5)	75	(39.3)	0.567
Dyslipidemia, *n* (%)	136	(72.3)	117	(61.3)	128	(67)	0.074
Smoking status							0.641
Current, *n* (%)	35	(18.3)	34	(17.8)	28	(14.7)	0.586
Former, *n* (%)	36	(18.8)	43	(22.5)	47	(24.6)	0.388
Never, *n* (%)	120	(62.8)	114	(59.7)	116	(60.7)	0.814
Family history of CHD, *n* (%)	65	(35.3)	75	(40.3)	75	(41.2)	0.460
Medication							
Aspirin, *n* (%)	33	(32.0)	35	(34.0)	35	(34.0)	0.949
Antihypertensive drugs, *n* (%)	131	(68.6)	142	(74.3)	141	(73.8)	0.381
Hypolipidemic drugs, *n* (%)	91	(47.6)	70	(36.6)	78	(40.8)	0.089
Insulin, *n* (%)	10	(5.2)	9	(4.7)	8	(4.2)	0.890
Oral hypoglycemic drugs, *n* (%)	40	(20.9)	46	(24.1)	45	(23.6)	0.736
Vitamin or minerals, *n* (%)	18	(9.5)	16	(8.5)	13	(6.9)	0.644
Educational level							
Primary school, *n* (%)	140	(74.1)	139	(73.5)	146	(76.8)	
High school, *n* (%)	32	(16.9)	28	(14.8)	28	(14.7)	0.793
University, *n* (%)	17	(9.2)	22	(11.6)	16	(8.4)	
Physical activity at leisure time (MET-min/d)	275	(212)	287	(204)	269	(183)	0.696
Polyphenol intake (mg/d)	853.4	(239.8)	831.2	(248.9)	882.7	(247.8)	0.135

BMI: body mass index; CHD: coronary heart disease; GAE: gallic acid equivalent; TPE: total polyphenol excretion.
Data are given as means (SD) for continuous variables and percentages for categorical variables; $P < 0.05$ indicates statistical significance.
*P values calculated by analysis of variance or χ^2 tests.

of higher polyphenol consumption on DBP were greater in women. In addition, when the P-14 was considered separately, higher scoring groups showed significant differences in plasma triglyceride concentration according to tertiles of changes in TPE.

4. Discussion

In this 5-year study of an elderly population at high cardiovascular risk living in a Mediterranean country, we observed that higher polyphenol intake, measured by TPE, was inversely associated with some cardiovascular risk factors. The observed benefits on CVDs were ascribable to a reduction in plasma glucose and triglyceride concentrations and a diminution of DBP. This may partly explain the decreased CVD risk shown by people following a polyphenol-rich diet such as the Mediterranean diet.

The beneficial effects of polyphenols consumption on major cardiovascular events in the PREDIMED cohort have been published before [20]. The difference between our findings and other reported results lies in the measurement of polyphenols in urine as biomarker of polyphenol intake. Given that more than 8000 phenolic structures exist in nature, beneficial effects from polyphenols depend on a variety of factors, including total intake, food cooking processes, digestion, absorption, metabolic pathways *in vivo*, or even differences between individuals [33]. Therefore, TPE, as a biomarker of total polyphenol intake, may provide a more accurate insight into the effects of polyphenols on CVDs than other dietary assessment methods.

Previous clinical studies on the benefits of polyphenols on the cardiovascular system have provided inconsistent results. A 12-week follow-up clinical trial conducted in Korea reported a strong inverse association between consumption of polyphenol extracts from *Ecklonia cava* and serum glucose, SBP, and HDL concentration [17]. In contrast, a recent randomized control trial performed with 67 elderly men at high cardiovascular risk found an increase in HDL

Table 2: Changes in daily intake of key foods after 5 years with energy adjustment categorized by tertile of changes in TPE[a].

		TPE (mg GAE/g creatinine)								
		Q_1 (ΔTP < −11.4)		Q_2 (−11.4 ≤ ΔTP ≤ 24.6)		Q_3 (ΔTP > 24.6)		P[b]		
		Mean	SD	Mean	SD	Mean	SD	Time[c]	Group[d]	Time ∗ Group[e]
Vegetables (g/d)	Baseline	302.1	117.5	293.9	109.0	289.6	118.4	<0.001	0.195	0.369
	5 years	366.0**	122.9	340.7**	115.8	354.3**	120.9			
Fruits (g/d)	Baseline	346.3	176.7	354.7	169.3	385.4	183.6	<0.001	0.477	0.103
	5 years	459.4**	181.4	456.4**	172.6	454.0**	158.8			
Legumes (g/d)	Baseline	18.7	7.2	19.2	7.2	19.69	8.8	0.445	0.149	0.736
	5 years	18.7	8.3	19.1	7.7	19.9	8.1			
Cereals (g/d)	Baseline	240.0	73.2	242.9	79.2	238.9	70.7	<0.001	0.867	0.712
	5 years	221.1**	63.4	216.4**	68.5	216.1**	63.0			
Milk (g/d)	Baseline	368.8	201.3	345.4	193.9	393.2	233.2	0.005	0.300	0.166
	5 years	402.2*	223.4	386.4**	204.3	395.6	196.6			
Meat (g/d)	Baseline	140.9	49.1	140.1	48.8	138.0	45.3	<0.001	0.992	0.431
	5 years	126.6**	41.9	126.9**	43.3	130.1	44.1			
Fish (g/d)	Baseline	94.7	37.2	90.4	39.1	91.7	39.2	<0.001	0.521	0.882
	5 years	101.4	45.6	98.8**	43.2	97.8*	36.2			
Pastries (g/d)	Baseline	26.1	26.1	25.7	27.1	26.6	25.1	0.001	0.846	0.485
	5 years	20.1	24.2	23.1	28.4	21.5	27.3			
EVOO (g/d)	Baseline	24.1	24.2	21.8	23.8	21.3	22.9	<0.001	0.848	0.346
	5 years	48.2**	22.8	48.1**	25.0	49.7**	23.1			
Nuts (g/d)	Baseline	11.0	12.1	9.8	13.1	10.6	13.1	<0.001	0.794	0.656
	5 years	16.0**	12.5	16.1**	13.1	16.7**	12.2			
Wine (g/d)	Baseline	98.3	140.1	105.2	157.2	96.8	136.1	0.002	0.781	0.979
	5 years	80.1**	130.5	89.0	130.8	80.7	123.4			
Folic acid (μg/d)	Baseline	376.7	83.8	379.4	81.1	381.9	87.6	<0.001	0.939	0.322
	5 years	432.6**	75.9	425.3**	87.3	424.5**	72.4			
Coffee (mL/d)	Baseline	38.4	57.0	33.2	44.2	33.9	47.2	0.004	0.902	0.258
	5 years	27.4*	48.0	28.6	46.4	30.7	48.8			
Chocolate (g/d)	Baseline	2.9	5.7	2.5	4.7	3.1	5.9	0.940	0.422	0.203
	5 years	2.2	4.2	3.1*	6.1	3.4	7.9			

[a]Data are given as means (SD); $P < 0.05$ indicates statistical significance. EVOO: extra virgin olive oil; GAE: gallic acid equivalent; TPE: total polyphenol excretion. Values with asterisks are statistically different from baseline values by the paired-samples t-test ($^*P < 0.05$; $^{**}P < 0.01$).
[b]Data analysed by repeated-measures 2-factor ANOVA.
[c]Comparison between the time before and after intervention.
[d]Comparison between tertiles of TPE changes.
[e]Comparison between measurements obtained before and after intervention and between tertiles of TPE changes.

after consumption of red wine, whereas fasting glucose was kept constant throughout the study, which differs from our observation [34]. Another contrasting result was found in participants with type-2 diabetes, who improved their HDL level and decreased total cholesterol after the consumption of polyphenol-rich chocolate [35]. In addition, a group of overweight participants consuming polyphenol-rich dark chocolate had lower plasma glucose, SBP, and DBP after the intervention, which partly agrees with our findings [18]. However, in the present study, we found no association between polyphenol intake and cholesterol profiles or SBP.

Participants who increased their polyphenol intake showed a reduction in plasma glucose concentrations, adding to the evidence that polyphenol-rich diets protect the cardiovascular system by improvements in glycemic control. A similar clinical trial performed on 78 participants at high cardiovascular risk, administration of polyphenol-rich foods, improved glucose metabolism by increasing early insulin secretion and insulin sensitivity [36]. Another cross-sectional study in an elderly population reported that green tea consumption was inversely associated with fasting blood glucose concentrations, though without adjusting for potential

Table 3: Changes in nutrient intake after 5 years with energy adjustment categorized by tertile of changes in TPE[a].

		TPE (mg GAE/g creatinine)								
		Q_1 (ΔTP < −11.4)		Q_2 (−11.4 ≤ ΔTP ≤ 24.6)		Q_3 (ΔTP > 24.6)		P[b]		
		Mean	SD	Mean	SD	Mean	SD	Time[c]	Group[d]	Time ∗ Group[e]
Total carbohydrates (g/d)	Baseline	235.6	36.5	238.4	43.2	239.9	35.9	0.736	0.964	0.41
	5 years	239.7	63.1	235.0	68.9	235.6	61.0			
Protein (g/d)	Baseline	88.4	36.5	91.2	43.2	92.7	35.9	0.12	0.649	0.498
	5 years	94.5	18.6	92.7	19.5	94.4	17.7			
Total fat (g/d)	Baseline	102.5	12.8	100.8	12.6	102.7	13.6	<0.001	0.528	0.331
	5 years	110.7**	23.1	112.9**	25.6	113.4**	24.4			
MUFA (g/d)	Baseline	53.5	13.6	52.3	17.4	51.6	15.2	<0.001	0.97	0.19
	5 years	58.3**	12.7	59.6**	13.5	59.8**	13.5			
SFA (g/d)	Baseline	25.5	9.0	24.6	10.1	24.0	9.7	0.949	0.887	0.114
	5 years	24.2	6.4	24.8	7.4	25.1	7.3			
PUFA (g/d)	Baseline	15.7	4.7	15.7	6.1	15.6	5.4	<0.001	0.716	0.675
	5 years	19.0**	5.9	19.0**	5.6	19.6**	5.5			
Alcohol (g/d)	Baseline	14.1	5.2	13.3	5.4	13.4	4.8	0.039	0.979	0.75
	5 years	11.9	14.6	12.4	15.2	12.2	14.7			
Fibre (g/d)	Baseline	24.2	6.0	24.6	5.6	25.2	6.4	<0.001	0.564	0.204
	5 years	26.6**	7.5	25.8	7.4	26.4	7.0			
Cholesterol (mg/d)	Baseline	352.4	84.6	353.1	94.6	350.5	94.0	0.2	0.946	0.975
	5 years	359.9	90.9	358.0	98.7	356.8	92.7			
Na (mg/d)	Baseline	2322.4	479.6	2273.1	528.7	2263.7	479.9	0.736	0.963	0.41
	5 years	2229.8	644.5	2230.8	728.0	2253.7	652.0			
K (mg/d)	Baseline	4230.9	723.7	4164.3	682.7	4300.6	796.1	<0.001	0.234	0.542
	5 years	4654.5**	826.8	4546.0**	963.7	4614.7**	805.9			
Mg (mg/d)	Baseline	359.5	62.7	358.4	58.1	367.1	61.8	<0.001	0.432	0.365
	5 years	398.5**	82.1	388.1**	86.4	394.3**	80.8			

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, GAE: gallic acid equivalent; TPE: total polyphenol excretion.
[a]Data are given as means (SD); $P < 0.05$ indicates statistical significance. Values with asterisks are statistically different from baseline by paired-samples t-test ($^*P < 0.05$; $^{**}P < 0.01$).
[b]Data analysed by repeated measures 2-factor ANOVA.
[c]Comparison between the time before and after intervention.
[d]Comparison between tertile changes in TPE.
[e]Comparison between measures obtained before and after intervention and between tertiles of TPE changes.

confounders [37]. Despite the abundance of results from different clinical trials, animal models, and *in vitro* tests, the mechanisms for hypoglycemic effects of polyphenols still warrant discussion. Potential explanations for these putative protective effects include reduced absorption of total carbohydrate in the intestine, modulation of enzymes related to glucose metabolism, stimulation of insulin secretion, improvement of β-cell function and insulin action, reduction in oxidative stress, inhibition of glucose transport, and enhanced vascular function [36, 38–40].

Triglycerides are considered the highest source of energy, and inhibition of triglyceride absorption also plays a role in the prevention of CVDs [41]. In the present study, increasing polyphenol intake was inversely associated with triglyceride levels, in agreement with some previous studies. Sugiyama et al. investigated the inhibitory effect of oligomeric procyanidins from apples on triglyceride absorption, explained by the inhibition of pancreatic lipase activity *in vivo* and in animal models [42]. Data from animal models indicated that such lowering effects could be attributed to the very low-density lipoprotein (VLDL) secretion rates and a decrease in apolipoprotein B secretion [43]. In addition, a study of haemodialysis patients fed with polyphenol-rich pomegranate juice also reported improvements in triglyceride levels, but this was explained by an inhibition of intestinal absorption and clearance of plasma triglycerides *in vivo*[19]. The variety of plausible mechanisms put forward to explain these effects, such as absorption, metabolism, and elimination during metabolic processes, reflect the highly varied chemical structure of polyphenols. Unlike the current study, most clinical trials have focused on a single

Table 4: Multivariate linear regression analyses with changes in cardiovascular risk factors as dependent variables and tertiles of changes in TPE in spot urine samples (mg GAE/g creatinine) as exposure variables, adjusted for potential confounders.

		β	SE	Beta	Sig.	95% CI	
Change in GLU (mg/dL)	Model 1	−4.164	1.979	−0.095	0.036	−8.053	−0.275
	Model 2	−4.316	1.981	−0.098	0.030	−8.208	−0.424
	Model 3	−4.355	1.949	−0.099	0.026	−8.186	−0.525
	Model 4	−4.372	1.953	−0.099	0.026	−8.209	−0.534
Change in COL (mg/dL)	Model 1	−2.51	2.001	−0.057	0.210	−6.442	1.421
	Model 2	−2.236	2.011	−0.050	0.267	−6.187	1.715
	Model 3	−1.845	2.013	−0.042	0.360	−5.800	2.109
	Model 4	−1.802	2.015	−0.041	0.372	−5.762	2.157
Change in HDL (mg/dL)	Model 1	0.102	0.448	0.010	0.820	−0.778	0.982
	Model 2	0.135	0.448	0.014	0.763	−0.744	1.015
	Model 3	0.133	0.456	0.014	0.771	−0.764	1.030
	Model 4	0.174	0.454	0.018	0.701	−0.718	1.067
Change in LDL (mg/dL)	Model 1	−0.205	1.775	−0.005	0.908	−3.693	3.283
	Model 2	−0.039	1.784	−0.001	0.983	−3.545	3.467
	Model 3	0.448	1.783	0.012	0.802	−3.056	3.952
	Model 4	0.469	1.786	0.012	0.793	−3.041	3.979
Change in TG (mg/dL)	Model 1	−8.356	3.06	−0.123	0.007	−14.369	−2.344
	Model 2	−8.563	3.058	−0.126	0.005	−14.572	−2.554
	Model 3	−8.627	3.094	−0.127	0.006	−14.708	−2.546
	Model 4	−8.572	3.099	−0.126	0.006	−14.662	−2.483
Change in SBP (mm Hg)	Model 1	−1.367	0.994	−0.058	0.169	−3.319	0.585
	Model 2	−1.222	1.001	−0.052	0.222	−3.188	0.744
	Model 3	−1.127	1.003	−0.048	0.262	−3.098	0.843
	Model 4	−1.098	1.005	−0.046	0.275	−3.071	0.876
Change in DBP (mm Hg)	Model 1	−1.316	0.531	−0.104	0.013	−2.359	−0.273
	Model 2	−1.254	0.532	−0.099	0.019	−2.298	−0.209
	Model 3	−1.153	0.532	−0.091	0.031	−2.198	−0.108
	Model 4	−1.156	0.533	−0.091	0.031	−2.203	−0.109
Change in HR	Model 1	−0.002	0.555	0.000	0.997	−1.091	1.087
	Model 2	0.043	0.559	0.003	0.938	−1.055	1.142
	Model 3	−0.011	0.567	−0.001	0.985	−1.125	1.103
	Model 4	−0.074	0.565	−0.006	0.895	−1.184	1.035

GLU: glucose, COL: total cholesterol, HDL: high-density lipoprotein, LDL: Low-density lipoprotein, TG: triglycerides, SBP: systolic blood pressure, DBP: diastolic blood pressure, and HR: heart rate.

β: nonstandardized coefficient (regression line coefficient); SE: standard error; Beta: standardized coefficient; CI: confidence interval; P: two-sided test of significance.

Model 1: unadjusted; Model 2 adjusted for sex, age, and intervention groups; Model 3 adjusted as in Model 2 plus BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, and medication use: antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin, or other antiplatelet drug; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score.

polyphenol-rich food such as dark chocolate, wine, or green tea. Therefore, considering that the Mediterranean diet is a constellation of several polyphenol-rich foods, it is difficult to draw a single mechanism to explain the lowering effects found on triglycerides.

Hypertension is a well-established risk factor for CVDs [44]. There is evidence from our study and others that increasing polyphenol intake is associated with lower BP. Both DASH (Dietary Approaches to Stop Hypertension) and SUN (Seguimiento Universidad de Navarra) studies emphasize that the consumption of plant-derived foods, particularly fruits, vegetables, nuts, and olive oil, is inversely associated with BP [45–47]. We previously reported that greater TPE was inversely associated with BP [13]. However, we found significant associations only for DBP, and not SBP. Another PREDIMED clinical substudy based on a 4-year intervention also supports our findings [48]. Mechanisms of the BP lowering effect could involve endothelial nitric oxide (NO) production. NO plays a fundamental role in the regulation of the vascular system, and vascular homeostasis is achieved only when NO levels are adequate [6]. Briefly, polyphenols induce NO production by promoting endothelial nitric oxide synthase (eNOS) expression, generating vascular relaxing factors such as prostacyclin (PGI2) and

Table 5: Sensitivity analysis of clinical cardiovascular risk factors.

			Change in TG (mg/dL)							Change in GLU (mg/dL)							Change in DBP (mm Hg)						
		N	Q_1		Q_2		Q_3		P^a	Q_1		Q_2		Q_3		P^a	Q_1		Q_2		Q_3		P^a
			Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD	
Gender	Male	236	6.41	43.58	−17.86	62.54	−14.80	44.68	0.007	2.35	38.03	−0.32	35.56	−6.12	35.99	0.36	−1.24	10.04	−2.43	9.73	−2.14	11.67	0.714
	Female	249	8.22	46.57	9.25**	53.14	−5.01	68.69	0.194	10.31	38.68	9.23	30.44	1.51	35.04	0.193	−1.46	10.24	−3.67	10.75*	−5.29	9.84	0.026
Age, years	≤67	242	8.71	47.80	−9.75	70.39	−8.77	62.27	0.089	7.08	41.13	1.56	30.86	−2.23	36.48	0.262	−0.64	9.70	−1.41	10.78	−2.77	11.73	0.396
	≥68	243	5.96	42.21	−2.06	46.74	−9.68	57.35	0.129	6.06	35.60	6.37	36.39	−1.36	34.91	0.285	−2.18	10.58	−4.50*	9.36	−4.99	9.75	0.123
P-14	<9	274	−0.89	57.27	−5.27	58.57	−10.63	67.61	0.676	7.29	28.72	5.65	34.89	−3.68	42.90	0.178	−1.36	11.63	−2.39	9.87	−4.98	10.05	0.094
	≥9	211	12.07	35.90	−6.49	61.19	−8.33	53.71	0.007	6.18	43.25	2.69	32.89	−0.43	29.55	0.428	−1.35	9.20	−3.36	10.40	−3.27	11.17	0.24

P-14: 14-point Mediterranean diet score test; GLU: glucose; TG: triglycerides; DBP: diastolic blood pressure.

[a] P value tested by ANOVA.

Values with asterisks are statistically different from the baseline by the paired-samples t-test ($^*P < 0.05$; $^{**}P < 0.01$).

inhibiting synthesis of the vasoconstrictor endothelin-1 (ET-1) in vascular endothelial cells [49]. Strong and positive association between polyphenol intake and plasma NO levels has been previously demonstrated by our group [8].

Some study limitations deserve to be noted. First, given that this substudy was conducted only among elderly subjects at high cardiovascular risk, it is difficult to extrapolate the results to the general population. Second, even though we adjusted potential confounders relative to CVD risk, residual confounding could still exist. Nonetheless, our study adds new evidence in support of a preventative effect of a long-term polyphenol intake on CVDs.

Compared with previous studies, the present study also has several strengths. Firstly, even though biomarkers are necessary to assess the compliance of the intervention, it is difficult to find a reliable and available biomarker. TPE in urine could be useful as a marker of compliance in intervention studies with foods with high-polyphenol content such as fruits, vegetables, wine, chocolate, tea, and coffee, while other markers are not suitable; moreover, in comparison with measuring the total polyphenol intake through self-reported information based on FFQ, the use of TPE, a biomarker of polyphenol intake, could provide more precise evidence [20, 50]. Secondly, the long duration of the intervention should also be considered as strength, since only few studies have tested associations between polyphenols and cardiovascular risk factors in such long-term intervention [8, 51, 52]. Thirdly, the selection of participants is a group of free-living individuals reproducing real-life conditions with home-prepared, energy-unrestricted foods. Fourthly, the Folin-Ciocalteu assay is a rapid, cheaper, and environmentally friendly measurement without requirement of dedicated instrumentation, which could be suggested to be applied in large intervention studies in the future.

In conclusion, in this 5-year study within the frame of the PREDIMED trial conducted in subjects at high cardiovascular risk, we found that polyphenol intake measured by TPE was inversely associated with some clinical cardiovascular risk factors, namely, plasma glucose and triglycerides concentrations and SBP, suggesting that intake of polyphenols provides protection against CVDs throughout these mechanisms. Further research is needed to confirm the current findings in the general population.

Disclosure

None of the funding sources played a role in the design, collection, analysis, or interpretation of the data or in the decision to submit the paper for publication.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Publication 2: Intake of Total Polyphenols and Some Classes of Polyphenols Is Inversely Associated with Diabetes in Elderly People at High Cardiovascular Disease Risk

Anna Tresserra-Rimbau, Marta Guasch-Ferré, Jordi Salas-Salvadó, Estefanía Toledo, Dolores Corella, Olga Castañer, **Xiaohui Guo**, Enrique Gómez-Gracia, José Lapetra, Fernando Arós, Miquel Fiol, Emili Ros, Lluis Serra-Majem, Xavier Pintó, Montserrat Fitó, Nancy Babio, Miguel A Martínez-González, Jose V Sorli, M Carmen López-Sabater, Ramón Estruch, Rosa M Lamuela-Raventós, on behalf of the PREDIMED study investigators. The Journal of Nutrition. First published ahead of print March 9, 2016 as doi: 10.3945/jn.115.223610.

Abstract: Higher consumption of some polyphenols has been associated with a reduced risk of diabetes. However, no studies have evaluated the relation between all polyphenol subclasses and the incidence of diabetes.

We aimed to prospectively examine the associations between the intake of total polyphenols and different groups of polyphenols (flavonoids, phenolic acids, stilbenes, lignans, and others) on the risk of incident diabetes in the PREDIMED (Prevención con Dieta Mediterranea) trial.

This was an observational cohort analysis of the nondiabetic participants in the PREDIMED trial. This study was a multicenter, controlled, randomized, parallel-group feeding trial to assess the effects of either a Mediterranean diet that was supplemented with extra-virgin olive oil or nuts or advice to adhere to a low-fat control diet on cardiovascular outcomes in elderly men and women at high cardiovascular disease risk. From the 7447 randomly assigned participants, 3430 were selected because they were free of diabetes at baseline and filled out the food-frequency questionnaires (FFQs). Polyphenol intake was calculated by matching food consumption data from repeated FFQs with the Phenol-Explorer database on the polyphenol content of each reported food. HRs and 95% CIs for diabetes according to tertiles of polyphenol intake were estimated with the use of time-dependent Cox proportional hazards models.

Over a mean of 5.51 y of follow-up (18,900 person-years), there were 314 new cases of diabetes. After multivariable adjustment, we observed a 28% reduction in new-onset diabetes in the highest compared with the lowest tertile of total polyphenol intake (HR: 0.72; 95% CI: 0.52, 0.99; P-trend = 0.05). The intake of subclasses of polyphenols also was inversely associated with diabetes risk, including for total flavonoids (HR: 0.67; 95% CI: 0.48, 0.93; P-trend =0.02), stilbenes (HR: 0.57; 95% CI: 0.38, 0.84; P-trend =

0.003), dihydroflavonols (HR: 0.59; 95% CI: 0.40, 0.88; P-trend =0.003), and flavanones (HR: 0.69; 95% CI: 0.49, 0.97; P-trend = 0.03).

A high intake of total polyphenols, total flavonoids (specifically flavanones and dihydroflavonols), and stilbenes is associated with a reduced risk of diabetes in elderly persons at high risk of cardiovascular disease.

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Intake of Total Polyphenols and Some Classes of Polyphenols Is Inversely Associated with Diabetes in Elderly People at High Cardiovascular Disease Risk[1–3]

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Abstract

Background: Higher consumption of some polyphenols has been associated with a reduced risk of diabetes. However, no studies have evaluated the relation between all polyphenol subclasses and the incidence of diabetes.

Objective: We aimed to prospectively examine the associations between the intake of total polyphenols and different groups of polyphenols (flavonoids, phenolic acids, stilbenes, lignans, and others) on the risk of incident diabetes in the PREDIMED (Prevención con Dieta Mediterránea) trial.

Methods: This was an observational cohort analysis of the nondiabetic participants in the PREDIMED trial. This study was a multicenter, controlled, randomized, parallel-group feeding trial to assess the effects of either a Mediterranean diet that was supplemented with extra-virgin olive oil or nuts or advice to adhere to a low-fat control diet on cardiovascular outcomes in elderly men and women at high cardiovascular disease risk. From the 7447 randomly assigned participants, 3430 were selected because they were free of diabetes at baseline and filled out the food-frequency questionnaires (FFQs). Polyphenol intake was calculated by matching food consumption data from repeated FFQs with the Phenol-Explorer database on the polyphenol content of each reported food. HRs and 95% CIs for diabetes according to tertiles of polyphenol intake were estimated with the use of time-dependent Cox proportional hazards models.

Results: Over a mean of 5.51 y of follow-up (18,900 person-years), there were 314 new cases of diabetes. After multivariable adjustment, we observed a 28% reduction in new-onset diabetes in the highest compared with the lowest tertile of total polyphenol intake (HR: 0.72; 95% CI: 0.52, 0.99; *P*-trend = 0.05). The intake of subclasses of polyphenols also was inversely associated with diabetes risk, including for total flavonoids (HR: 0.67; 95% CI: 0.48, 0.93; *P*-trend = 0.02), stilbenes (HR: 0.57; 95% CI: 0.38, 0.84; *P*-trend = 0.003), dihydroflavonols (HR: 0.59; 95% CI: 0.40, 0.88; *P*-trend = 0.003), and flavanones (HR: 0.69; 95% CI: 0.49, 0.97; *P*-trend = 0.03).

Conclusions: A high intake of total polyphenols, total flavonoids (specifically flavanones and dihydroflavonols), and stilbenes is associated with a reduced risk of diabetes in elderly persons at high risk of cardiovascular disease. This trial was registered at http://www.controlled-trials.com as ISRCTN35739639. *J Nutr* doi: 10.3945/jn.115.223610.

Keywords: chronic disease, cox regression, epidemiology, glucose, observational study

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Introduction

In 2014, the global prevalence of diabetes was estimated to be 9% in adults, and it was the direct cause of 1.5 million deaths in 2012. In recent decades, the prevalence of this disease and its modifiable risk factors (overweight/obesity, dyslipidemia, hypertension, and physical inactivity) has been increasing globally, particularly in low- and middle-income countries (1). The incidence of type 2 diabetes could be reduced by the adoption of a healthier lifestyle. Weight loss, regular exercise, a healthy diet, and abstinence from smoking are all recognized as important lifestyle factors that condition the risk of diabetes. A diet including whole grains, fruits, vegetables, legumes, nuts, and moderate alcohol consumption has been shown to decrease the risk of the onset of type 2 diabetes, whereas consumption of refined grains, red or processed meats, and sugar-sweetened beverages increases the risk (2). Polyphenol dietary intake has been associated with a reduced incidence of type 2 diabetes in humans (3–5). The protective activity of these bioactive compounds, widespread in foods from plants, also has been demonstrated in animal models (6–10). Thus, polyphenols may influence glycemia through different mechanisms, including the inhibition of glucose absorption in the gut or inhibition of its uptake in peripheral tissues (11). However, the influence of the different subgroups of polyphenols on diabetes in humans has not been completely studied. Indeed, epidemiologic and clinical studies have focused attention only on lignans, flavanols, flavonols, flavones and anthocyanins, or individual polyphenols such as resveratrol or quercetin.

To our knowledge, no prospective research has comprehensively quantified the association between the intake of all polyphenol subgroups and the risk of diabetes; therefore, we aimed to prospectively examine whether polyphenol intake is associated with a risk of incident diabetes and which polyphenol subgroups may be involved in the possible association.

Methods

The study design was an observational cohort analysis of the nondiabetic participants in the PREDIMED (Prevención con Dieta Mediterránea) trial. This study, which took place from October 2003 to December 2010, was a multicenter, controlled, randomized, parallel-group feeding trial to assess the effects of either a Mediterranean diet (MedDiet) supplemented with extra-virgin olive oil or nuts or advice to adhere to a low-fat control diet on cardiovascular outcomes in individuals at high cardiovascular disease risk. Details of the recruitment method and study design have been described elsewhere (12) and also are available at www.predimed.es. The trial was stopped after a median follow-up of 4.8 y because of the benefit of the MedDiets on the prevention of major cardiovascular events (myocardial infarction, stroke, or death from cardiovascular disease) compared with the control low-fat group (13). Although the trial was completed in 2010, the ascertainment of the endpoints was extended until June 2012, and the results of the present analysis are based on an extended follow-up that used the same methods as those used during the trial to obtain updated information on diabetes. The study protocol was approved by the institutional review boards of the participating centers (ISRCTN35739639).

Study population. From the 7447 randomly assigned PREDIMED participants, we excluded 3614 who reported diabetes (including types 1 and 2) at baseline. We also excluded 371 participants who did not complete the FFQs at baseline, and 27 who had an extreme energy intake (i.e., energy intake <500 or >3500 kcal/d for women and <800 or >4000 kcal/d for men) (14). When some dietary data were missing, the imputed values were the mean between the previous and the following available FFQs. The percentages of missing dietary data in different years were as follows: 8% (year 1), 13% (year 2), 7% (year 3), 4% (year 4), 2% (year 5), and 0% (year 6). Finally, we also excluded 5 participants for whom the PREDIMED Clinical Event Ascertainment Committee had not confirmed the diagnosis of diabetes. After exclusions, data from 3430 participants were available for this analysis.

Assessment of diet, polyphenol intake, and lifestyle. At baseline and yearly, participants filled out the following validated questionnaires: a 137-item semiquantitative FFQ (15), a 14-point score questionnaire on adherence to the traditional MedDiet (16), and the Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire (17). In addition, participants filled out a general questionnaire to provide data on lifestyle habits, concurrent diseases, and medication use.

Total energy and nutrient intake were calculated on the basis of Spanish food composition tables (18). Individual polyphenol intake was calculated by multiplying the content of each polyphenol in a particular food item (milligrams per gram) by the consumption of this food item (grams per day) and then summing the product across all food items. We obtained the polyphenol content of foods with the use of the Phenol-Explorer database (www.phenol-explorer.eu). The

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[3] Supplemental Table 1 is from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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correspondence between food items in the FFQ and the database was previously described (19). In previous studies, our group validated the FFQ to assess total polyphenol intake in both clinical ($r = 0.48$, $P < 0.01$) and cross-sectional ($r = 0.26$, $P = 0.04$) studies (20, 21).

Assessment of diabetes. For the present analysis, the main endpoint was incidence of type 2 diabetes diagnosed according to the American Diabetes Association criteria (22), namely, fasting plasma glucose concentrations of ≥7.0 mmol/L (≥126.1 mg/dL) or 2-h plasma glucose concentrations of ≥11.1 mmol/L (≥200.0 mg/dL) after an oral dose of 75 g glucose. A review of all medical records of participants was completed yearly in each center by physician investigators who were blinded to the intervention. When new-onset diabetes cases were identified on the basis of a medical diagnosis reported in the medical charts or by a glucose test during routine biochemical analyses (done ≥1 time/y), these reports were sent to the PREDIMED Clinical Events Ascertainment Committee, whose members also were blinded to treatment allocation. Only when a second test that used the same criteria and repeated within the next 3 mo was available and confirmed the new diabetes case was the endpoint definitively confirmed by the adjudication committee (23).

Statistical analysis. Baseline characteristics are presented as means ± SDs for continuous variables and frequencies (and percentages) for categorical variables across tertiles of total polyphenol intake at baseline adjusted for energy intake (with the use of the residual method) (24). Differences between tertiles were tested by a 1-factor ANOVA test for continuous variables and by the chi-square test for categorical variables.

We calculated person-years of follow-up for each individual from the date of inclusion to the date of diagnosis of type 2 diabetes, death, or end of the follow-up, whichever came first. We used time-dependent Cox proportional hazards regression models with updated diet and covariate information to estimate the HRs for polyphenol intake in relation to type 2 diabetes risk, while using the lowest tertiles of intake as the reference group. Tertiles were used to avoid assumptions about linearity and also to reduce the effect of potential outliers. Then, the median intake value and the 25th and 75th percentiles, the number of cases, and the median years of follow-up (with the 25th and 75th percentiles) were assigned to each tertile. A test for linear trend was performed with the use of the resulting variable as a continuous one.

Total polyphenols and subclasses were previously adjusted for total energy intake with the use of the residual method (24). To assess long-term polyphenol intake and reduce within-person variation, we also calculated the weighted cumulative mean of polyphenol intake at each yearly visit, i.e., polyphenol intake for a given year was the mean between the intake for that year and the mean of the previous years. Nondietary covariates such as smoking, BMI, physical activity, and medication use, as well as dietary covariates, were updated yearly.

In multivariable models, we adjusted for age (<60, 60–64.9, 65–69.9, 70–74.9, and ≥75 y), BMI (continuous), smoking status (never, current, or former), physical activity (continuous), education (primary education, secondary education, or academic/graduate), fasting blood glucose concentrations at baseline (continuous), prevalence of dyslipidemia (yes/no) and hypertension (yes/no), alcohol consumption (continuous in grams per day, adding a quadratic term), energy intake (continuous), and adherence to the traditional MedDiet (14-point score). We also stratified for sex, recruitment center, and intervention group in all models. Stratification allows for the assessment of modifying effects, as well as controlling for confounding factors. The stratums were then pooled by the software (SAS) to give an overall estimate of the RR adjusted for other potential confounders.

We conducted additional stratified analyses for sex, age, alcohol intake, smoking, physical activity, intervention group, and fasting glucose concentrations at baseline to evaluate potential effect modification. We present the HRs and 95% CIs for each risk factor category, comparing the third tertile with the first tertile and using the fully-adjusted model, taking out the risk factor that we were evaluating. We also included the number of cases and median years of follow-up for each category. To test for linearity, we used the median intake in each tertile as a continuous variable. To test for statistical interactions, we also added to the model interaction terms between total polyphenol intake and each of these factors.

All statistical analyses were conducted with the use of SAS software, version 9. All t tests were 2-sided and P values below 0.05 were considered to be significant.

Results

The present study was conducted on 3430 subjects: 1314 men aged 65.2 ± 6.3 y and 2116 women aged 67.5 ± 5.6 y. We present baseline characteristics by tertiles of baseline total polyphenol intake in **Table 1**. At baseline, participants in the third tertile were more likely to be men ($P < 0.001$), younger ($P < 0.001$), and current ($P < 0.001$) or former smokers ($P = 0.03$); have a lower BMI (in kg/m^2; $P = 0.02$); and be more physically active ($P < 0.001$). They also had a higher adherence to the traditional MedDiet ($P < 0.001$) and tended to consume foods with a high polyphenol content, such as fruits and vegetables, nuts, coffee, and wine ($P < 0.001$). Those with a lower intake of polyphenols had a lower education level ($P < 0.001$), were more hypertensive ($P = 0.003$), and had a higher waist-to-height ratio ($P < 0.001$).

During a median of 5.5 ± 2.0 y of follow-up (18,900 person-years), a total of 314 incident cases of diabetes were diagnosed (9.1%). The Cox proportional HRs for type 2 diabetes according to tertiles of cumulative intake of total polyphenols (adjusted for calories) and the main polyphenol groups are shown in **Table 2**. After adjustment for anthropometric, sociodemographic, lifestyle, and dietary variables (fully adjusted model) and stratifying by sex, recruitment center, and intervention group, significant and linear inverse associations were found for total polyphenols (HR: 0.72; 95% CI: 0.52, 0.99; P-trend = 0.05), total flavonoids (HR: 0.67; 95% CI: 0.48, 0.93; P-trend = 0.02), and stilbenes (HR: 0.57; 95% CI: 0.38, 0.84; P-trend = 0.003), whereas nonsignificant results were found for other polyphenol groups.

The HRs for new-onset type 2 diabetes and tertiles of cumulative flavonoid class intake are shown in **Table 3**. Dihydroflavonols and flavanones were significantly associated with the risk of type 2 diabetes in the fully adjusted model when comparing the third with the first tertile (HR: 0.59; 95% CI: 0.40, 0.88; P-trend = 0.003; and HR: 0.69; 95% CI: 0.49, 0.97; P-trend = 0.03, respectively). Nevertheless, it is worth mentioning that, for catechins, the middle tertile was significantly associated with the risk of type 2 diabetes compared with the first tertile, even in the fully adjusted model (HR: 0.61; 95% CI: 0.44, 0.85). This association was not observed for the group with the highest intake (HR: 0.84; 95% CI: 0.60, 1.17; P-trend = 0.45).

There were substantial changes from model 2 to model 3; for instance, HRs for stilbenes changed from 0.84 to 0.57, and for dihydroflavonols, from 0.87 to 0.59, when comparing the third to the first tertile, and flavonols changed from 0.93 to 0.77 when comparing the second to the first tertile. This was due to the inclusion of both alcohol and fasting glucose concentrations at baseline in the model.

The HRs and 95% CIs of diabetes risk, comparing the highest with the lowest tertile of intake of total polyphenols and subclasses after adjustment for all potential confounders, are shown in **Figure 1**.

We also conducted stratified analyses by different predictors of diabetes (results shown in **Table 4**) and total polyphenol

TABLE 1 Baseline characteristics of the PREDIMED study cohort according to tertiles of calorie-adjusted total polyphenol intake at baseline[1]

Characteristics	T1 (n = 1143)	T2 (n = 1144)	T3 (n = 1143)	P^2
Polyphenol intake (cutoff values), mg/d	554 ± 103 (<701)	805 ± 63 (701–914)	1131 ± 203 (>914)	
Female	722 (63.2)[b]	778 (68.0)[b]	616 (53.9)[a]	<0.001
Age, y	66.9 ± 6.1[a]	67.1 ± 5.9[a]	66.0 ± 5.9[b]	<0.001
BMI, kg/m^2	30.2 ± 3.5[a]	29.8 ± 3.4[b]	29.8 ± 3.5[b]	0.02
Leisure-time physical activity, MET-min/d	211 ± 214[b]	223 ± 205[b]	263 ± 248[a]	<0.001
Smoking				<0.001
Never	746 (65.3)[b]	774 (67.7)[b]	613 (53.6)[a]	
Current	196 (17.1)[b]	174 (15.2)[b]	283 (24.8)[a]	
Former	201 (17.6)[b]	196 (17.1)[b]	247 (21.6)[a]	
Education				<0.001
Primary	906 (79.3)[b]	885 (77.3)[b]	802 (70.2)[a]	
Secondary	174 (15.2)	179 (15.7)	201 (17.6)	
Academic/graduate	63 (5.5)[b]	80 (7.0)[b]	104 (12.2)[a]	
Intervention group				<0.001
MedDiet–EVOO	384 (33.6)	380 (33.2)	364 (31.9)	
MedDiet–nuts	374 (32.7)[a]	387 (33.8)[a,b]	437 (38.2)[b]	
Low-fat diet (control group)	385 (33.7)	377 (33.0)	342 (29.9)	
Drug use				
Hypolipidemic	539 (47.2)[a]	591 (51.7)[b]	579 (50.9)[a,b]	0.07
Antihypertensive	907 (79.5)[a]	878 (76.8)[a,b]	863 (75.8)[b]	0.09
Aspirin	174 (15.3)	211 (18.4)	201 (17.7)	0.11
Multivitamins	152 (13.3)	165 (14.5)	142 (12.5)	0.36
Mean intake				
Total energy intake, kcal/d	2372 ± 621[a]	2196 ± 513[b]	2310 ± 558[c]	<0.001
Carbohydrates, g/d	258 ± 87[a]	235 ± 71[b]	248 ± 75[c]	<0.001
Protein, g/d	94.5 ± 22.4[a]	89.3 ± 19.0[b]	91.0 ± 20.7[c]	<0.001
SFAs, g/d	26.6 ± 9.7[a]	23.9 ± 7.7[b]	23.9 ± 8.7[b]	<0.001
MUFAs, g/d	49.9 ± 15.4[a]	46.6 ± 13.6[b]	47.3 ± 14.4[b]	<0.001
PUFAs, g/d	16.2 ± 6.7[a]	14.9 ± 6.0[b]	15.3 ± 6.3[b]	<0.001
Fiber, g/d	23.2 ± 7.6[a]	24.7 ± 7.7[b]	28.7 ± 9.7[c]	<0.001
Total cholesterol, mg/d	378 ± 135[a]	357 ± 117[b]	352 ± 112[b]	<0.001
Alcohol, g/d	6.2 ± 10.9[a]	7.4 ± 12.2[b]	14.4 ± 19.3[c]	<0.001
Vegetables, g/d	303 ± 125[a]	322 ± 132[b]	359 ± 152[c]	<0.001
Fruits, g/d	279 ± 152[a]	359 ± 170[b]	480 ± 227[c]	<0.001
Legumes, g/d	20.8 ± 15.7	20.3 ± 11.3	20.5 ± 12.3	0.63
Cereals, g/d	256 ± 117[a]	220 ± 95[b]	217 ± 94[b]	<0.001
Dairy products, g/d	389 ± 228[b]	371 ± 212[b]	350 ± 220[a]	<0.001
Meat or meat products, g/d	139 ± 61[a]	129 ± 51[b]	129 ± 52[b]	<0.001
Fish, g/d	96.0 ± 44.5[b]	96.3 ± 45.3[b]	101 ± 45.5[a]	0.01
Sugar-sweetened soft drinks, g/d	28.9 ± 88.9[a]	21.1 ± 69.4[b]	17.9 ± 60.1[b]	0.001
Nuts, g/d	9.7 ± 13.1[b]	8.7 ± 1.9[b]	11.4 ± 14.0[a]	0.003
Coffee, mL/d	45.2 ± 42.6[a]	65.8 ± 44.9[b]	90.8 ± 58.8[c]	<0.001
Tea, mL/d	4.9 ± 20.1	5.2 ± 18.0	6.5 ± 25.2	0.17
Wine, mL/d	36.5 ± 74.0[a]	52.3 ± 94.2[b]	110.3 ± 154.0[c]	<0.001
14-point MedDiet score	8.24 ± 1.89[a]	8.67 ± 1.93[b]	9.08 ± 1.84[c]	<0.001
Clinical variables				
Hypertension	1076 (94.1)[a]	1049 (91.7)[b]	1034 (90.5)[b]	<0.001
Hypercholesterolemia	925 (80.9)[a]	999 (87.3)[b]	991 (86.7)[b]	<0.001
Waist-to-height ratio	0.63 ± 0.06[a]	0.62 ± 0.06[b]	0.62 ± 0.06[b]	<0.001
Systolic BP, mm Hg	149 ± 19[a]	148 ± 19[b]	148 ± 18[b]	0.02
Diastolic BP, mm Hg	84 ± 10	84 ± 10	84 ± 10	0.41
Glucose,[3] mg/dL	98 ± 15	98 ± 16	99 ± 16	0.57
Total cholesterol,[3] mg/dL	210 ± 37	214 ± 39	214 ± 38	0.09
HDL cholesterol,[3] mg/dL	52 ± 12	53 ± 11	53 ± 11	0.05
LDL cholesterol,[3] mg/dL	139 ± 34	140 ± 33	140 ± 34	0.83
TGs,[3] mg/dL	128 ± 73	129 ± 71	129 ± 63	0.94

[1] Values are frequencies (percentages) for categorical variables or means ± SDs for continuous variables; n = 3430. Values in a row without a common superscript letter are significantly different, $P < 0.05$. BP, blood pressure; MedDiet–EVOO, Mediterranean diet supplemented with extra-virgin olive oil; MedDiet–nuts, Mediterranean diet supplemented with nuts; MET, metabolic equivalent task; PREDIMED, Prevención con Dieta Mediterránea, T, tertile.
[2] Calculated by ANOVA or χ^2 tests.
[3] Measured in plasma.

TABLE 2 Cox proportional HRs for new-onset diabetes in the PREDIMED cohort by cumulative intake of polyphenols, adjusted for energy intake and divided into tertiles[1]

	T1	T2	T3	*P*-trend[2]	*P*[3]
Total polyphenols, mg/d	600 (518, 653)	781 (739, 825)	1002 (929, 1119)		
Cases, *n*	117	103	94		
Person-years, *n*	5910	6785	6205		
Follow-up, y	5.6 (4.0, 7.2)	5.4 (4.0, 7.2)	5.3 (3.9, 7.2)		0.70
Incidence, %	10.9	8.4	8.3		0.06
Model 1	1.00 (ref)	0.82 (0.62, 1.07)	0.81 (0.61, 1.08)	0.15	
Model 2	1.00 (ref)	0.78 (0.59, 1.04)	0.74 (0.55, 0.99)	0.04	
Model 3	1.00 (ref)	0.74 (0.54, 1.00)	0.72 (0.52, 0.99)	0.05	
Flavonoids, mg/d	291 (236, 334)	425 (392, 462)	596 (533, 698)		
Cases, *n*	133	91	90		
Person-years, *n*	5659	6685	6556		
Follow-up, y	5.0 (3.9, 7.1)	5.9 (4.0, 7.3)	5.8 (4.0, 7.3)		<0.0001
Incidence, %	12.4	7.7	7.7		<0.0001
Model 1	1.00 (ref)	0.66 (0.50, 0.87)	0.69 (0.52, 0.92)	0.01	
Model 2	1.00 (ref)	0.64 (0.48, 0.85)	0.69 (0.51, 0.93)	0.02	
Model 3	1.00 (ref)	0.60 (0.44, 0.82)	0.67 (0.48, 0.93)	0.02	
Phenolic acids, mg/d	164 (130, 192)	256 (234, 279)	381 (342, 442)		
Cases, *n*	101	109	104		
Person-years, *n*	6577	6555	6767		
Follow-up, y	6.0 (4.1, 7.3)	5.8 (4.0, 7.2)	4.9 (3.8, 7.1)		<0.0001
Incidence, %	8.7	9.3	9.5		0.83
Model 1	1.00 (ref)	1.03 (0.78, 1.36)	1.03 (0.78, 1.37)	0.84	
Model 2	1.00 (ref)	1.06 (0.80, 1.41)	0.96 (0.71, 1.29)	0.73	
Model 3	1.00 (ref)	0.89 (0.65, 1.21)	0.85 (0.62, 1.17)	0.34	
Stilbenes, mg/d	0.04 (0, 0.17)	1.01 (0.73, 1.35)	3.89 (2.77, 6.95)		
Cases, *n*	102	115	97		
Person-years, *n*	3141	6519	6238		
Follow-up, y	5.4 (4.0, 7.3)	5.1 (4.0, 7.1)	6.0 (4.0, 7.3)		0.003
Incidence, %	9.2	9.5	8.8		0.85
Model 1	1.00 (ref)	1.08 (0.82, 1.42)	0.81 (0.60, 1.09)	0.09	
Model 2	1.00 (ref)	1.01 (0.76, 1.34)	0.84 (0.62, 1.14)	0.23	
Model 3	1.00 (ref)	0.90 (0.64, 1.27)	0.57 (0.38, 0.84)	0.003	
Lignans, mg/d	0.42 (0.35, 0.47)	0.59 (0.56, 0.63)	0.78 (0.73, 0.88)		
Cases, *n*	111	96	107		
Person-years, *n*	5401	6345	7153		
Follow-up, y	4.9 (3.6, 7.0)	5.4 (4.0, 7.1)	6.1 (4.4, 7.4)		<0.0001
Incidence, %	10.5	8.3	8.8		0.17
Model 1	1.00 (ref)	0.73 (0.55, 0.97)	0.78 (0.58, 1.05)	0.12	
Model 2	1.00 (ref)	0.68 (0.51, 0.92)	0.75 (0.55, 1.01)	0.08	
Model 3	1.00 (ref)	0.68 (0.48, 0.94)	0.82 (0.58, 1.15)	0.35	
Others,[4] mg/d	41.3 (32.8, 47.3)	63.3 (58.1, 69.5)	96.5 (85.3, 115.0)		
Cases, *n*	90	113	111		
Person-years, *n*	5701	7143	6055		
Follow-up, y	5.1 (3.9, 7.2)	5.9 (4.0, 7.3)	5.4 (4.0, 7.2)		0.004
Incidence, %	8.5	8.9	10.1		0.43
Model 1	1.00 (ref)	0.97 (0.72, 1.29)	1.08 (0.81, 1.45)	0.51	
Model 2	1.00 (ref)	0.95 (0.71, 1.28)	1.06 (0.79, 1.44)	0.60	
Model 3	1.00 (ref)	0.98 (0.71, 1.36)	0.97 (0.70, 1.36)	0.89	

[1] Values are HRs (95% CIs), unless otherwise indicated. Polyphenol intake and follow-up values are medians (25th, 75th percentiles). Model 1 is adjusted for age and stratified by sex, recruitment center, and intervention group. Model 2 is adjusted for factors in model 1, in addition to smoking, BMI, physical activity, dyslipidemia, hypertension, and education level. Model 3 is adjusted for factors in model 2, in addition to total energy intake, alcohol intake, adherence to the Mediterranean diet, and fasting glucose concentrations at baseline. PREDIMED, Prevención con Dieta Mediterránea; ref, reference; T, tertile.

[2] Based on tests for trend across tertiles of polyphenol intake by assigning the median value of each tertile.

[3] Calculated by ANOVA (continuous variables) or χ^2 tests (categorical variables).

[4] Includes alkylmethoxyphenols, alkylphenols, curcuminoids, furanocoumarins, hydroxybenzaldehydes, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxycoumarins, hydroxyphenylpropenes, methoxyphenols, naphtoquinones, phenolic terpenes, and tyrosols.

TABLE 3 Cox proportional HRs for new-onset diabetes in the PREDIMED cohort by cumulative intake of flavonoid classes, adjusted for energy intake and divided into tertiles[1]

	T1	T2	T3	*P*-trend[2]	*P*[3]
Anthocyanidins, mg/d	14.9 (8.6, 19.6)	30.9 (26.9, 35.3)	58.9 (48.3, 77.1)		
Cases, *n*	104	97	113		
Person-years, *n*	6642	6485	5772		
Follow-up, y	5.2 (3.9, 7.2)	5.9 (4.0, 7.3)	5.6 (4.0, 7.2)		0.02
Incidence, %	8.5	8.4	10.7		0.11
Model 1	1.00 (ref)	0.84 (0.63, 1.12)	0.99 (0.75, 1.30)	0.89	
Model 2	1.00 (ref)	0.84 (0.63, 1.13)	0.96 (0.72, 1.27)	0.89	
Model 3	1.00 (ref)	0.82 (0.59, 1.13)	0.88 (0.62, 1.24)	0.54	
Catechins, mg/d	13.8 (10.5, 16.4)	23.2 (20.8, 26.2)	39.4 (33.7, 48.2)		
Cases, *n*	125	83	106		
Person-years, *n*	6080	6593	6227		
Follow-up, y	5.4 (4.0, 7.3)	5.5 (4.0, 7.2)	5.4 (4.0, 7.2)		0.35
Incidence, %	11.3	7.0	9.3		0.002
Model 1	1.00 (ref)	0.64 (0.48, 0.85)	0.85 (0.64, 1.11)	0.37	
Model 2	1.00 (ref)	0.64 (0.47, 0.85)	0.84 (0.63, 1.11)	0.35	
Model 3	1.00 (ref)	0.61 (0.44, 0.85)	0.84 (0.60, 1.17)	0.45	
Dihydrochalcones, mg/d	0.99 (0.41, 1.38)	2.40 (2.00, 2.77)	3.96 (3.48, 6.18)		
Cases, *n*	117	97	100		
Person-years, *n*	5477	6906	6516		
Follow-up, y	5.1 (3.9, 7.1)	6.0 (4.1, 7.4)	5.3 (3.9, 7.2)		<0.0001
Incidence, %	11.3	8.1	8.4		0.22
Model 1	1.00 (ref)	0.77 (0.58, 1.01)	1.00 (0.75, 1.01)	0.99	
Model 2	1.00 (ref)	0.79 (0.60, 1.06)	1.00 (0.74, 1.35)	0.92	
Model 3	1.00 (ref)	0.88 (0.64, 1.19)	1.15 (0.83, 1.61)	0.44	
Dihydroflavonols, mg/d	0 (0, 0.16)	1.48 (1.03, 2.03)	6.09 (4.31, 11.0)		
Cases, *n*	100	117	97		
Person-years, *n*	6132	6546	6220		
Follow-up, y	5.6 (4.0, 7.3)	5.1 (3.9, 7.1)	6.0 (4.0, 7.3)		0.002
Incidence, %	9.0	9.6	8.8		0.8
Model 1	1.00 (ref)	1.13 (0.86, 1.49)	0.83 (0.62, 1.12)	0.12	
Model 2	1.00 (ref)	1.05 (0.79, 1.39)	0.87 (0.64, 1.17)	0.27	
Model 3	1.00 (ref)	0.99 (0.70, 1.38)	0.59 (0.40, 0.88)	0.003	
Proanthocyanidins, mg/d	74.5 (54.3, 87.4)	122 (111, 134)	187 (164, 228)		
Cases, *n*	122	102	90		
Person-years, *n*	5778	6831	6290		
Follow-up, y	5.1 (3.9, 7.2)	5.8 (4.0, 7.3)	5.6 (4.0, 7.2)		0.003
Incidence, %	11.3	8.4	7.9		0.01
Model 1	1.00 (ref)	0.78 (0.59, 1.02)	0.73 (0.55, 0.97)	0.04	
Model 2	1.00 (ref)	0.80 (0.61, 1.06)	0.70 (0.52, 0.95)	0.02	
Model 3	1.00 (ref)	0.75 (0.55, 1.02)	0.75 (0.54, 1.04)	0.09	
Flavanones, mg/d	43.4 (15.9, 63.8)	114 (96.4, 132)	197 (166, 292)		
Cases, *n*	121	105	88		
Person-years, *n*	5206	6670	7024		
Follow-up, y	5.1 (3.9, 7.1)	6.0 (4.1, 7.3)	5.6 (3.9, 7.3)		<0.0001
Incidence, %	12.3	8.9	7.0		<0.0001
Model 1	1.00 (ref)	0.77 (0.59, 1.01)	0.73 (0.55, 0.98)	0.04	
Model 2	1.00 (ref)	0.81 (0.61, 1.07)	0.74 (0.54, 1.00)	0.05	
Model 3	1.00 (ref)	0.87 (0.65, 1.17)	0.69 (0.49, 0.97)	0.03	
Flavones, mg/d	21.5 (16.6, 25.1)	34.7 (31.5, 38.0)	56.8 (48.4, 71.2)		
Cases, *n*	116	94	104		
Person-years, *n*	5328	6474	7098		
Follow-up, y	5.1 (3.9, 7.1)	5.9 (4.0, 7.2)	5.8 (4.0, 7.3)		<0.0001
Incidence, %	11.5	8.1	8.3		0.009
Model 1	1.00 (ref)	0.78 (0.59, 1.03)	0.87 (0.66, 1.15)	0.46	
Model 2	1.00 (ref)	0.79 (0.49, 1.06)	0.87 (0.65, 1.17)	0.46	
Model 3	1.00 (ref)	0.78 (0.56, 1.06)	0.98 (0.71, 1.35)	0.92	

(Continued)

TABLE 3 *Continued*

	T1	T2	T3	*P*-trend[2]	*P*[3]
Flavonols, mg/d	57.5 (46.8, 64.5)	82.9 (76.9, 87.9)	106 (99.1, 122)		
Cases, *n*	114	100	100		
Person-years, *n*	6242	6279	6378		
Follow-up, y	5.0 (3.9, 7.0)	5.7 (4.0, 7.3)	6.0 (4.2, 7.4)		<0.0001
Incidence, %	9.6	8.9	9.0		0.84
Model 1	1.00 (ref)	0.94 (0.71, 1.25)	1.03 (0.75, 1.42)	0.88	
Model 2	1.00 (ref)	0.93 (0.69, 1.24)	1.04 (0.75, 1.45)	0.96	
Model 3	1.00 (ref)	0.77 (0.56, 1.06)	0.97 (0.68, 1.39)	0.77	

[1] Values are HRs (95% CIs), unless otherwise indicated. Polyphenol intake and follow-up values are medians (25th, 75th percentiles). Model 1 is adjusted for age and stratified by sex, recruitment center, and intervention group. Model 2 is adjusted for factors in model 1, in addition to smoking, BMI, physical activity, dyslipidemia, hypertension, and education level. Model 3 is adjusted for factors in model 2, in addition to total energy intake, alcohol intake, adherence to the Mediterranean diet, and fasting glucose concentrations at baseline. PREDIMED, Prevención con Dieta Mediterránea; ref, reference; T, tertile.
[2] Based on tests for trend across tertiles of polyphenol intake by assigning the median value of each tertile.
[3] Calculated by ANOVA (continuous variables) or χ^2 tests (categorical variables).

intake. None of the stratified results had a significant interaction term; therefore, we cannot draw conclusions.

When we removed fasting glucose concentrations at baseline from the model (**Supplemental Table 1**), some associations were no longer significant (dihydroflavonols and flavanones), whereas others became significant (proanthocyanidins and lignans).

Discussion

In this observational and longitudinal study within the PREDIMED trial, we found that a higher intake of total polyphenols, total flavonoids, stilbenes, and some flavonoid subclasses (dihydroflavonols and flavanones) was inversely and linearly associated with incidence of type 2 diabetes. Even though previous studies have investigated the association between the intake of specific groups of polyphenols and type 2 diabetes, to our knowledge, this is the first study that comprehensively quantified the association between the intake of all polyphenol subgroups and the risk of type 2 diabetes.

Some of our results agree with previous studies, whereas others are contradictory or cannot be compared because of a lack of previously reported data. In 1983, Thompson et al. (25) found for the first time, to our knowledge, an inverse correlation between the glycemic index of the diet and total intake of polyphenols in both healthy and diabetic individuals, especially the large polymeric type or condensed tannins. Other epidemiologic studies that mainly focused on lignans, flavanols, flavonols, anthocyanins and individual polyphenols have associated these polyphenols with a lower incidence of diabetes (3–5).

Several mechanisms have been invoked to explain the inverse associations between polyphenol consumption and diabetes incidence. Long-term cellular inflammation plays an important role in the metabolic consequences of diabetes and other chronic diseases. Indeed, classic cases of hormonal disruption are insulin resistance, which causes hyperinsulinemia, or the β cell burnout in type 2 diabetes that results in chronic hyperglycemia (26). Some polyphenols can inhibit cellular inflammation through the activation of PPARγ and AMPK (Adenosine Monophosphate-activated Protein Kinase), an upstream activator of the anti-inflammatory gene transcription factors SIRT1 (Sirtuin 1) and FOX (Forkhead box) (26, 27). Two flavanones, naringin and hesperidin, showed antidiabetic properties partially mediated through the regulation of PPARγ (6). In a prospective study conducted in 2 cohorts of US women, urinary excretion of hesperetin, another flavanone, was associated with a decreased risk of type 2 diabetes. Other polyphenol metabolites, including naringenin, quercetin, isorhamnetin, and caffeic acid, were associated only during the early follow-up period (28). Consistent with these results, the intake of flavanones in our population was inversely associated with diabetes risk in the fully adjusted model. It is important to note that 99% of flavanone intake came from the consumption of oranges and orange juice (19).

Previous results from animal and cell-cultured studies have shown that flavan-3-ols, especially epigallocatechin gallate, which belongs to the family of catechins, have antidiabetic effects. According to these studies, epigallocatechin gallate acts through multiple signaling pathways, leading to improvements in insulin secretion, glucose uptake, insulin resistance, glucose tolerance, oxidative stress, inflammation, and mitochondrial function (6). A substantial reduction in estimated peripheral insulin resistance and an improvement in insulin sensitivity have also been demonstrated after the consumption of flavonoid-enriched chocolate (containing flavan-3-ols and isoflavones) in individuals with type 2 diabetes (29). In our study, catechins also were significantly associated with a decreased risk of type 2 diabetes when comparing the second to the first tertile. However,

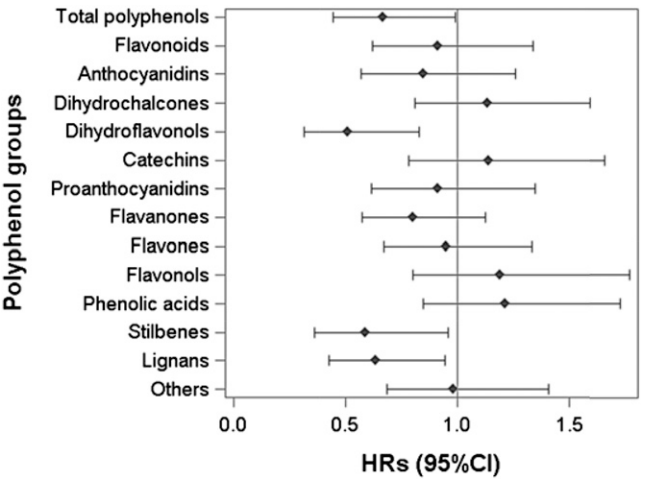

FIGURE 1 HRs (95% CIs) of diabetes incidence for the highest compared with the lowest tertile of polyphenol intake (fully adjusted model) in the Prevención con Dieta Mediterránea study cohort (*n* = 3430).

TABLE 4 Cox proportional HRs for new-onset diabetes in the PREDIMED cohort by cumulative intake of total polyphenols, adjusted for energy intake and stratified by risk factors[1]

Risk factors	Cases, *n*	Person-years	Follow-up,[2] y	T3 vs. T1 multivariable-adjusted HR (95% CI)[3]	*P*-trend[4]	*P*-interaction
Sex						0.96
M	140	7169	5.4 (3.9, 7.3)	0.73 (0.44, 1.19)	0.18	
F	174	11,731	5.5 (4.0, 7.2)	0.77 (0.49, 1.20)	0.22	
Age, y						0.30
<65	154	8608	5.1 (3.9, 7.2)	0.79 (0.48, 1.29)	0.30	
≥65	160	10,292	5.9 (4.0, 7.3)	0.69 (0.43, 1.09)	0.09	
Alcohol intake						0.11
Nondrinkers[5]	123	7117	5.3 (4.0, 7.3)	0.55 (0.30, 0.88)	0.04	
Drinkers	191	11,783	5.6 (4.0, 7.2)	0.89 (0.60, 1.34)	0.59	
Smoking						0.16
Never	173	12,190	5.8 (4.0, 7.3)	0.59 (0.37, 0.94)	0.03	
Former	63	3857	5.1 (3.9, 7.2)	0.97 (0.46, 2.04)	0.94	
Current	78	2853	5.2 (3.8, 7.2)	1.40 (0.69, 2.88)	0.35	
Physical activity						0.25
<median	200	11,940	5.1 (3.9, 7.1)	0.80 (0.49, 1.30)	0.82	
≥median	114	6945	6.0 (4.1, 7.4)	0.47 (0.28, 0.79)	0.006	
Intervention group						0.74
MedDiet–EVOO	91	6435	6.0 (4.2, 7.3)	0.84 (0.45, 1.55)	0.57	
MedDiet–nuts	107	6497	5.2 (3.8, 7.2)	0.77 (0.41, 1.43)	0.38	
Low-fat diet	116	5968	5.2 (3.9, 7.3)	0.83 (0.49, 1.41)	0.48	
Fasting glucose concentrations at baseline, mg/dL						0.54
≤100	78	13,109	6.0 (4.2, 7.3)	0.87 (0.45, 1.68)	0.67	
>100	236	5791	4.9 (3.6, 7.1)	0.63 (0.44, 0.91)	0.02	

[1] MedDiet–EVOO, Mediterranean diet supplemented with extra-virgin olive oil; MedDiet–nuts, Mediterranean diet supplemented with nuts; PREDIMED, Prevención con Dieta Mediterránea; T, tertile.
[2] Medians (25th, 75th percentiles).
[3] Analyses were stratified by sex, recruitment center, and intervention group and adjusted for age, smoking, BMI, physical activity, dyslipidemia, hypertension, education level, fasting glucose concentrations at baseline, total energy intake, alcohol intake, and adherence to the Mediterranean diet.
[4] Highest compared with lowest groups with the use of continuous variables.
[5] Alcohol intake of 0 g/d.

proanthocyanidins, which are polymers of the flavan-3-ols found in grapes, red wine, apples, berries, chocolate, seeds, and legumes, were only inversely associated with diabetes risk when glucose concentrations were taken out of the model.

Similar results were found for anthocyanidins. These colorful compounds traditionally have received special attention because of their antioxidant capacity. Although their bioavailability seems to be low compared with other flavonoids, results from human and animal trials have shown that anthocyanidins improve glucose homeostasis through different mechanisms (5, 6).

In a cross-sectional study conducted by Jennings et al. (30), the intake of different flavonoids was calculated with the use of FFQs and the USDA database. After adjusting for potential confounders, higher anthocyanin and flavone intake and consumption of anthocyanin-rich food were associated with improvements in insulin resistance and high-sensitivity C-reactive protein. Flavones also increased adiponectin secretion, as described by Liu et al. (31).

There is a lack of consensus on the antidiabetic properties of flavonols, the most consumed flavonoids, and flavones. We did not find any relation between flavonols or flavones and diabetes in our study, and neither did Kataja-Tuomola et al. (32) in a large cohort of male smokers aged 50–69 y. On the contrary, flavonols and myricein were significantly associated with a lower risk of developing type 2 diabetes in participants of the European Prospective Study into Cancer and Nutrition study (3, 33). Flavonol intake was also associated with a lower incidence of type 2 diabetes in participants of the Framingham Heart Study Offspring Cohort (34). Moreover, some animal and cell-culture studies support the hypoglycemic effects of quercetin (7, 8).

Despite having a similar structure, dihydroflavonols and flavonols have different solubilities and antioxidant capacities and, therefore, different bioavailability and properties (35). We found a strong inverse association between dihydroflavonols and diabetes, which has been previously demonstrated in animal and in vitro models (9, 10).

Stilbenes, a group of polyphenols that includes the well-known resveratrol, were also strongly and inversely associated with type 2 diabetes in this PREDIMED cohort. The antidiabetic effects of resveratrol evidenced from in vitro and clinical studies have multiple mechanisms: improving insulin sensitivity, enhancing GLUT4 (Glucose transporter type 4) translocation, reducing oxidative stress, regulating carbohydrate metabolizing enzymes, activating SIRT1 and AMPK, and decreasing adipogenic genes (36, 37).

It is noteworthy that the main source of dihydroflavonols and stilbenes in the PREDIMED population was red wine (19). Indeed, Nettleton et al. (38) previously found that women who reported drinking red wine >1 time/wk had a 16% reduced risk of diabetes compared with those drinking wine less often. Nevertheless, these authors could not find any association for total flavonoid intake in this cohort of postmenopausal women. In a crossover trial with men at high risk of cardiovascular

disease, plasma insulin and HOMA-IR decreased after consumption of red wine and dealcoholized red wine, but not gin, suggesting that the nonalcoholic fraction of red wine (mainly polyphenols) was responsible for the effect (39).

Finally, our results suggest an inverse association between lignan intake and diabetes incidence, but only when glucose concentrations at baseline were not added to the model. Enterodiol and enterolactone, which are gut microbiota metabolites of dietary lignans, were associated with a lower type 2 diabetes incidence in US women after multivariable adjustment (4). In the European Prospective Study into Cancer and Nutrition study, however, intake of lignans was not associated with type 2 diabetes (33). The main sources of lignans in our population were olive oil and whole-grain wheat bread (19). These results agree with those from a randomized, placebo-controlled crossover trial in middle-aged overweight men who received capsules with olive-leaf extract or a placebo for 12 wk. The supplementation improved insulin sensitivity and pancreatic β cell secretory capacity (40).

Results from stratified analyses suggested that the association between total polyphenol intake and diabetes could be affected by alcohol intake and smoking. Those who did not drink alcohol and had never smoked appeared to have higher inverse associations. Indeed, an increasing number of epidemiologic studies show that the relation between the risk of the onset of type 2 diabetes and alcohol intake is *U*-shaped. Heavy alcohol consumption is related to obesity and impaired liver function, both of which are associated with an increased risk of diabetes, and active smoking is also positively associated with diabetes in a dose-dependent manner (41).

Foods high in polyphenols may provide multiple other beneficial food components, such as fiber, unsaturated FAs or magnesium (42), which have been associated with a decreased risk of type 2 diabetes. For instance, red wine was the main source of stilbenes and dihydroflavonols in the PREDIMED, but red wine also contains alcohol, and moderate wine or alcohol consumption was inversely associated with diabetes risk in an intervention (43) and 2 meta-analysis (44, 45). Muraki et al. (46) studied whether individual fruits were differentially associated with risk of type 2 diabetes in 3 prospective longitudinal cohort studies, and found that blueberries, grapes, and apples were inversely associated with type 2 diabetes. Grapes and blueberries are a good source of stilbenes, and apples were the third contributor to total polyphenol intake in the PREDIMED cohort (19). Olive oil is the main source of fat in the MedDiet, and it is rich in MUFAs and polyphenols (lignans, flavones, and other classes of polyphenols). It has been demonstrated that extra-virgin olive oil in the framework of a MedDiet can reduce the risk of diabetes in persons with a high risk of cardiovascular disease (23). Diets that are rich in whole grains and fiber can also decrease the risk of diabetes, according to different studies (47–49).

The main strengths of this study include the prospective design, a relatively large sample size, blinded assessment of the endpoints, and comprehensive information about risk factors and confounders for diabetes. Moreover, the use of a weighted cumulative mean of polyphenol intake, calculated with validated FFQs, is the best approach for reducing measurement error in nutritional epidemiology (50), and allowed us to control changes in the diet from the intervention. Finally, we also used the most comprehensive database currently available: the Phenol-Explorer database.

The study also has some limitations. First, this is an observational study within an intervention trial. Because polyphenol-rich foods, mainly extra-virgin olive oil and nuts, were recommended in both MedDiet intervention groups but not in the control group, the dietary advice could affect the accuracy of reporting the consumption of polyphenol-rich foods, mainly by over-reporting some of them. However, the distribution of intervention groups between tertiles was uniform ($P > 0.05$), because the foods that primarily contributed to polyphenol intake in the PREDIMED study were fruits, vegetables, and coffee, and there were no statistically significant differences in the intake of these foods after 5 y of follow-up. Moreover, even though we controlled for several confounders in multivariable models, other unknown or unmeasured confounders could exist. Other limitations refer to the estimation of polyphenol intake. Data were indirectly derived from FFQs; therefore, the bioavailability of the molecules was not taken into account. Moreover, intake could have been underestimated because not all foods from the FFQs were available in the database (e.g., honey) and not all polyphenol-rich foods were recorded in the FFQs (e.g., spices and seasonings). We also should mention that the Phenol-Explorer database contains data on foods from various countries that may lead to a misclassification of Spanish foods. We also did not take into account other factors that modify polyphenol content in foods, including ripeness, environmental factors, processing and storage, and variety (51). Because the intake of theaflavins and isoflavones was very low in our population, we could not study the associations for these polyphenol groups. Finally, these results might not be generalizable to other populations other than middle-aged to elderly people at high risk of cardiovascular disease.

In sum, our data suggest an inverse association between the intake of total polyphenols, total flavonoids (specifically dihydroflavonols and flavanones), and stilbenes and the risk of type 2 diabetes in an elderly Mediterranean population at high risk of cardiovascular disease. These associations were independent of other dietary and nondietary risk factors. Our findings could be the starting point of future randomized, controlled trials to clarify the promising benefits deriving from long-term consumption of polyphenol-rich diets, and to establish dietary recommendations.

Acknowledgments

AT-R, MG-F, JS-S, XG, and RML-R carried out the statistical analyses, interpreted the data, and drafted the manuscript; JS-S, ET, DC, OC, EG-G, JL, FA, M Fiol, ER, LS-M, XP, M Fitó, NB, MAM-G, JVS, RE, MCL-S, and RML-R participated in the design of the study and acquisition of the data, and contributed to the critical review of the paper; and RML-R is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

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Publication 3: Polyphenol levels in urine are inversely correlated with body weight and obesity in an elderly population after 5 years of follow-up (the PREDIMED study)

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Abstract: Overweight and obesity, major independent risk factor for various chronic diseases, have been steadily increasing in recent years and currently represent a serious threat to public health. Only few human studies have assessed the effects of polyphenol intake in body weight control; therefore our aim was to study the relationship between urinary polyphenol levels and body weight.

A longitudinal study was performed with 573 participants, (612 joined at baseline and 39 were excluded) randomly selected from the PREDIMED cohort (ISRCTN35739639). Total polyphenol excretion (TPE), determined by the Folin-Ciocalteu method in urine samples was used as a reliable biomarker of polyphenol intake. Participants were categorized into quintiles according to TPE at 5 years. Multiple linear regression models were used to assess the relationships between TPE and obesity parameters [body weight (BW), body mass index (BMI), waist circumference (WC) and waist-to-height ratio (WHtR)]. Multiple logistic regression analyses were used to calculate the odds ratio (OR) for quintiles of TPE and obesity (BMI>30 kg/m^2).

Significant inverse correlations were observed between TPE at 5 years of follow-up and BW (β=-1.004; 95% CI: -1.634 to -0.375, P=0.002), BMI (β=-0.320; 95% CI: -0.541 to -0.098, P=0.005), WC (β=-0.742; 95% CI: -1.326 to -0.158, P=0.013) and WHtR (β=-0.408; 95% CI: -0.788 to -0.028, P=0.036) after adjustment for potential confounders. Compared with those in the lowest quintile, participants in the top TPE

quintile showed a lower prevalence of obesity (OR=0.346, 95% CI: 0.176 to 0.178; P-trend= 0.039).

The main limitations are the following: the results cannot be extrapolated to the general population; we cannot exclude residual confounding from measurements; and the lack of specific measurements of polyphenol metabolism in vivo.

Greater polyphenol intake may contribute to reduce the risk of obesity in elderly people at high cardiovascular risk.

PLOS ONE

Polyphenol levels are inversely correlated with body weight and obesity in an elderly population after 5 years of follow-up (the PREDIMED study)

--Manuscript Draft--

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Article Type:	Clinical Trial
Full Title:	Polyphenol levels are inversely correlated with body weight and obesity in an elderly population after 5 years of follow-up (the PREDIMED study)
Short Title:	Polyphenols and body weight
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Keywords:	Folin-Ciocalteu, waist, BMI, weight, obesity, urine
Abstract:	Background: Overweight and obesity have been steadily increasing in recent years and currently represent a serious threat to public health. Few human studies have investigated the relationship between polyphenol intake and body weight, even though obesity is considered as a major independent risk factor for various chronic diseases. Our aim was to assess the relationship between urinary polyphenol levels and body weight. Methods and Findings: A longitudinal study was performed with 573 participants from the PREDIMED (Prevención con Dieta Mediterránea) trial (ISRCTN35739639), a large, parallel-group, randomized, multicenter, controlled, clinical trial designed to assess the effects of the Mediterranean diet on the primary prevention of cardiovascular disease in Spain. Total polyphenol levels were measured by a reliable biomarker, total urinary polyphenol excretion (TPE), determined by the Folin-Ciocalteu method in urine samples. Participants were categorized into five groups according to TPE at 5 years. Multiple linear regression models were used to assess the relationships between TPE and obesity parameters [body weight (BW), body mass index (BMI), waist circumference (WC) and waist-to-height ratio (WHtR)]. After 5-year follow-up, significant inverse correlations were observed between 5-year TPE and BW (β=-1.004; 95% CI: -1.634 to -0.375, P=0.002), BMI (β=-0.320; 95% CI: -0.541 to -0.098, P=0.005), WC (β=-0.742; 95% CI: -1.326 to -0.158, P=0.013) and WHtR (β=-0.408; 95% CI: -0.788 to -0.028, P=0.036) after adjustments for potential confounders. Compared with those in the lowest quintile, participants in the top TPE quintile showed a lower prevalence of obesity (OR=0.346, 95% CI: 0.176 to 0.178; P-trend= 0.039). The major limitation of this analysis is the results cannot be extrapolated to the general population and lack of specific measurements of polyphenol metabolism in vivo. Conclusion: A greater polyphenol intake may thus contribute to reducing the risk of obesity in elderly people at high cardiovascular risk.
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Polyphenol levels are inversely correlated with body weight and obesity in an elderly population after 5 years of follow-up (the PREDIMED study)

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Abstract

Background: Overweight and obesity have been steadily increasing in recent years and currently represent a serious threat to public health. Few human studies have investigated the relationship between polyphenol intake and body weight, even though obesity is considered as a major independent risk factor for various chronic diseases. Our aim was to assess the relationship between urinary polyphenol levels and body weight.

Methods and Findings: A longitudinal study was performed with 573 participants from the PREDIMED (Prevención con Dieta Mediterránea) trial (ISRCTN35739639), a large, parallel-group, randomized, multicenter, controlled, clinical trial designed to assess the effects of the Mediterranean diet on the primary prevention of cardiovascular disease in Spain. Total polyphenol levels were measured by a reliable biomarker, total urinary polyphenol excretion (TPE), determined by the Folin-Ciocalteu method in urine samples. Participants were categorized into five groups according to TPE at 5 years. Multiple linear regression models were used to assess the relationships between TPE and obesity parameters [body weight (BW), body mass index (BMI), waist circumference (WC) and waist-to-height ratio (WHtR)]. After 5-year follow-up, significant inverse correlations were observed between 5-year TPE and BW (β=-1.004; 95% CI: -1.634 to -0.375, P=0.002), BMI (β=-0.320; 95% CI: -0.541 to -0.098, P=0.005), WC (β=-0.742; 95% CI: -1.326 to -0.158, P=0.013) and WHtR (β=-0.408; 95% CI: -0.788 to -0.028, P=0.036) after adjustments for potential confounders. Compared with those in the lowest quintile, participants in the top TPE quintile showed a lower prevalence of obesity (OR=0.346, 95% CI: 0.176 to 0.178; P-trend= 0.039). The major limitation of this analysis is the results cannot be extrapolated to the general population and lack of specific measurements of polyphenol metabolism *in vivo*.

58 **Conclusion:** A greater polyphenol intake may thus contribute to reducing the risk of
59 obesity in elderly people at high cardiovascular risk.
60

Introduction

Overweight and obesity have been steadily increasing in recent years and currently represent a serious threat to public health [1]. In 2014, more than 1.9 billion adults were overweight worldwide, and of these over 600 million were obese [2]. With nearly 3 million adults dying each year as a result of being overweight or obese, the impact of obesity on morbidity, mortality, and health care costs is very high [3]. Lifestyle and dietary habits are key determinants in the prevalence of obesity [4–6].

Polyphenols, the most abundant antioxidants in nature, are widely distributed in plant-derived foods such as vegetables, fruits, seeds, coffee, wine and tea [7]. Only a few human studies have reported a relationship between polyphenol intake and body weight, even though obesity is considered as a major independent risk factor for various chronic diseases [8,9]. Evidence for the effects of polyphenols on obesity parameters in humans is inconsistent, possibly due to divergence among study designs, characteristics of the participants, and metabolic pathways. Although some intervention clinical trials with polyphenol-enriched food or polyphenol extracts do not show any effect on weight or waist circumference [10–12], other studies have reported that polyphenols reduce body weight and increase energy expenditure [13–16]. Oral bioavailability of polyphenols is particularly important because, after being modified and metabolized by enzymes, their concentration in tissues and biological fluids is quite low [9,12,15,17]. There is therefore a need for a biomarker to accurately reflect polyphenol concentration after their absorption and metabolism.

Polyphenol plasma levels or total urinary polyphenol excretion, considered in recent years as a reliable biomarker of total polyphenol intake, has been correlated with dietary polyphenol intake, and has been applied to explore associations between polyphenol intake and several chronic disease risk parameters [18–21]. Thus, the objective of the

current study was to assess the associations between total polyphenol intake, measured by TPE, and obesity parameters in an elderly population at high cardiovascular risk after 5 years of follow-up.

Methods

Subjects

Participants were from the PREDIMED Study ("Prevención con Dieta Mediterránea" (Prevention with the Mediterranean Diet), www.predimed.es), which is a large, parallel-group, randomized, multicenter, controlled, clinical trial designed to assess the effects of the Mediterranean diet on the primary prevention of cardiovascular disease in Spain (ISRCTN35739639). The protocol and recruitment method are reported in detail elsewhere [22]. Briefly, 7447 men aged 55-80 years and women aged 60-80 years were randomly divided into three intervention groups: two Mediterranean diet groups supplemented with either extra virgin olive oil or nuts and a control low-fat diet group. Participants were free of cardiovascular disease at baseline but at high risk. The trial was stopped after a median follow-up of 4.8 years due to the benefits of the Mediterranean diet with respect to major cardiovascular events: myocardial infarction, stroke or death from cardiovascular causes (analysis performed by the Drug and Safety Monitoring Board of the trial), compared with a control low-fat group [23].

The 573 participants in the present study were come from two recruitment centers, Clinic Hospital of Barcelona and University of Valencia, all in Spain. All participants provided written informed consent, and the protocol was approved by the Institutional Review Boards of the participating centers and registered [22].

Nutritional Measurements

Selected participants were asked to complete some questionnaires: a validated 137-item

food frequency questionnaire (FFQ) to assess dietary habits [24]; a 47-item general questionnaire aimed to summarize information about lifestyle, health condition, education, history of illnesses and medication use; a 14-point questionnaire evaluating the degree of adherence to the Mediterranean diet [25]; and a validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire to record physical activity [26]. Nutrient intake was adjusted by calories using the residual method [27]. All questionnaires were administered and yearly repeated during the follow-up by trained staff in face-to-face interviews.

TPE measurements

Urine samples were collected and coded, and then immediately shipped to a central laboratory, to be stored at -80ºC until analyzed. The Folin-Ciocalteu method was applied to determine the content of TPE, using a clean-up procedure with solid phase extraction (SPE) performed in 96-well plate cartridges (Oasis MAX), which helped to remove urinary interferences. Finally, TPE was expressed as milligrams gallic acid equivalent (GAE)/g of creatinine. All details have been previously described by Medina-Remón et al. [19].

Anthropometric measurements

Weight and height were measured with calibrated scales and a wall-mounted stadiometer, respectively. BMI was calculated as weight in kilograms divided by the square of height in meters. WC was measured midway between the lowest rib and the iliac crest. WHtR was calculated as the waist in centimeters divided by the height in meters. Blood pressure was determined using a validated semi-automatic sphygmomanometer (Omron HEM-705CP) by trained nurses. Measurements were taken at 5-minute intervals with participants in a seated position. Data were collected as an average of 2 measurements in each arm, repeated twice [28]. Obesity is defined as

BMI more than 30 kg/m^2.

Statistical analysis

Results were expressed as mean ± SD for continuous variables or percentages for categorical variables. Kolmogorov and Levene tests were applied to examine the normality distribution and skewness. All participants were divided into five categories (roughly quintiles) according to the TPE at 5 years of follow-up. Changes in nutrient intakes and key food consumption according to the FFQs were assessed with yearly repeated-measures analysis during the follow-up period. A Bonferroni post-hoc test and paired T-test were used to compare each variable within and between groups.

Multivariate linear regression models were used to assess the relationship between anthropometric parameters (BW, BMI, WC, and WHtR) and quintiles of TPE at 5 years, adjusted for potential confounders, including sex, age, intervention groups, smoking status (never, current, former), family history of coronary heart disease (CHD), physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) and recruitment centers, 14-unit Mediterranean diet score and energy intake at baseline. Multiple logistic regression analyses were used to calculate the odds ratio (OR) for quintiles of TPE and obesity (BMI>30 kg/m^2). Models were adjusted for potential confounders as in linear regression analyses. Sensitivity analyses were used to further assess the relationship between polyphenol urinary excretion and weight in subcategories (gender and age).

All analyses were performed using SPSS software V21.0 (Chicago, USA). All models were tested for the detection of outliers, multicollinearity, homoscedasticity, and normality and independence of errors. All statistical tests were two-tailed, and the

significance level was $P < 0.05$.

Results

From 612 participants randomly selected for the present study among the participants from the Hospital Clinic of Barcelona and University of Valencia who collected urinary samples after 5 years of follow-up. However, 39 were excluded because they had extreme TPE values; hence a total of 573 participants were finally included.

Baseline characteristics of participants grouped by quintiles of TPE at 5 years of follow-up are shown in Table 1. There were a total of 277 men and 296 women with a mean age of 66.2±6.1 years and 68.3±5.4 years, respectively. Of those participants, 41.5% had diabetes, 80.5% had hypertension, 66.8% had dyslipidemia, 16.9% were current smokers, and 37.5% had a family history of CHD. Compared with participants with the lowest TPE, those with higher TPE were more likely to be women, older, less likely to smoke and to be married, and also had lower body weight. We did not find any significant differences for other variables.

174

Table 1. Baseline characteristics of participants according to quintiles of TPE after 5 years of follow-up.

	TPE (mg GAE/ g creatinine)										
	Q1 (<79.02)		Q2 (79.03-99.50)		Q3 (99.51-124.53)		Q4 (124.54-160.06)		Q5 (>160.07)		P
No. of subjects	114		115		115		115		114		
Women, n (%)	31	27.2	54	47.0	59	51.3	70	60.9	82	71.9	<0.001
Age (y), mean (SD)	64.9	5.5	67.0	6.0	66.6	5.8	69.3	5.5	68.8	5.7	<0.001
Weight (kg), mean (SD)	80.0	11.5	75.2	10.1	76.4	11.5	72.5	10.4	71.5	9.5	<0.001
BMI (kg/m^2), mean (SD)	29.5	2.9	29.1	3.1	29.4	3.4	29.1	3.4	28.9	3.4	0.549
Systolic BP (mm Hg), mean (SD)	150.1	17.9	151.3	15.7	150.2	18.1	152.7	18.6	152.7	18.7	0.658
Diastolic BP (mm Hg), mean (SD)	86.3	9.5	84.4	10.5	85.8	11.3	84.8	9.5	84.8	9.4	0.596
Hypertension, n (%)	92	80.7	93.0	80.9	94.0	81.7	88	76.5	94.0	82.5	0.816
Diabetes, n (%)	43	37.7	47.0	40.9	48.0	41.7	51	44.3	49.0	43.0	0.881
Dyslipidemia, n (%)	71	62.3	77.0	67.0	85.0	73.9	77	67.0	73.0	64.0	0.396
Smoking status, n (%)											0.003
Current	34	29.8	21	18.3	17	14.8	11	9.6	14	12.3	
Former	27	23.7	20	17.4	25	21.7	28	24.3	26	22.8	
Never	53	46.5	74	64.3	73	63.5	76	66.1	74	64.9	
Family history of CHD, n (%)	37	3.5	42	2.6	42	3.5	47	4.3	47	4.4	0.890

Medication, n (%)											
Aspirin	24	21.1	16	13.9	21	18.3	23	20.0	19	16.8	0.655
Antihypertensive drugs	84	73.7	86	74.8	83	72.2	75	65.2	86	75.4	0.420
Hypolipidemic drugs	46	40.4	41	35.7	52	45.2	45	39.1	55	48.2	0.371
Insulin	5	4.4	9	7.8	2	1.7	4	3.5	7	6.1	0.224
Oral hypoglycemic drugs	22	19.3	25	21.7	23	20.0	30	26.1	31	27.2	0.504
Vitamin or minerals	6	5.3	4	3.5	11	9.6	13	11.4	13	11.6	0.088
Education level, n (%)											0.574
University	13	11.5	11	9.7	12	10.6	9	7.8	10	8.8	
High school	24	21.2	13	11.5	19	16.8	15	13.0	17	14.9	
Primary school	76	67.3	89	78.8	82	72.6	91	79.1	87	76.3	
Marital status, n (%)											0.019
Single	7	6.3	5	4.4	6	5.3	3	2.7	4	3.5	
Married	99	89.2	90	78.9	92	80.7	84	75.0	85	75.2	
Widowed	5	4.5	19	16.7	16	14.0	25	22.3	24	21.2	
Physical activity at leisure time (MET-min/d), mean (SD)	272.8	271.1	307.5	271.5	262.9	223.4	261.0	208.1	244.2	200.1	0.341

175 TPE: total polyphenol excretion; GAE: gallic acid equivalent; BMI: body mass index; BP: blood pressure; CHD: coronary heart diseases.

176 Data are given as means (SD) for continuous variables and percentages for categorical variables; $P < 0.05$ indicates statistical significance.

177 *P-values calculated by analysis of variance or $\chi 2$ tests.

178 Comparison of total urinary polyphenol excretion between baseline and 5-year of follow
179 up by quintiles of TPE at 5 years is shown in Fig 1. For the first two quintiles, TPE at
180 baseline was significantly higher than at 5-year. By contrast, TPE at top two categories
181 were higher than at baseline.

182 **Fig 1. Total polyphenol excretion at baseline and at 5-years of follow-up by**
183 **quintiles of TPE (at the 5th year).**

184 Table 2 summarizes information on key changes in food consumption during the
185 intervention according quintiles of TPE. As shown, at the end of the intervention, the
186 consumption of most of the items belonging to a Mediterranean dietary pattern had
187 increased significantly, including vegetables, fruits, fish, milk, extra virgin olive oil,
188 olive oil, nuts, and coffee. However, intake of wine decreased significantly, as well as
189 intakes of cereals, meat, and pastries. Table 2 also shows changes in nutrient intake and
190 degrees of adherence to a Mediterranean diet. Significant increments were observed in
191 the consumption of total fat, fiber, polyunsaturated fatty acids (PUFA),
192 monounsaturated fatty acids (MUFA), folic acid, potassium (K) and magnesium (Mg),
193 while total carbohydrates, protein, cholesterol, sodium (Na) and saturated fatty acids
194 (SFA) remained similar throughout.

Table 2. Changes in key food intake and nutrients according to the FFQs after energy adjustment categorized by quintile of TPE at 5 years [a]

		TPE (mg GAE/ g creatinine)													
		Q1 (<79.02)		Q2 (79.03-99.50)		Q3 (99.51-124.53)		Q4 (124.54-160.06)		Q5 (>160.07)		P[b]	P[c]		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	ANOVA	TIME	GROUP	INTERACTION
Vegetables (g/d)	baseline	302.6	126.5	295.2	122.6	283.3	114.1	312.9	142.4	297.0	107.0	0.484	<0.001	0.916	0.440
	changes	47.7 **	131.5	53.1 **	130.2	76.3 **	150.4	46.0 **	162.3	59.0 **	131.6	0.514			
Fruits (g/d)	baseline	328.9	184.8	361.6	179.7	358.6	182.3	389.5	162.5	394.5	189.3	0.064	<0.001	0.051	0.530
	changes	89.1 **	220.1	82.9 **	222.7	111.0 **	220.6	80.6 **	208.4	67.9 **	194.8	0.658			
Legumes (g/d)	baseline	18.5	8.4	19.3	7.4	20.0	9.2	19.2	7.2	19.0	6.6	0.634	0.446	0.251	0.045
	changes	0.3	10.7	1.7	11.3	-3.2	29.1	-1.0	8.5	1.0	8.3	0.160			
Cereals (g/d)	baseline	246.8	83.8	246.3	81.2	232.8	74.2	242.9	71.9	237.7	61.9	0.636	<0.001	0.530	0.386
	changes	-22.2 *	91.1	-21.7 **	85.1	-14.5	91.5	-31.4 **	80.1	-25.6 **	77.5	0.673			
Milk (g/d)	baseline	322.8	191.3	370.1	202.2	365.8	210.0	354.5	203.7	422.5	231.6	0.010	0.005	0.009	0.728
	changes	38.3	207.4	21.4	200.9	10.3	198.6	40.2 *	188.3	20.6	208.9	0.776			
Meat (g/d)	baseline	138.1	50.4	136.2	43.9	145.6	56.5	139.3	41.7	141.6	47.1	0.613	<0.001	0.198	0.983
	changes	-9.9	52.1	-13.9 **	44.6	-12.1 *	52.5	-13.8 **	51.5	-11.7 *	47.7	0.975			
Fish (g/d)	baseline	92.0	39.6	92.5	36.9	90.4	38.0	97.0	41.3	93.0	39.0	0.787	0.005	0.970	0.481

	changes	8.2 *	40.4	6.2	42.2	11.5**	44.2	4.2	39.4	7.3	43.3	0.764			
Pastries (g/d)	baseline	29.6	32.1	23.6	22.4	24.1	24.7	23.9	22.4	26.6	26.5	0.379	0.006	0.291	0.920
	changes	-4.8	32.4	-3.0	29.4	-4.3	27.2	-3.2	24.3	-3.9	29.2	0.991			
EVOO (g/d)	baseline	22.7	25.6	20.5	22.4	22.6	23.6	22.9	23.7	22.8	23.3	0.960	<0.001	0.961	0.626
	changes	24.9 **	29.6	27.6 **	27.6	25.4**	28.2	26.4 **	30.1	26.9 **	25.6	0.955			
Olive oil (g/d)	baseline	45.4	17.6	46.3	14.2	45.3	13.5	46.3	14.5	44.2	15.3	0.791	<0.001	0.575	0.161
	changes	7.8 **	18.2	10.5 **	17.5	9.8 **	16.8	9.3 **	18.7	11.2**	16.8	0.654			
Nuts (g/d)	baseline	10.7	14.2	11.2	12.6	10.0	12.7	10.0	13.6	10.9	11.5	0.904	<0.001	0.634	0.192
	changes	3.1	16.4	5.3 **	17.1	5.5 **	15.8	9.5 **	17.3	4.2 **	14.0	0.039			
Wine (g/d)	baseline	104.9	144.8	97.0	138.3	103.9	171.5	98.2	136.2	81.2	125.6	0.739	0.013	0.647	0.984
	changes	-8.4	124.2	-13.3	129.4	-17.1	111.3	-18.1 *	95.3	-14.5	91.9	0.971			
Tea (ml)	baseline	4.8	14.5	4.6	15.1	6.4	17.0	5.2	12.5	7.6	21.1	0.605	0.401	0.479	0.172
	changes	0.1	16.6	-1.9	14.5	-1.8	16.7	3.2	24.9	-2.0	22.4	0.204			
Coffee (ml)	baseline	39.1	58.4	36.7	52.4	30.7	43.0	35.0	47.1	30.9	43.0	0.717	0.002	0.546	0.098
	changes	-11.5 *	50.1	-1.8	50.2	-7.3 *	36.8	-13.1 **	47.0	3.7	51.3	0.048			
Total carbohydrates (g/d)	baseline	237.5	43.2	242.1	38.0	237.0	41.7	235.8	36.3	239.7	34.8	0.682	0.769	0.114	0.025
	changes	4.5	78.6	-10.3	71.6	5.7	72.4	-13.2 *	68.1	-0.4	64.6	0.166			
Protein (g/d)	baseline	90.3	43.2	94.9	38.0	89.7	41.7	88.5	36.3	92.5	34.8	0.682	0.274	0.307	0.474
	changes	2.3	47.6	-3.0	41.4	6.1	46.9	2.5	40.9	4.6	39.6	0.592			
Total Fat (g/d)	baseline	100.4	13.6	100.9	12.7	102.8	13.7	102.7	12.9	104.5	13.6	0.132	<0.001	0.235	0.981

	changes	10.0 **	30.8	9.2 **	28.5	10.9 **	28.0	8.5 **	26.9	9.4 **	31.5	0.980			
Fiber (g/d)	baseline	24.2	6.0	24.5	6.5	24.2	6.5	25.8	5.8	25.5	5.6	0.256	0.006	0.632	0.013
	changes	1.1	8.2	1.4	9.7	3.2 **	8.4	0.2	8.2	1.0	7.8	0.107			
Alcohol (g/d)	baseline	13.5	5.6	13.6	5.2	13.7	5.3	13.3	4.7	14.2	4.9	0.780	0.032	0.961	0.765
	changes	-0.2	17.8	-1.6	15.0	-1.2	16.7	-1.9	14.4	-3.2 *	14.2	0.707			
SFA (g/d)	baseline	24.9	11.2	24.2	9.7	24.5	8.3	25.7	9.1	23.9	9.9	0.663	0.541	0.772	0.266
	changes	-0.5	12.9	-0.1	10.6	0.7	9.8	-1.5	11.1	1.6	11.3	0.301			
MUFA (g/d)	baseline	52.1	16.8	51.3	15.4	52.4	16.0	53.6	14.5	52.4	15.0	0.828	<0.001	0.941	0.694
	changes	6.0 **	20.3	7.5 **	19.2	7.4 **	17.5	4.8 **	18.1	7.1 **	19.0	0.798			
PUFA (g/d)	baseline	15.6	5.2	15.4	5.7	15.5	5.8	16.0	5.2	15.5	5.5	0.887	<0.001	0.575	0.670
	changes	3.0 **	7.7	3.1 **	7.8	3.4 **	7.1	3.4 **	8.1	4.2 **	8.9	0.800			
Folic acid (μg/d)	baseline	379.3	89.5	373.3	90.5	369.8	76.5	394.4	98.8	394.2	85.7	0.152	<0.001	0.447	0.086
	changes	42.7 **	91.3	46.5 **	99.7	65.7 **	92.6	39.2 **	91.6	38.7**	100.4	0.196			
Cholesterol (mg/d)	baseline	354.9	92.4	340.0	92.2	351.4	93.3	353.5	81.6	359.1	95.5	0.505	0.234	0.406	0.329
	changes	0.2	115.0	11.9	101.3	11.4	109.2	-3.5	105.5	15.2	112.6	0.644			
Na (mg/d)	baseline	2331.2	570.5	2254.1	499.7	2272.1	480.3	2286.1	491.3	2298.5	447.1	0.815	0.257	0.258	0.319
	changes	-64.0	941.6	-83.1	728.6	10.6	730.0	-184.2 **	713.4	10.8	752.6	0.306			
K (mg/d)	baseline	4130.5	769.6	4218.7	756.2	4208.9	696.0	4312.6	808.1	4410.2	748.0	0.069	<0.001	0.246	0.067
	changes	379.8 **	1070.7	286.0**	1042.2	497.3 **	1007.2	249.1 *	1116.2	268.1 *	1100.4	0.389			
Mg (mg/d)	baseline	355.0	64.0	361.1	68.6	356.9	56.6	368.6	58.2	374.8	61.9	0.147	<0.001	0.474	0.056

	changes	30.7 **	97.9	26.8 **	99.2	42.9 **	87.9	22.0 **	87.3	23.9 *	95.9	0.481			
P-14 score	baseline	8.9	1.8	9.0	1.8	9.2	1.9	8.8	1.9	9.1	1.8	0.441	<0.001	0.370	0.571
	changes	1.6 **	2.5	1.8**	1.9	1.7 **	2.1	1.9**	2.0	1.6 **	2.1	0.626			
Total Energy intake (Kcal/d)	baseline	2508.8	582.3	2285.5	578.5	2338.5	463.0	2262.2	510.7	2191.7	470.7	<0.001	0.034	<0.001	0.234
	changes	-25.9	627.6	32.0	497.4	100.2*	503.9	8.9	564.0	157.8**	520.1	0.089			

197 TPE: total polyphenol excretion; GAE: gallic acid equivalent; EVOO: Extra Virgin Olive Oil; SFA: saturated fatty acids; MUFA:
198 monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; Na: sodium; K: potassium; Mg: magnesium; P-14: 14-point Mediterranean diet
199 score test.

200 a. Data are given as means (SD); $P < 0.05$ indicates statistical significance. Values with asterisks are statistically different from baseline by
201 paired-samples T test (*$P < 0.05$; **$P < 0.01$).

202 b. Data analysed by one-way ANOVA.

203 c. Data analysed by repeated-measures 2-factor ANOVA.

204

205 Changes in obesity parameters between baseline and end of follow-up were observed
206 (see Table 3). Subjects in the highest TPE category had the lowest BW (70.29±10.25
207 Kg) and BMI (28.40±3.75 Kg/m^2) after the intervention. Inversely, those participants in
208 the first quintile of TPE had significantly higher WC (101.41±9.35 cm) and WHtR
209 (61.80±5.15) compared with baseline values.
210

Table 3. Comparisons of obesity parameters during follow-up categorized by quintile of TPE (mg GAE/ g creatinine) at 5 years [a]

		Q1 (<79.02)		Q2 (79.03-99.50)		Q3 (99.51-124.53)		Q4 (124.54-160.06)		Q5 (>160.07)		P[b]	P[c]	P[d]
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Q1VsQ5	ANOVA	
BW (kg)	baseline	79.98	11.52	75.24	10.07	76.42	11.55	72.50	10.44	71.52	9.50	<0.001	<0.001	0.101
	5-year	80.50	11.13	75.07	11.10	76.04	11.72	71.92	11.29	70.29**	10.25	<0.001	<0.001	
	changes	0.72	5.06	-0.17	5.06	-0.39	5.04	-0.57	4.23	-1.23**	4.57	0.024	0.045	
BMI (Kg/m^2)	baseline	29.53	2.92	29.07	3.07	29.44	3.41	29.09	3.43	28.90	3.44	1.000	0.549	0.092
	5-year	29.81	3.04	28.98	3.31	29.30	3.63	28.85	3.76	28.40**	3.75	0.027	0.039	
	changes	0.30	1.88	-0.09	2.10	-0.13	1.86	-0.24	1.73	-0.50**	1.87	0.015	0.031	
WC (cm)	baseline	99.96	9.64	96.44	8.88	98.03	10.14	95.18	9.83	94.15	8.60	<0.001	<0.001	<0.001
	5-year	101.41**	9.35	97.13	9.90	98.78	9.78	95.89	10.97	94.50	9.50	<0.001	<0.001	
	changes	0.72**	5.06	-0.17	5.06	-0.39	5.04	-0.57	4.23	-1.23	4.57	0.024	0.045	
WHtR (cm/m)	baseline	60.84	5.09	60.07	5.80	60.95	5.83	60.37	5.98	59.92	5.74	1.000	0.572	0.001
	5-year	61.80**	5.15	60.46	5.94	61.37	5.71	60.80	6.61	60.12	6.36	0.360	0.216	
	changes	1.02**	3.83	0.38	3.89	0.45	3.53	0.51	4.35	0.25	3.60	1.000	0.618	

TPE: total polyphenol excretion; GAE: gallic acid equivalent; BW: body weight; BMI: body mass index. WC: waist circumference; WtHR: waist to height ratio.

a. Data are given as means (SD); $P < 0.05$ indicates statistical significance. Values with asterisks are statistically different from baseline by

paired-samples T-test ($P < 0.05$): *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

b. Data analyzed by Bonferroni post-hoc comparisons.

c. Data analyzed by ANOVA.

d. Data analyzed by paired-samples T-test.

219 The associations between TPE and adiposity indexes were analyzed by linear regression
220 models (see Table 4). Significant inverse associations were found between quintiles of
221 TPE at 5 years and BW (β=-1.004; P=0.002), BMI (β=-0.320; P=0.005), WC (β=-0.742;
222 P=0.013) and WHtR (β=-0.408; P=0.036) after adjustment for potential confounders.

223 **Table 4. Multivariate linear regression analyses with obesity indexes and quintiles**
224 **of TPE at 5-year**

		β	SE	Beta	P	95%CI	
BW (Kg)	Model 1	-2.350	0.331	-0.285	<0.001	-3.000	-1.700
	Model 2	-1.070	0.315	-0.130	0.001	-1.689	-0.451
	Model 3	-1.148	0.323	-0.139	<0.001	-1.783	-0.513
	Model 4	-1.004	0.320	-0.124	0.002	-1.634	-0.375
BMI (Kg/m^2)	Model 1	-0.295	0.104	-0.118	0.005	-0.499	-0.090
	Model 2	-0.328	0.110	-0.131	0.003	-0.544	-0.111
	Model 3	-0.358	0.113	-0.143	0.002	-0.580	-0.136
	Model 4	-0.320	0.113	-0.129	0.005	-0.541	-0.098
WC (cm)	Model 1	-1.500	0.296	-0.208	<0.001	-2.082	-0.918
	Model 2	-0.721	0.293	-0.100	0.014	-1.296	-0.147
	Model 3	-0.877	0.302	-0.122	0.004	-1.471	-0.283
	Model 4	-0.742	0.297	-0.104	0.013	-1.326	-0.158
WHtR(cm/m)	Model 1	-0.298	0.178	-0.070	0.094	-0.648	0.051
	Model 2	-0.367	0.189	-0.087	0.052	-0.739	0.004
	Model 3	-0.474	0.195	-0.112	0.016	-0.857	-0.090
	Model 4	-0.408	0.194	-0.097	0.036	-0.788	-0.028

225 BW: body weight; BMI: body mass index; WC: waist circumference; WHtR: waist to
226 height ratio. TPE: total polyphenol excretion
227 β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta:
228 Standardized coefficient; CI: Confidence interval; P: two-sided test of significance.

Model 1. unadjusted; Model 2 was adjusted for sex, age and intervention groups; Model 3 adjusted as in Model 2 plus smoking status (never, current, former), family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) and recruitment centers; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score and energy intake at baseline.

Table 5 shows the OR (95% confidence interval) for obesity according to the quintile of TPE at 5 years. In fully-adjusted models, participants in the category of highest TPE had a lower prevalence of obesity (odds ratio (OR) = 0.346, 95% confidence interval (CI) 0.176 to 0.178; P-trend, 0.039) than those in the lowest category.

Table 5. Multivariate adjusted odds ratios (95% CI) for prevalent obesity (213 cases) after 5-year follow-up.

	Q1	Q2	95% CI		Q3	95% CI		Q4	95% CI		Q5	95% CI		P-trend
Model 1	1 (ref.)	0.639	0.375	1.089	0.769	0.454	1.302	0.664	0.390	1.129	0.450	0.259	0.782	0.073
Model 2	1 (ref.)	0.597	0.344	1.035	0.691	0.400	1.192	0.618	0.350	1.091	0.383	0.211	0.694	0.036
Model 3	1 (ref.)	0.559	0.314	0.995	0.649	0.367	1.147	0.543	0.296	0.996	0.318	0.166	0.606	0.015
Model 4	1 (ref.)	0.604	0.332	1.100	0.720	0.399	1.300	0.560	0.298	1.054	0.346	0.176	0.678	0.039

Obesity was defined as BMI>30 kg/m^2.

Model 1. unadjusted; Model 2 was adjusted for sex, age and intervention groups; Model 3 adjusted as in Model 2 plus smoking status (never, current, former), family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) and recruitment

centers; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score and energy intake at baseline.

252 Finally, we performed sensitivity analyses (**S1 Table**) to account for differences in sex
253 and age. In fully-adjusted models, only males showed significant inverse associations
254 with BW (β=-1.004; P=0.031) and BMI (β=-0.298; P=0.036). For age categories (<67
255 years and ≥67 years), all adiposity parameters [BW (β=-1.358; P=0.002), BMI (β=-
256 0.466; P=0.003), WC (β=-1.061; P=0.012), WHtR (β=-0.623; P=0.023))] were lower in
257 the older group (age≥67).

258 Discussion

259 In this 5-year study conducted in elderly participants at high cardiovascular risk, a
260 higher total polyphenol intake, expressed as TPE, was inversely associated with weight
261 parameters including BW, BMI, WC, and WHtR, as well as with the prevalence of
262 obesity after 5-year follow-up, suggesting that polyphenols could be considered as an
263 independent contributor to the weight-losing effects of a Mediterranean diet.

264 Several PREDIMED sub-trials have reported a range of mechanisms for the weight-
265 losing effects of a Mediterranean diet, including a high ingestion of dietary fiber,
266 antioxidants, unsaturated fatty acids, extra virgin olive oil, and nuts, and moderate wine
267 consumption [29–33]. The reduction we observed in weight parameters might be partly
268 attributed to intake of the aforementioned food items; however, in the fully adjusted
269 models, we removed their effects by adjusting for adherence to the Mediterranean diet
270 (14-unit MedDiet questionnaire). Furthermore, even though the intake of these foods
271 increased after 5 years of follow-up, none of them showed significant differences within
272 quintile categories at the end of the intervention; therefore, polyphenol intake could be
273 considered as an independent factor.

274 The present findings are consistent with previous reports on the inverse associations
275 between polyphenol intake and weight parameters. A 16-year longitudinal study from
276 the Netherlands associated a higher intake of total flavonols/flavones and catechins with

277 a lower increase in BMI [34]. Other supporting evidence showed a significant decrease
278 of 1.9 cm in WC and 1.2 Kg in BW after supplementation of catechin-rich green tea for
279 90 days, although at a much higher dose than habitual intakes [35]. Two 12-week
280 intervention studies also demonstrated anti-obesity effects of green tea intake, finding a
281 considerable reduction in BW, BMI, WC and total abdominal fat area [36,37].
282 The Mediterranean diet could be considered as rich in polyphenol content because it is
283 characterized by a high consumption of fruit and vegetables, virgin olive oil, legumes,
284 and nuts, and a moderate consumption of wine [38]. Results from a meta-analysis of 16
285 randomized controlled trials with a Mediterranean diet showed an average reduction in
286 participant weight of 1.75 kg and a reduction in BMI of 0.57 kg/m^2, as well as a greater
287 reduction in BW of 3.88 kg under conditions of energy restriction, suggesting that
288 adherence to a Mediterranean diet helps to control weight [39]. Both the EPIC-Spain
289 cohort and the SUN cohort also in Spain, have shown in the long-term a significantly
290 lower risk of overweight/obesity associated with better Mediterranean diet adherence
291 [40,41]. Furthermore, in a prior PREDIMED study, it was observed that BW and BMI
292 decreased slightly, but without differences among groups, after 3 months of intervention
293 [42]. We observed a 1.22 kg decrease in BW and 0.50 in BMI in the highest TPE
294 quintile, which partly agrees with previous studies reporting a similar reduction in body
295 weight parameters.
296 Indexes of abdominal obesity, namely WC and WHtR, were significantly lower in the
297 highest TPE quintile. These parameters are more accurate discriminators of
298 cardiovascular risk than BMI due to the closer relationship between cardiovascular
299 disease and abdominal obesity [43]. In agreement with our findings, in a PREDIMED
300 and several other studies, the Mediterranean diet was negatively associated with WC

and WHtR [44–46]. Additionally, two feeding trials with green tea polyphenol extracts also showed beneficial effects on abdominal obesity parameters [37,47].

Potential explanations of the observed inverse association between polyphenol intake and weight-loss likely involve several mechanisms, due to the diversity of polyphenol chemical structures, complex metabolic pathways and oral bioavailability. Excess adipose mass and adipose tissue expansion results from adipocyte hypertrophy and hyperplasia[48]. Common plausible mechanisms include: suppression of fat absorption and anabolic pathways; inhibition of adipogenesis and lipogenesis; stimulation of catabolic pathways with increment of lipolysis, apoptosis of mature adipocytes and acid β-oxidation; reduction of chronic inflammatory response relative to adiposity; increment in energy expenditure through up-regulating uncoupling protein (UCP1-3) [8,9]. However, knowledge of the anti-obesity effects of polyphenols is limited and only a few specific compounds have been analyzed in this context. For instance, it has been demonstrated that resveratrol, widely present in red grapes and red wine, exerts an anti-obesity action by reducing adipogenesis and increasing apoptosis in mature adipocytes, and inhibiting fat accumulation processes and stimulating lipolytic and oxidative pathways in *in vivo* studies [49,50]. Anthocyanins, water-soluble plant pigments in blue, purple, and red fruits, have also been found to significantly reduce body weight. This effect may be due to suppression of lipid synthesis, up-regulation of adiponectin, which enhances insulin sensitivity, and reduction in of serum triglycerides and leptin levels [8,51]. The anti-obesity effects of flavonoids, which are a large group of polyphenols found in a wide range of Mediterranean diet foods [52,53], have been mainly attributed to improvement in adipocyte functionality and fatty oxidation[54]. Also playing a key role in weight control is the down-regulation of a variety of pro-inflammatory adipocytokines, particularly tumor necrosis factor alpha (TNF-α) [55]. In summary,

even though intake of some specific polyphenols has been associated with body weight management, there is still not enough evidence for the effect of total polyphenols or some classes of polyphenols, and further studies are needed to explore the mechanisms involved as well as potential synergistic effects among them.

Some limitations of this study should be noted. First, given that the study was conducted among elderly subjects at high cardiovascular risk, the results cannot be extrapolated to the general population. Second, even though we adjusted for major potential confounders, we still cannot exclude residual confounding from measurements. Third, even though WC and WHtR may reflect abdominal obesity more accurately, they cannot differentiate between fat distribution in visceral adipose tissue and subcutaneous abdominal adipose tissue; hence we cannot conclude if a reduction in abdominal obesity parameters is beneficial to visceral or subcutaneous fat mass, or both [56]. Another limitation is the lack of specific measurements of polyphenol metabolism *in vivo*.

The present study also has several strengths. Its main strong point is the use of TPE, a biomarker of polyphenol intake, which could provide more precise data than measuring total polyphenol intake through self-reported information in FFQ or databases. Another strength is its prospective design. Only a few studies have analyzed the association between total polyphenol intake and weight control, and the current work is the first to associate anti-obesity effects with total polyphenol intake in individuals at high cardiovascular risk [8,57]. In addition, the long-term duration of the intervention provides more robust results compared with other short-term trials.

In summary, with 5 years of follow-up, the present study shows that polyphenol levels expressed as TPE in urine, was inversely associated with BW, BMI, WC, and WHtR in an elderly population at high cardiovascular risk. Therefore, we confirmed that a long-

term polyphenol-rich diet contributes to BW loss, which can offer protection from several chronic diseases. For future research, similar studies should be conducted in the general population and specific mechanisms need to be explored by further clinical trials.

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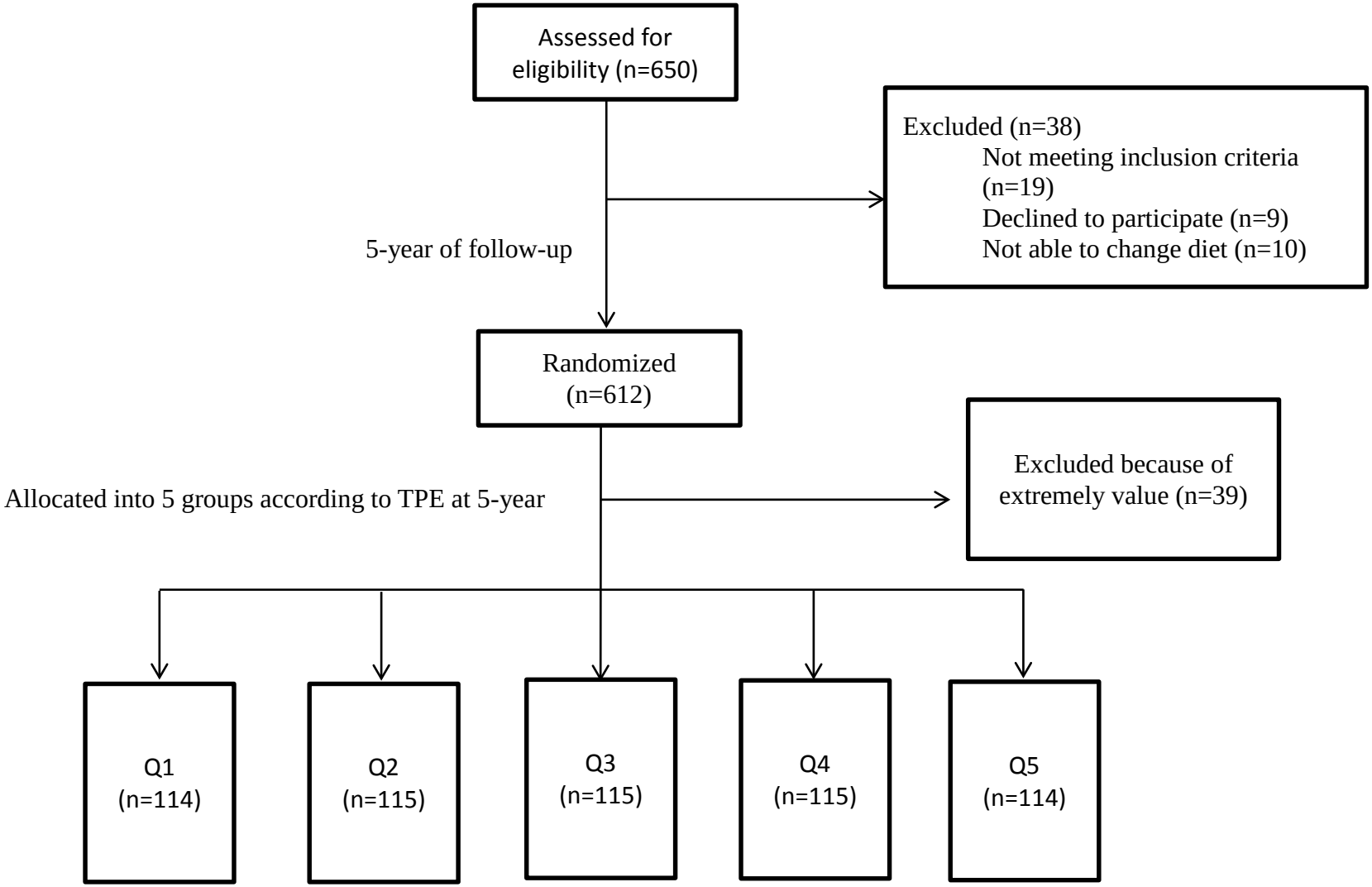

Figure 1. Flowchart of study participants. The diagram includes detailed information on the excluded participants.

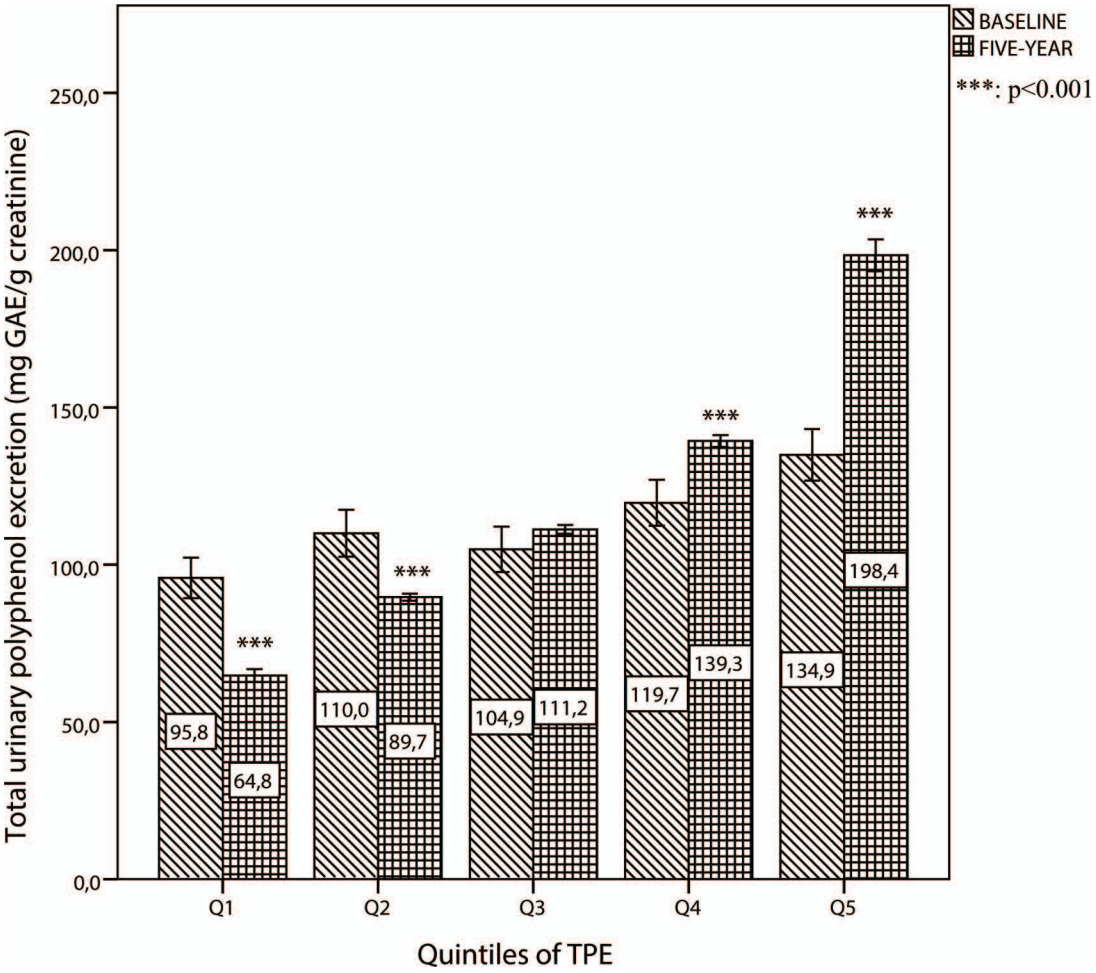

Fig 2. Total polyphenol excretion at baseline and at 5-years of follow-up by quintiles of TPE

Supporting Information

S1 Table. Sensitivity analyses of obesity indexes with linear regression analyses.

Supplementary table 1. Sensitivity analyses of obesity indexes with linear regression analyses.

		BW						BMI						WC						WHtR					
		β	SE	Beta	sig.	95%CI		β	SE	Beta	sig.	95%CI		β	SE	Beta	sig.	95%CI		β	SE	Beta	sig.	95%CI	
Sex	Male	-1.004	0.462	-0.133	0.031	-1.915	-0.094	-0.298	0.142	-0.130	0.036	-0.577	-0.020	-0.502	0.379	-0.082	0.187	-1.249	0.246	-0.230	0.230	-0.063	0.319	-0.684	0.224
	Female	-0.747	0.458	-0.101	0.104	-1.649	0.155	-0.265	0.176	-0.094	0.134	-0.612	0.082	-0.766	0.463	-0.105	0.099	-1.677	0.145	-0.483	0.314	-0.097	0.125	-1.100	0.135
Age (year)	<67	-0.638	0.495	-0.081	0.198	-1.614	0.337	-0.174	0.167	-0.073	0.299	-0.502	0.155	-0.351	0.432	-0.053	0.417	-1.203	0.500	-0.152	0.280	-0.039	0.588	-0.704	0.400
	≥67	-1.358	0.430	-0.164	0.002	-2.204	-0.512	-0.466	0.157	-0.176	0.003	-0.774	-0.158	-1.061	0.418	-0.137	0.012	-1.883	-0.239	-0.623	0.272	-0.136	0.023	-1.157	-0.088

BW: body weight; BMI: body mass index; WC: waist circumference; WHtR: waist to height ratio.

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance

Model was adjusted for sex, age, intervention groups, smoking status (never, current, former), family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, marital status (single, married, widowed), education level (primary school, high school, university), medication used (antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month) recruitment centers, 14-unit Mediterranean diet score and energy intake at baseline.

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	5-6
	2b	Specific objectives or hypotheses	5-6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	6
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NO
Sample size	7a	How sample size was determined	8
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NO
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Protocol S1
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Protocol S1
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Protocol S1
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Protocol S1
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	Protocol S1

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	Protocol S1
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	8
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	8
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	9
	13b	For each group, losses and exclusions after randomisation, together with reasons	9
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Protocol S1
	14b	Why the trial ended or was stopped	NR
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	10
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Diagram
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	18-20
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	NO
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Supporting table1
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	No harms
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	26
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	26
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	23-26
Other information			
Registration	23	Registration number and name of trial registry	6
Protocol	24	Where the full trial protocol can be accessed, if available	Protocol S1
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Funding section

NR: Not report

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Conferences

Poster 1

Title: Association between urinary total polyphenols excretion and clinical cardiovascular risk factors after 5 years of follow up in a Mediterranean population at high cardiovascular risk

Authors: Xiaohui Guo, Anna. Tresserra-Rimbau, Jordi. Salas-Salvadó, Miguel Ángel. Martínez-González, Dolores Corella, R. Estruch, and Rosa M. Lamuela-Raventós, on behalf of the PREDIMED Study Investigators.

Conference: 7th International Conference on Polyphenols and Health. Congress Center Tours, France, 2015

Association between urinary total polyphenols excretion and clinical cardiovascular risk factors after 5 years of follow up in a Mediterranean population at high cardiovascular risk

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Background

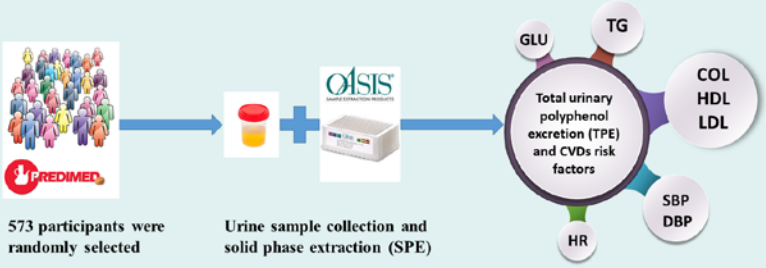
Several epidemiological studies have shown an inverse association between the consumption of polyphenol-rich foods and the risk of cardiovascular disease. Urinary total polyphenols excretion was considered as a reliable biomarker for polyphenols intake.

Objective

Our aim was to assess the association between urinary total polyphenols excretion (TPE) and clinical cardiovascular risk factors (glucose, cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, systolic blood pressure, and diastolic blood pressure) in a population at high cardiovascular risk.

Methods

A longitudinal study was performed with 573 men and women (aged 67.3±5.9) from the PREDIMED trial. TPE was measured using Rapid Folin-Ciocalteu method with a precleaning step using solid phase extraction (SPE) performed in 96-well plate cartridges (Oasis MAX). Anthropometric and clinical measurements were collected yearly during follow-up. Participants were categorized into three groups according to changes in TPE. Multiple linear regression models were used to assess the relationships between TPE and clinical cardiovascular risk factors, adjusting for potential confounders.



573 participants were randomly selected

Urine sample collection and solid phase extraction (SPE)

GLU: Glucose, COL: Total cholesterol, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, TG: triglycerides, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate.

Results

After 5 years of follow-up, significant inverse correlations were observed between changes in TPE, a biomarker of TP intake, and triglycerides (β=-8.563; P=0.007), glucose (β=-4.164; P=0.036), and diastolic blood pressure (β=-1.316; P=0.013).

Multivariate linear regression analyses with changes in cardiovascular risk factors as dependent variables, and tertiles of changes in TPE in spot urine samples (mg GAE/g creatinine) as exposure variables, adjusted for potential confounders.

		β	SE	Beta	sig.	95% CI	
Change in GLU (mg/dL)	Model 1	-4.164	1.979	-0.095	0.036	-8.053	-0.275
	Model 2	-4.316	1.981	-0.098	0.030	-8.208	-0.424
	Model 3	-4.355	1.949	-0.099	0.026	-8.186	-0.525
	Model 4	-4.372	1.953	-0.099	0.026	-8.209	-0.534
Change in COL (mg/dL)	Model 1	-2.51	2.001	-0.057	0.210	-6.442	1.421
	Model 2	-2.236	2.011	-0.050	0.267	-6.187	1.715
	Model 3	-1.845	2.013	-0.042	0.360	-5.800	2.109
	Model 4	-1.802	2.015	-0.041	0.372	-5.762	2.157
Change in HDL (mg/dL)	Model 1	0.102	0.448	0.010	0.820	0.778	0.982
	Model 2	0.135	0.448	0.014	0.763	-0.744	1.015
	Model 3	0.133	0.456	0.014	0.771	-0.764	1.030
	Model 4	0.174	0.454	0.018	0.701	-0.718	1.067
Change in LDL (mg/dL)	Model 1	-0.205	1.775	-0.005	0.908	-3.693	3.283
	Model 2	-0.039	1.784	-0.001	0.983	-3.545	3.467
	Model 3	0.448	1.783	0.012	0.802	-3.056	3.952
	Model 4	0.469	1.786	0.012	0.793	-3.041	3.979
Change in TG (mg/dL)	Model 1	-8.356	3.06	-0.123	0.007	-14.369	-2.344
	Model 2	-8.563	3.058	-0.126	0.005	-14.572	-2.554
	Model 3	-8.627	3.094	-0.127	0.006	-14.708	-2.546
	Model 4	-8.572	3.099	-0.126	0.006	-14.662	-2.483
Change in SBP (mm Hg)	Model 1	-1.367	0.994	-0.058	0.169	-3.319	0.585
	Model 2	-1.222	1.001	-0.052	0.222	-3.188	0.744
	Model 3	-1.127	1.003	-0.048	0.262	-3.098	0.843
	Model 4	-1.098	1.005	-0.046	0.275	-3.071	0.876
Change in DBP (mm Hg)	Model 1	-1.316	0.531	-0.104	0.013	-2.359	-0.273
	Model 2	-1.254	0.532	-0.099	0.019	-2.298	-0.209
	Model 3	-1.153	0.532	-0.091	0.031	-2.198	-0.108
	Model 4	-1.156	0.533	-0.091	0.031	-2.203	-0.109
Change in HR	Model 1	-0.002	0.555	0.000	0.997	-1.091	1.087
	Model 2	0.043	0.559	0.003	0.938	-1.055	1.142
	Model 3	-0.011	0.567	-0.001	0.985	-1.125	1.103
	Model 4	-0.074	0.565	-0.006	0.895	-1.184	1.035

GLU: Glucose, COL: Total cholesterol, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, TG: triglycerides, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate.
β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance
Model 1. unadjusted; Model 2 was adjusted for sex, age and intervention groups; Model 3 adjusted as in Model 2 plus BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidaemia, medication use: antihypertensive drugs, vitamins, insulin, oral hypoglycaemic drugs, aspirin or other antiplatelet drug; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score.

Conclusion

Our results suggest that higher polyphenol intakes, measured as TPE in urine, exert a protective effect on cardiovascular risk factors.

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References

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2. Medina-Remón, A.; Barrionuevo-González, A.; Lamuela-Raventos, R. M, et al. Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. Anal. Chim. Acta 2009, 634, 54–60.
3. Tresserra-Rimbau, A.; Rimm, E. B.; Lamuela-Raventós, R. M. et al. Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study. Nutr. Metab. Cardiovasc. Dis. 2014, 24, 639–647.