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Polyphenol intake from a Mediterranean diet decreases inflammatory biomarkers related to atherosclerosis: A sub-study of The PREDIMED trial.

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ABSTRACT

Background and Aims: High dietary polyphenol intake is associated with reduced all-cause mortality and lower incidence of cardiovascular events. However, the mechanisms involved are not fully understood. The aim of this sub-study of the PREDIMED (Prevention with Mediterranean diet) trial was to analyze the relationship of polyphenol intake measured by total urinary polyphenol excretion (TPE), with circulating inflammatory biomarkers and cardiovascular risk factors in elderly individuals.

Materials and methods: A sub-study of 1139 high-risk participants was carried out within the PREDIMED trial. The subjects were randomly assigned to a low-fat control diet or to two Mediterranean diets, supplemented with either extra-virgin olive oil or nuts. Dietary intake, anthropometrics, clinical and laboratory assessments including inflammatory biomarkers, and urinary TPE were measured at baseline and after one-year intervention.

Results: Participants in the highest tertile of changes in urinary TPE (T3) showed significant lower plasma inflammatory biomarkers [VCAM-1 (–9.47 np/mL), ICAM-1 (–14.71 np/mL), IL-6 (–1.21 pg/mL), TNF-α (–7.05 pg/mL), and MCP-1 (–3.36 pg/mL)] than those in the

lowest tertile (T1, P<0.02; all). A significant inverse correlation existed between urinary TPE and plasma concentration of VCAM-1(r=–0.301; P<0.001). In addition, systolic and diastolic blood pressure (BP) decreased and plasma HDL-cholesterol increased in parallel with increasing urinary TPE (T3 vs T1)(P≤0.005 and P=0.004, respectively).

Conclusions: Increases in polyphenol intake measured as urinary TPE are associated with decreased inflammatory biomarkers, suggesting a dose-dependent anti-inflammatory effect of polyphenols. In addition, high polyphenol intake improves cardiovascular risk factors, mainly BP and the lipid profile.

What is known about this subject?

Atherosclerosis is a low-grade inflammatory disease of cardiovascular system.

Polyphenol-rich foods such as cocoa, fruits, vegetables, tea, olive oil and wine have been associated inversely with the risk of overall mortality, cardiovascular disease and cardiovascular risk factors in numerous epidemiological and clinical studies.

No previous study has evaluated the relationship between dietary polyphenol intake measured by urinary total polyphenol excretion (TPE), plasma inflammatory biomarkers related to atherosclerosis, and main cardiovascular risk factors.

What this study adds?

This is the first intervention study to assess the association of polyphenol intake using biochemical analyses of urine TPE, classical vascular risk factors and plasma inflammatory biomarkers related to arteriosclerosis.

Increases in polyphenol intake measured as urinary TPE are associated with decreased inflammatory biomarkers

High polyphenol intake improves cardiovascular risk factors, mainly blood pressure and the lipid profile.

Introduction

Atherosclerosis, the leading cause of death worldwide, is considered a low-grade inflammatory disease of the cardiovascular system (1). In the earliest stage, vascular inflammation is activated by pro-inflammatory stimuli such as saturated fat intake, hypercholesterolemia, obesity, hyperglycemia, and hypertension, stimulating the secretion of inflammatory cytokines that promote the formation of endothelial adhesion molecules. These molecules are subsequently released into the circulation, where they mediate the adhesion of circulating monocytes and lymphocytes to the vascular endothelium (2,3).

The first step in the management of hypertension and other cardiovascular risk factors is to follow a healthy diet such as the traditional Mediterranean diet (Med-diet) (4) or the DASH (Dietary-Approaches-to-Stop-Hypertension) diets (5) and to improve additional lifestyle measures, such as reducing body weight and increasing physical activity (6). The Med- and DASH diets are both rich in fruits and vegetables (F&V), which have been considered rich sources of phytochemicals, and are inversely associated with high blood pressure (BP) and incidence of hypercholesterolemia, among other effects (7,8).

Consequently, numerous epidemiological studies have suggested an inverse association between adherence to the traditional Med-diet and a reduction in coronary heart diseases; this protective effect has been attributed in part to the richness of this diet in antioxidants (9). Polyphenol-rich foods such as cocoa, F&V, tea, olive oil and wine have been associated inversely with the risk of overall mortality or cardiovascular disease in numerous epidemiological studies (7,10-16).

At least part of the beneficial effects of Med-diet or its main components such as extra virgin olive oil, nuts, fruits, vegetables and wine has been attributed to their anti-oxidant and anti-inflammatory effects (17). On the other hand, previous clinical and laboratory studies have pointed out that dietary polyphenols may exert anti-inflammatory effects (18,19). In this setting, we undertook a sub-study of the PREDIMED trial to evaluate the relationship between dietary polyphenols intake measured by urinary TPE, plasma inflammatory biomarkers related to atherosclerosis, and main cardiovascular risk factors. To our knowledge this is the first intervention study to assess these associations using biochemical analyses of TPs in spot urine samples, and directly measuring classical vascular risk factors or plasma inflammatory biomarkers in a large sample of older individuals at high cardiovascular risk.

Materials and methods

Subjects

The PREDIMED (*PREvención con DIetaMEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled clinical trial of 4.8-year duration aimed to assess the effects of the Med-Diet on cardiovascular events (www.predimed.es). The detailed study protocol have been described previously (20,21).

We randomly selected 1170 participants from primary health centres affiliated with three University hospitals in Spain. Eligible participants were community-dwelling men aged 55-80 years and women aged 60-80 years and those included in the current sub-study were similar to the overall PREDIMED population. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of the participating centres. This trial has been registered with the International Standard Randomised Controlled Trial Number (ISRCTN of London, England: 35739639).

Assessments and intervention

At baseline all participants completed a validated semiquantitative food frequency questionnaire (FFQ) including 137 items, the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire, a validated 14-point Med-diet score and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use (20). Trained dieticians were responsible for all aspects of the intervention. Participants in both Med-diet intervention groups were given personalized advice for dietary changes directed to archive a diet closes to the traditional Med-diet. The participants from the group of Med-diet supplemented with extra virgin olive oil (EVOO) received free EVOO (1 L/wk) for all the family and those from the Med-diet plus nuts group were provided with mixed nuts (30 g/d, as 15 g walnuts, 7.5 g almonds, and 7.5 g hazelnuts). Participants assigned to the control diet received personal dietary advice in order to follow a low-fat diet according to the American Heart Association guidelines (22). In the three groups, the general guideline recommendation included increasing the intake of fresh fruit (≥ 3 servings/d), vegetable (≥ 2 servings/d), legumes, fish or seafood (≥ 3 servings/wk), and white meat; negatively recommendation included eliminating and/or reduction of detrimental foods (red and processed meats, fat-rich dairy products, commercial pastries, snacks and sugar-sweetened beverages). No specific recommendation was given in relation to wine and beer intake.

Clinical measurements

Trained nurses measured the height and weight with a wall-mounted stadiometer and calibrated scales, respectively, as well as the BP in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP (23); Hoofddorp, The Netherlands). Waist circumference was measured midway between the lowest rib and the iliac crest with an anthropometric tape. Urine and blood samples were obtained after an overnight fast; they were coded, shipped to a central laboratory and frozen at -80ºC until analysis. Analyses determined in frozen samples

from the participants of whole serum or plasma as appropriate were blood glucose level by the glucose-oxidase method; serum insulin level by radioimmunoassay, cholesterol and triglyceride level by enzymatic procedures; HDL cholesterol level after precipitation with phosphor-tungstic acid and magnesium chloride. We performed all the analysis by duplicate. Analysis of TP and creatinine in urine samples were performed following the procedure described by Medina-Remón *et al.* (24); TPE was expressed as mg gallic acid equivalent (GAE)/ g of creatinine. Energy and nutrients intake were derived from Spanish food composition tables (25).

The concentrations of soluble adhesion molecules were measured by xMAP technology on the Luminex platform (Luminex Corporation), according to the manufacturer's instructions and analysed with the Bio-Plex Manager TM Software (Bio-Rad Laboratorios, Inc.). We determine the concentration of 5 potential biomarkers: plasma soluble Vascular Cell Adhesion Molecule-1 (VCAM-1), Inter-Cellular Adhesion Molecule-1 (ICAM-1), Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α) and Monocyte Chemotactic Protein-1 (MCP-1), by using a Human Cytokine Plex (Bio-Rad, Hercules, CA) which is based on magnetic bead-based multiplex assays designed to measure multiple cytokines, adhesion molecules and chemokine in matrices of plasma. Samples were thawed and analyzed at a 1:3 dilution for every molecule except for ICAM and VCAM which are analyzed at a 1:100 dilution. For all assays, the procedures were performed by a single blinded researcher following manufacturer's instructions.

We used as little as 12.5 μL of sample. The principle behind the 96-well plate-formatted, bead-based assays is similar to a capture sandwich immunoassay. An antibody directed against the desired cytokine or chemokine target is covalently bound to internally dyed beads. The bound beads are allowed to react with the sample containing the target biomolecules. After a series of washes to remove unbound protein, a biotinylated detection antibody

specific to an epitope different from that of the capture antibody is added to the reaction. This results in the formation of a sandwich of antibodies around the cytokine, chemokine, or adhesion molecules. A streptavidin-phycoerytrin reporter complex is then added to bind to the biotinylated detection antibodies on the bead surface. The plate is then analyzed on a Luminex 100™ instrument (Luminex, Austin, TX) using Bio-Plex Manager Software (Bio-Rad, Hercules, CA). A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity and target concentration on Bio-Plex Manager™. Analytes concentrations were obtained by standard calibration curves. Results are shown in pg/mL or ng/mL. All measurements were performed in duplicate.

Statistical analyses.

Analyses were performed using SPSS software v19.0 (Chicago, USA). Baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD). Variables were examined for normality and skewness (Kolmogorov and Levene tests). ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. Changes in all outcomes were assessed with repeated-measures analysis of variance for the 2 factors: tertiles of changes of urinary TPE [T1 (<-14.03), T2 (-14.04 to 31.56) and T3 (>31.57)] and time (baseline and 1-year) and their interaction, with the Bonferroni post hoc test to compare differences in the effects of each tertile within and between groups. Within- and between-group differences are expressed as mean percent difference [95% confidence interval (CI)].

We used the General Linear Model (GLM) approach to ANCOVA to determine the effects of the tertiles 2 and 3 of changes in TPE (fixed factors), compared with tertile 1, VCAM-1, ICAM-1, IL-6, TNF-α and MCP-1 concentration after one year (dependent variables), using the baseline measurements as covariates and others as additional covariates. Model 1 was unadjusted; Model 2, adjusted by baseline inflammatory biomarkers, intervention group, sex,

age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) supplements taken in the last month, and intake of sodium, potassium, total energy, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA). *P*-values<0.05 (two-tailed) were considered statistically significant.

Results

We excluded 31 of 1170 eligible participants before randomization for different reasons: not meeting inclusion criteria (n=9), not accepting to change their dietary habits (n=6), food allergies (n=5) or refusing to participate (n=11). Baseline characteristics of the three group are shown in **Table 1** (511 men and 628 women); most of them were overweight or obese (>90%), and with a sizeable burden of cardiovascular risk factors (more than the 75% of the patients had hypertension; more than 40% were diabetic and/or had family history of cardiovascular disease, and more than 60% had dyslipidemia). Most of them were taking antihypertensive drugs, nearly one-half were taking statins or other hypolipidemic drugs, and nearly one-quarter took oral hypoglycemic drugs and aspirin or other antiplatelet drugs. Subsequent data refer only to the 1139 participants who completed the study.

Food, energy and nutrient intake

After one year of intervention, the main dietary changes that were observed in the three groups were a substantial increase in the consumption of EVOO, total nuts, fruit, vegetables and legumes in the three groups, and a reduced consumption of meat and meat products, and, pastries, cakes and sweets (**Table 2**). All groups significantly increased the Med-diet score. **Table 3** shows changes in total energy intake, energy expenditure in physical activities and daily nutrient intake at baseline and after one year in each tertile of urinary TPE. Fiber and total fat intake significantly increased in all groups, the latter to the expense of an increased consumption of MUFA), attributable in part to increased use of olive oil, and PUFA, due to

increased nut consumption. SFA and total cholesterol intake significantly decreased in all groups, as well as sodium intake. In all groups magnesium and potassium intake were significantly increased.

Cardiovascular risk factors and Inflammation biomarkers

Table 4 shows the changes from baseline, in urine total polyphenols, anthropometric measurements and, cardiovascular risk factors. By definition of the groups, urinary TPE, expressed as mg GAE/ g creatinine, was significantly increased in T2 and T3, and decreased in T1. Participants in all groups significantly decreased systolic and diastolic BP. No significant changes were observed in blood glucose levels, total cholesterol, or triglycerides. LDL-cholesterol was significantly reduced and HDL-cholesterol was significantly increased in all, groups. Inflammatory biomarkers were significantly reduced in the groups with higher changes of urinary TPE (T2 and T3), with higher decreases in the T3 group. **Figure 1** shows the change from baseline values in VCAM-1, ICAM-1, IL-6, TNF-α and MCP-1 concentrations in the three groups.

After the intervention with Med-diet+EVOO and Med-diet+nuts the participants showed a significant increases of urinary TPE, 22.82 mg GAE/g creatinine (95% CI: 16.63 to 29.01; $P<0.001$) and 21.83 mg GAE/g creatinine (CI: 12.68 to 30.98; $P<0.001$), respectively. **Figure 2** shows the changes from baseline values in VCAM-1, ICAM-1, IL-6, TNF-α and MCP-1 concentrations in the three intervention groups. In the covariate analysis with VCAM-1, ICAM-1, IL-6, TNF-α and MCP-1 concentration at one-year as dependent variables, circulating VCAM-1, ICAM, IL-6, TNF-α and MCP-1 concentrations decreased in both Med-diets and increased in the control diet group.

Table 5 shows the 1-year changes in VCAM-1, ICAM-1, IL-6, TNF-α and MCP-1 concentrations, associated with tertiles of changes in urinary TPE (T1 compared with T2 and T3). After the covariate analysis of the inflammatory biomarkers changes, one year measures

with respect to baseline, with inflammatory biomarkers at year one as the dependent variables, tertiles of changes in TPE as the fixed factor, and other measurements as additional covariates. We observed the effects of the higher changes of urinary TPE (T2 and T3) with respect to T1. Non-standardized coefficient (B) represents the differences in the T2 and T3 with respect to the T1. In model 2, adjusted by all possible confounders, participants with the similar VCAM levels at baseline experienced a statistically significant reduction of –8.45 np/mL (95% CI: –16.73 to –0.18) and –9.47 np/mL (95% CI: –17.38 to –1.56) for those in the T2 and T3 respectively, compared with T1. In this model participants in T2 and T3 experienced a reduction of –10.18 np/mL (95% CI: –19.60 to –0.77) and –14.71 np/mL (95% CI: –23.88 to –5.53) of ICAM–1, respectively. IL–6 was reduced significantly –0.99 pg/mL (95% CI: –2.03 to –0.40) and –1.21 pg/mL (95% CI: –1.85 to –0.21), in T3 and T2 compared with T1, respectively. Statistically significant reductions of –5.88 pg/mL (95% CI: –9.17 to –2.58) and –7.05 pg/mL (95% CI: –10.35 to –3.75) in TNF–α level were observed in T2 and T3, respectively, compared with T1. Participants in T2 and T3 experienced a reduction of –3.55 np/mL (95% CI: –4.68 to –0.82) and –3.36 np/mL (95% CI: –4.55 to –0.92) of MCP–1, respectively. Finally, a significantly inverse correlation was observed between changes in urinary TPE and changes in the 5 inflammatory biomarkers analyzed (**Table 6**).

Discussion

In this cohort of older participants at high cardiovascular risk, we observed a significant inverse correlation between changes in polyphenol intake, as measured by the objective biomarker urinary TPE, and circulating inflammatory molecules related to atherosclerosis. Thus, participants with higher increases in urinary TPE showed significant reductions in the plasma concentrations of VCAM-1, ICAM, IL-6, TNF-α and MCP-1 compared with participants with lesser changes of urinary TPE. In addition, other cardiovascular risk factors

like systolic and diastolic BP were significantly decreased and HDL-cholesterol increased when polyphenol intake increased (T2 and T3).

Polyphenols are the most abundant antioxidants in human diets. They are secondary metabolites of plants. Fruits and vegetables, particularly seeds, and derived foods and beverages, make up the main sources of polyphenols (26). The most common polyphenols in the human diet are not necessarily the most active *in vivo* (26), since most dietary polyphenols (75–99%) are not found in urine and the quantities detected intact vary from one phenolic compound to another (27). This fact may be due to their reduced absorption through the gut barrier, their excretion to the bile or their metabolization by the colonic microflora or our own tissues.

EVOO, one of the supplemental foods given in our study, contains considerable amount of polyphenols that have a great effect on the stability and nutritional characteristics of the oil. Some of the most representative simple phenols are hydroxytyrosol and tyrosol. However, most phenolic compounds are removed when the oil is refined (26). The anti-inflammatory properties of EVOO have been attributed to its content in polyphenols (28,29) and some phenolic compounds, such as oleocanthal, exhibit strong anti-inflammatory properties (30). Epidemiological studies have related olive oil consumption to various health indexes. A recent comprehensive meta-analysis of 32 cohort studies relating exposure to MUFA (of both plant and animal origin), olive oil, oleic acid, and the MUFA:SFA ratio to various health outcomes indicated that, when comparing the upper to the lower tertile of consumption, olive oil, but not MUFA, was associated with reduced risk of all-cause mortality, cardiovascular disease events, and stroke (31). When focusing on virgin olive oil consumption, an additional inverse association with CHD risk was observed. A recent report from the prospective cohort of the Nurses' Health Study, a US population with low average olive oil consumption, suggests a modest inverse relationship between exposure to olive oil and the risk of type-2

diabetes (32). Nevertheless, recent evidence from the PREDIMED study indicates that the Med-Diet enriched with EVOO protects from incident diabetes (33).

Cardiovascular protection from olive oil is attributable in part to effects on cardiovascular risk factors. In fact, there is evidence that polyphenol-rich olive oils decrease blood pressure and improve the lipid profile (34), having a significant HDL-cholesterol raising effect (12). The beneficial effect of virgin olive oil on HDL-cholesterol is due in part to MUFA and in part to polyphenols, but the latter appear to have additional salutary effects on HDL functionality by promoting reverse cholesterol transport (35) and also are implicated in improving endothelial function (36). Clinical trials have also demonstrated beneficial effects of olive oil on markers of endothelial function and inflammation such as C-reactive protein and interleukin-6 (37). All of these effects might be attributed to the antioxidant and anti-inflammatory effects of EVOO components, particularly polyphenols (34).

Nuts, the other supplemented food in our study, also contain highly bioactive molecules, including unsaturated fatty acids, high-quality protein, fiber, tocopherols, non-sodium minerals, phytosterols and phenolic compounds (38). Remarkably, in all nuts, most of the antioxidants are located in the pellicle or outer soft shell, and more than 50% of them are lost when the skin is removed (39). Nut consumption also plays a role in the prevention of chronic age-related diseases (40). There is a growing body of evidence suggesting a beneficial role of nut consumption on CHD and mortality (41-43). A pooled analysis of epidemiologic studies showed that subjects in the highest quantiles of nut consumption had an approximately 35% reduced risk of CHD incidence, with a reduction in fatal CHD that was due primarily to a decrease in sudden cardiac death (40).

Several feeding trials have also analyzed the mechanisms of the health effects of nuts. Thus, diets enriched with nuts improve the lipid profile (44,45), reduce endothelial dysfunction (46,47), and ameliorate glycemic control in diabetes (48). While cholesterol lowering by nut

diets can be ascribed to both unsaturated fatty acids and phytosterols (45), improvement of vascular reactivity is likely to be due to their richness in polyphenols, besides their content in L-arginine, the precursor of the endogenous vasodilator nitric oxide (46). In addition, nuts ameliorate inflammatory status at the vascular level reducing ICAM-1, VCAM-1 and E-selectin, which are released from the activated endothelium and circulating monocytes (47). Berry *et al.* (49) showed that oxidation of plasma and LDL lipids in healthy volunteers was less after an almond diet compared with a low-fat diet. Jenkins *et al.* (50) in a dose-response study compared two doses of almonds with a low-fat diet in hyperlipidemic subjects. The full-dose almonds produced the greatest reduction in levels of blood lipids. Significant reductions from baseline were seen on both half- and full-dose almonds for LDL cholesterol and LDL:HDL cholesterol and on full-dose almonds alone for lipoprotein and oxidized LDL concentrations, with no significant reductions on the control diet. Other previous studies have also reported that some components of the Med-diet such as EVOO or nuts may down-regulate inflammatory markers related to atherosclerosis such as VCAM-1, ICAM-1, E- and P-Selectin, C-reactive protein and IL-6 (51-53).

Finally, the role of dietary patterns on the prevention of chronic diseases should be undertlined. The Med-diet and the DASH diet have been considered as healthy dietary patterns, useful in the prevention of cardiovascular disease [4,5]. Both diets include a high intake of fruits, vegetables, whole grain cereals, legumes, and nuts, together with a moderate consumption of fish and low-fat dairies, and low consumption of meat, meat products, sweets and commercial bakery. The Med-diet also includes a high intake of olive oil and a moderate consumption of wine, mainly with meals, as specific and distinguishing features. Given that the Med-diet is plant-based and enriched in olive oil and wine, it is by definition a polyphenol-rich dietary pattern, and this is likely to play a role in its anti-inflammatory effect

and down-regulation of cellular and circulating inflammatory biomarkers related to atherosclerosis (54,55).

Our study has limitations. First, since the study subjects included were older people at high risk of cardiovascular disease, the results may not be generalized to other populations. A second limitation is the size of the study population, which was relatively small in comparison with other studies. The present study also has strengths, including the design as a randomized controlled clinical trial, which is considered the most rigorous method of determining whether a cause-effect relationship exists between an intervention and an outcome. In randomized studies the conclusions reached achieve the highest level of scientific evidence. Another strength was the use of TPE as a biomarker of total polyphenol intake, since this is more precise than self-reported information based on recalled dietary assessments, thus providing a more objective measurement of specific nutrient intake than the subjective information obtained by a FFQ.

In conclusion, our results suggest that a Med-diet intervention is associated with increased TPE in spot urine samples. After adjustment for potential confounders, higher compared with lower changes of TPE in urine samples are associated with decreased inflammatory biomarkers and improvement of cardiovascular risk factors such as LDL-cholesterol, HDL-cholesterol and systolic and diastolic BP. Thus, a polyphenol-rich diet may help reduce cardiovascular risk.

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Conflicts of interest

Dr. Ros reports grants, non-financial support and other from California Walnut Commission, grants, personal fees, non-financial support and other from Merck, Sharp & Dohme, grants, personal fees, non-financial support and other from Alexion, personal fees, non-financial support and other from Aegerion, grants and personal fees from Sanofi Aventis, grants, personal fees, non-financial support and other from Ferrer International, grants from Amgen, grants from Pfizer, outside the submitted work. Dr. Estruch reports grants from Spanish Institute of Health "Carlos III", non-financial support from Patrimonio Comunal Olivarero, Spain, non-financial support from California Walnut Commission, Spain, non-financial support from Borges SA, Spain, grants and non-financial support from FIS, Government of Spain, non-financial support from Fundacion Bosch i Gimpera, Spain, during the conduct of the study; personal fees from Fundación Dieta Mediterránea, Spain, personal fees from FIVIN, Spain, personal fees from Cerveceros de España, Spain, personal fees from Brewers of Europe, Belgium, grants from Bicentury, SA, Spain, grants from Grand Fountaine, Spain, grants from Novartis Farmaceutica, SA, grants from Amgen SA, personal fees from Lilly Laboratories, Spain, personal fees from Instituto Cervantes, Alburquerque, USA, personal fees from Instituto Cervantes, Milan, Italy, personal fees from Wine and Culinary International Forum, non-financial support from Harvard School of Public Health, Boston, USA , from University of Columbia, NYC, USA, outside the submitted work.

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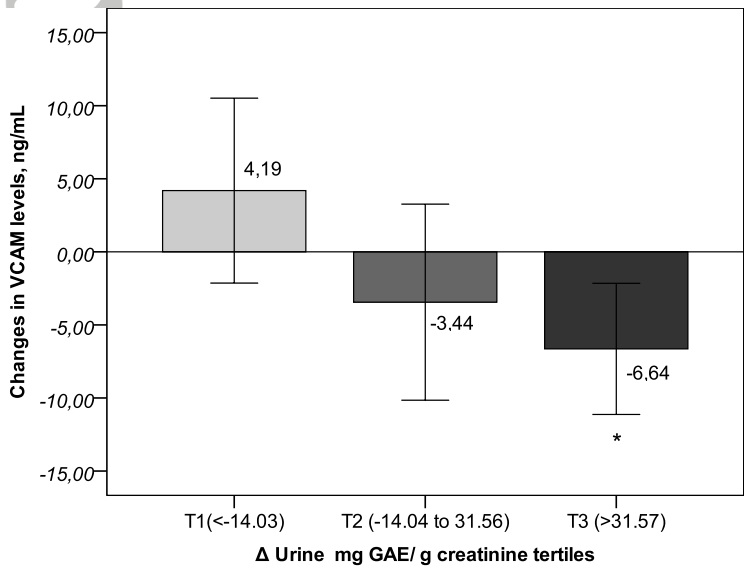
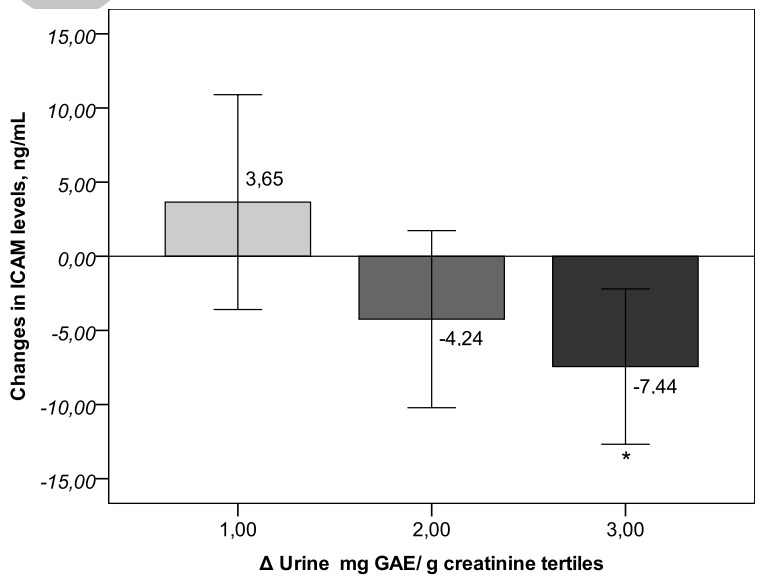
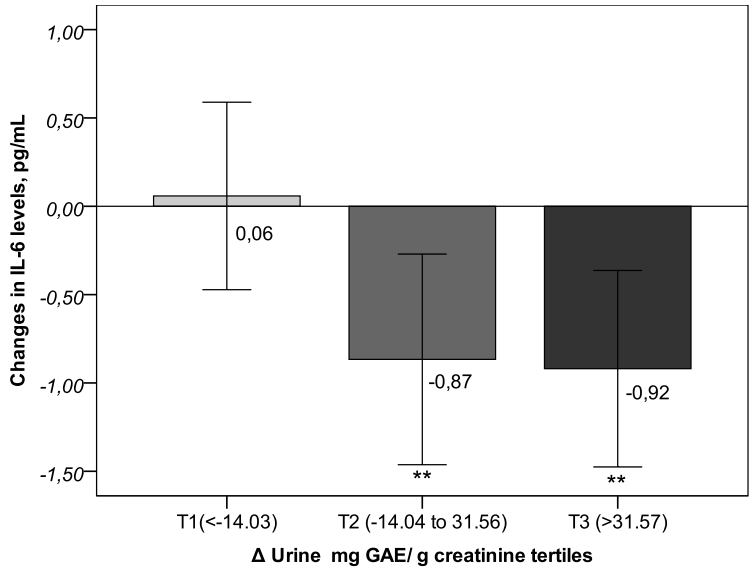
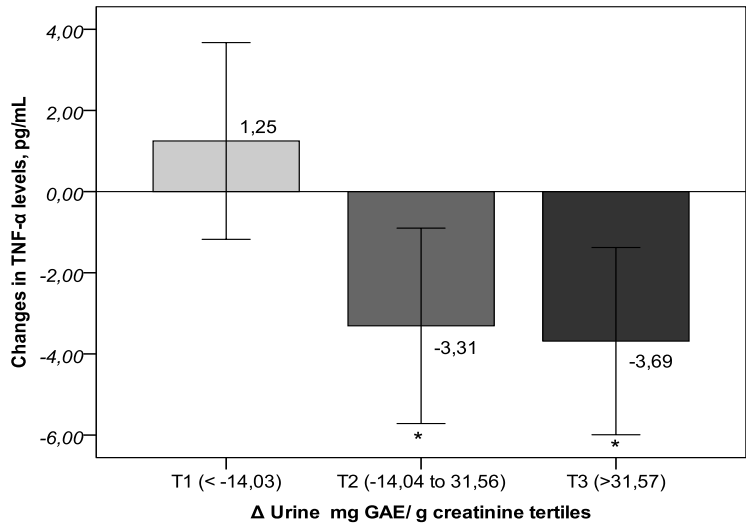
Figure legends

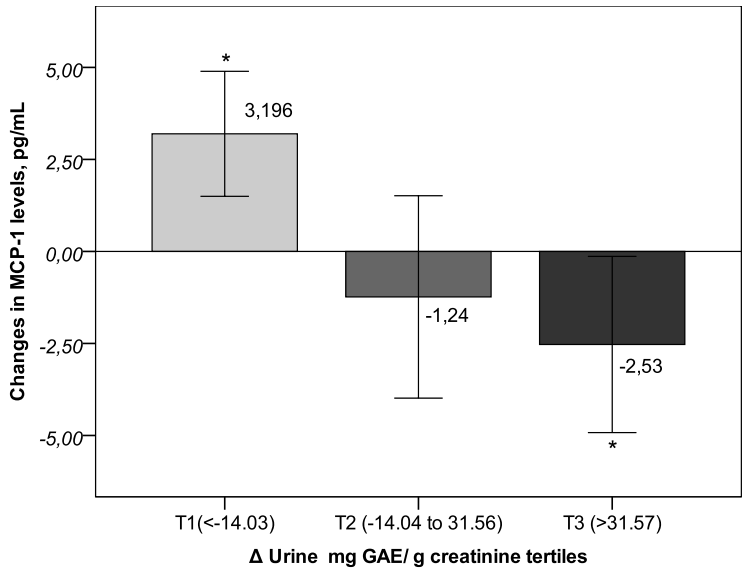
Figure 1: Changes from baseline after 1-year in plasma concentration of the inflammatory biomarker, according to tertiles of changes of total urinary polyphenols excreted.





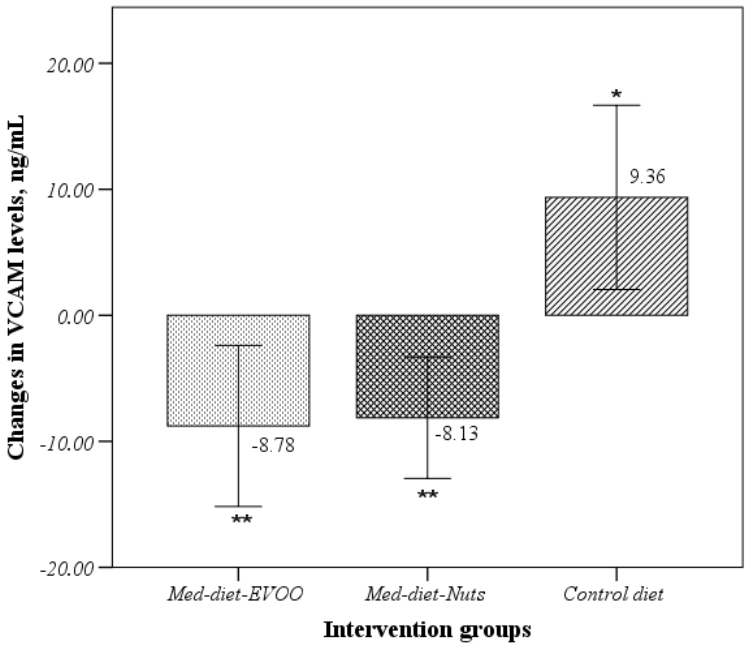


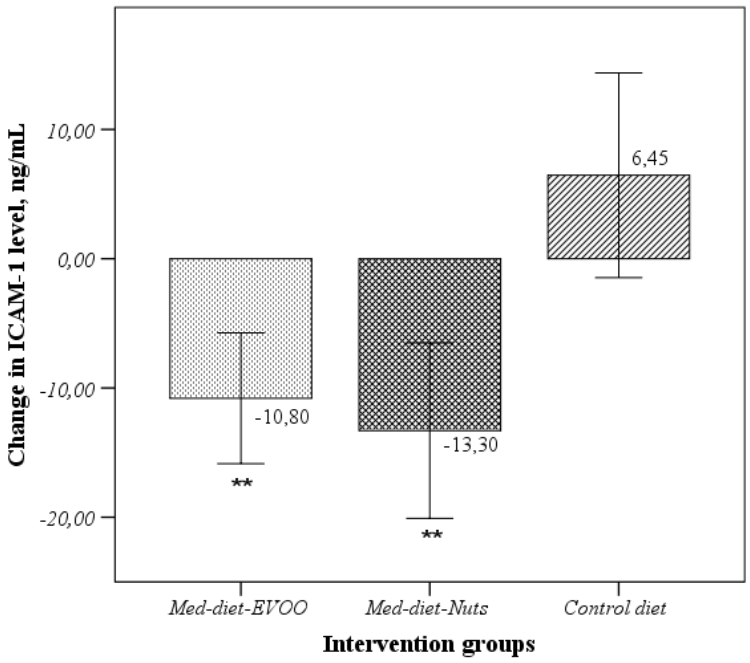


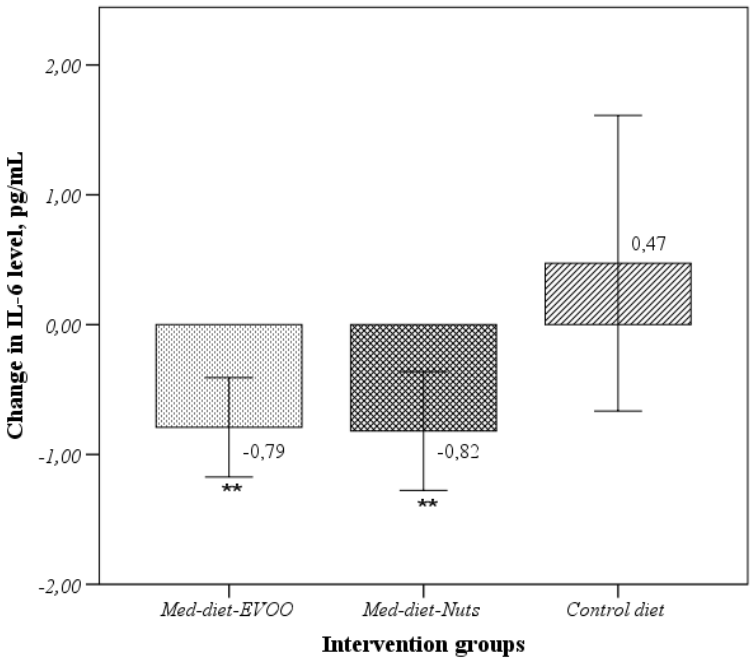


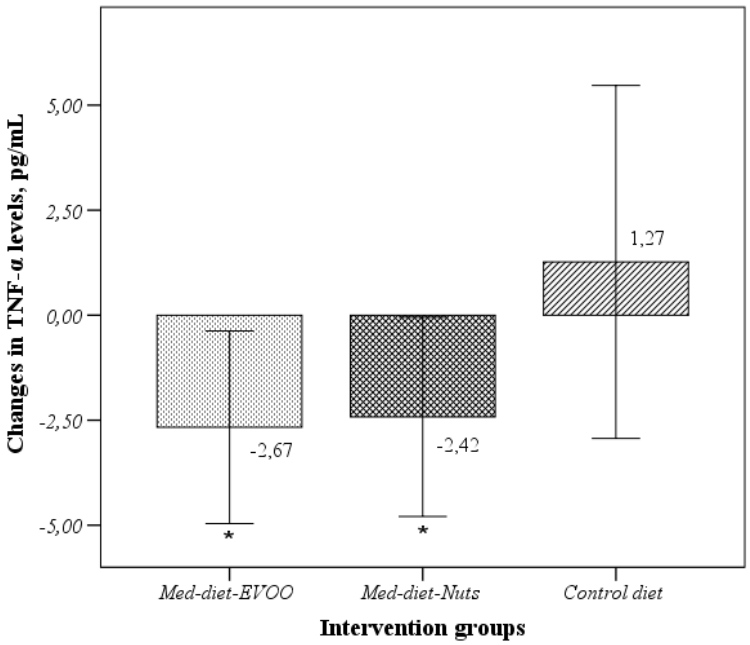
*P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%. VCAM-1: Vascular Cell Adhesion Molecule-1; ICAM-1: soluble Inter-Cellular Adhesion Molecule-1; IL-6: Plasma Interleukin-6; TNF-α: Tumor Necrosis Factor Alpha; MCP-1: Monocyte Chemotactic Protein-1.

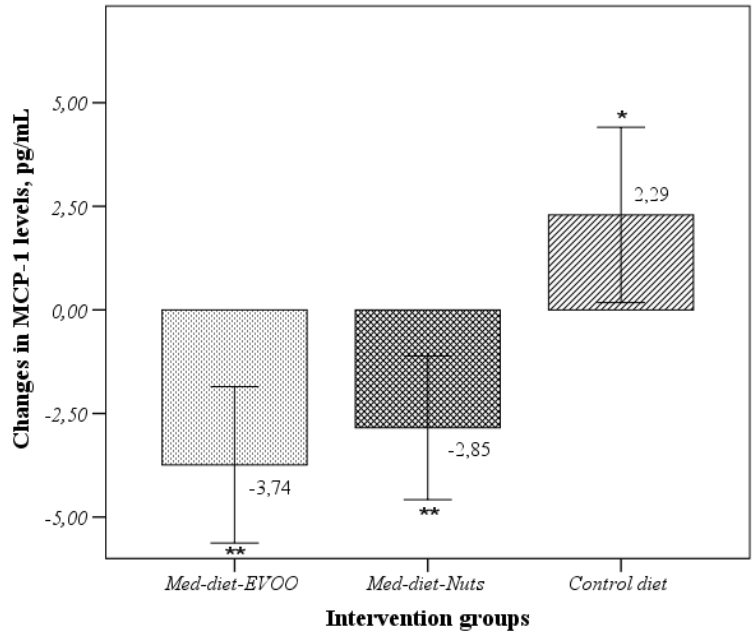
Figure 2: Changes from baseline after 1-year in plasma concentration of the inflammatory biomarkers, according to the intervention groups.











Med-diet: Mediterranean diet; EVOO: extra virgin olive oil;**P<0.01, *P<0.05 indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%. VCAM-1: Vascular Cell Adhesion Molecule-1; ICAM-1: soluble Inter-Cellular Adhesion Molecule-1; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor-α; MCP-1: Monocyte Chemotactic Protein-1.

Table 1: Baseline of the study participants, according to tertiles of changes of total urinary polyphenols excreted, expressed as mg GAE /g creatinine.

	Δ Urine mg GAE/ g creatinine tertiles			
	T1 (<-14.03)	**T2 (-14.04 to 31.56)**	**T3 (>31.57)**	***P*[1]**
No. of subjects	379	380	380	
Age (y), mean (SD)	67.4 (6.2)	67.5 (5.9)	68.0 (5.7)	0.318
Women, n (%)	217 (57.3)	186 (48.9)	225 (59.2)	0.010
BMI (kg/m^2), mean (SD)	29.3 (3.5)	29.1 (3.3)	29.5 (3.4)	0.281
Overweight or obese (BMI ≥25 Kg/m^2), n (%)	329 (86.8)	348 (91.6)	353 (92.9)	0.011
Hypertension, n (%)	303 (79.9)	300 (78.9)	302 (79.5)	0.943
Diabetes, n (%)	183 (48.3)	172 (45.3)	148 (38.9)	0.030
Dyslipidemia, n (%)	253 (66.8)	234 (61.6)	254 (66.8)	0.304
Current smoker, n (%)	55 (14.5)	79 (20.8)	53 (13.9)	0.018
Family history of CHD, n (%)	165 (44.0)	174 (46.5)	194 (51.2)	0.026
Medication, n (%)				
Antihypertensive drugs	287 (76.7)	252 (70.2)	278 (74.9)	0.115
Statins (hypolipidemic drugs)	179 (47.2)	149 (39.5)	153 (40.4)	0.063
Insulin	25 (6.6)	14 (3.7)	21 (5.5)	0.192
Oral hypoglycemic drugs	118 (31.2)	104 (27.5)	96 (25.3)	0.190
Aspirin or other antiplatelet drugs	75 (19.9)	69 (18.3)	72 (19.0)	0.678
Vitamins or supplements, n (%)	40 (10.7)	27 (7.4)	47 (12.6)	0.068
Educational level, n (%)				
Primary school	279 (73.6)	274 (72.1)	277 (72.9)	0.896
High school	61 (16.1)	49 (12.9)	64 (16.8)	0.275
University	27 (7.1)	36 (9.5)	33 (8.7)	0.495
Energy expenditure in physical activity (kcal/d), mean (SD)	284.3 (235.2)	293.5 (236.2)	271.2 (196.5)	0.390

[1]ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); CHD: coronary heart disease; GAE: gallic acid equivalent; SD: standard deviation.

Table 2: Changes in daily intake of selected foods and Mediterranean diet score after 1-year, according to tertiles of changes of total urinary polyphenols excreted[1].

	Δ Urine mg GAE/ g creatinine tertiles			Repeated measures ANOVA[2]	*P* value for differences[3]	
	T1 (<-14.03)	**T2 (-14.04 to 31.56)**	**T3 (>31.57)**	*Time x Group*	T2 *vs.* T1	T3 *vs.* T1
No. of subjects	379	380	380			
Total nuts (g)						
Baseline	9.6 (12.6)	10.4 (13.8)	10.7 (11.6)	0.651	0.217	0.877
1 year	17.8 18.6 ()**	17.1 (17.1)**	18.2 (15.6)**			
EVOO (g)						
Baseline	18.2 (23.0)	17.7 (22.3)	22.0 (23.9)	<0.001	0.017	<0.001
1 year	29.6 (27.0)**,a	34.9 (27.6)**,b	39.5 (27.9)**,b			
Fruits (g)						
Baseline	372.3 (204.0)	370.0 (194.2)	374.6 (181.3)	0.630	0.077	0.669
1 year	450.6 (218.5)**	429.8 (185.6)**	445.8 (185.9)**			
Vegetables (g)						
Baseline	301.6 (127.5)	314.0 (132.7)	288.0 (104.7)	0.007	0.700	0.437
1 year	328.6 (139.2)**	341.2 (149.2)**,a	313.8 (112.1)**,b			
Legumes (g)						
Baseline	18.7 (8.8)	18.8 (8.8)	17.8 (7.8)	0.084	0.289	0.878
1 year	20.6 (8.9)**	21.5 (9.6)**	20.1 (7.5)**			
Fish or seafood (g)						
Baseline	99.8 (41.3)	96.7 (40.9)	87.3 (36.2)	<0.001	0.661	0.565
1 year	99.8 (39.1)a	100.1 (41.0)a	91.9 (36.1)*,b			
Meat or meat products (g)						
Baseline	142.8 (56.0)	142.5 (49.3)	135.8 (51.4)	0.198	0.511	0.626
1 year	127.1 (45.2)**	129.9 (43.9)**	125.8 (44.5)**			
Cereals (g)						
Baseline	240.6 (105.6)	258.9 (114.7)	232.6 (102.9)	0.006	0.069	0.019

1 year	239.3 (98.7)	239.3 (93.5)**	223.8 (89.9)			
Milk and dairy products (g)						
Baseline	370.8 (212.7)	349.5 (185.0)	393.1 (254.2)	0.004	0.380	0.005
1 year	355.1 (204.9)[a]	356.7 (185.3)[a]	404.7 (245.7)[b]			
Pastries, cakes or sweets (g)						
Baseline	21.9 (27.8)	26.0 (28.7)	23.7 (26.9)	0.048	0.168	0.882
1 year	16.7 (19.5)**	20.7 (25.9)**	17.5 (20.7)**			
Alcohol (g)						
Baseline	10.2 (16.7)	14.2 (19.2)	11.0 (17.5)	0.025	0.490	0.915
1 year	9.8 (16.4)	12.2 (16.4)**	10.3 (14.8)			
Tea (mL)						
Baseline	3.4 (13.3)	3.9 (15.2)	6.4 (20.9)	0.023	0.218	0.931
1 year	4.0 (14.2)	2.9 (11.6)	5.8 (22.3)			
Coffee (mL)						
Baseline	29.3 (48.1)	30.1 (46.1)	34.3 (49.8)	0.406	0.386	0.970
1 year	31.8 (51.5)	30.8 (47.9)	34.2 (50.7)			
Med Diet score						
Baseline	8.8 (1.9)	8.8 (1.8)	9.0 (1.7)	<0.001	0.709	<0.001
1 year	10.2 (1.9)**,[a]	10.2 (2.0)**,[a]	10.8 (1.8)**,[b]			

[1]Data are given as means (SD). Different letters in rows shows significant difference between groups by Bonferroni post hoc test ($P<0.05$). Values with asterisks are statistically different from baseline by Bonferroni post hoc test ($P<0.05$): *$P<0.05$; **$P<0.01$. EVOO: Extra Virgin Olive Oil.

[2]Data analyzed by repeated-measures 2-factor ANOVA ($P<0.05$).

[3]Data analyzed by ANCOVA ($P<0.05$), and adjusted for energy intake and baseline value of each variable.

Table 3: Changes in energy and, daily nutrient intake after 1 year, according to tertiles of changes of total urinary polyphenols excreted[1].

	Δ Urine mg GAE/ g creatinine tertiles			Repeated measures ANOVA[2]	*P* value for differences[3]	
	T1 (<-14.03)	T2 (-14.04 to 31.56)	T3 (>31.57)	*Time x Group*	T2 *vs.* T1	T3 *vs.* T1
No. of subjects	379	380	380			
Total energy, Kcal/d						
Baseline	2250.7 (536.1)	2345.5 (588.6)	2236.8 (584.1)	0.039	0.996	0.813
1 year	2282.8 (519.6)	2335.6 (516.1)	2268.0 (492.9)			
Total protein (g)						
Baseline	92.5 (19.2)	93.0 (20.1)	89.1 (22.4)	0.069	0.760	0.752
1 year	90.8 (19.0)	91.8 (18.8)	89.8 (18.3)			
Total Carbohydrate (g)						
Baseline	233.5 (71.1)	245.9 (80.8)	231.9 (78.0)	0.056	0.041	0.030
1 year	234.7 (67.3)	236.4 (68.2)*	227.4 (63.5)			
Fibre (g)						
Baseline	24.4 (8.4)	24.8 (7.7)	23.7 (6.8)	0.225	0.117	0.465
1 year	26.6 (9.1)**	26.4 (7.4)**	25.8 (7.1)**			
Total fat (g)						
Baseline	97.3 (26.5)	99.0 (27.6)	97.3 (27.3)	0.424	0.570	0.048
1 year	101.3 (26.4)**	104.1 (25.1)**	102.9 (25.2)**			
SFA (g)						
Baseline	24.7 (8.0)	25.3 (8.4)	24.2 (8.7)	0.048	0.443	0.374
1 year	23.4 (7.2)**	24.2 (7.0)**,a	22.8 (7.0)**,b			
MUFA (g)						
Baseline	48.7 (14.5)	49.1 (14.2)	49.7 (14.0)	0.131	0.251	<0.001
1 year	51.9 (14.3)**,a	53.7 (13.5)**	54.5 (13.6)**,b			
PUFA (g)				0.115	0.488	0.403

Baseline	15.5 (6.6)	16.0 (6.9)	15.0 (6.3)			
1 year	17.2 (6.9)**	17.5 (6.6)**	16.7 (5.6)**			
Cholesterol (g)						
Baseline	361.5 (108.9)	363.9 (114.4)	343.1 (117.5)	0.030	0.686	0.520
1 year	339.9 (115.9)**	342.4 (107.1)**	328.3 (86.9)*			
Magnesium (mg)						
Baseline	367.8 (105.1)	374.8 (101.0)	354.6 (91.7)	0.040	0.112	0.822
1 year	385.6 (107.9)**	386.2 (91.3)*	375.0 (82.8)**			
Potassium (mg)						
Baseline	4289.2 (1053.9)	4350.0 (1033.9)	4197.8 (1026.3)	0.172	0.220	0.784
1 year	4473.3 (1071.8)**	4480.8 (960.0)**	4391.4 (855.3)**			
Sodium (mg)						
Baseline	2349.1 (846.0)	2438.5 (921.6)	2164.5 (924.2)	<0.001	0.223	0.003
1 year	2251.8 (719.8)*,a	2273.5 (818.9)**,a	2072.1 (697.5)*,b			
Energy expenditure in physical activity (kcal/d)						
Baseline	284.3 (235.2)	293.5 (236.2)	271.2 (196.5)	0.527	0.310	0.871
1 year	303.1 (240.9)	299.6 (228.0)	290.3 (202.1)*			

[1]Data are given as means (SD). Different letters in rows shows significant difference between groups by Bonferroni post hoc test (P<0.05). Values with asterisks are statistically different from baseline by Bonferroni post hoc test (P<0.05): *P<0.05; **P<0.01. MUFA: monounsaturated fat acids; PUFA, polyunsaturated fat acids and SFA: saturated fat acid.

[2] Data analyzed by repeated-measures 2-factor ANOVA (P<0.05).

[3]Data analyzed by ANCOVA (P<0.05), and adjusted for energy intake and baseline value of each variable.

Table 4: Baseline level and 1-year changes in total polyphenol excreted, lipid profiles, blood pressure and inflammatory biomarkers, according to tertiles of changes of total urinary polyphenols excreted[1].

	Δ Urine mg GAE/ g creatinine tertiles			Repeated measures ANOVA[2]	*P* value for Differences[3]	
	T1 (<-14.03)	T2 (-14.04 to 31.56)	T3 (>31.57)	*Time x Group*	T2 *vs.* T1	T3 *vs.* T1
No. of subjects	379	380	380			
Urine total polyphenol (mg GAE/ g creatinine)						
Baseline	166.1 (84.7)	102.4 (40.6)	107.5 (47.3)	<0.001	<0.001	<0.001
1 year	102.9 (51.6)**,a	110.2 (41.7)**,a	209.0 (95.9)**,b			
Systolic BP (mmHg), mean (SD)						
Baseline	150.2 (17.3)	151.6 (17.4)	153.2 (18.5)	0.594	0.034	0.005
1 year	149.8 (18.1)	147.8 (16.2)**	148.6 (17.6)**			
Diastolic BP (mmHg), mean (SD)						
Baseline	83.3 (9.3)	84.9 (10.2)	85.2 (9.8)	0.409	0.044	0.004
1 year	83.4 (9.4)	83.0 (10.1)**	83.4 (9.4)**			
Fasting glucose, mg/dl						
Baseline	122.5 (36.8)	117.6 (37.2)	114.1 (33.4)	0.024	0.118	0.455
1 year	121.5 (39.0)	116.4 (34.4)	114.7 (35.9)			
Total cholesterol, mg/dl						
Baseline	204.3 (37.7)	206.4 (35.2)	207.9 (35.4)	0.250	0.734	0.648
1 year	206.1 (36.6)	208.0 (34.2)	211.4 (34.5)			
LDL cholesterol, mg/dl						
Baseline	132.3 (33.6)	134.9 (31.4)	136.8 (32.7)	0.313	0.539	0.163
1 year	124.1 (32.3)**	126.8 (29.4)**	126.8 (30.2)**			
HDL cholesterol, mg/dl						
Baseline	53.4 (11.0)	53.3 (11.2)	53.9 (10.9)	0.086	0.712	0.004
1 year	56.6 (13.4)**,a	55.9 (13.5)**,a	59.5 (14.3)**,b			
Triglyceride, mg/dl						

Baseline	126.5 (58.9)	127.5 (69.6)	132.9 (66.3)	0.122	0.382	0.553
1 year	128.8 (58.8)	124.8 (59.6)[a]	138.4 (80.5)[b]			
VCAM, ng/mL						
Baseline	176.1 (44.1)	177.5 (49.8)	179.8 (46.2)	0.877	0.045	0.019
1 year	180.3 (47.2)	174.1 (46.8)	173.2 (46.3)*			
ICAM-1, ng/mL						
Baseline	183.9 (64.4)	170.8 (78.3)	180.9 (68.8)	0.039	0.034	0.002
1 year	187.5 (73.3)[a]	166.6 (64.7)[b]	173.5 (64.2)*			
IL-6, pg/mL						
Baseline	6.0 (6.7)	6.0 (5.6)	5.3 (6.4)	0.031	0.019	0.004
1 year	6.1 (8.7)[a]	5.1 (3.9)**	4.4 (4.6)**[,b]			
TNF-α, pg/mL						
Baseline	15.5 (21.5)	15.7 (21.8)	12.9 (22.2)	0.040	<0.001	<0.001
1 year	16.7 (27.2)[a]	12.4 (16.8)**	9.2 (13.7)**[,b]			
MCP-1, pg/mL						
Baseline	37.0 (17.5)	38.9 (26.2)	41.7 (25.2)	0.306	0.012	0.017
1 year	40.2 (18.4)*	37.7 (18.5)	39.2 (17.0)*			

[1]Data are given as means (SD). Different letters in rows shows significant difference between groups by Bonferroni post hoc test (P<0.05). Values with asterisks are statistically different from baseline by Bonferroni post hoc test (P<0.05): *P<0.05; **P<0.01. BP: blood pressure; GAE: gallic acid equivalent; ICAM-1: soluble Inter-Cellular Adhesion Molecule-1; IL-6: Plasma Interleukin-6; MCP-1: Monocyte Chemotactic Protein-1; Med diet: Mediterranean diet; OxLDL: oxidized low-density lipoprotein; TNF-α: Tumor Necrosis Factor Alpha; VCAM-1: Vascular Cell Adhesion Molecule-1.

[2] Data analyzed by repeated-measures 2-factor ANOVA (P<0.05).

[3]Data analyzed by ANCOVA (P<0.05), and adjusted by baseline value of each variable, intervention group, sex, age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) supplements taken in the last month, sodium, potassium, total energy, monounsaturated fat acids, polyunsaturated fat acids and saturated fat acid intake.

Table 5: Changes in inflammatory biomarkers after one year, associated with tertiles of changes in total

	Model	B	*P*	95 % CI
VCAM, ng/mL	*Model 1*			
	T2 *vs.* T1	-7.18	0.066	-14.84 to 0.48
	T3 *vs.* T1	-9.64	0.016	-17.46 to -1.82
	Model 2			
	T2 *vs.* T1	-8.45	0.045	-16.73 to -0.18
	T3 *vs.* T1	-9.47	0.019	-17.38 to -1.56
ICAM-1, ng/mL	*Model 1*			
	T2 *vs.* T1	-10.91	0.009	-19.10 to -2.72
	T3 *vs.* T1	-11.78	0.006	-20.20 to -3.36
	Model 2			
	T2 *vs.* T1	-10.18	0.034	-19.60 to -0.77
	T3 *vs.* T1	-14.71	0.002	-23.88 to -5.53
IL-6, pg/mL	*Model 1*			
	T2 *vs.* T1	-0.95	0.009	-1.67 to -0.24
	T3 *vs.* T1	-1.23	0.001	-1.96 to -0.51
	Model 2			
	T2 *vs.* T1	-0.99	0.019	-2.03 to -0.40
	T3 *vs.* T1	-1.21	0.004	-1.85 to -0.21
TNF-α, pg/mL	*Model 1*			
	T2 *vs.* T1	-4.46	0.003	-7.39 to -1.53
	T3 *vs.* T1	-5.93	<0.001	-8.92 to -2.94
	Model 2			
	T2 *vs.* T1	-5.88	<0.001	-9.17 to -2.58
	T3 *vs.* T1	-7.05	<0.001	-10.35 to -3.75
MCP-1, pg/mL	*Model 1*			

	T2 *vs.* T1	-3.32	0.009	-4.20 to 0.75
	T3 *vs.* T1	-2.98	0.021	-3.84 to 1.20
	Model 2			
	T2 *vs.* T1	-3.55	0.012	-4.68 to 0.82
	T3 *vs.* T1	-3.36	0.017	-4.55 to 0.92

polyphenol excreted, T2 (-14.04 to 31.56) and T3 (>31.57) compared with T1(<-14.03).

B: Non-standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by baseline inflammatory biomarkers, intervention group, sex, age, BMI, smoking status, physical activity, medication use (antihypertensive, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) supplements taken in the last month, sodium, potassium, total energy, monounsaturated fat acids, polyunsaturated fat acids and saturated fat acid intake.

Table 6: Correlation between changes in total polyphenol excreted (mg gallic acid/g creatinine) and changes in inflammatory biomarkers, after one year.

Inflammatory biomarkers	***r***	***P***
VCAM, ng/mL	-0.301	<0.001
ICAM-1, ng/mL	-0.159	<0.001
IL-6, pg/mL	-0.092	0.006
TNF-α, pg/mL	-0.138	0.001
MCP-1, pg/mL	-0.077	0.019