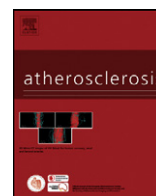




Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis



Effect of Mediterranean diet on the expression of pro-atherogenic genes in a population at high cardiovascular risk

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ARTICLE INFO

Article history:

Received 3 June 2009

Received in revised form 30 July 2009

Accepted 3 August 2009

Available online xxx

Keywords:

Virgin olive oil

Nuts

Inflammation

Lipoprotein receptors

Thrombosis

Genes

ABSTRACT

Experimental and epidemiological studies have demonstrated the beneficial effects of the traditional Mediterranean diet (TMD) on the incidence and progression of atherosclerosis. Several genes play a major role in determining atherosclerosis susceptibility. We compared the short-term effects of two TMD diets versus a control diet on the expression of pro-atherogenic genes. One TMD diet was supplemented with virgin olive oil (VOO) (TMD + VOO) and the other with nuts (TMD + nuts). Gene expression was analyzed in monocytes from 49 asymptomatic high cardiovascular-risk participants (23 men, 26 women), aged 55–80 years. Monocytes were isolated from blood before and 3 months after dietary intervention. We analyzed the expression of genes involved in inflammation [cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and monocyte chemoattractant protein (MCP-1)], genes involved in foam cell formation [low-density lipoprotein receptor-related protein (LRP1), LDL receptor and CD36], and genes involved in thrombosis [tissue factor (TF) and tissue factor pathway inhibitor (TFPI)]. We found that TMD + VOO intervention prevented an increase in COX-2 and LRP1, and reduced MCP-1 expression compared to TMD + nuts or control diet interventions. TMD + nuts specifically increased the expression of CD36 and TFPI compared to TMD + VOO and control diet intervention.

Our findings showed that the Mediterranean diet influences expression of key genes involved in vascular inflammation, foam cell formation and thrombosis. Dietary intervention can thus actively modulate the expression of pro-atherothrombotic genes even in a high-risk population.

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1. Introduction

The “Seven Countries” study showed that coronary mortality was lower in Mediterranean populations than in United States or Northern European inhabitants, a finding that was attributed to the variation in food patterns among countries [1–3].

Southern European populations had a large intake of monounsaturated fatty acids (MUFA), mainly as oleic acid from olive oil, while the other countries consumed a high amount of saturated fatty acids (SFA) [3]. Olive oil is the main component of the traditional Mediterranean diet (TMD) and is a rich source of MUFA,

antioxidants and minor bioactive components. Clinical studies in smaller samples have demonstrated its beneficial effects on surrogate markers of cardiovascular disease. The intake of virgin olive oil (VOO) (olive oil obtained from the first pressing of olives) reduces blood pressure [4], improves lipid profile [5] and decreases plasma levels of von Willebrand factor, tissue factor pathway inhibitor and tissue plasminogen activator type-1 [6,7]. The healthy benefits of VOO may be partly due to its anti-inflammatory actions [8].

Tree nuts are also typical in the TMD. They have a favorable fatty acid profile and are a rich source of nutrients and other bioactive compounds—such as fiber, phytosterols, folic acid, and antioxidants—that may beneficially influence the risk of coronary heart disease (CHD). Frequent nut intake has been associated with decreased CHD rates in prospective studies [9]. Walnuts differ from other nuts because of their high content of polyunsaturated fatty acids, particularly α -linolenic acid, a plant-based n-3

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fatty acid that may confer additional antiatherogenic properties [10,11].

Many genes are involved in the development of atherothrombosis; cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and monocyte chemoattractant protein (MCP-1) play a role in inflammation [12–15], while low-density lipoprotein receptor-related protein (LRP1) and CD36 participate in intracellular lipid accumulation [16–20] and tissue factor (TF) and tissue factor pathway inhibitor (TFPI) [21,22] regulate thrombosis. Modulation of gene expression undoubtedly plays a key role in the control of atherosclerosis. In this study, we investigated whether two TMD diets, one supplemented with VOO and another supplemented with nuts, could modulate pro-atherothrombotic genes. We aimed to evaluate the effects of the TMD on monocyte gene expression in a substudy of a larger feeding trial (PREDIMED Study) [23]. This substudy was designed to assess the effects of two Mediterranean diets, TMD + VOO and TMD + nuts, compared with those of a control diet, on pro-atherothrombotic gene expression. We report, the results after 3 month's intervention in the first 49 participants recruited in the Barcelona center of the PREDIMED trial.

2. Methods

2.1. Study design

The PREDIMED study is a large, parallel-group, multicenter, randomized, controlled, 5-year clinical trial aimed at assessing the effects of the TMD on the primary prevention of cardiovascular disease (<http://www.predimed.org>). It is currently underway and has been registered in the Current Controlled Trials, London, with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639. An estimated sample of 7500 participants at high risk for CHD has been assigned to one of three intervention groups: (1) TMD + VOO; (2) TMD + nuts; or (3) a control diet. The present study was designed to assess the effects of 3 month's intervention on pro-atherothrombotic genes in the first 49 participants in the Barcelona center (Table 1). The Institutional Review Board at the Hospital Clinic approved the

Table 1
Participants's characteristics^a.

	TMD + VOO (n = 16)	TMD + nuts (n = 18)	Control (n = 15)	<i>p</i> ^b
Age, years	67 ± 4 ^c	66 ± 7	68 ± 6	0.31
Men, n (%)	6 (37.5)	12 (66.7)	5 (33.3)	0.08
Family history of CHD, n (%)	4 (25.0)	5 (27.7)	4 (26.6)	0.28
Current smokers, n (%)	2 (12.5)	4 (22.2)	2 (13.3)	0.27
BMI, kg/m ²	29.0 ± 2.7	27.7 ± 2.5	29.7 ± 5.4	0.30
BMI ≥ 25 kg/m ² , n (%)	15 (93.7)	15 (83.3)	13 (86.7)	0.88
Type 2 diabetes, n (%)	10 (62.5)	12 (66.6)	9 (60.0)	0.94
Hypertension, n (%)	12 (75.0)	10 (55.5)	13 (81.2)	0.14
Dyslipidemia, n (%)	10 (62.5)	8 (44.4)	10 (66.7)	0.41
Medications, n (%)				
ACE inhibitors	7 (43.7)	10 (55.5)	6 (40.0)	0.52
Diuretics	4 (25.0)	3 (16.6)	7 (46.6)	0.12
Other antihypertensive agents	5 (31.2)	4 (22.2)	6 (40.0)	0.63
Statins	6 (37.5)	5 (27.7)	4 (26.6)	0.68
Other lipid-lowering agents	0 (0.0)	1 (5.5)	2 (0.0)	0.79
Insulin	0 (0.0)	3 (16.6)	1 (6.6)	0.22
Oral hypoglycemic drugs	7 (43.7)	9 (50.0)	8 (53.3)	0.74
Aspirin or antiplatelet drugs	3 (18.7)	3 (16.6)	2 (13.3)	0.95

^a TMD = traditional Mediterranean diet; VOO = virgin olive oil; CHD = coronary heart disease; BMI = body mass index; ACE = angiotensin-converting enzyme.

^b *P* from Fisher for categorical variables and one-way ANOVA for continuous variables.

^c Mean ± SD or *N* (%), when indicated.

study protocol and all participants signed an informed consent form.

2.2. Participants

Eligible subjects were community-dwelling adults, aged 55–80 years for men and 60–80 years for women, who fulfilled at least one of the two following criteria: (a) type 2 diabetes mellitus, or (b) 3 or more of the following CHD risk factors: current smoking, hypertension (blood pressure >140/90 mmHg, or treatment with antihypertensive drugs), hypercholesterolemia (LDL cholesterol ≥160 mg/dL or treatment with hypolipidemic drugs), low HDL cholesterol (≤40 mg/dL), body mass index (BMI) ≥25 kg/m² or family history of premature CHD. Exclusion criteria were history of cardiovascular disease, severe chronic illness, drug or alcohol addiction, low-predicted likelihood of following Prochaska and DiClemente's stages of change in behavior concerning dietary habits, history of food allergy or intolerance to olive oil or nuts, and any condition that might impair participation in the study.

2.3. Randomization and intervention

After a screening visit, participants were randomly assigned to one of the three diets by means of a computer-generated random-number sequence. Allocation was concealed by the use of opaque sealed envelopes.

At baseline, participants completed a 14-item questionnaire, an extension of a previously validated questionnaire [24], which assessed the degree of adherence to the TMD. We also administered a 137-item validated food frequency questionnaire [25]; the validated Spanish version [26] of the Minnesota Leisure Time Physical Activity Questionnaire; and a 47-item questionnaire about education, lifestyle, history of illnesses, and medication use. We performed anthropometric and blood pressure measurements, and obtained samples of fasting blood and spot urine. All examinations were repeated at 3 months.

After explaining the intervention to each of the three dietary groups, the same dietitian interviewed participants individually and gave advice about specific food intake. Participants in the control group were recommended to reduce all types of fat and were given an American Heart Association leaflet [27]. Participants in the two TMD intervention groups were given individual advice on dietary changes needed to achieve a TMD diet [28]. TMD groups received instructions to increase the intake of vegetable fat and oils. The dietitian provided written material to participants in all groups, with descriptions of target foods and seasonal shopping lists, meal plans, and recipes. All diets were *ad libitum*.

TMD + VOO participants were given a 3-month supply of VOO (1 L/wk). TMD-nuts participants received a 3-month supply of mixed nuts (30 g/d, as 15 g walnuts, 7.5 g almonds and 7.5 g hazelnuts). The fatty acid composition of the olive oil and nuts used in the trial has been reported previously [23]. We estimated energy and nutrient intakes from Spanish food-composition tables [29].

2.4. Intervention evaluation

Biological tests were performed in 75% of participants (*n* = 37) to assess compliance with the intervention. These participants were selected at random, and matched by age and sex. In the TMD + VOO group, tyrosol and hydroxytyrosol concentrations, the main phenolic compounds in VOO [30], were measured in urine by gas chromatography-mass spectrometry. In the TMD + nuts group, plasma α-linolenic content was used as a biomarker and measured by gas chromatography.

Table 2Changes in weight, adiposity, blood pressure, and other cardiovascular-risk factors^a.

Variable	TMD+VOO	TMD+nuts	Control	P time ^b	P group ^c	P interaction ^d
Weight, kg						
Baseline	73.6 ± 11.6 ^e	76.9 ± 6.6	74.9 ± 13.1	0.488	0.761	0.373
Final	74.0 ± 11.1	76.0 ± 6.3	74.6 ± 13.5			
BMI, kg/m ²						
Baseline	28.8 ± 2.7	27.7 ± 2.5	29.9 ± 5.5	0.567	0.175	0.539
Final	28.8 ± 2.5	27.4 ± 2.3	29.9 ± 5.4			
Waist, cm						
Baseline	102 ± 10	101 ± 5	105 ± 16	0.007	0.706	0.533
Final	101 ± 8	98 ± 6 ^f	100 ± 10			
Systolic BP, mmHg						
Baseline	153 ± 10	149 ± 18	161 ± 17	0.043	0.006	0.145 ^{g,h}
Final	147 ± 11 ^f	142 ± 15 ⁱ	161 ± 11			
Diastolic BP, mmHg						
Baseline	82 ± 9	83 ± 8	87 ± 12	0.021	0.153	0.743
Final	80 ± 9	80 ± 8 ^f	86 ± 11			
Glucose, mg/dL						
Baseline	156 ± 59	144 ± 47	156 ± 59	0.018	0.418	0.011 ^g
Final	132 ± 40 ⁱ	128 ± 36 ^f	165 ± 79			
Cholesterol, mg/dL						
Baseline	231 ± 31	218 ± 23	205 ± 28	0.014	0.472	0.050 ^g
Final	208 ± 40 ⁱ	205 ± 18	209 ± 43			
LDL-cholesterol, mg/dL						
Baseline	148 ± 28	143 ± 29	125 ± 29	0.003	0.202	0.207
Final	129 ± 41 ⁱ	135 ± 19	121 ± 31			
HDL-cholesterol, mg/dL						
Baseline	52.3 ± 12.9	48.1 ± 11.1	48.5 ± 9.9	0.154	0.252	0.201
Final	56.2 ± 14.1 ^f	48.6 ± 10.0	48.4 ± 10.5			
Triglycerides, mg/dL						
Baseline	147 ± 67	127 ± 78	145 ± 68	0.215	0.275	0.405
Final	126 ± 50	106 ± 38	152 ± 84			
Cholesterol/HDL ratio						
Baseline	4.6 ± 1.0	4.7 ± 1.2	4.1 ± 0.68	0.004	0.682	0.041 ^g
Final	3.9 ± 1.1 ⁱ	4.3 ± 0.8 ^f	4.1 ± 0.73			

^a TMD = traditional Mediterranean diet; VOO = virgin olive oil; BMI = body mass index; BP = blood pressure; LDL = low-density lipoprotein; HDL = high-density lipoprotein.^b P time = comparison between before and after intervention (repeated-measures ANOVA).^c P group = comparison between the three diet groups (repeated-measures ANOVA).^d P interaction = comparison between measures obtained before and after intervention and in the three diet groups (repeated-measures ANOVA).^e Mean ± SD (all values).^f P < 0.05 for changes from baseline by simple effect analysis (Bonferroni's multiple contrast).^g P < 0.05 between TMD+VOO and Control (simple effect analysis by Bonferroni's multiple contrast).^h P < 0.01 between TMD+nuts and Control (simple effect analysis by Bonferroni's multiple contrast).ⁱ P < 0.01 for changes from baseline by simple effect analysis (Bonferroni's multiple contrast).

2.5. Measurements and biochemical determinations

Trained personnel measured waist circumference midway between the lowest rib and the iliac crest using an anthropometric tape. Weight and height were recorded using a calibrated scale and a wall-mounted stadiometer, respectively; and blood pressure was measured in triplicate with a validated semiautomatic oscilometer (Omrom HEM-705CP, Hoofddorp, the Netherlands). At the 3-month visit and whenever consulted by participants, dietitians assessed any adverse effects related to the dietary interventions by administering a checklist of symptoms and gave advice on how to remedy these. The checklist included oral symptoms, bloating, fullness, indigestion, altered bowel habits, and any diet-related symptoms.

Samples of serum, EDTA plasma and urine were coded, shipped to the central laboratory and stored at -80°C until assay. All clinical investigators and laboratory technicians were blinded to the interventions. Plasma glucose levels were analyzed using the glucose-oxidase method, cholesterol and triglyceride levels were measured by enzymatic procedures. HDL cholesterol was

determined using precipitation with phosphotungstic acid and magnesium chloride.

2.6. Monocyte isolation

Human monocyte-derived macrophages (HMDM) were isolated by standard protocols from buffy-coats (35–40 mL) of participants before and after the 3-month diet intervention. Cells were applied on 15 mL of Ficoll-Hypaque and centrifuged at $400 \times g$, 40 min, 22°C with no brake. Mononuclear cells were obtained from the central white band of the gradient, exhaustively washed in DPBS, and resuspended in RPMI medium (Gibco) supplemented with 20% human serum AB (Immunogenetics).

2.7. Determination of gene expression by real time PCR

Monocytes were washed with cold PBS and total RNA and protein were isolated by using the TripureTM isolation reagent (Roche Molecular Biochemicals) according to the manufacturer. TaqMan fluorescent real time PCR primers and probes (6'FAM-MGB)

Table 3
Changes in inflammatory, lipoprotein receptor and thrombotic gene expression^a.

Variable	TMD + VOO	TMD + nuts	Control	P time ^b	P group ^c	P interaction ^d
COX-1						
Baseline	1.16 ± 1.03 ^e	1.02 ± 0.94	0.73 ± 0.48	0.289	0.676	0.057
Final	0.80 ± 0.68	1.04 ± 6.87	0.82 ± 0.49			
COX-2						
Baseline	1.09 ± 0.81	1.18 ± 0.87	0.88 ± 0.93	0.003	0.532	0.612
Final	1.75 ± 1.29	2.67 ± 1.67 ^f	2.20 ± 1.97 ^g			
MCP-1						
Baseline	1.07 ± 1.11	0.52 ± 0.56	0.33 ± 0.40	1.000	0.178	0.013
Final	0.67 ± 0.75 ^f	0.71 ± 0.36	0.54 ± 0.42			
LDLR						
Baseline	0.68 ± 0.46	0.68 ± 0.51	0.54 ± 0.47	0.001	0.787	0.880
Final	0.92 ± 0.54	0.97 ± 0.44 ^f	0.90 ± 0.59 ^f			
LRP1						
Baseline	0.96 ± 0.60	0.75 ± 0.51	0.52 ± 0.61	0.001	0.303	0.017 ^h
Final	1.06 ± 0.65	1.09 ± 0.51 ^f	0.90 ± 0.70 ^f			
CD36						
Baseline	0.93 ± 0.57	0.70 ± 0.53	0.61 ± 0.48	0.011	0.210	0.047 ^h
Final	0.95 ± 0.49	1.08 ± 0.51 ^f	0.69 ± 0.42			
TF						
Baseline	0.68 ± 0.43	0.63 ± 0.55	0.52 ± 0.65	0.064	0.675	0.946
Final	0.86 ± 0.68	0.88 ± 0.72	0.69 ± 0.58			
TFPI						
Baseline	0.99 ± 0.60	0.61 ± 0.47	0.72 ± 0.56	0.789	0.392	0.048 ⁱ
Final	0.80 ± 0.63	0.85 ± 0.50 ^j	0.74 ± 0.42			

^a TMD = traditional Mediterranean diet; VOO = virgin olive oil; COX = cyclooxygenase enzyme; MCP-1 = monocyte chemoattractant protein; LDLR = low-density lipoprotein receptor; LRP1 = low-density lipoprotein receptor-related protein 1; TF = tissue factor; TFPI = tissue factor pathway inhibitor.

^b P time = comparison between before and after intervention (repeated-measures ANOVA).

^c P group = comparison between the three diet groups (repeated-measures ANOVA).

^d P interaction = comparison between measures obtained before and after intervention and in the three diet groups (repeated-measures ANOVA).

^e Mean ± SD (all values).

^f P < 0.01 for changes from baseline by simple effect analysis (Bonferroni's multiple contrast).

^g P < 0.05 between TMD + VOO vs TMD + nuts and Control (simple effect analysis by Bonferroni's multiple contrast).

^h P < 0.05 between TMD + nuts vs TMD + VOO and Control (simple effect analysis by Bonferroni's multiple contrast).

ⁱ P < 0.05 between TMD + nuts vs Control (simple effect analysis by Bonferroni's multiple contrast).

^j P < 0.05 for changes from baseline by simple effect analysis (Bonferroni's multiple contrast).

were selected and introduced in a microfluidic card specifically designed to analyze the expression of genes involved in atherothrombosis. We analyzed pro-inflammatory genes such as MCP-1 (Hs00234140-m1), COX-1 (Hs0000377721-m1) and COX-2 (Hs00153133), genes involved in the regulation of thrombosis such as TF (Hs00175225-m1) and TFPI (Hs00196731-m1) and lipoprotein receptors such as LRP1 (Hs00233899-m1), CD36 (Hs00169627-m1) and LDL receptor (Hs00181192-m1). Human 18S rRNA (4319413E) was used as endogenous control. TaqMan real time PCR was performed with 5 µL well ⁻¹ of RT products (1 µg of total RNA) in 45 µL TaqMan PCR Master Mix (PE Biosystems). PCR was performed at 95 °C for 10 min (for AmpliTaq Gold activation) and then run for 40 cycles at 95 °C for 15 s and 60 °C for 1 min on the ABIPRISM 7900 HT Detection system (PE Biosystems). Relative quantification (Rq) was used for the calculations.

2.8. Statistical analysis

Results are expressed as means ± standard deviation (SD). One-factor analysis of variance or chi-square tests, as appropriate, were used to determine differences in baseline characteristics between the three study groups. Changes in all outcomes were assessed with repeated-measures analysis of variance for the two factors diet and time, and their interactions. Significant interactions were analyzed by the simple effects test with Bonferroni corrections for multiple testing. All statistical tests were 2-tailed, and the significance level was set at P < 0.05. Analyses were performed by using SPSS (version 14.0; SPSS Inc., Chicago, IL).

3. Results

The baseline characteristics of the 49 study participants (23 men and 26 women) who were randomly assigned to one of three dietary interventions are detailed in Table 1. The groups were well balanced regarding demographic characteristics, adiposity and risk factors. Medication taken and occupation and educational levels were also similar in the three groups. Two participants (4%) in the TMD + nuts group reported adverse effects, but these were minor. They complained that particle of nuts lodged between teeth but in both cases this was solved by crushing the nuts and adding them to low-fat yoghurt. No adverse effects were reported by participants in the TMD + VOO and control diet groups.

3.1. Food, energy, and nutrient intake

The main dietary changes observed were the large increases in consumption of VOO and nuts in the corresponding Mediterranean diet groups. In addition, a significant reduction of common olive oil consumption was observed in the TMD + VOO and control diet groups (Table 1 *on-line*). Legume consumption increased significantly in the TMD + VOO group and cereal consumption decreased significantly in the TMD + nuts group. Intake of vegetables, fruit, fish, pastries, meat and meat products, and dairy products did not vary significantly between the three intervention groups. However, the 14-point Mediterranean diet score increased significantly in both TMD groups and remained unchanged in the control group.

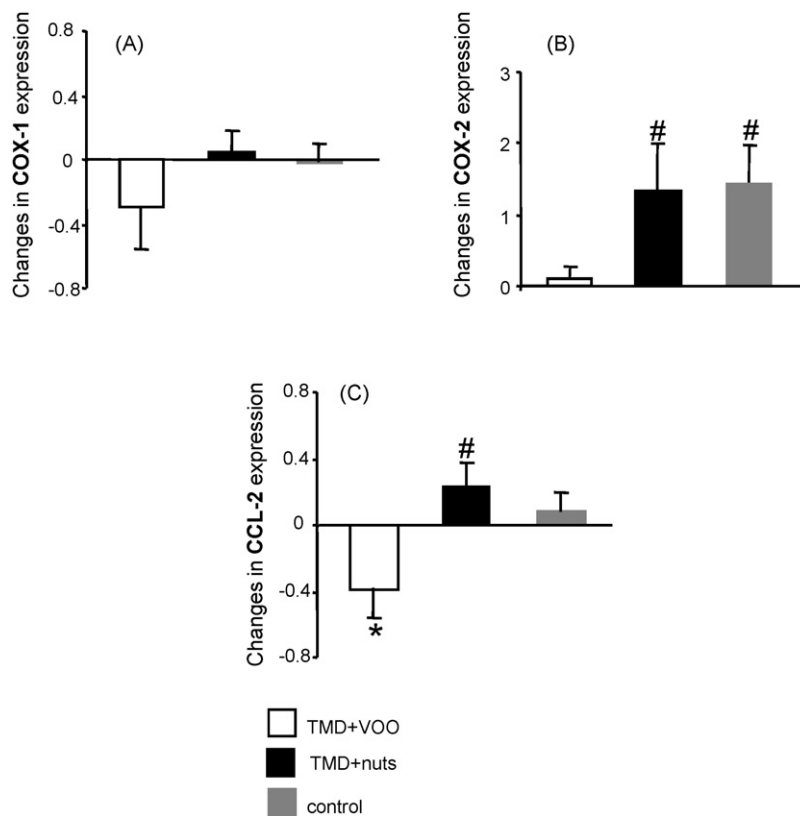


Fig. 1. Changes from baseline in pro-inflammatory genes in the 3 intervention groups. Changes in COX-1 (A) COX-2 (B) and MCP-1 expression (C) in TMD+VOO (white bars), TMD + nuts (black bars) and control diet (gray bars) intervention groups. Results are expressed as the mean \pm SD. * $P < 0.05$ vs control diet. # $P < 0.05$ vs TMD + VOO. TMD indicates traditional Mediterranean diet; VOO, virgin olive oil; COX, cyclooxygenase enzyme; MCP-1, monocyte chemoattractant protein.

Energy intake was reportedly reduced in all three dietary groups (Table II on-line). In the TMD+nuts group, the percentage of total fat significantly increased and that of carbohydrates significantly decreased. Similarly, intake of polyunsaturated fatty acids (PUFA) and α -linolenic acid significantly increased in this group. Participants in the control group reported only a marginal decrease in the percentage of total fat and saturated fatty acids intake.

Biochemical measurements in plasma and urine samples from a random group of 37 participants (75%) showed good adherence to supplemental foods in both TMD groups. TMD+VOO participants showed an increase of 25 ng/mL in urinary tyrosol levels (95% CI, 8–42 ng/mL; $P = 0.007$) and of 63 ng/mL in hydroxytyrosol levels (2–123 ng/mL; $P = 0.043$). TMD+nuts participants showed an increase in plasma α -linolenic acid level of 0.09 mol (0.03–0.14 mol%; $P = 0.005$). Urinary phenolics and plasma α -linolenic concentrations in both TMD groups were significantly higher than in the control group ($P < 0.01$, both).

3.2. Cardiovascular-risk factors

Changes in cardiovascular-risk factors are shown in Table 2. Compared to control group participants, those in the two TMD groups had decreased systolic blood pressure levels. Interestingly, waist circumferences decreased in the three groups, but only the reduction observed in the TMD+nuts group achieved statistical significance. Plasma glucose concentration and cholesterol/HDL ratio decreased in both TMD groups. Total and LDL-cholesterol decreased and HDL-cholesterol increased in the TMD+VOO group only. The remaining parameters did not change in any group.

3.3. Inflammatory genes

As shown in Table 3, there were no significant differences between groups regarding baseline expression of COX-1 and COX-2 genes. However, baseline MCP-1 expression was significantly higher in the TMD+VOO group than in the other two groups. As expected, COX-1 constitutive gene was not altered by any dietetic intervention (Fig. 1A). In contrast, COX-2 expression was significantly increased from baseline in the TMD+nuts group (by 2.26-fold) and the control diet group (2.50-fold) (Table 3) and COX-2 increase was significantly higher in TMD+nuts and control diet than in TMD+VOO group (TMD+nuts: 1.41 ± 0.51 and control diet: 1.43 ± 0.56 versus TMD+VOO: 0.10 ± 0.18 , $P < 0.05$) (Fig. 1B). TMD+VOO not only prevented the increase in MCP-1 expression observed with the control diet (Table 3), but also significantly decreased MCP-1 expression (TMD+VOO: -0.39 ± 0.17 versus TMD+nuts: 0.23 ± 0.15 and control diet: 0.08 ± 0.11 , $P < 0.05$) (Fig. 1C).

3.4. Genes involved in foam cell formation

There was a significantly higher baseline LRP1 and CD36 expression in the TMD+VOO than in the other two groups. Intra-group comparison showed that while the LDL receptor and LRP1 expression were significantly increased in both TMD+nuts and control groups, CD36 expression was only significantly increased in the TMD+nuts group (Table 3). There were no differences in the classical LDL receptor changes from baseline among the three intervention groups (Fig. 2A). However, LRP1 increase in TMD+nuts and control groups was significant versus the TMD+VOO group

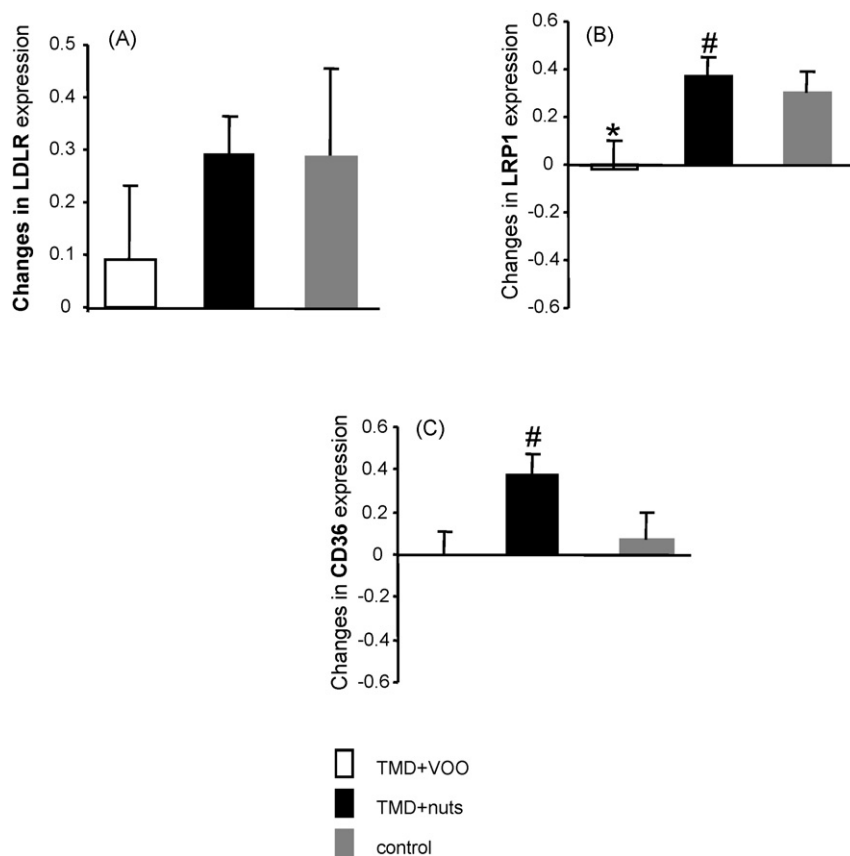


Fig. 2. Changes from baseline in lipoprotein receptor genes in the 3 intervention groups. Changes in LDLR (A), LRP1 (B) and CD36 expression in TMD+VOO (white bars), TMD+nuts (black bars) and control diet (gray bars) intervention groups. Results are expressed as the mean \pm SD. * P <0.05 vs control diet. # P <0.05 vs TMD+VOO. TMD indicates traditional Mediterranean diet; VOO, virgin olive oil; LDLR, low-density lipoprotein receptor; LRP1, low-density lipoprotein receptor-related protein-1.

(TMD+nuts: 0.38 ± 0.07 and control diet: 0.30 ± 0.08 versus TMD+VOO: -0.02 ± 0.12 , P <0.05) (Fig. 2B). CD36 was significantly upregulated in the TMD+nuts group versus TMD+VOO and control groups (TMD+nuts: 0.37 ± 0.10 versus TMD+VOO: 0.00 ± 0.12 and control: 0.08 ± 0.06 , P <0.05) (Fig. 2C).

3.5. Genes involved in thrombosis

TF expression was not significantly altered by any dietary intervention (Table 3, Fig. 3C). However, TFPI was significantly increased in the TMD+nuts (TMD+nuts: 0.24 ± 0.03 versus TMD+VOO: -0.16 ± 0.22 and control diet: -0.08 ± 0.11 ; P <0.05) (Fig. 3B). As shown in Fig. 3C, TMD+VOO and TMD+nuts prevented the rise of the TF/TFPI ratio observed in the control diet.

3.6. Subgroup analyses and correlations

There were no differences in outcomes between subgroups for age, gender, baseline body weight, smoking, drug intake or physical activity. However, at the 3-month evaluation, participants with hypertension showed a significantly higher increase in diastolic blood pressure ($P=0.048$) and COX-2 expression ($P=0.040$). Diabetics showed a significantly higher increase in COX-2 expression than non-diabetics ($P=0.049$).

Patients with dyslipidemia did not show significant changes in LRP1 or LDLR expression after 3 months of dietetic intervention. In the TMD+VOO group, changes in systolic blood pressure correlated positively with changes in LRP1 expression ($r=0.562$; $P=0.036$) and changes in diastolic blood pressure correlated positively with changes in LRP1 expression ($r=0.643$, $P=0.013$), in COX-2 ($r=0.676$,

$P=0.022$) and in MCP-1 ($r=0.637$, $P=0.026$). In the TMD+nuts group, changes in HDL-cholesterol concentration correlated negatively with changes in LRP1 expression ($r=-0.418$, $P=0.040$) and positively with changes in MCP-1 expression ($r=0.588$, $P=0.021$). In this group, changes in plasmatic triglyceride concentration correlated negatively with changes in MCP-1 ($r=-0.678$, $P=0.006$) and TFPI expression ($r=-0.783$, P <0.001). In the control group, no significant correlations were observed between clinical or biochemical parameters and changes in gene expression.

4. Discussion

Our results showed that COX-2 expression was significantly increased with time in monocytes from high cardiovascular-risk patients. These results are in agreement with the high expression of COX-2 previously reported in monocyte-derived macrophages from atherosclerotic vessels [31,32]. Interestingly, VOO specifically prevented the overexpression of COX-2 and MCP-1 in the study groups on a traditional Mediterranean diet with virgin olive oil for a period of 3 months. In atherosclerotic lesions, monocyte MCP-1 is a potent regulator of leukocyte trafficking [33,34]. The inhibition of leukocyte recruitment seems to be pivotal in treating inflammation associated with myocardial infarction [15,35]. Results from another PREDIMED substudy showed that VOO specifically downregulated plasmatic CRP levels [23]. Taken together, these findings support an anti-inflammatory role for VOO. Some of the anti-inflammatory effects of VOO might be attributed to oleocanthal, whose properties overlap with those attributed to non-steroidal anti-inflammatory drugs [8]. The VOO prevention of pro-inflammatory gene overexpression may play a key role for cardiovascular protection.

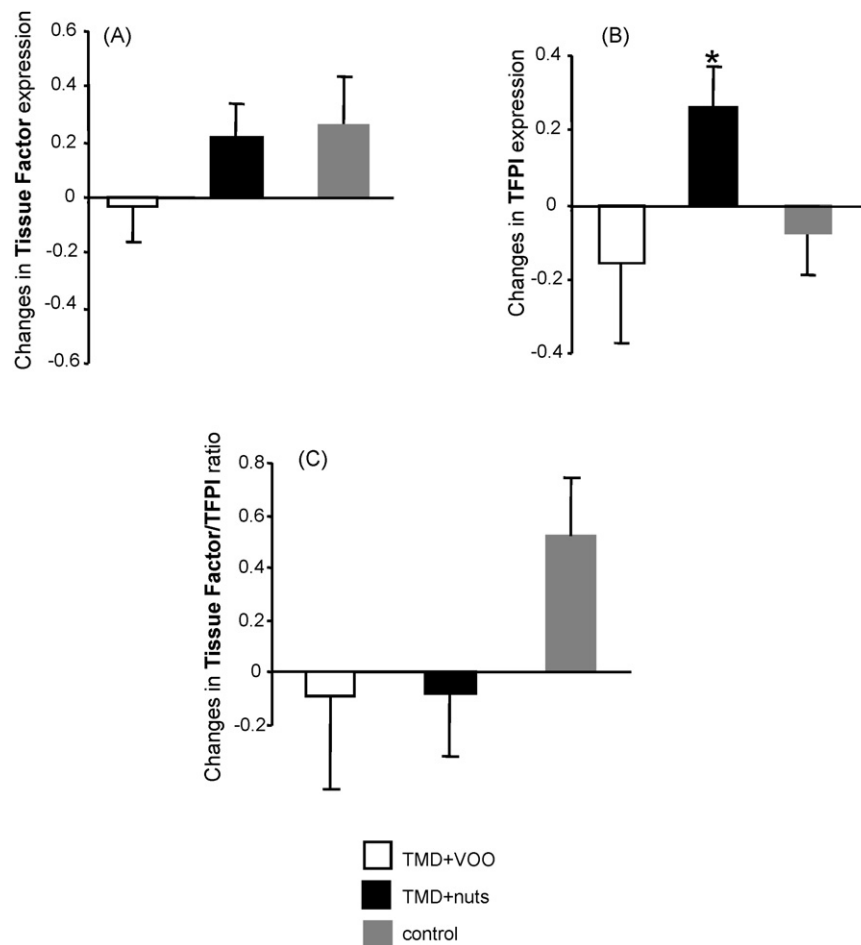


Fig. 3. Changes from baseline in thrombotic genes in the 3 intervention groups. Changes in TF (A) and TFPI (B) expression in TMD + VOO (white bars), TMD + nuts (black bars) and control diet (gray bars) intervention groups. Results are expressed as the mean \pm SD. * $P < 0.05$ vs control diet. TMD indicates traditional Mediterranean diet; VOO, virgin olive oil; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

Our results indicate that VOO specifically prevented monocyte LRP1 overexpression in a population with a high cardiovascular risk. Both *in vivo* [36,37] and *in vitro* studies [38,39] have shown that LRP1 plays a major role in macrophage-foam cell formation. We have previously demonstrated that LRP1 is also a key receptor for the prothrombotic transformation of the vascular wall [40,41] and for the migratory capacity of vascular cells [42,43]. By preventing LRP1 overexpression, VOO might therefore influence some of the most important mechanisms involved in atherothrombosis. Altered LRP1 expression has been shown in blood mononuclear cells from patients with coronary obstruction [44,45]. One of the atherosclerotic risk factor contributing to LRP1 overexpression in the vascular cells and in blood nuclear cells is hypercholesterolemia [46,47]. In the present work, we found no correlation between monocyte LRP1 expression and LDL cholesterol levels. These results suggest that the effects of VOO on LRP1 expression are not caused by reduction in plasma cholesterol. In contrast, our results showed a positive correlation between systolic blood pressure and LRP1 expression in the TMD + VOO group. We have previously demonstrated *in vivo* that vascular LRP1 expression correlates positively with systolic blood pressure [48]. Although TMD + nuts also decreased blood pressure, it did not exert any effect on LRP1 expression. Nuts are highly enriched in PUFA, considered endogenous PPAR activators [49,50]. It has been previously described that PUFA upregulate LRP1 expression through a PPRE sequence located in the LRP1 promoter [51]. Therefore, the down-regulatory effect of systolic blood pressure on LRP1 expression

might be compensated by the upregulatory effect of PUFA in the TMD + nuts group. Further investigation is required to determine which properties of VOO are responsible for the modulation of LRP1 expression.

The specific upregulatory effect of nuts on CD36 expression might be also associated with their high PUFA content since PUFA stimulate CD36 expression through a PPAR-dependent mechanism [52,53]. Reports concerning the role of CD36 on atherosclerosis progression are controversial. CD36 appears to play a role in intracellular lipid accumulation in cultured macrophages [54,55], and monocyte CD36 deletion *in vivo* seems to decrease atherogenesis [56,57]. However, in other studies, hyperlipidemic CD36 $^{-/-}$ -ApoE $^{-/-}$ has shown increased aortic sinus lesion areas [58]. The effect of nuts on CD36 thus remains open.

TF and TFPI proteins play opposing roles in the process of thrombosis. The effect of nuts increasing TFPI expression also has important implications for atherosclerosis since it has been reported that TFPI gene delivery markedly reduces restenosis in atherosclerotic arteries [59]. This finding implies that diet might influence vascular remodeling.

Although in this PREDIMED substudy the number of participants is small, our results demonstrate that both TMD + VOO and TMD + nuts beneficially modified blood pressure, cholesterol and glucose in a group of overweight people at risk of cardiovascular events. These results are in agreement with those previously reported in other PREDIMED substudies including larger number of patients [60,61]. Remarkably, in this substudy, as in other PRED-

IMED substudy [62], waist circumference was significantly reduced in the TMD + nuts group. Taken together, these results support the possibility of an association between nuts consumption and lower prevalence of metabolic syndrome.

We can conclude that a Mediterranean-type dietary intervention in a high-risk cardiovascular population influences key genes involved in inflammation, vascular foam cell formation and vascular remodeling. These results further increase the evidence for recommending the TMD to prevent atherosclerotic plaque progression in a high-risk cardiovascular population.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

The authors thank Laura Nasarre for technical help. CIBEROBN is an initiative of the Instituto de Salud Carlos III. This study was made possible by funds provided by REDINSCOR RD06/0003/0015, RD06/0045 and PI070473 from the Instituto de Salud Carlos III, SAF 2006/10091 from MEC and AGL2006-14228-C03-01/ALI from MICINN, Spain.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.atherosclerosis.2009.08.004](https://doi.org/10.1016/j.atherosclerosis.2009.08.004).

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