In vivo transcriptomic profile after a Mediterranean diet in high–cardiovascular risk patients: a randomized controlled trial[1–3]

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ABSTRACT

Background: Nutrients can exert healthy effects through nutrigenomic modulation. Data are scarce concerning the in vivo effect of a sustained traditional Mediterranean diet (TMD) pattern on the whole transcriptomic response.

Objective: We explored the overall nutrigenomic effect associated with a TMD.

Design: We focused on biological pathways related to cardiovascular disease (CVD) in a subsample (n = 34) of the Prevención Con Dieta Mediterránea (PREDIMED) study, which was a large, parallel-group, multicenter, randomized controlled trial that aimed to assess the effects of TMD on the primary prevention of CVD in individuals with high cardiovascular risk. Participants were randomly assigned to a low-fat diet control group or TMD intervention groups [traditional Mediterranean diet supplemented with virgin olive oil (TMD+VOO) or traditional Mediterranean diet supplemented with nuts (TMD+Nuts)] in equal proportions. Three-month changes in whole genome peripheral blood mononuclear cells were assessed by using whole transcriptome microarray analyses.

Results: A functional annotation analysis was performed on 241 selected responder genes after the TMD+VOO (139 upregulated and 102 downregulated genes), 312 selected responder genes after the TMD+Nuts (165 upregulated and 147 downregulated genes), and 145 selected responder genes after the low-fat (100 upregulated and 45 downregulated genes) diets. Of 18 cardiovascular canonical pathway analyses, 12 pathways were differentially expressed, and 43% of pathways were modulated by both TMDs; the most prevalent pathways were related to atherosclerosis and hypertension. After simultaneous testing adjustment, 9 pathways were modulated by the TMD+VOO diet, and 4 pathways were modulated by the TMD+Nuts diet.

Conclusion: One of the mechanisms by which TMD, particularly if supplemented with virgin olive oil, can exert health benefits is through changes in the transcriptomic response of genes related to cardiovascular risk. This trial was registered at the London-based Current Controlled Trials register as ISRCTN35739639. *Am J Clin Nutr* 2013;98:845–53.

INTRODUCTION

The Mediterranean diet has been associated with reduced risk of cardiovascular mortality despite its high MUFA content (1). The traditional Mediterranean diet (TMD)[4] has the following characteristics: *1*) high consumption of vegetables, legumes, fruit, and cereals; *2*) regular but moderate wine intake; *3*) moderate consumption of fish; *4*) low consumption of meat; and *5*) from low to moderate intake of dairy products. It also provides all essential micronutrients, fiber, and other plant-food substances to promote health (2). Olive oil is the main source of fat in the TMD, which explains a higher ratio of MUFA to SFA in regions that follow a Mediterranean diet pattern than elsewhere (3). Indeed, olive oil consumption increases MUFA intake without a significant rise in SFAs and guarantees an appropriate intake of PUFAs. Adherence to the TMD has been associated with reduced risk of overall mortality, cardiovascular morbidity and mortality (4), cancer incidence and mortality, and incidence of neurodegenerative diseases (5–7). Within the framework of the Prevención Con Dieta Mediterránea (PREDIMED) study, we recently published first-level evidence of the role of TMD in reducing the incidence of cardiovascular events (8).

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[2] Supported by the CNIC06 project of the Instituto Nacional de Investigaciones Cardiovasculares, and grant GVACOMP2011-151 from the Generalitat Valenciana, and partially funded by the Instituto Carlos III (ISCIII; PI11/01647 and CP06/00100), Sistema Nacional de Salud contract Rio Hortega CM08/00054. MF was supported by a joint contract of the ISCIII and Health Department of the Catalan Government (Generalitat de Catalunya).

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[4] Abbreviations used: CVD, cardiovascular disease; eNOS, endothelial nitric oxide synthase; LFD, low-fat diet; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PREDIMED, Prevención Con Dieta Mediterránea; TMD, traditional Mediterranean diet; TMD-14, 14-item questionnaire to assess traditional Mediterranean diet adherence; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD+VOO, traditional Mediterranean diet supplemented with virgin olive oil; VOO, virgin olive oil.

Received February 11, 2013. Accepted for publication June 14, 2013.
First published online July 31, 2013; doi: 10.3945/ajcn.113.060582.

The exact mechanisms by which TMD and olive oil produce their health effects are not yet fully understood. One characteristic feature of the TMD is its richness in antioxidants. The PREDIMED study determined the capacity of TMD to reduce lipid (9) and DNA oxidative damage (10) as well as inflammatory status (11). It is becoming more evident that antioxidant molecules, such as phenolic compounds, exert cellular protection by interacting with intracellular signaling pathways involved in pathologic processes (12). The gene-environment and gene-diet interaction also could play an important role in the development and protection of chronic degenerative diseases. Although data on the in vivo effect of sustained consumption of a Mediterranean diet pattern on human gene expression are limited (13–15), the available data point to a modulation of inflammation-related genes toward a protective mode. However, to the best of our knowledge, the effect of a TMD pattern on the overall transcriptomic response related to cardiovascular risk has not been previously assessed. In the context of the large-scale PREDIMED study, we designed a nutrigenomic study that focused on the most relevant pathways for cardiovascular diseases (CVDs). Therefore, the aim of the current work was to explore changes in the whole transcriptomic response of peripheral blood mononuclear cells (PBMCs) in biological pathways related to CVDs that might be promoted by TMD interventions compared with a low-fat diet (LFD) (ie, the control condition).

SUBJECTS AND METHODS

Study design

The PREDIMED study was a large, parallel-group, multicenter, controlled, randomized, 5-y clinical trial conducted in Spain that was aimed at assessing the effects of the TMD on the primary prevention of CVDs (16). The trial included 7447 participants at high risk of coronary artery disease who were assigned at random to the following 3 intervention groups: *1*) a traditional Mediterranean diet supplemented with virgin olive oil (TDM+VOO); *2*) a traditional Mediterranean diet supplemented with mixed nuts (TMD+Nuts); and *3*) an LFD-control group. The current study was designed to assess the 3-mo effects of the dietary interventions on changes in gene expression and biological pathways related to CVD in a random subsample (n = 34) of participants. Institutional review boards approved the study protocol, and participants signed an informed consent. Before and after 3 mo of intervention, biological samples were obtained after an overnight fast, coded, shipped to central laboratories, and frozen at −80°C until assay.

Participants

Eligible participants were men aged 55–80 y and women aged 60–80 y who fulfilled at least one of the following criteria: *1*) type 2 diabetes or *2*) ≥3 cardiovascular disease risk factors [current smoking, hypertension (blood pressure >140/90 mm Hg or treatment with antihypertensive drugs), LDL cholesterol concentration >160 mg/dL (or treatment with hypolipidemic drugs), HDL cholesterol concentration <40 mg/dL, BMI (in kg/m^2) >25, or a family history of premature coronary artery disease. Exclusion criteria were a history of CVD, any severe chronic illness, drug or alcohol addiction, history of allergy or intolerance to olive oil or nuts, a low predicted likelihood of changing dietary habits according to the stages of change model (17), or any condition that could impair study participation. Eligibility to participate was based on a screening visit by the study physician. Eligible subjects were centrally randomly assigned to 1 of 3 dietary intervention groups in equal proportions by the coordinating center, Hospital Clinic, by using a computer-generated random-number sequence.

Baseline assessments

The baseline examination included the administration of the following 2 instruments: *1*) a validated 14-item questionnaire to assess traditional Mediterranean diet adherence (TMD-14), with values of 0 or 1 assigned to each item (18); and *2*) a 47-item general questionnaire that assessed lifestyle, health conditions, sociodemographic variables, history of illness, and medication use.

Intervention

On the basis of the initial TMD-14 score, each participant received personalized dietary advice from the dietitian during a 30-min session. Participants allocated to the LFD-control group were advised to reduce all types of fat and were given written recommendations according to American Heart Association guidelines (19). TMD intervention groups received instructions that would increase their TMD-14 score, including the following: *1*) use of olive oil for cooking and dressings; *2*) increased consumption of vegetables, nuts, and fish products; *3*) consumption of white meat instead of red or processed meats; *4*) preparation of homemade sauce by simmering tomato, garlic, onion, and aromatic herbs with olive oil to dress vegetables, pasta, rice, and other dishes; and *5*) if drinking alcohol, to follow a moderate pattern of red wine consumption. No calorie limitations were suggested for TMD groups. Participants in the enriched TMD groups were given a free 3-mo supply of VOO (15 L) or nuts (packets of 1350 g walnuts = 15 g/d; 675 g hazelnuts = 7.5 g/d; and 675 g almonds = 7.5 g/d), respectively. To improve compliance and account for family needs, additional VOO or nuts were made available to participants in the corresponding TMD groups. One week after inclusion, the dietitian delivered a 1-h group session to each TMD group and the LFD-control group. Each session consisted of an informative talk and written material with detailed descriptions of recommended foods (typical Mediterranean foods for TMD groups and American Heart Association recommendations for the LFD-control group) and seasonal shopping lists, meal plans, and recipes. All participants had free and unlimited access to their center's dietitian throughout the study.

Evaluation of the intervention

After the 3-mo intervention, all baseline procedures were repeated. A biological assessment of dietary compliance was performed, and results were compared with baseline values. In the TMD+VOO group, tyrosol and hydroxytyrosol (the major phenolic compounds present in olive oil) were measured in urine by using gas chromatography–mass spectrometry. In the TMD+Nuts group, plasma α-linolenic acid was measured by using gas chromatography (20).

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Outcome measures

Systemic variables

Anthropometric data were obtained by standardized methods (11). Serum glucose, total and HDL cholesterol, and triglyceride concentrations were measured by using standard enzymatic automated methods. Apolipoprotein A1 and B100 were determined by using immunoturbidimetry (PENTRA-400; ABX-Diagnostics). When triglyceride concentrations were <300 mg/dL, LDL cholesterol was calculated by using Friedewald's formula. EDTA plasma oxidized LDL was determined with an ELISA procedure that used the murine monoclonal antibody mAb-4E65 (Mercodia AB).

Gene expression

PBMCs were isolated from peripheral blood by using cell preparation tubes (Becton Dickinson). Whole blood was centrifuged at 1690 × *g* for 30 min; cells were washed with phosphate-buffered saline, centrifuged at 970 × *g* for 15 min, resuspended in Ultraspec RNA Isolation Reagent (Bioteck Laboratories), and finally stored at −80°C until RNA isolation. The RNA concentration (A260) and purity were calculated spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies). RNA integrity was assessed by using microcapillary gel electrophoresis (Bioanalyzer, NanoChip; Agilent Technologies) and the RNA integrity number value was calculated with Agilent 2100 Expert Software (Agilent Technologies). Gene expression was assessed at baseline and after 3-mo intervention by using the Affymetrix GeneChip Human Genome U133A 2.0 (Affymetrix Inc), which is a commercial microarray platform that analyzes the expression level of 18,400 transcripts and variants, including 14,500 well-characterized human genes. Microarray data are registered as GSE28358 in the Gene Expression Omnibus, which is a public functional genomics data repository.

After the bioinformatics analysis, several upregulated and downregulated genes were validated by using quantitative real-time polymerase chain reaction (PCR) analysis. A total of 100 ng transfer RNA in a 20-μL reaction was reverse-transcribed by using the High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems) according to manufacturer's protocols. TaqMan Low Density Array for gene-expression analysis was performed in triplicate by using 384-well Micro-Fluidic cards (TaqMan Low Density Array by design) on the ABI Prism 7900HT Sequence Detection System (SDS 2.1 software; Applied Biosystems). The selected genes to be verified were *IL1β*, *ICAM1*, *IGF2R*, *TNF-α*, *PTGS2*, and *VEGF*. The human *GADPH* gene was used as an endogenous control to correct for any differences in total complementary DNA amounts added to each reaction. Results from each run were analyzed separately by using a software-defined baseline and a C_t threshold of 0.20, with C_t being the point at which the fluorescence crosses the threshold. Changes in gene expression were calculated by using the relative quantification method and applying the $2^{-\Delta\Delta Ct}$ formula (with ΔCt as the difference between Ct values). Each gene expression was first normalized to the endogenous reference gene

$$\Delta\text{Ct} = \text{C gene} - \text{Ct reference gene} \qquad (1)$$

and then to its untreated control (baseline) ($\Delta\Delta$Ct) (21). Data obtained were analyzed with SDS 2.1 software.

Microarray bioinformatic analyses

The microarray experiment was performed in the Príncipe Felipe Research Centre, Valencia, Spain. The whole transcriptome data obtained were analyzed at the Centre and by the Microarrays Analysis Service (Servei d'Anàlisi de Microarray) of the Hospital del Mar Medical Research Institute, Barcelona, Spain. At both centers, raw data were quality-checked and normalized by using a robust microarray analysis methodology (22). Intensity data were standardized and $\log_2$ transformed. One microarray was discarded from the analysis because it was considered an outlier on the basis of clustering and principal component analysis plots, and therefore, its paired sample was discarded as well. After blind cluster analyses, the difference between posttreatment values and pretreatment values was applied, together with the *T* statistic, to assess changes in gene expression. At the Servei d'Anàlisi de Microarray, 2888 transcripts were included in the comparison analyses; transcripts with <75% interarray variability were eliminated. In contrast, the Príncipe Felipe Research Centre included all 22,277 probe sets represented in the array in the comparison analyses and adjusted the results by age, sex, and diabetes status. All statistical analyses were performed with R open-source software version 2.15.1 (R Core Team, 2012).

Functional analysis

In both bioinformatics analyses, a *T* statistic less than or equal to −2 or ≥2 was selected to assign responder genes. A gene was considered a responder only when the *T* statistic reached the established cutoff in the analyses done by both centers; this method yielded a total of 697 genes. The expression changes of responder genes were selected to study cardiovascular canonical pathways. Pathways with more eligible genes than expected by random occurrence were considered significant. The *P* value associated with a pathway was considered a measure of its statistical significance with respect to the pathways' eligible molecules for the dataset and a reference set of molecules. The *P* value was calculated by using the right-tailed Fisher's exact test. To establish group comparisons within each canonical pathway, we performed Fisher's exact test. To control for the false-discovery rate, a Benjamini-Hochberg procedure was used for simultaneous testing of multiple independent hypotheses (23). A functional annotation analysis was performed with Ingenuity Pathways Analysis 8.7 software (Ingenuity Systems). The bioinformatics analysis procedure is specified in the flowchart shown in **Figure 1**.

Sample size and power analysis

A total sample of 34 participants allowed ≥80% power to detect a significant difference in *JUN* gene expression between intervention groups of 0.003 U of the relative quantification $\log_2$ ratio, assuming a dropout rate of 10% and a 2-sided type error of 0.05. Calculations were based on previous data concerning the SD of *JUN* gene expression in healthy volunteers (24).

Statistical analyses

The normality of continuous variables was assessed by kurtosis and skewness measures and normal probability plots. Nonnormally distributed variables were log transformed before the analysis. We used the single-factor ANOVA to determine differences in baseline

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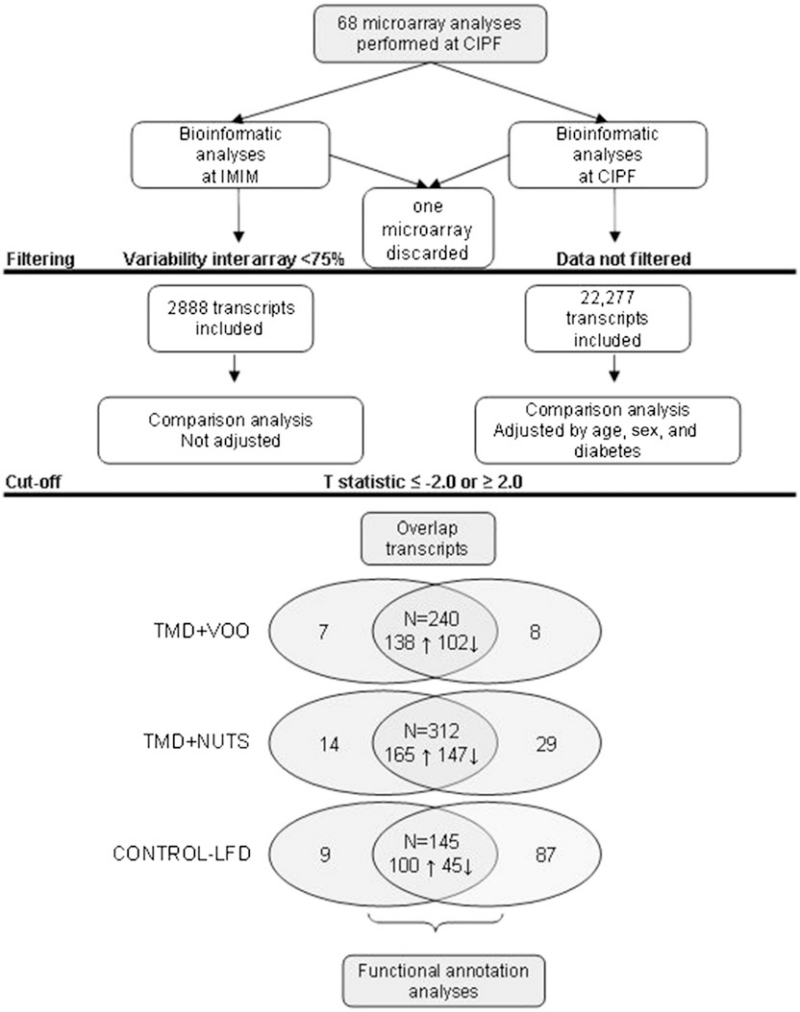

FIGURE 1. Flowchart of the 2 bioinformatics analysis approaches and procedures. CIPF, Príncipe Felipe Investigation Center; IMIM, Hospital del Mar Research Institute; LFD, low-fat diet; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD+VOO, traditional Mediterranean diet supplemented with virgin olive oil; ↓, downregulated; ↑, upregulated.

characteristics. A paired Student's *t* test was applied to assess differences between preinterventions and postinterventions for normal variables, and the Kruskal-Wallis test was used for nonnormal ones. The 3-mo change comparisons were studied by using ANCOVA. Correlation analyses were performed by using Spearman's correlation coefficient test. $P \leq 0.05$ was considered statistically significant. All statistical analyses were conducted with SPSS 12.3 software (SPSS Inc) for Windows XP (Microsoft).

RESULTS

Subject characteristics and systemic variables

A subsample of 34 PREDIMED participants randomly assigned to TMD+Nuts ($n = 11$), TMD+VOO ($n = 11$), and LFD-control ($n = 12$) groups was selected at the Hospital del Mar Medical Research Institute for the current study. Baseline participant characteristics by intervention group are shown in **Table 1**. No differences were observed between intervention groups in assessed variables except that the TMD+VOO group was younger. Changes in classical and novel cardiovascular risk factors after interventions are shown in **Table 2**. A decrease in BMI and waist circumference was observed after the TMD+Nuts intervention; waist circumference was significantly lower in the TMD+Nuts group than in the other 2 intervention groups, and the change in BMI was significantly greater than in the TMD+VOO group. A decrease in systolic blood pressure was observed after the TMD+VOO. No other differences were observed in lipid profile or anthropometric variables and blood pressure.

Within the framework of the PREDIMED study, 3 mo of dietary interventions produced significantly ($P \leq 0.05$) higher urinary hydroxytyrosol concentrations after the TMD+VOO and higher α-linolenic acid concentrations after the TMD+Nuts in a representative subsample of 275 participants (25). Participants in the current study showed a similar trend (Table 2), without reaching significance because of the large span of reference values of compliance biomarkers. The TMD-14 score, which reflected TMD adherence, was significantly higher ($P \leq 0.01$) in both TMD groups than in the LFD-control group (data not shown). Oxidized LDL and high-sensitivity C-reactive protein did not change after interventions.

Microarray gene-expression analyses

The flowchart of bioinformatics analysis procedures is shown in Figure 1. The *t* test showed a Spearman's correlation coefficient between the 2 bioinformatics approaches of 0.61 in

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TABLE 1
Baseline characteristics of the participants by intervention group[1]

	TMD+VOO (n = 11)	TMD+Nuts (n = 11)	LFD-control (n = 12)
Age (y)	62 ± 8[*,2]	63 ± 6	68 ± 5
Weight (kg)	78 ± 15	81 ± 11	76 ± 11
BMI (kg/m^2)	29.04 ± 4.29	30.53 ± 3.39	28.67 ± 3.53
Waist circumference (cm)	98 ± 13	104 ± 13	99 ± 11
Systolic blood pressure (mm Hg)	158 ± 22	167 ± 16	158 ± 22
Diastolic blood pressure (mm Hg)	84 ± 9	87 ± 7	82 ± 9
Tobacco use (%)			
Regular smoker	36.4	9.1	8.3
Former smoker (>1 y)	27.3	27.3	33.3
Never smoker	36.4	44.44	58.3
Diabetes (%)	45.5	63.6	58.3
Sex (F) (%)	45	45	40

[1] ANOVA test was used for group comparisons. *P = 0.020 compared with the LFD-control group. LFD, low-fat diet; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD+VOO, traditional Mediterranean diet supplemented with virgin olive oil.

[2] Mean ± SD (all such values).

TMD+VOO, 0.66 in TMD+Nuts, and 0.33 in LFD-control groups ($P \leq 0.001$ in all cases). Verification of the microarray gene expression in a set of 6 genes by using quantitative real-time PCR showed that gene-expression changes followed similar trends in both methods and differed only when small changes were present after the intervention. Correlations between microarray and quantitative real-time PCR data for each of the 6 genes are shown in **Figure 2**. t Test analyses were performed to assess differences between microarray and quantitative real-time PCR data. No differences were observed between data sets. Correlations were significant ($P \leq 0.05$) in all cases except for *VEGF* in all groups and *IL1β* in the TMD+Nuts group.

Functional analysis of cardiovascular canonical pathways

Of 18 CVD pathways analyzed, 12 pathways were differentially expressed after interventions. In crude analyses, 9 pathways were modulated by the TMD+VOO, 7 pathways were modulated by the TMD+Nuts, and 2 pathways were modulated by the LFD-control diet. Angiopoietin signaling was differentially expressed in the TMD+VOO group, and aldosterone signaling was differentially expressed in the TMD+Nuts group compared in the other 2 groups. When the analysis was performed for each intervention group by comparing the number of expressed and not expressed genes within each cardiovascular system pathway, only renin-angiotensin, endothelial nitric oxide synthase (eNOS), P2Y-purigenic receptor signaling, and hypoxia signaling were modulated by both TMDs, which surpassed the threshold for within-group comparisons (**Figure 3**).

After adjustment by multiple hypotheses, 9 pathways remained modulated by the TMD+VOO, 4 pathways remained modulated by the TMD+Nuts, and none of the pathways remained modulated by the LFD. Nutritional interventions, in which canonical CVD pathways reached significance, and the direction of changes for associated genes are shown in **Figure 4**.

TABLE 2
Changes in classical and novel cardiovascular risk factors after interventions[1]

	TMD+VOO (n = 11)		TMD+Nuts (n = 11)		LFD-control (n = 12)		
	Postintervention		Postintervention		Postintervention		
	Mean ± SD	Change	Mean ± SD	Change	Mean ± SD	Change	P between groups
BMI (kg/m^2)	29.0 ± 4.29	0.049 (−0.526, 0.624)[2]	29.7 ± 3.00	−0.812 (−1.37, to −0.255)[*,†]	28.7 ± 3.64	−0.051 (−0.623, 0.522)	0.064
Waist (cm)	97.6 ± 12.4	0.643 (−0.557, 1.84)	101 ± 12.1	−2.67 (−3.78, −1.55)[*,†,‡]	9.63 ± 11.04	−0.265 (−0.917, 1.46)	0.001
Systolic blood pressure (mm/Hg)	154 ± 20.3	−9.66 (−21.5, −2.17)[*]	159 ± 13.2	−7.52 (−18.5, 3.46)	157 ± 22.2	−4.78 (−16.4, 6.85)	0.847
Diastolic blood pressure (mm/Hg)	84.7 ± 5.50	2.02 (−3.57, 7.61)	87 ± 12.2	−0.214 (−5.41, 4.98)	81.7 ± 7.53	−1.08 (−6.58, 4.42)	0.722
Glucose (mg/dL) (log)	2.09 ± 0.130	0.038 (−0.028, 0.104)	2.07 ± 0.144	−0.044 (−0.105, 0.016)	2.04 ± 0.07	−0.058 (−0.126, 0.010)	0.099
Total cholesterol (mg/dL)	204 ± 38.8	−3.13 (−24.8, 18.5)	196 ± 55.1	−3.68 (−23.7, 16.4)	210 ± 33.2	−13.9 (−36.3, 8.43)	0.746
LDL cholesterol (mg/dL)	128 ± 35.1	2.35 (−16.6, 21.3)	130 ± 35.1	5.19 (−12.4, 22.7)	133 ± 31.2	−12.6 (−32.2, 6.96)	0.382
HDL cholesterol (mg/dL)	52.7 ± 3.29	−1.49 (−4.74, 1.77)	42.0 ± 10.1	−0.32 (−3.33, 2.69)	48.6 ± 7.80	−0.317 (−3.68, 3.05)	0.835
Triglycerides (mg/dL) (log)	2.06 ± 0.151	−0.036 (−0.155, 0.083)	2.10 ± 0.211	−0.122 (−0.230, −0.012)	2.03 ± 0.104	−0.005 (−0.128, 0.118)	0.323
Apolipoprotein A-I (g/L)	1.38 ± 0.153	−0.036 (−0.108, 0.037)	1.32 ± 0.261	−0.028 (−0.096, 0.036)	1.48 ± 0.139	0.033 (−0.042, 0.108)	0.373
Apolipoprotein B100 (g/L)	1.03 ± 0.197	0.020 (−0.078, 0.117)	0.974 ± 0.257	0.006 (−0.084, 0.096)	1.04 ± 0.186	−0.084 (−0.185, 0.017)	0.304
Oxidized LDL (U/L)	35.9 ± 16.1	−0.964 (−19.2, 17.3)	48.7 ± 27.3	−13.9 (−30.8, 2.98)	43.6 ± 25.5	−10.4 (−29.2, 8.52)	0.554
CRP (mg/dL)	0.338 ± 0.246	−0.243 (−0.652, 0.166)	0.169 ± 0.175	−0.065 (−0.428, 0.299)	0.440 ± 0.837	0.167 (−0.18, 0.510)	0.331
Hydroxytyrosol (μg/L) (log)	1.79 ± 0.291	0.202 (−0.066, 0.470)	1.55 ± 0.430	−0.349 (−0.724, 0.025)	2.08 ± 0.447	0.112 (−0.535, 0.760)	0.065

[1] A covariance model adjusted for age, sex, and diabetes was used to assess differences in groups. *$P \leq 0.05$ between preintervention and postintervention values; [†]$P \leq 0.05$ for TMD+Nuts compared with TMD+VOO groups; [‡]$P \leq 0.05$ for TMD+Nuts compared with LFD-control groups. ANCOVA test was used to compare changes across groups. CRP, C-reactive protein; LFD, low-fat diet; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD+VOO, traditional Mediterranean diet supplemented with virgin olive oil.

[2] Mean; 95% CI in parentheses (all such values).

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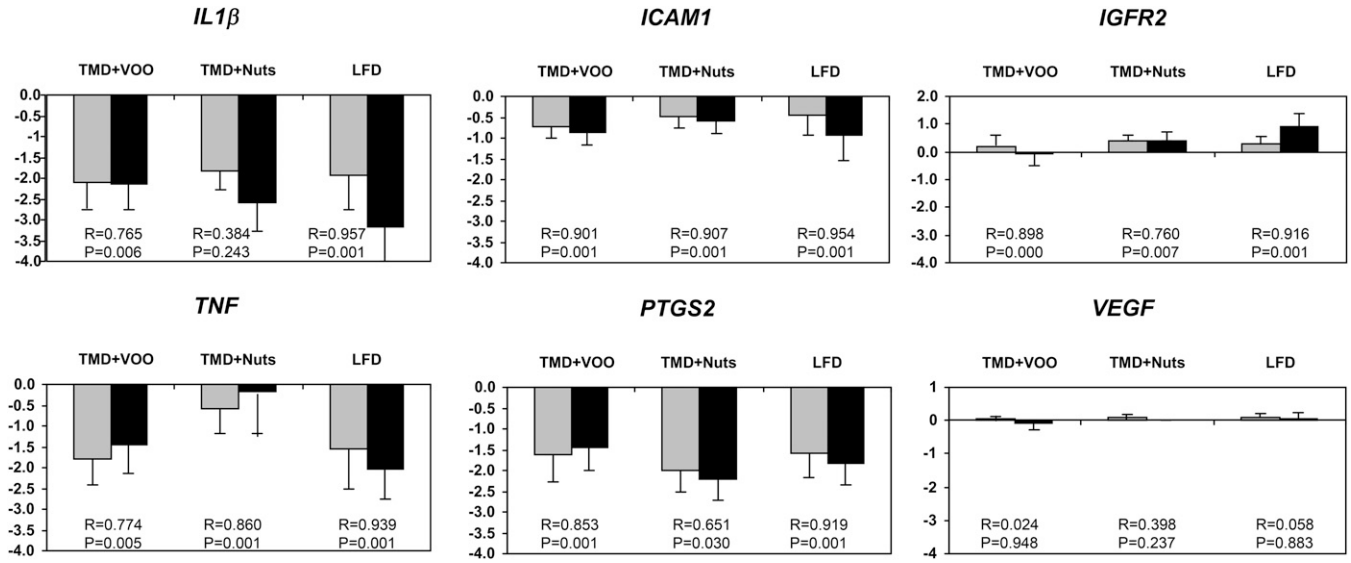

FIGURE 2. Microarray (black) validation by using qRT-PCR (gray). Means ± SEMs of relative expression values after the 3 interventions are shown. Relative expressions are shown as the $\log_2$ ratio. Spearman's correlation analyses were performed. TMD+VOO: n = 11; TMD+Nuts: n = 11; LFD, n = 12. LFD, low-fat diet; qRT-PCR, quantitative real-time polymerase chain reaction; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD +VOO, traditional Mediterranean diet supplemented with virgin olive oil.

DISCUSSION

Briefly, we examined changes in cardiovascular system canonical pathways of patients with cardiovascular risk factors after 3 mo of dietary interventions, including 2 enriched TMD groups and a LFD-control group. Nine pathways were modified by the TMD+VOO and 4 pathways were modulated by the TMD+Nuts. Two different bioinformatics approaches and procedures were used to ensure a more robust analysis and strengthen the conclusions of the current study.

Previous nutrigenomic intervention studies have revealed that real-life daily doses of olive oil, which is the main source of fat and MUFA in the Mediterranean diet, have a protective effect on the expression of genes related to insulin sensitivity and atherosclerosis development in PBMCs (26–28). Also, in PBMCs, we recently reported a downregulation of the expression of proatherosclerotic genes in a dose-dependent manner, depending on the phenolic content of the olive oil administered (14, 29). Nuts are rich in α-linolenic acid, which has been shown to promote changes in genes related to lipid metabolism (30).

In our study, the atherosclerosis signaling pathway was significantly downregulated after the TMD+VOO intervention. Downregulated genes within the pathway were *IL1β*, *IL1RN*, *TNF-α*, and *ICAM1*. In agreement with our results, a postprandial study has reported greater downregulation of *IL1β* in human PBMCs after a breakfast with a high polyphenol content compared with after a low-polyphenol breakfast (24). In addition, hydroxytyrosol, which is one of the main phenolic compounds of VOO, inhibits proinflammatory cytokines such as *TNF-α* in human monocytes ex vivo (31). *TNF-α* was downregulated after a TMD compared with a low-fat, high-carbohydrate diet enriched in n−3 PUFA (15). We previously reported a downregulation of *ICAM1*, *CD40L*, and *IL8RA* expression in human PBMCs (29) linked to the polyphenol content of the olive oil consumed. *CD40* ligation may increase *ICAM1* expression through *TRAF* recruitment and *MAPK* activation (32). *ICAM1*, which is a cell adhesion molecule, is considered an independent predictor of developing type 2 diabetes, which is a major risk factor for cardiovascular mortality and morbidity (33).

Downregulation of *VEGF* was observed in the following 3 pathways modulated by the TMDs: hypoxia, eNOS, and nitric oxide signaling. *HIF1α*, which is closely related to *VEGF*, was also downregulated by the TMD+Nuts within the hypoxia signaling pathway. Dihydroxyphenil 2-(3,4-dihydroxyphenil)-ethanol, which is a polyphenol present in olive oil, promotes a reduction of *VEGF* expression through a decrease in *HIF-α* expression (34). *HIF1α* is associated with an advanced plaque phenotype with abundant inflammation and the presence of an extracellular lipid pool (35). *HIF1α* is present in the atherosclerotic plaque, which induces the expression of its main target gene *VEGF* (35). *VEGF* expression in atherosclerotic plaques has been described in connection with plaque angiogenesis and progression (35). Within the TMD+VOO group, the downregulation of *VEGF* occurred together with *NF-κβ* in the hypoxia and angiopoietin signaling pathways. *NF-κβ* contributes to an altered hypoxia-induced signal-transduction through the expression of target genes that encode interleukins and chemokines, such as *IL1, IL8,* and *TNF-α*, inflammatory enzymes, cell-adhesion molecules, and inducible nitric oxide synthase (36, 37). A proinflammatory signal involving *NF-κβ*, *IL1β*, and *COX-2* can be translated into an angiogenic one by upregulating the *VEGF* signal through *HIF-1α* secretion (38). *TNF-α* can also cause the accumulation of *HIF1α* through an *NF-κβ*–dependent pathway (39). In our results, *TNF-α, NF-κβ*, and their downstream products *HIF-1α* and *VEGF* were among the genes that appeared downregulated within the cardiovascular signaling pathways. These results agree with the reports of several nutritional intervention clinical trials that used PBMCs. Compared with a carbohydrate-PUFA diet, the TMD has been shown to downregulate *NF-κβ* and *TNF-α* (40). In addition, compared with an olive oil–rich breakfast, a butter-rich breakfast has been shown to upregulate *NF-κβ* (41) and *TNF-α* (42).

The following 7 CVD pathways related to hypertension were modulated after TMD interventions: hypoxia, nitric oxide, eNOS,

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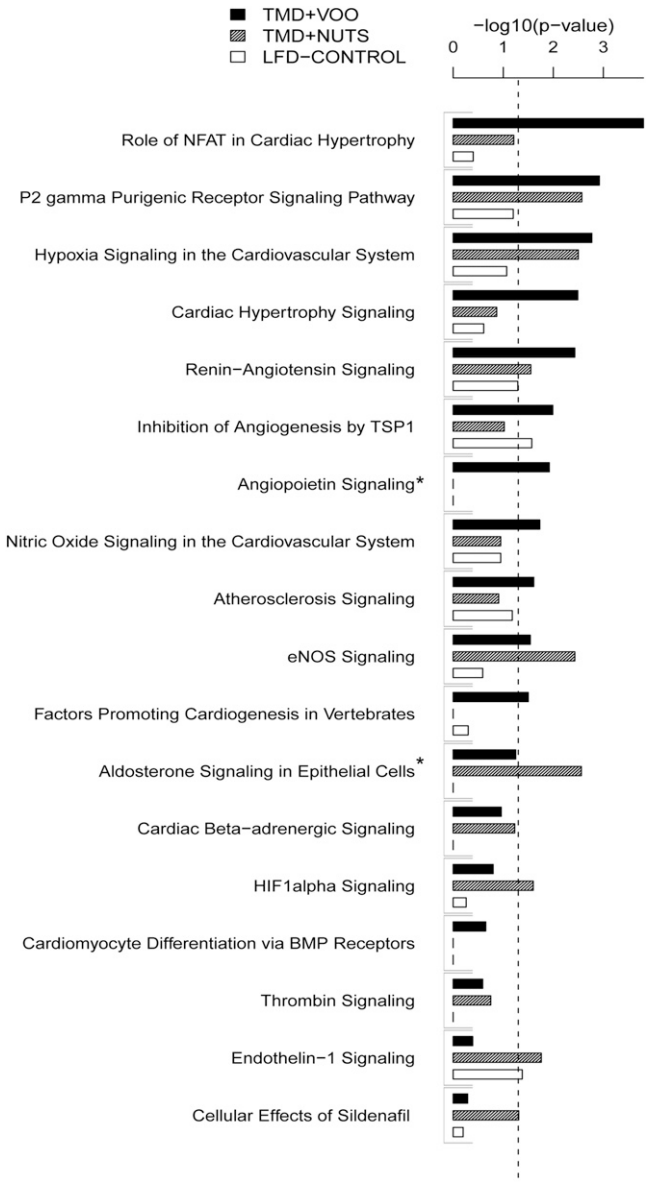

FIGURE 3. Crude analyses of cardiovascular canonical pathways affected by interventions. Only overrepresented pathways (those that have more eligible molecules than expected by randomness) are significant (threshold of [–log10 (*P*)] ≥1.3). TMD+VOO: $n = 11$; TMD+Nuts: $n = 11$; LFD: $n = 12$. *$P \leq 0.05$ between intervention groups (Fisher's exact test). BMP, basic protein membrane; eNOS, endothelial nitric oxide synthase; LFD, low-fat diet; NFAT, nuclear factor of activated T cells; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD+VOO, traditional Mediterranean diet supplemented with virgin olive oil.

renin-angiotensin, aldosterone, P2Y purigenic, and cardiac hypertrophy signaling. A key feature within these pathways is the downregulation of the *JUN* gene, which has been observed after a polyphenol-rich olive oil intervention (24). Mechanisms by which olive oil polyphenols could exert their health effects involve the modulation, toward a protective mode, of pathways related to *NF-κB/AP-1* (24). Activator protein 1 is a heterodimer *c-JUNc-FOS,* which is activated by angiotensin II (Agt2). Through angiotensin receptor 2, angiotensin II promotes an increase in ceramide production involved in *NF-κB* activation, and *NF-κB* enhances *iNOS* expression (36). In keeping with this observation, olive oil polyphenols have been shown to enhance endothelial function in vivo in human intervention studies (43). Previous PREDIMED data with a larger population supported a greater effect of TMDs compared with a LFD in lowering systolic blood pressure (44). An inverse association between TMD adherence and blood pressure also has been reported in a cross-sectional study within the Greek European Prospective Investigation into Cancer and Nutrition Cohort (45). Walnut consumption for 4 wk improved vascular reactivity in hypercholesterolemic patients (46). In our subset of the PREDIMED study, systolic blood pressure decreased significantly after the TMD+VOO intervention.

One of the strengths of the study was its design. Randomized controlled trials are able to provide first-level scientific evidence, which allows nutritional recommendations to be made at a population level (47). Furthermore, the design reproduced real-life conditions, such as home-prepared foods. The performance of bioinformatics analyses in 2 different centers by using different approaches as a quality-control procedure added robustness to the results obtained. Our study also had limitations. First, participants were encouraged to adhere to the TMD compliance solely by means of dietary instructions. Second, a 3-mo period provided no

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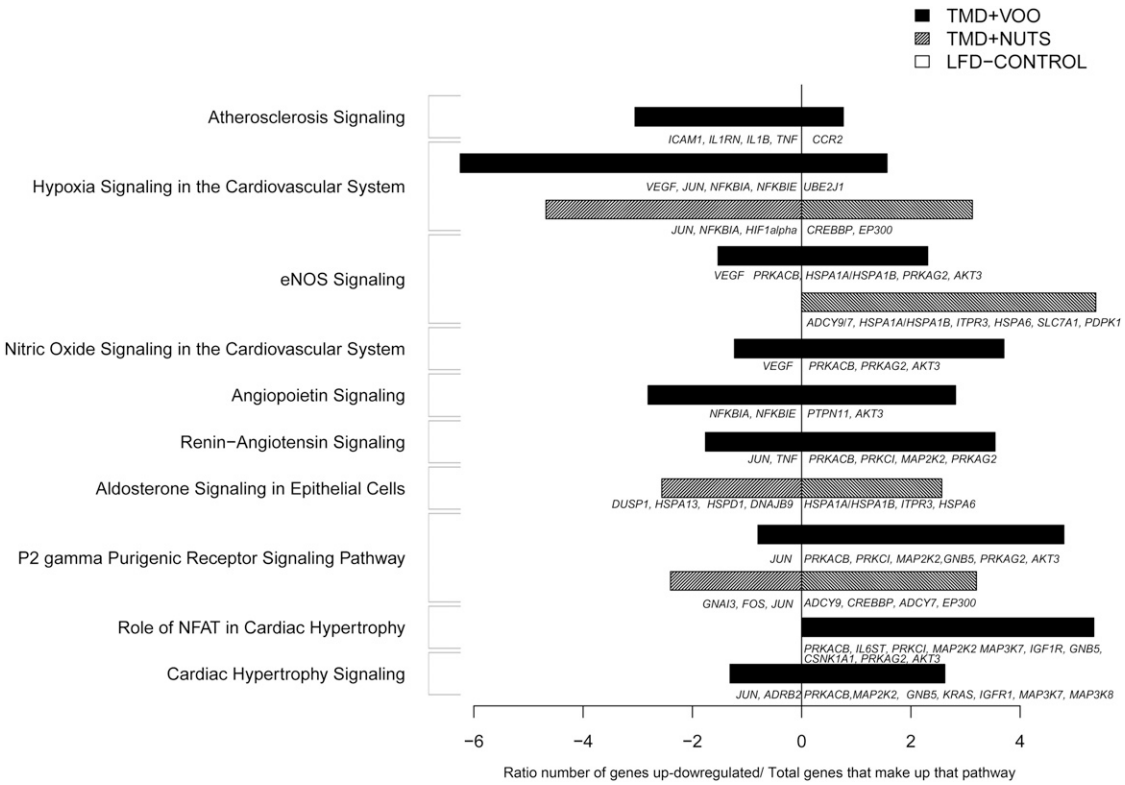

FIGURE 4. Cardiovascular canonical pathways significantly modulated after Benjamini-Hochberg correction and direction of the changes for the associated genes. TMD+VOO: n = 11; TMD+Nuts: n = 11; LFD, n = 12. eNOS, endothelial nitric oxide synthase; LFD, low-fat diet; NFAT, nuclear factor of activated T cells; TMD+Nuts, traditional Mediterranean diet supplemented with nuts; TMD+VOO, traditional Mediterranean diet supplemented with virgin olive oil.

information about the long-term nutrigenomic effects of diets. However, 2 wk is the established timeframe for fat-rich diets to reach equilibrium in the plasma lipid profile; longer intervention periods do not modify lipid concentrations (48). Our study was carried out in PBMCs because these cells have been reported as being useful to study cardiovascular biomarkers such as inflammation and peripheral cholesterol efflux. Endothelial cells, fibroblasts, hepatocytes, and adipocytes are also closely related to cardiovascular risk but could not be taken into consideration because their collection in not viable for population-based studies.

In conclusion, key pathways in the physiopathology of cardiovascular events, such as atherosclerosis, renin-angiotensin, nitric oxide and angiopoietin signaling, were modulated by the TMD+VOO, whereas hypoxia and eNOS signaling pathways were modified by both TMDs. On the basis of our results, one of the mechanisms by which the Mediterranean diet, particularly a TMD rich in VOO, can exert health benefits is by changes in the transcriptomic response of genes related to cardiovascular risk.

We thank Stephanie Londsdale and Elaine Lilly for English-language revision. The Centros de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición is an initiative of the Instituto de Salud Carlos III, Madrid, Spain.

The authors' responsibilities were as follows—OC: carried out sample analyses in the laboratory and data analyses and wrote the manuscript; M-IC: conceived the study, elaborated its design and coordination, and supervised the manuscript; M-IC, DC, JVS, and OP: participated in the design of the experiment and data collection; RdlT: participated in the design of the experiment and data collection as well as samples analyses; GF-M and MB: collaborated in data collection; LN: carried out bioinformatics analyses; IS: provided methodologic and statistical assistance; MF: carried out the collection of data, data analysis, and supervision of the manuscript; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

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