Mediterranean Diet improves thrombosis biomarkers in high cardiovascular risk individuals: a randomized controlled trial

Short title: Mediterranean Diet and thrombosis mechanisms

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- Following a Mediterranean diet improves thrombosis-related biomarkers in high cardiovascular risk individuals
ABSTRACT

Following a Traditional Mediterranean diet (TMD) decreases cardiovascular risk. However, its anti-thrombotic effects are unexplored. We aimed to assess whether this dietary pattern improved thrombosis-related biomarkers in high cardiovascular risk subjects. In 358 random PREDIMED Study volunteers (Prevención con Dieta Mediterránea), we assessed: 1) 1-year effects of a TMD, enriched in virgin olive oil (TMD-VOO; \( N = 120 \)) or nuts (TMD-Nuts; \( N = 119 \)) versus a low-fat diet (\( N = 119 \)), on thrombosis biomarkers; 2) the differences between individuals with greater TMD compliance increments (\( \geq 3 \) adherence score points) and those with compliance decrements; and 3) associations between 1-year changes in key TMD food groups and thrombosis signals. TMD-VOO and TMD-Nuts attenuated pro-thrombotic status (-5.1\% \( -P = 0.045 \) and -5.8\% \( -P = 0.025 \), respectively). TMD-VOO decreased HDL-bound \( \alpha_1 \)-antitrypsin levels (HDL pro-inflammatory/pro-thrombotic properties, -6.3\%, \( P = 0.034 \)) and increased HDL platelet activating factor-acetylhydrolase activity (+7.5\%, \( P = 0.044 \)). TMD-Nuts diminished non-esterified fatty acid levels (-10.3\%, \( P = 0.022 \)). Individuals with greater TMD adherence increments presented decreases in their pro-thrombotic status (-9.5\%, \( P = 0.032 \)), fibrinogen levels (-9.1\%, \( P = 0.030 \)), and non-esterified fatty acid concentrations (-18.1\%, \( P = 0.011 \)). Only the low-fat diet was associated with increased platelet factor-4 concentrations (\( P = 0.012 \)) and thrombin activation (higher prothrombin factor\(_{1+2} \) levels, \( P = 0.003 \)) versus baseline. Finally, in the secondary analyses, nut intake increases were associated with antithrombin increments, fruit and vegetable consumption increases were linked to decreases of thrombin activation, and processed meat intake decrements were related to increases in antithrombin and nitrite + nitrate levels (\( P < 0.05 \)). Summarizing, TMD and some of its typical food items were associated with improved thrombosis biomarkers in high cardiovascular risk individuals.
INTRODUCTION

Following a traditional Mediterranean Diet (TMD) prevents the development of cardiovascular outcomes\textsuperscript{1,2}. As observed in the PREDIMED Study (\textit{Prevención con Dieta Mediterránea}), TMD effects on cardiovascular protection may be mediated by improvements in risk factors related to blood pressure, glucose metabolism, oxidative stress, and low-grade inflammation\textsuperscript{3}. However, very scarce evidence related to TMD effects on other mechanisms such as thrombosis is available.

Thrombosis consists of the formation of a clot to avoid blood loss in the case of a vessel disruption. However, its excessive activation is also linked to an increased risk of atherosclerotic outcomes\textsuperscript{4}. Thrombus generation is based on the occurrence of four phenomena (apparition of an endothelial lesion, platelet aggregation, activation of the coagulation cascade, and fibrinolysis) which, in turn, are constituted by a chain of intertwined biochemical responses\textsuperscript{4}. Excessive levels of biomarkers related to these responses (or defective, in the case of fibrinolysis) are linked to an increased risk of suffering atherosclerotic events. This association has been particularly reported for plasma biomarkers of endothelial damage (von Willebrand factor\textsuperscript{5}), platelet aggregation (P-selectin\textsuperscript{6}, platelet factor-4\textsuperscript{7}), activation of coagulation (fibrinogen\textsuperscript{8}, thrombin formation – directly proportional to the levels of prothrombin fragment 1+2\textsuperscript{9}, antithrombin\textsuperscript{10}), and fibrinolysis (plasminogen activator inhibitor-1 –PAI1–\textsuperscript{11}, D-dimer\textsuperscript{12}). Other substances (such as non-esterified fatty acids and nitrite + nitrate levels) have been shown to be involved in the development of cardiovascular diseases through their capacity to modulate endothelial stress and platelet activation\textsuperscript{13,14}. Finally, high-density lipoproteins (HDLs) have also been attributed antithrombotic properties\textsuperscript{15}. On the one hand, HDLs are deeply involved in the regulation of inflammatory responses, which have been shown to promote thrombosis\textsuperscript{8}. On the other hand, HDLs carry enzymes able to metabolize pro-thrombotic
signals such as the platelet activating factor\textsuperscript{16} (the platelet activating factor acetylhydrolase, PAF-AH).

Very few interventions with healthy dietary patterns have been associated with changes in thrombosis biomarkers. Following a TMD has only been associated with a decrease in P-selectin levels in high cardiovascular risk subjects in the context of the PREDIMED Trial\textsuperscript{17}. Beyond this study, a cross-sectional study reported an inverse association between adherence to a Mediterranean-like diet and platelet levels\textsuperscript{18}. Finally, other small-scale prospective analysis with 21 young, healthy male volunteers assessed the impact of a TMD-like diet for 90 days on thrombosis biomarkers, and reported that this dietary pattern was associated with lower fibrinogen levels and a moderation of the coagulation response\textsuperscript{19}. However, no extensive analysis of the long-term TMD effects on thrombosis mechanisms has been performed to date in a large-scale intervention trial.

Our main aim was to assess whether a 1-year intervention with TMD improved a comprehensive set of endothelial stress, platelet, coagulation, and fibrinolysis-related biomarkers in high cardiovascular risk subjects. Our secondary aim was to determine whether there were associations between 1-year changes in the consumption of key food group whose intake was modified in the TMD intervention (virgin olive oil, nuts, fruits and vegetables, legumes, fish, and processed meat) and thrombosis biomarkers.

**MATERIALS AND METHODS**

**Study protocol and population**

Study subjects belonged to the PREDIMED Study. It was a large-scale, parallel, multicenter, randomized controlled trial assessing the long-term effects of following a TMD on the prevention of primary cardiovascular disease in a population at high cardiovascular...
risk. Volunteers were free of cardiovascular disease at baseline but presented type 2 diabetes mellitus or at least three of the following risk factors: high low-density lipoprotein cholesterol levels, low HDL cholesterol concentrations, hypertension, overweight/obesity, current smoking, or family history of premature heart disease. Individuals were randomly assigned to follow one of the three following intervention groups (in a 1:1:1 ratio): 1) a TMD supplemented with virgin olive oil (TMD-VOO); 2) a TMD supplemented with mixed nuts (TMD-Nuts); and 3) a low-fat control diet. Further details of inclusion and exclusion criteria and the dietary intervention are available in Supplemental Methods.

We selected a random subsample of 358 subjects (4.8% of the total PREDIMED population, belonging to Hospital Clinic and IMIM study sites, with biological samples at baseline and after 1 year of dietary intervention) for the present analyses. 120 volunteers were allocated to the TMD-VOO intervention group, 119 to the TMD-Nuts one, and 119 to the low-fat diet. In these individuals, age, sex and educational level data were gathered at baseline. At baseline and after 1 year of intervention, trained clinical personnel collected the following information: 1) body mass index, blood pressure, smoking, and medication use; 2) physical activity levels (using a validated Minnesota Leisure-Time Physical Activity Questionnaire); 3) adherence to a Mediterranean diet (the 14-item Mediterranean Diet adherence score, used as a continuous variable); and 4) intake of 137 food items in the previous year (using a validated food frequency questionnaire). From the information in the food frequency questionnaires, we calculated the intakes of key TMD food groups (Supplemental Methods). In addition, trained nurses collected plasma and serum samples, and stored at -80°C upon use and to quantify the levels of fasting glucose, total and HDL cholesterol, and triglycerides by automatized methods.

The trial protocol was approved by local institutional ethic committees, registered under the International Standard Randomized Controlled Trial Number ISRCTN35739639 (http://www.isrctn.com/ISRCTN35739639), and thoroughly described in previous
All participants provided written informed consent before joining the study. A.H. analyzed the data of the present work and all authors had access to primary data results. Finally, the CONSORT checklist regarding our study is available in Supplemental Table 1.

**Thrombosis biomarkers**

We determined thrombosis biomarkers in biological samples collected at baseline and after 1 year of intervention. We used ELISA kits to quantify the levels of: fibrinogen (*Human Fibrinogen SimpleStep ELISA kit*, ref.: ab208036, abcam, UK), plasminogen activator inhibitor-1 (PAI1; *Human PAI1 SimpleStep ELISA kit* (SERPINE1), ref.: ab184863, abcam, UK), platelet factor-4 (PF4 (CXCL4) *Human SimpleStep ELISA kit*, ref.: ab189573, abcam, UK), prothrombin fragment 1+2 (*Human F1+2 (prothrombin fragment 1+2) ELISA kit*, ref.: E-EL-H1793, Elabscience, USA), and von Willebrand factor (*Human von Willebrand factor SimpleStep ELISA kit*, ref.: ab223864, abcam, UK). We assessed the concentrations of antithrombin (*Antithrombin III*, ref.: SR-1102016, Spinreact, Spain) and D-dimer (*D-Dimer*, ref.: SR-1709231, Spinreact, Spain) by immunoturbidimetry, and of non-esterified fatty acids (*Non-Esterified Fatty Acids*, ref.: FA115, Randox, Spain) using a colorimetric technique in an ABX-Pentra 400 autoanalyzer (Horiba-ABX, France). We quantified nitrates + nitrites by means of a colorimetric kit (*Nitrate/Nitrite Colorimetric Assay Kit*, ref.: CAY-780001, Cayman Chemical, USA). All the previous analyses were performed in citrate plasma samples. In parallel, in apolipoprotein B-depleted plasma samples (specimen in which all lipoproteins but high-density lipoproteins –HDLs– were eliminated, prepared from citrate plasma samples), we determined the levels of total cholesterol by colorimetry (*Cholesterol Enzymatic-Colorimetry*, ref.: SR-41022, Spinreact, Spain) and of α1-antitrypsin (*α-1-antitrypsin*, ref.: SR-1102054, Spinreact, Spain) by immunoturbidimetry in an ABX-Pentra 400 autoanalyzer (Horiba-ABX, France). With this
data, we calculated the α1-antitrypsin/cholesterol ratio. We also determined in these samples the activity of the PAF-AH enzyme by a colorimetric kit (PAF Acetylhydrolase Activity Assay Kit, ref.: K765-1000, Cayman Chemical, USA). An extended explanation of the quality control, inter-assay coefficients of variation, and the number of missing values of each determination is available in Supplemental Methods and Supplemental Table 2.

**Sample size**

Since the only thrombosis-related biomarker assessed in the PREDIMED Study was P-selectin, we assumed that its variability would be similar to that in our variables. Thus, a sample size of 119 individuals per group (total sample size: \( N = 357 \)) allowed ≥80% power to detect a 10% change relative to the mean baseline value of P-selectin levels (7.65 ng/mL) between post-and pre-intervention values, and a 14% change relative to the mean baseline value (10.8 ng/mL) in the differences among the three diets, considering a 2-sided type I error of 0.05, a loss rate of 5%, and the standard deviation of the differences in P-selectin values in this intervention (SD=28.9 ng/mL)\(^{17}\).

**Statistical analyses**

We first checked the distribution of continuous variables in normality plots and by the Shapiro-Wilk test. We assessed whether there were differences in baseline values among the three intervention groups with one-way ANOVA tests for normally distributed continuous variables, Kruskal-Wallis tests for non-normally distributed continuous ones, and chi-squared tests (or exact Fisher tests when expected frequencies were <5) for categorical parameters.

We first calculated a continuous score reflecting the general activation of the thrombosis mechanisms. In biomarkers where high concentrations were associated with a more pro-thrombotic state (von Willebrand factor, platelet factor-4, fibrinogen, prothrombin fragment
1+2, D-dimer, PAI-1, non-esterified fatty acids, and α1-antitrypsin in HDL), we assigned values from 1 to 5 to the volunteers located in increasing quintiles. On the contrary, in biomarkers where high levels were linked to a less pro-thrombotic state (antithrombin, nitrites+nitrates, and PAF-AH activity in HDL), we assigned values from 5 to 1 to the volunteers located in increasing quintiles. For each biomarker, the same cut points were used at baseline and after 1 year of intervention. By summing these scores, we obtained a general pro-thrombotic score ranging from 11 (all biomarkers located in the least pro-thrombotic quintile) to 55 (all biomarkers located in the most pro-thrombotic quintile), in which higher scores indicated a higher pro-thrombotic state. These scores were only calculated in those volunteers with no missing values in any of the thrombosis biomarker variables at both time points (N=299).

We analyzed differences between pre- and post-intervention values in every intervention by paired t tests in normally distributed variables and Wilcoxon signed rank tests in non-normally distributed parameters. We checked whether there were differences in the 1-year changes in thrombosis biomarkers (calculated by subtracting baseline to post-intervention values) in the TMD interventions relative to the low-fat control diet by multivariate linear regressions (intention-to-treat analyses). We also compared with these regression models the differences in the 1-year changes in the volunteers with greater increases in TMD adherence (individuals incrementing their compliance in ≥3 points of the 14-item Mediterranean Diet score, N=66) relative to those who decreased their adherence in relation to baseline values (high versus low compliance analyses). Finally, in a secondary exploratory approach, we studied whether there were associations between 1-year changes in the intake of food groups whose consumption was significantly modified in the TMD interventions relative to the low-fat diet (virgin olive oil, nuts, fruits and vegetables, legumes, fish, and processed meat; see the “Study population” paragraph in the Results section) and thrombosis biomarkers by additional multivariate linear regression analyses.
To promote the interpretability of data, results were presented as percentage changes relative to baseline values (to calculate them, we divided the adjusted difference coefficients obtained in the regression models by the baseline value of the variable in each of the groups). Models were adjusted for: age, sex, study site, educational level (primary education or lower/secondary or equivalent/higher education), baseline value of the variable, fasting glucose levels, use of antidiabetic drugs (yes/no), cholesterol concentrations, triglyceride values (log-transformed), use of lipid-lowering drugs (yes/no), systolic blood pressure, use of antihypertensive drugs (yes/no), use of antithrombotic drugs (yes/no), tobacco use (never/former/actual smoker), body mass index, leisure-time physical activity (log-transformed), and two propensity scores to correct for the theoretical deviations in the randomization process (calculated from 30 baseline variables)\textsuperscript{1}. High versus low compliance and food group intake analyses were additionally adjusted for the TMD adherence score or the intake of each particular food group at baseline, respectively, and for the PREDIMED intervention group. Models were plotted using the “lme” package in R Software\textsuperscript{23}. We considered any two-sided \textit{P}-value<0.05 as significant and performed statistical analyses with R software version 3.5.0\textsuperscript{24}.

\textbf{Data sharing statement}

Considering that we do not have the explicit written consent of the study volunteers to yield their deidentified data at the conclusion of the study, individual participant data cannot be shared. The study protocol is available in the main publication of the study\textsuperscript{1} and in the study website (http://www.predimed.es/uploads/8/0/5/1/8051451/_1estr_protocol_olf.pdf), and a summary of the dietary intervention is also available in \textbf{Supplemental Methods}. 


RESULTS

Study population and dietary intervention

We found no significant differences in clinical variables at baseline among the individuals assigned to the three intervention groups (Table 1). Regarding the non-included PREDIMED Study subjects, there were 6.2% more women and 7.4% less antithrombotic drug users among our volunteers and they presented higher glucose (6 mg/dL), total cholesterol (5 mg/dL), and leisure-time physical activity levels (22 METs-min/d) ($P<0.05$) (Supplemental Table 3). Physical activity did not change throughout the study ($P>0.05$). Volunteers’ compliance to the dietary intervention was correct according to the Mediterranean diet adherence score: we observed a 1.10 and 1.35-point increment in score values after 1 year of intervention in the TMD-VOO and TMD-Nuts interventions, respectively ($P<0.001$, relative to the low-fat diet) (Supplemental Table 4). Volunteers allocated in the TMD-VOO intervention group increased their compliance due to increments in the consumption of virgin olive oil (+3.5 servings/day), fruits and vegetables (+0.8 servings/d), legumes (+0.2 servings/day), and fish (+0.2 servings/d), whilst those in the TMD-Nuts group incremented their adherence due to increases in the intake of nuts (+1.2 servings/d), legumes (+0.1 servings/d –only marginally–), and fatty fish (+0.2 servings/d), and decreases in the consumption of processed meat (-0.2 servings/d) ($P<0.05$, relative to the low-fat diet). Volunteers with greater compliance increments presented a 4.63 point increase in the TMD adherence score due to increases in the consumption of virgin olive oil (+1.8 servings/d), nuts (+0.6 servings/d), fruits and vegetables (+2.4 servings/d), legumes (+0.2 servings/d), and fish (+0.6 servings/d), and decreases in red meat intake (-0.3 servings/d) ($P<0.05$, in relation to the volunteers with decrements in TMD adherence). Finally, low-fat diet compliance was observed as a reduction in the consumption of fats, based on a decrease of the intake of mixed nuts and
the substitution of red for white meat (red meat consumption decreased while meat did not) and the substitution of fatty for non-fatty fish (total fish intake increased while fatty fish did not) \( (P<0.05, \text{relative to baseline}). \)

**TMD and thrombosis biomarkers**

The thrombosis score (a measurement of the pro-thrombotic status) was attenuated by both TMD-VOO and TMD-Nuts interventions (-5.1\% \( -P=0.045\)– and -5.8\% \( -P=0.025\)–, respectively, relative to the low-fat diet) \( (\text{Figure 1A}). \) TMD-VOO decreased the levels of \( \alpha_1 \)-antitrypsin (-6.3\%, \( P=0.034, \text{Figure 1K} \)) and increased the activity of the platelet activating factor-acetylhydrolase in HDLs (+7.5\%, \( P=0.044, \text{Figure 1L} \)) in relation to the control intervention. TMD-Nuts particularly diminished the concentrations of non-esterified fatty acids versus the low-fat diet (-10.3\%, \( P=0.022, \text{Figure 1J} \)). Volunteers with greater TMD adherence increments decreased their overall pro-thrombotic status (-9.5\%, \( P=0.032, \text{Figure 1A} \), fibrinogen levels (-9.1\%, \( P=0.030, \text{Figure 1D} \)), and non-esterified fatty acid concentrations (-18.1\%, \( P=0.011, \text{Figure 1J} \)) relative to those who decreased their TMD adherence. Finally, when assessing post- vs. pre-intervention changes, only the low-fat diet was associated with increases in platelet factor-4 concentrations \( (P=0.012) \) and thrombin activation \( (\text{higher prothrombin factor}_{1+2} \text{levels, } P=0.003) \) \( (\text{Table 2}). \) Exact coefficients are available in **Supplemental Table 5** (expressed as percentage changes relative to baseline values) and **Supplemental Table 6** (expressed as linear differences).

In the secondary exploratory approach regarding food group intake: 1) increases in nut intake were associated with increments in antithrombin levels; 2) increases in the consumption of fruit and vegetables were linked to decreases in thrombin activation \( (\text{lower prothrombin factor}_{1+2} \text{levels}); \) and 3) decrements in the intake of processed meats were related to increases in antithrombin and nitrite + nitrate levels \( (P<0.05) \). Exact coefficients for these analyses are available in **Supplemental Table 7** (expressed as percentage
changes relative to baseline values) and **Supplemental Table 8** (expressed as linear differences).

**DISCUSSION**

Our results show that 1 year of intervention with TMD improves pro-thrombotic state in high cardiovascular risk individuals. To the best of our knowledge, this trial is the largest (N=358) and more comprehensive study on the effects of a healthy dietary pattern on the molecular mechanisms related to thrombosis.

Thrombosis is deeply affected by inflammation and oxidative stress\textsuperscript{25,26}. TMD is a lifestyle intervention known to improve these last two risk factors\textsuperscript{3}. Our data indicate that following this dietary pattern decreased the overall pro-thrombotic response of high cardiovascular risk subjects (around 5-6% in the subjects in the TMD-VOO and TMD-Nuts groups, and up to 10% in those with greater increases in TMD adherence) and we hypothesize that this effect could be partially explained by a decrease in oxidative stress and low-grade inflammation. On the one hand, following a TMD has been associated in our study with beneficial changes in thrombosis biomarkers linked as well to low-grade inflammation such as fibrinogen and \(\alpha_1\)-antitrypsin levels in HDL\textsuperscript{8,27}. On the other hand, some particular dietary modifications of the TMD that have been associated with beneficial changes in thrombosis biomarkers in our data (increases in the consumption of nuts, fruits and vegetables; decreases in the intake of processed meat) have been broadly related to antioxidant and anti-inflammatory effects in previous human studies\textsuperscript{28–31}.

HDLs have been shown to be able to induce beneficial effects on some hemostatic mechanisms\textsuperscript{15}. In this regard, the TMD-VOO intervention led to a particular decrease in \(\alpha_1\)-antitrypsin levels and an increase in PAF-AH activity in HDL particles, two potential HDL
antithrombotic properties. It is not the first time that we report that the TMD-VOO intervention promotes HDL functionality in the PREDIMED Study\textsuperscript{22}. However, our data extend this protection to anti-inflammatory and anti-thrombotic HDL functions. Antioxidant protection of typical TMD food items on HDL components could contribute to explaining an improved activity of the lipoprotein protective enzymes (due to a greater preservation of their non-oxidized structure)\textsuperscript{32}. In addition, the capacity of this dietary pattern to decrease the levels of inflammation biomarkers in circulation may be related to a decrement in the concentrations of pro-inflammatory molecules bound to HDL particles\textsuperscript{33}.

We observed a decrease in non-esterified fatty acid levels after 1 year of TMD-Nuts intervention (also present in subjects with greater TMD adherence increments). Non-esterified fatty acids promote endothelial stress and platelet aggregation and, therefore, are considered an emergent pro-thrombotic risk factor in some cardiovascular risk states (e.g., obesity, hypertriglyceridemia)\textsuperscript{13}. The capacity of polyunsaturated fatty acids (whose intake was particularly increased in the TMD-Nuts group among individuals with greater TMD compliance) to bind to GPR120 receptors in adipose tissue in order to decrease lipolysis could be an explanation for this effect\textsuperscript{34,35}. In addition, TMD is a fiber-rich dietary pattern, whose intestinal metabolism leads to blood peaks of short-chain fatty acids (known to be able to bind other GPR family receptors with a similar anti-lipolytic effect\textsuperscript{36}). Thus, a synergistic effect between these two nutrients could be expected.

Our study has several strengths. First, it presents a large sample size ($N=358$). Second, it is based on a randomized design with an active comparator (low-fat control diet) and a long-term duration (1 year). Last, it is the first analysis of the effects of a dietary pattern on a comprehensive, hypothesis-driven set of thrombosis-related biomarkers, performed after a strict laboratory quality control. However, it also presents limitations. First, our volunteers were elderly people at high cardiovascular risk and, therefore, the extrapolation of our conclusions to other populations is hindered. Second, we report moderate changes after
the interventions. However, the PREDIMED trial is based on modest real-life dietary modifications and the control diet is already a well-known healthy dietary pattern. Finally, it was not possible to calculate the pro-thrombotic score in a random 16.5% of the volunteers due to the missing values in the individual determinations of thrombosis biomarkers (each marker presented missing values that were not overlapping with the others). Nevertheless, the amount of missing values was low for individual parameters (0% for fibrinogen and antithrombin; ≤1% for the von Willebrand factor, PAF-AH activity in HDLs, and non-esterified fatty acids; and ≤5% for the rest of the determinations excepting nitrites + nitrates –6.1%–) and the order in which samples were analyzed was randomly assigned before starting the laboratory procedures. Thus, we may assume that missing data occurred at random and did not consistently affect the rest of determinations.

In conclusion, 1 year of intervention with TMD improved the pro-thrombotic status in high cardiovascular risk individuals. The TMD-VOO intervention enhanced HDL antithrombotic properties, whilst the TMD-Nuts one decreased the levels of non-esterified fatty acids. High TMD adherences particularly improved the pro-thrombotic status and decreased fibrinogen and non-esterified fatty acid concentrations. The low-fat diet was the only dietary group linked to increments in the levels of platelet factor-4 and prothrombin activation. TMD effects could be particularly based on increments in the intake of nuts, fruits, and vegetables, and decreases in processed meat consumption. As far as we know, this is the largest, most comprehensive, hypothesis-based analysis of the effects of a healthy dietary pattern on thrombosis mechanisms in high cardiovascular risk subjects. Our results support the improvement of thrombosis status after following a TMD. Further studies are needed to confirm whether these improvements mediate the reported cardioprotective benefits of this dietary pattern.
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AUTHORSHIP

Contribution: A.H. and M. Fitó designed the research; A.H., O.C. A.T.-R., X.P., and R.E were involved in funding search; X.P., M. Fitó, M.-Á. M.-G., D.C., J.S.-S., J.L., E.G.-G., F.A., M. Fiol, L.S.-M., E.R., and R.E. conducted the clinical trial and provided biological samples; A.H., O.C., M. Fitó, and R.C. developed the experimental part of the project; A.H. analyzed results and wrote the paper; O.C. A.T.-R., X.P., M. Fitó, R.C., M.-Á. M.-G., D.C., J.S.-S., J.L., E.G.-G., F.A., M. Fiol, L.S.-M., E.R., and R.E. revised the manuscript and approved its final version.

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### Table 1. Description of the study population

|                         | TMD-VOO (N=120) | TMD-Nuts (N=119) | Low-fat diet (N=119) | P-value |
|-------------------------|------------------|-------------------|----------------------|---------|
| Sex (female) (n, %)     | 78 (65.0%)       | 75 (63.0%)        | 74 (62.2%)           | 0.898   |
| Age (years)             | 67.3 (5.34)*     | 66.6 (5.79)       | 66.5 (6.35)          | 0.530   |
| Educational level:      |                  |                   |                      | 0.228   |
| Primary or less (n, %)  | 95 (79.2%)       | 79 (66.4%)        | 81 (68.1%)           |         |
| Secondary (n, %)        | 15 (12.5%)       | 25 (21.0%)        | 21 (17.6%)           |         |
| Higher (n, %)           | 9 (7.50%)        | 14 (11.8%)        | 13 (10.9%)           |         |
| Non-available (n, %)    | 1 (0.83%)        | 1 (0.84%)         | 4 (3.36%)            |         |
| Glucose (mg/dL)         | 125 (39.9)       | 123 (46.7)        | 131 (46.4)           | 0.321   |
| Glucose-lowering drug users (n, %) | 41 (34.2%) | 35 (29.4%)       | 44 (37.0%)           | 0.458   |
| Total cholesterol (mg/dL) | 213 (34.6)     | 214 (36.3)        | 207 (33.5)           | 0.298   |
| HDL cholesterol (mg/dL) | 53.4 (9.03)      | 51.2 (9.88)       | 51.0 (11.1)          | 0.134   |
| LDL cholesterol (mg/dL) | 134 (32.5)       | 137 (33.1)        | 131 (29.5)           | 0.414   |
| Triglycerides (mg/dL)   | 114 [92.9; 156]† | 109 [80.7; 156]   | 111 [84.5; 146]      | 0.672   |
| Lipid-lowering drug users (n, %) | 62 (51.7%)  | 51 (42.9%)       | 51 (42.9%)           | 0.287   |
| Systolic blood pressure (mmHg) | 154 (18.3)  | 155 (21.6)       | 153 (20.9)           | 0.668   |
| Diastolic blood pressure (mmHg) | 83.0 (10.5) | 85.6 (11.3)      | 83.9 (8.90)          | 0.141   |
| Antihypertensive drug users (n, %) | 86 (71.7%) | 79 (66.4%)      | 76 (63.9%)           | 0.423   |
| Antithrombotic drug users (n, %) | 19 (15.8%) | 13 (10.9%)      | 14 (11.8%)           | 0.479   |
| Tobacco use:            |                  |                   |                      | 0.445   |
| Never smoker (n, %)     | 83 (69.2%)       | 77 (64.7%)        | 69 (58.0%)           |         |
|                                    | 16 (13.3%) | 15 (12.6%) | 20 (16.8%) |
|------------------------------------|------------|------------|------------|
| Actual smoker (n, %)               |            |            |            |
| Former smoker (n, %)               | 21 (17.5%) | 27 (22.7%) | 30 (25.2%) |
| Body mass index (kg/m²)            | 29.7 (3.37)| 29.9 (3.50)| 29.5 (3.34)| 0.690 |
| Leisure-time physical activity (METs·min/d) | 215 [103; 367] | 179 [76.9; 335] | 202 [59.4; 377] | 0.447 |
| Mediterranean diet adherence score | 8.61 (1.68)| 8.69 (1.85)| 8.99 (1.64)| 0.197 |

*: Mean (standard deviation)
: Median [first quartile; third quartile]

**HDL**: high-density lipoprotein; **LDL**: low-density lipoprotein; **METs**: metabolic equivalents of task.
Table 2. Changes in thrombosis biomarkers throughout the 1-year intervention relative to baseline.

| Normally distributed variables (paired t-tests) | Low-fat control diet (N=119) | TMD-VOO (N=120) | TMD-Nuts (N=119) |
|-----------------------------------------------|-------------------------------|-----------------|------------------|
|                                               | Post-int. value | Difference | P-value | Post-int. value | Difference | P-value | Post-int. value | Difference | P-value |
| Thrombosis score (μg/mL)                      | 30.3 (6.54)     | 0.50 (6.52) | 0.434   | 30.2 (5.69)     | 0.057 (5.32) | 0.913   | 29.3 (5.82)     | -0.52 (5.87) | 0.378   |
| Von Willebrand factor (μg/mL)                 | 31.2            | -2.03      | 0.139   | 32.2 (14.5)     | 0.22 (13.6)  | 0.859   | 30.9 (12.9)     | 1.00 (11.9)  | 0.364   |
| Fibrinogen (mg/dL)                            | 275 (65.1)      | 7.40 (64.2)| 0.211   | 277 (63.5)      | -0.094 (68.0)| 0.988   | 272 (62.1)      | 1.92 (57.8)  | 0.718   |
| Antithrombin (mg/dL)                          | 36.1 (4.44)     | 0.38 (4.14)| 0.321   | 37.0 (4.37)     | 0.33 (3.19)  | 0.254   | 36.1 (5.29)     | -0.15 (4.40) | 0.718   |
| Plasminogen activator (mg/dL)                 | 3.68 (1.56)     | 0.080 (2.12)| 0.683   | 3.60 (1.66)     | 0.009 (1.97) | 0.962   | 3.68 (1.73)     | 0.098 (2.10) | 0.619   |
| inhibitor-1 (mg/dL)                           | 1.12 (0.54)     | 0.057 (0.67)| 0.360   | 1.13 (0.59)     | 0.063 (0.77) | 0.378   | 1.25 (0.70)     | 0.089 (0.83) | 0.251   |

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|                                | Pre-int. value | Post-int. value | P-value | Pre-int. value | Post-int. value | P-value | Pre-int. value | Post-int. value | P-value |
|--------------------------------|----------------|-----------------|---------|----------------|-----------------|--------|----------------|-----------------|--------|
| **Platelet factor-4 (ng/mL)**  |                |                 |         |                |                 |        |                |                 |        |
|                                | 268            | 351             | 0.012   | 287            | 372             | 0.168  | 288            | 323             | 0.901  |
|                                | [183; 431]     | [211; 565]      |         | [164; 498]     | [202; 563]      |         | [186; 486]     | [205; 514]      |         |
| **Prothrombin fragment 1+2 (pg/mL)** |                |                 |         |                |                 |        |                |                 |        |
|                                | 261            | 319             | 0.005   | 246            | 312             | 0.114  | 275            | 289             | 0.573  |
|                                | [149; 433]     | [209; 538]      |         | [162; 415]     | [223; 487]      |         | [165; 439]     | [194; 443]      |         |

**HDL**: high-density lipoprotein; **PAF-AH**: platelet activating factor acetylhydrolase; **Pre-int.**: pre-intervention; **Post-int.**: post-intervention;

**TMD-Nuts**: traditional Mediterranean diet enriched with mixed nuts; **TMD-VOO**: traditional Mediterranean diet enriched with virgin olive oil.
**FIGURES**

**Figure 1.** TMD effects on thrombosis biomarkers.

![Graphs showing TMD effects on thrombosis biomarkers](https://example.com/graphs.png)

- **A:** Thrombosis score (adjusted difference, %)
- **B:** Von Willebrand factor (adjusted difference, %)
- **C:** Platelet factor-4 (adjusted difference, %)
- **D:** Fibrinogen (adjusted difference, %)
- **E:** Prothrombin fragment 1+2 (adjusted difference, %)
- **F:** Antithrombin (adjusted difference, %)
- **G:** Plasminogen activator inhibitor-1 (adjusted difference, %)
- **H:** D-dimer (adjusted difference, %)
- **I:** Nitrites + nitrates (adjusted difference, %)
- **J:** Non-esterified fatty acids (adjusted difference, %)
- **K:** Alpha-1 antitrypsin in HDLs (adjusted difference, %)
- **L:** PAF-AH activity in HDLs (adjusted difference, %)
Effects of TMD-VOO (first forest plot of each panel) and TMD-Nuts interventions (second forest plot of each panel) (versus the low-fat control diet), and of subjects with greater increases in TMD adherence (versus those showing a decrease in their compliance to the TMD intervention): thrombosis score (A), and levels of von Willebrand factor (B), platelet factor-4 (C), fibrinogen (D), prothrombin fragment 1+2 (E), antithrombin (F), plasminogen activator inhibitor-1 (G), D-dimer (H), nitrites + nitrates (I), non-esterified fatty acids (J), α1-antitrypsin in HDLs (K), and platelet activating factor acetylhydrolase activity in HDLs (L). Results are shown as adjusted coefficients (percentage changes relative to baseline values) with 95% confidence intervals.

*: P<0.05.

HDL: high-density lipoprotein; PAF-AH: platelet activating factor acetylhydrolase; TMD: traditional Mediterranean diet; TMD-Nuts: traditional Mediterranean diet enriched with mixed nuts; TMD-VOO: traditional Mediterranean diet enriched with virgin olive oil.