The Mediterranean Diet improves HDL function in high cardiovascular risk individuals: a randomized controlled trial

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ABSTRACT

Background. The biological functions of high-density lipoproteins (HDLs) contribute to explaining the cardioprotective role of the lipoprotein beyond quantitative HDL cholesterol levels. A few small-scale interventions with a single antioxidant have improved some HDL functions. However, to date, no long-term, large-scale, randomized, controlled trial has been conducted to assess the effects of an antioxidant-rich dietary pattern (such as a Traditional Mediterranean Diet, TMD) on HDL function in humans.

Methods. This study was performed in a random sub-sample of volunteers from the PREDIMED Study (*Prevención con Dieta Mediterránea*) (*N*=296) after a 1-year intervention. We compared the effects of two TMDs, one enriched with virgin olive oil (TMD-VOO, *N*=100) and another with nuts (TMD-Nuts, *N*=100), with respect to a low-fat control diet (*N*=96). We assessed the effects of both TMDs on the role of HDL particles on reverse cholesterol transport (cholesterol efflux capacity, HDL ability to esterify cholesterol, and cholesteryl ester transfer protein activity), HDL antioxidant properties (paraoxonase-1 arylesterase activity and total HDL antioxidant capacity on low-density lipoproteins), and HDL vasodilatory capacity (HDL ability to induce the release of nitric oxide in endothelial cells). We also studied the effects of a TMD on several HDL quality-related characteristics (HDL particle oxidation, resistance against oxidative modification, main lipid and protein composition, and size distribution).

Results. Both TMDs increased cholesterol efflux capacity relative to baseline (*P*=0.018 and *P*=0.013, respectively). The TMD-VOO intervention decreased cholesteryl ester transfer protein activity (relative to baseline, *P*=0.028), and increased HDL ability to esterify cholesterol, paraoxonase-1 arylesterase activity, and HDL vasodilatory capacity (relative to control, *P*=0.039, *P*=0.012, and *P*=0.026, respectively). Adherence to a TMD induced these beneficial changes by improving HDL oxidative status and composition. The three diets increased the percentage of large HDL particles (relative

to baseline, *P*<0.001).

Conclusions. The TMD, especially when enriched with virgin olive oil, improved HDL atheroprotective functions in humans.

Clinical Trial Registration. ISRCTN35739639 (http://www.controlled-trials.com/ISRCTN35739639).

Key words: high-density lipoprotein, diet, antioxidant, lipids and lipoprotein metabolism, randomized controlled trial

CLINICAL PERSPECTIVE

What is new?

- The biological functions of high-density lipoproteins (HDLs) contribute to explaining the cardioprotective role of the lipoprotein beyond HDL cholesterol levels.
- HDL functions improved after some small-scale interventions with antioxidants.
- In this trial, an antioxidant-rich dietary pattern, a Traditional Mediterranean Diet (especially when enriched with virgin olive oil), enhanced the key HDL functions (reverse cholesterol transport pathway, HDL antioxidant properties, and vasoprotective effects) in humans.
- To date, this randomized controlled trial was the largest (*N*=296) and the longest duration (1 year) study assessing change in HDL functionality in humans.

What are the clinical implications?

- Adherence to a Traditional Mediterranean Diet, particularly when enriched with virgin olive oil, improves HDL function in humans.
- The present work could contribute to the discovery of novel therapeutic targets that may improve HDL function in humans (new antioxidant-rich functional foods, nutraceuticals, or new drug families).

INTRODUCTION

A growing and consistent body of evidence (from observational and randomized controlled trials) supports that the Traditional Mediterranean Diet (TMD) protects against the development of cardiovascular diseases[1]. The PREDIMED Study (*Prevención con Dieta Mediterránea*), a multi-center, parallel, randomized controlled trial, has been crucial to show this protection in primary cardiovascular disease prevention[2,3]. Several mechanisms may contribute to explain the protection of this traditional food pattern against atherosclerosis, such as its ability to preserve DNA and systemic lipids against oxidative modifications, its anti-inflammatory effects, its modulatory capacity on the metabolomic profile, and its capacity to modulate gene expression related to cardiovascular diseases[4–7]. The TMD has also improved the lipid profile related to high density lipoproteins (HDLs)[8,9]. However, it is becoming increasingly more accepted that HDL function may reflect the anti-atherogenic role of the lipoprotein better than HDL cholesterol (HDL-C) levels[10]. Several foods and nutrients present in the TMD have been shown to improve a number of HDL functions in humans in previous trials[11–14]. To date, however, no evidence of the effects of the whole TMD on HDL properties has been reported.

The aim of the present study was to determine, in a random sub-sample of the PREDIMED Study, whether the long-term consumption of a TMD, enriched with virgin olive oil or nuts, was able to improve the different HDL functional properties in humans.

METHODS

An extended description of methods is available in the online-only Data Supplement.

Study population

Considering the available samples of the PREDIMED Study[2,15] and the sample size calculation estimated before the experiments, we performed a random selection of individuals with biological samples at baseline and after one year of dietary intervention

(*N*=296, 4.14% of the volunteers of the total trial). Volunteers had been randomly assigned in the beginning of the study to one of the following three interventions: 1) a TMD enriched with virgin olive oil (TMD-VOO, *N*=100); 2) a TMD enriched with nuts (TMD-Nuts, *N*=100); and 3) a low-fat control diet (*N*=96). We also registered the changes in general clinical and sociodemographic variables, adherence to the TMD, a food frequency questionnaire of the previous year, and physical activity[15]. The local Research and Ethics Committee approved the protocol of the study. Volunteers gave informed consent before joining the trial.

HDL functions and quality-related properties

We determined the volunteers' biochemical profile (glucose, triglycerides, total cholesterol, HDL-C, apolipoprotein A-I –ApoA-I–, and apolipoprotein B –ApoB–) in an ABX-Pentra 400 autoanalyzer (Horiba ABX)[11]. We calculated LDL cholesterol levels with the Friedewald formula (whenever triglycerides were <300 mg/dL)[16], and the HDL-C/ApoA-I and ApoB/ApoA-I ratios. We isolated HDL particles from the plasma of the participants by density gradient ultracentrifugation[11] or by polyethylene glycol precipitation of apolipoprotein B-containing lipoproteins[17]. We measured cholesterol efflux capacity in a model of human THP-1 monocyte-derived macrophages[11]. We calculated the capacity of HDL particles to esterify cholesterol (the HDL cholesterol esterification index) as follows: percentage of esterified cholesterol in HDL/mass of lecithin-cholesterol transfer protein (LCAT, measured in plasma as previously reported[18]). We assessed the activity of cholesteryl ester transfer protein (CETP) in plasma and the arylesterase activity of paraoxonase-1 (PON1) in serum in commercial kits[11,18]. We measured HDL antioxidant capacity as the HDL ability to decrease the formation of conjugated dienes in LDLs in a pro-oxidant environment[16], in a random sub-selection of 90 volunteers. HDL inflammatory index (HII) was based on the oxidation of the fluorescent 2',7'-dichlorodihydrofluorescein[19]. We determined HDL vasodilatory capacity as the HDL-induced increase in nitric oxide production in human

umbilical vein endothelial cells[20]. The level of oxidative modifications in HDL particles (the HDL oxidation index) was measured as follows: equivalents of malondialdehyde in HDL[21]/cholesterol in HDL. We assessed the resistance of HDL lipoprotein against oxidation (HDL oxidation lag time in a pro-oxidant environment) in a random sub-sample of 90 volunteers[16]. We assessed HDL composition (total cholesterol, free cholesterol, esterified cholesterol, triglycerides, phospholipids, and apolipoproteins A-I, A-II –ApoA-II– and C-III –ApoC-III–) in isolated HDL samples in an ABX-Pentra 400 autoanalyzer (Horiba ABX)[11]. From these data, we calculated the amount of each apolipoprotein per mg of cholesterol, and the following ratios: HDL triglycerides/HDL esterified cholesterol ("triglycerides in HDL core"), and HDL phospholipids/HDL free cholesterol ("phospholipids in HDL surface")[11,18]. Finally, we determined HDL size distribution by the LipoPrint technology (Quantimetrix)[11].

Sample size

Main sample. A sample size of 96 participants per group allowed ≥ 80% power to detect a significant difference of 0.025 points in normalized cholesterol efflux capacity values between pre-and post-intervention values, and of 0.035 points among the three interventions, considering a 2-sided type I error of 0.05, a loss rate of 5%, and the standard deviation of the differences in cholesterol efflux capacity (SD=0.084) after an analogous dietary intervention[11].

Sub-group for HDL antioxidant capacity and HDL resistance against oxidation. As we had no previous data available on these properties, we calculated this sample size considering the variability of the differences in the activity of the main HDL antioxidant enzyme (PON1) after an analogous dietary intervention[22]. A sample size of 28 participants per group allowed ≥ 80% power to detect a significant difference of 0.057 points in the previous normalized capacity between pre-and post-intervention, and of 0.08 points among the three interventions, considering a 2-sided type I error of

0.05, a loss rate of 5%, and the standard deviation of the differences in this property (SD=0.104)[22].

Statistical analyses

We checked the distribution of continuous variables in normality plots and by the Shapiro-Wilk test, and log-transformed the non-normally distributed ones. We looked for differences between our subsample and the whole PREDIMED population by a T-test, and for differences in the baseline values among the three intervention groups by a Chi-square test (in categorical variables) or a one-way ANOVA (in continuous variables).

We analyzed differences between pre- and post-intervention values in every intervention by a paired T-test. We checked the effects of the three interventions on the changes in the variables of interest in a multivariate linear regression analysis (using two dummy variables, one for each intervention group), in which we included as co-variables: sex, age, participant's center of origin (*k*-1 dummy variables), baseline value of the variable, and changes in the presence of dyslipidemia, type-2 diabetes mellitus, hypertension, and smoking habit throughout the study.

To assess the relationships among HDL functional parameters in baseline values, we performed Spearman's correlation analyses among variables and a principal component analysis. Moreover, to study the relationships among the changes in HDL functional parameters after the TMD-VOO intervention (the one in which most of the changes in HDL functions took place), we performed Spearman's correlation analyses among these differences, a principal component analysis, and multivariate linear regression analyses adjusted for the changes in other HDL functions (together with the variables described in the previous paragraph).

Any two-sided *P*-value <0.05 was considered significant. We did not correct *P*-values for multiple comparisons because our analyses were hypothesis-driven and the

phenotypes of interest were highly correlated and non-independent. Analyses were performed with R Software, version 3.0.2[23].

RESULTS

Participants

Study design is available in **Supplemental Figure 1**. We found no significant differences among groups in the baseline characteristics of the volunteers (**Table 1**). When comparing the baseline characteristics between our subsample and the whole population of the PREDIMED trial (**Supplemental Table 1**), our volunteers were on average 1.1 year older and there were 6.8% more males. However, no other differences in their main clinical characteristics were observed. Energy expenditure in leisure time physical activity and caloric intake did not change throughout the study, and volunteers' compliance with the interventions was correct according to biomarkers of compliance, TMD adherence scores, and the 1-year food frequency questionnaire (**Supplemental Tables 2-3**). Increased adherence to TMD after the TMD-VOO intervention was due to: 1) increases in the consumption of virgin olive oil, legumes, and fish; 2) substitution of red/processed meat for white meat; and 3) decreases in the consumption of precooked foods and industrial sweets ($P<0.05$). Increased adherence to TMD after the TMD-Nuts intervention was due to: 1) increases in the consumption of nuts, virgin olive oil (less than in the TMD-VOO intervention), fruits, vegetables, and oily fish; and 2) decreases in the consumption of red and processed meat, precooked meals, and industrial sweets ($P<0.05$). Finally, adherence to a low-fat diet was observed as a decrease in total fat intake (particularly saturated fats), due to decreases in the consumption of high-fat dairy products, red and processed meat, precooked meals, and industrial sweets ($P<0.05$).

Lipids

Regarding the biochemical profile of the participants (**Supplemental Table 4-5**), we observed an expected decline in total cholesterol levels after the low-fat control diet (*P*=0.039 and *P*=0.007, relative to baseline and the TMD-VOO intervention, respectively). This was mainly due to a decrease in LDL cholesterol levels (*P*=0.019 and *P*=0.004, relative to baseline and the TMD-VOO intervention, respectively). Regarding the HDL-related biochemical profile, we did not observe significant changes in HDL cholesterol or ApoA-I levels in any intervention relative to baseline or the low-fat diet (**Figures 1A-1D**). However, there was a significant decrease in the HDL cholesterol/ApoA-I ratio values in plasma in the TMD interventions relative to baseline (*P*=0.031 and *P*<0.001 in the TMD-VOO and the TMD-Nuts interventions, respectively) but not relative to the low-fat diet.

Cholesterol efflux capacity

The cholesterol efflux capacity of the volunteers increased with both TMD interventions relative to baseline (*P*=0.018, and *P*=0.013, for TMD-VOO and TMD-Nuts respectively) (**Figures 1E-1F**) but not relative to the low-fat diet.

HDL role in other steps of reverse cholesterol transport

The ability of HDL particles to esterify cholesterol (the HDL cholesterol esterification index) increased significantly after the TMD-VOO intervention relative to baseline (*P*=0.007) and compared to the low-fat control intervention (*P*=0.039) (**Figures 1G-1H**). CETP activity (**Figure 1I-1J**) decreased significantly after the TMD-VOO intervention relative to baseline (*P*=0.008) but not compared to the low-fat diet.

HDL antioxidant capacities

PON1 arylesterase activity did not change significantly relative to baseline after any intervention. However, when compared with the low-fat diet, it increased after the TMD-VOO intervention (*P*=0.012) (**Figures 2A-2B**).

The HDL capacity to directly counteract LDL oxidation rose after the TMD-VOO intervention, relative to baseline values (*P*=0.004) but not compared to the low-fat diet (**Figures 2C-2D**).

With respect to other antioxidant/anti-inflammatory properties of HDL, the HII increased significantly after the low-fat control diet relative to baseline (*P*=0.025), whilst it did not rise after the TMD interventions (**Figures 2E-2F**). This led to a borderline significant decrease in HII values after the TMD-Nuts intervention relative to the low-fat diet (*P*=0.060).

HDL vasodilatory capacity

HDL ability to promote the production of nitric oxide in endothelial cells (the HDL vasodilatory capacity) did not change significantly relative to baseline after any intervention. However, it increased significantly after the TMD-VOO intervention relative to the low-fat diet (*P*=0.026) (**Figures 3A-3B**).

HDL oxidation

The level of oxidation of HDL particles (the HDL oxidation index) decreased after the TMD-VOO and the low-fat control diet, relative to their baseline values (*P*=0.028 and *P*=0.011, respectively) (**Figures 4A-4B**).

HDL dynamic resistance against oxidation (HDL oxidation lag time) increased after the TMD-VOO intervention, relative to baseline values (*P*=0.006) but not compared to the low-fat diet (**Figures 4C-4D**).

HDL composition

When compared with the low-fat diet, the content of triglycerides in HDL core decreased significantly after both the TMD-VOO and the TMD-Nuts interventions (*P*=0.027 and *P*=0.035, respectively) (**Figures 4E-4F**).

After the TMD-VOO intervention, the content of phospholipids in the HDL surface increased relative to baseline (P=0.003) and the low-fat control diet (P=0.036) (**Figures 4G-4H**).

We found no significant changes in the levels of apolipoproteins A-I, A-II and C-III in HDL in any of the interventions.

HDL size distribution

The three interventions increased the levels of large HDL particles relative to their baseline values (P<0.001 for the three interventions) (**Figures 4I-4J**). We observed no differences among the three of them in the multivariate regression model.

Other data

All values of the comparisons between post- and pre-intervention values, and between the changes in the TMD interventions relative to the low-fat diet, are available in **Supplemental Tables 6** and **7**, respectively.

Pearson's correlations among baseline values, and the differences after the TMD-VOO intervention in HDL functional parameters, are available in **Supplemental Tables 8** and **9**, respectively. Bi-dimensional plots of the distribution of baseline values and the differences in HDL functional traits after the TMD-VOO intervention, according to the two main components of the respective principal component analysis, are available in **Supplemental Figures 2** and **3**, respectively. Independent effects of the TMD-VOO intervention on each HDL function (eliminating the effect of the diet on the rest of the HDL functional capacities) are available in **Supplemental Tables 10** and **11**. A further description of the relationships among baseline and changes in HDL functionality traits is available in the **Supplemental Results** section.

DISCUSSION

Our results show that one year of intervention with a TMD improves several HDL functions (cholesterol efflux capacity, cholesterol metabolism, antioxidant/anti-inflammatory properties, and vasodilatory capacity) in individuals at high cardiovascular risk. Thus far, this randomized controlled trial is the largest (*N*=296) and the longest duration (1.13±0.21 years, mean±SD) study assessing change in HDL functionality in humans.

Cholesterol efflux capacity is a key biological function of HDL: HDL particles extract excess cholesterol from peripheral cells for delivery to the liver for metabolization or excretion[24]. Macrophage-specific cholesterol efflux capacity is inversely related to higher cardiovascular risk[17] and greater incidence of coronary events[25]. In the present trial, both TMD interventions increased cholesterol efflux capacity relative to baseline. We assessed cholesterol efflux capacity in a human THP-1 macrophage-based model, as these cells do not require a chemical induction of the expression of the cholesterol transporters prior to the experiments[26] and reflect more physiologically the efflux phenomenon and the overall human atherosclerotic process[24]. An improved HDL oxidative status[27] (as has been associated in our data), lipid composition[12], and HDL-related gene expression[28] may contribute to explaining these changes. Similar increments in cholesterol efflux capacity using the same human macrophage model have been observed in clinical trials with virgin olive oil[11] and trials with supplements of polyunsaturated fatty acids[14].

HDL lipoproteins esterify and internalize accepted cholesterol from the periphery, continuing the reverse cholesterol transport pathway[29]. This property can be measured by LCAT activity[29] or the HDL cholesterol esterification index. In our study, the TMD-VOO intervention significantly increased the HDL cholesterol esterification index. As LCAT is very sensitive to oxidative attacks[30], dietary antioxidants in the TMD may contribute to maintaining LCAT non-oxidized and functional. A fruit and vegetable-rich diet[31] and a supplement of the antioxidant lycopene[13] have induced similar effects in previous studies.

CETP is a key enzyme in the cholesterol transfer from HDL particles to triglyceride-poor lipoproteins (and subsequently to the liver). CETP activity is frequently increased in high cardiovascular risk states[32]. However, inhibition of CETP with pharmacological agents has not resulted in improved cardiovascular outcomes[33]. It has been hypothesized that this lack of effects could be due to the futility of drastically increasing HDL-C levels above physiological concentrations or to harmful off-target effects (e.g. hypertensive ones)[33]. Nevertheless, a slight decrease in the CETP activity (such as the one present after the TMD-VOO intervention in this trial –2.90%– or after a lycopene-rich diet –5.87%–[13]), accompanied by a general improvement in the lipid metabolism profile and without off-target effects, may be protective since it could contribute to maintaining HDL homeostatic function.

Another key atheroprotective role of HDL is its antioxidant capacity. HDL lipoproteins are able to counteract the oxidation of LDL, one of the main biochemical triggers of the development of the atherosclerotic plaque[10]. This antioxidant capacity is coordinated through the action of several proteins, among which PON1 is the central enzyme[34]. Whilst low PON1 arylesterase activity has been postulated as a potential predictor of cardiac events in some trials[35], its predictive role is still controversial in other human studies[36]. In the present trial, the TMD-VOO intervention increased the overall antioxidant protection of HDL particles on LDLs, and also incremented PON1 arylesterase activity. This augmentation in PON1 function seemed independent from the benefits of the TMD-VOO intervention on other HDL functions. Although not directly assessed in this study, the different bioactive compounds in the TMD (olive oil phenolic compounds[18], carotenoids such as lycopene[13], and omega-3 polyunsaturated fatty acids[14]) may act synergistically and could: 1) induce local antioxidant functions; 2) preserve other dietary antioxidants in HDL lipoproteins (such as vitamin E); and 3) protect PON1 against oxidative modifications and/or enhance its function[37,38].

HDL particles can also counteract the pro-inflammatory effects of pro-oxidant substances, such as modified LDL, due to the combined action of HDL-associated

proteins[19,34]. Low HII values reflect an increase in HDL anti-inflammatory properties[19]. HII has been shown to be abnormally elevated in subjects with cardiovascular pathologies and can discriminate patients with acute coronary syndrome from those with stable angina or healthy controls[19]. In the present study, the low-fat control diet increased HII values, whilst the TMD interventions did not. Further studies are warranted since to date no diet-based intervention has been shown to be able to improve the HDL inflammatory index significantly.

In addition to cholesterol transport, anti-oxidative, and anti-inflammatory functions, HDL lipoproteins also have endothelial vasoprotective properties[39], which are impaired in high cardiovascular risk states[40]. In the present trial, the TMD-VOO intervention increased the capacity of isolated HDL to induce the production of nitric oxide from endothelial cells, a vasodilatory trait (which can also be considered as vasoprotective). This increase in HDL endothelial protection seemed independent from the benefits of the TMD-VOO intervention on other HDL functions, as in the case of the improvement in the PON1 function. HDL particles with a high content of sphingosine-1-phosphate, a low content of oxidized lipids, and stronger capacity to perform ABCG1-dependent cholesterol efflux have been associated with a greater ability to maintain endothelial homeostasis[39]. Massive weight losses due to gastric surgery[20] and an aerobic exercise training program[41] have increased the HDL ability to induce the release of nitric oxide from endothelial cells in previous trials. However, TMD is the first dietary modification that has been shown to promote HDL vasoprotective properties.

A potential explanation for the TMD-mediated increase in HDL functionality could be an improvement in the oxidative status of the lipoprotein[27]. In our trial, whilst the TMD-VOO intervention and the low-fat diet led to a decreased oxidation of HDL particles, the lipoproteins were more resistant against dynamic oxidation only after the TMD-VOO intervention. Both the TMD-VOO and the low-fat intervention were rich in fruit and vegetables[15]. Some antioxidants, such as carotenoids, are highly present in both, bind to HDL lipoproteins, and protect them locally against oxidative modifications[13].

However, antioxidant protection could be greater in the TMD due to a higher content of its particular antioxidants, such as olive oil phenolic compounds[11,38].

Another mechanism that could explain the benefits of the TMD on HDL function could be a better HDL composition profile. Both TMD interventions decreased the content of triglycerides in the HDL core. Low-fat diets are known to induce transient hypertriglyceridemias[42] which could lead to an indirect hypertriglyceridemic state in HDL particles. Nevertheless, HDL hypertriglyceridemias appeared to be reversed by the TMD in our trial. In the specific case of the TMD-VOO intervention, the decreased CETP activity could help to explain the reduced triglyceride content in HDL lipoproteins, as observed in our data. In the particular case of the TMD-Nuts intervention, the nut consumption may have decreased systemic triglyceride levels[43] and, indirectly, reduced HDL triglyceride content. These decrements in the HDL triglyceride content after the TMD interventions are associated with a more stable conformation of ApoA-I in the HDLs[44] and could partially explain the improved HDL role in cholesterol efflux capacity and antioxidant functions.

Continuing with HDL composition, after the TMD-VOO intervention, HDL particles had more phospholipids in their surface. As has been associated in our data, the improved HDL capacity to esterify cholesterol may explain this change: when the free cholesterol content in the surface decreases, the relative content of phospholipids increases. This could be linked to a greater HDL fluidity, which has already been observed after the consumption of virgin olive oil[11,38] and could contribute to explaining an increased HDL functionality.

Some parameters related to the structure of HDL, such as the HDL-C/ApoA-I ratio in plasma, also improved after the TMD interventions. A decrease in the HDL-C/ApoA-I balance could be beneficial since high values of this ratio have been associated with increased cardiovascular and all-cause mortality[45].

Finally, size is also a major but controversial trait of lipoproteins. Large HDL particles may be atheroprotective due to their ability to mediate ABCG1- and SRBI-dependent

386 cholesterol efflux capacity, whilst small and lipid-free HDL lipoproteins could also be
387 beneficial through their ability to promote the ABCA1-dependent efflux
388 phenomenon[24,46]. The ability of the TMD and some of its bioactive components (olive
389 oil phenolic compounds) to increase the levels of large HDL particles has already been
390 described in previous trials[11,47].
391 The present study has several strengths. First, it involves a large sample size (*N*=296).
392 Second, it presents a randomized design with the presence of an active comparator
393 (low-fat diet). Third, its duration is long (one year of follow-up). Finally, it
394 comprehensively assesses diverse HDL functions and HDL quality-related
395 characteristics. Nevertheless, it also has limitations. The participants of the trial were
396 elderly people at high cardiovascular risk which hinders the extrapolation of our results
397 to the general population. As expected, we found only slight differences since: 1) the
398 trial is based on modest real-life modifications of the diet; and 2) the control diet is
399 already a well-known healthy dietary pattern. The use of cellular models, although it is
400 a non-invasive alternative to test relevant physiological functions, may not have
401 demonstrated the effect of contra-regulatory mechanisms, which can modify the final *in*
402 *vivo* outcome in humans. Due to availability issues, in the samples from two of the
403 study centers (*N*=67) we could not perform the analyses related to HDL main
404 enzymatic proteins (CETP and PON1 activities, and LCAT mass). In the samples from
405 one study center (*N*=37) we were also unable to assess HDL size. Finally, we could not
406 perform HDL vasodilatory capacity analyses in 16% of the individuals in all the
407 interventions due to technical issues in cell cultures.
408 In conclusion, we report the effects of an integral dietary intervention on HDL
409 functionality. To date, this is the first randomized controlled trial to study simultaneously
410 four key HDL functional traits (cholesterol efflux capacity, HDL cholesterol metabolism,
411 HDL antioxidant/anti-inflammatory properties, and vasoprotective effects), in a large
412 sample size and with an extended duration of the intervention. Adherence to a TMD,
413 especially when enriched with virgin olive oil, was associated with increases in these

four key HDL functions. The TMD could have induced these benefits through improvements in HDL oxidative status, composition, and size. Our data support the improvement in HDL function after following a TMD. Further studies are warranted to investigate the mechanism by which the TMD improves HDL function and whether these properties convey cardioprotective effects.

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DISCLOSURES

None.

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633 **TABLES**

634

635 **TABLE 1**

636 Baseline characteristics of the volunteers in the three intervention groups of the study.

VARIABLES	TMD-VOO *N*=100	TMD-Nuts *N*=100	Low-fat diet *N*=96	*P*-value
Age (years)	66.3 (5.78)*	66.4 (6.93)	65.0 (6.49)	0.247
Sex (% male)	56.0%	47.0%	50.0%	0.432
Body Mass Index (kg/m^2)	30.1 (3.85)	29.0 (3.76)	29.9 (3.87)	0.087
Waist Circumference (cm)	100 (10.7)	101 (10.3)	102 (11.2)	0.469
Leisure-time physical activity (MET·min/day)	176 [69.3;284]†	175 [68.3;408]	174 [41.3;362]	0.870
Smoking status (% of smokers)	15.0%	10.0%	12.5%	0.565
Type 2 diabetes (% of diabetic patients)	48.0%	52.0%	46.9%	0.751
Hypertension (% of hypertensive patients)	78.0%	78.0%	80.2%	0.910
Dyslipidemia (% of dyslipidemic patients)	79.0%	70.0%	83.3%	0.074
Glucose (mg/dL)	110 [93.8;137]	108 [92.5;140]	108 [94.0;131]	0.982
Triglycerides (mg/dL)	207 (36.8)	196 (36.2)	204 (36.8)	0.112
Total cholesterol (mg/dL)	110 [93.2;158]	100 [72.5;144]	113 [83.0;140]	0.160
HDL cholesterol (mg/dL)	50.2 (12.3)	49.8 (10.3)	49.1 (11.6)	0.777
LDL cholesterol (mg/dL)	130 (28.5)	123 (30.4)	130 (31.7)	0.181
Apolipoprotein A-I (mg/dL)	138 (23.5)	134 (20.2)	131 (19.4)	0.232
Apolipoprotein B (mg/dL)	106 (21.6)	98.2 (18.9)	103 (21.8)	0.062

637

638 *: Mean (SD). †: Median [1st-3rd quartile].

639 *MET*: metabolic equivalent of task. *TMD-VOO*: Traditional Mediterranean Diet enriched

640 with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet enriched with mixed

641 nuts.

FIGURE LEGENDS

FIGURE 1

Post- vs. pre-intervention changes (A, C, E, G, I) and inter-intervention changes in a multivariate linear regression model (B, D, F, H, J) in HDL variables related to reverse cholesterol transport: HDL cholesterol levels (A-B), apolipoprotein A-I levels (C-D), cholesterol efflux capacity (E-F), HDL cholesterol esterification index (G-H), and activity of cholesteryl ester transfer protein (I-J). Results are shown as means with 95% CI (A, C, E, G, I) or adjusted coefficients with 95% CI (B, D, F, H, J). CETP: cholesteryl ester transfer protein; TMD-VOO: Traditional Mediterranean Diet enriched with virgin olive oil; TMD-NUTS: Traditional Mediterranean Diet enriched with nuts. *: $P<0.05$.

FIGURE 2

Post- vs. pre-intervention changes (A, C, E) and inter-intervention changes in a multivariate linear regression model (B, D, F) in variables related to HDL antioxidant capacity: paraoxonase-1 arylesterase activity (A-B), HDL capacity to increase LDL lag time (C-D), and HDL inflammatory index (E-F). Results are shown as means with 95% CI (A, C, E) or adjusted coefficients with 95% CI (B, D, F). PON1: paraoxonase-1; TMD-VOO: Traditional Mediterranean Diet enriched with virgin olive oil; TMD-NUTS: Traditional Mediterranean Diet enriched with nuts. *: $P<0.05$; **: $P<0.01$.

FIGURE 3

HDL vasodilatory capacity: post- vs. pre-intervention changes (A) and inter-intervention changes in a multivariate linear regression model (B). Results are shown as means with 95% CI (A) or adjusted coefficients with 95% CI (B). TMD-VOO: Traditional Mediterranean Diet enriched with virgin olive oil; TMD-NUTS: Traditional Mediterranean Diet enriched with nuts. *: $P<0.05$.

FIGURE 4

Post- vs. pre-intervention changes (A, C, E, G, I) and inter-intervention changes in a multivariate linear regression model (B, D, F, H, J) in variables related to HDL oxidation, composition and size: HDL oxidation index (A-B), HDL resistance against oxidation (HDL lag time) (C-D), quantity of triglycerides in HDL core (E-F), quantity of phospholipids in HDL surface (G-H), and percentage of large HDL particles (I-J). Results are shown as means with 95% CI (A, C, E, G, I) or adjusted coefficients with 95% CI (B, D, F, H, J). TMD-VOO: Traditional Mediterranean Diet enriched with virgin olive oil; TMD-NUTS: Traditional Mediterranean Diet enriched with nuts. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.