The Mediterranean Diet decreases LDL atherogenicity in high cardiovascular risk individuals: a randomized controlled trial

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Abbreviations

ApoB: apolipoprotein B

LDL-C: low-density lipoprotein cholesterol

TMD: Traditional Mediterranean Diet

TMD-VOO: TMD intervention enriched with virgin olive oil

TMD-Nuts: TMD intervention enriched with nuts

Keywords: “LDL cytotoxicity”, “LDL oxidation”, “LDL size”, “low density lipoproteins”, “Mediterranean Diet”

ABSTRACT

Scope. Traditional Mediterranean Diet (TMD) protects against cardiovascular disease through several mechanisms such as decreasing LDL cholesterol levels. However, evidence regarding TMD effects on LDL atherogenic traits (resistance against oxidation, size, composition, cytotoxicity) is scarce.

Methods and results. We assessed the effects of a 1-year intervention with a TMD on LDL atherogenic traits in a random sub-sample of individuals from the PREDIMED Study (*N*=210). We compared two TMDs: one enriched with virgin olive oil (TMD-VOO, *N*=71) and another with nuts (TMD-Nuts, *N*=68), versus a low-fat control diet (*N*=71). After the TMD-VOO intervention, LDL resistance against oxidation increased (+6.46%, *P*=0.007), the degree of LDL oxidative modifications decreased (-36.3%, *P*<0.05), estimated LDL particle size augmented (+3.06%, *P*=0.021), and LDL particles became cholesterol-rich (+2.41% *P*=0.013) relative to the low-fat control diet. LDL lipoproteins became less cytotoxic for macrophages only relative to baseline (-13.4%, *P*=0.019). No significant effects of the TMD-Nuts intervention on LDL traits were observed versus the control diet.

Conclusion. Adherence to a TMD, particularly when enriched with virgin olive oil, decreased LDL atherogenicity in high cardiovascular risk individuals. The development of less atherogenic LDLs could contribute to explaining some of the cardioprotective benefits of this dietary pattern.

INTRODUCTION

Adherence to a Traditional Mediterranean Diet (TMD) protects against the development of cardiovascular diseases as observed in a consistent body of evidence coming from observational and randomized controlled trials [1]. The PREDIMED Study (*Prevención con Dieta Mediterránea*), a multi-center, parallel, randomized controlled trial, has demonstrated with a high degree of scientific evidence that a TMD has protective effects on primary cardiovascular disease prevention [2, 3]. Due to its richness in antioxidants and other bioactive molecules (this dietary pattern is based on the consumption of virgin olive oil, nuts, fruit, vegetables, whole grains, legumes, fish, poultry, and moderate quantities of wine at meals) [2], the TMD protects against atherosclerosis by improving blood lipid levels, oxidative/inflammatory status, and gene expression associated with the development of cardiovascular diseases [4–7]. The TMD has also been shown to enhance some characteristics related to low-density lipoproteins (LDLs), such as the levels of total and oxidized LDL particles [8, 9]. Besides these properties there are other characteristics that make LDL especially atherogenic including: 1) LDL resistance against oxidative modifications; 2) LDL content of triglycerides, cholesterol, and various proteins; 3) LDL cytotoxic potential on different cell types; and 4) LDL ability to transfer cholesterol to hepatocytes. Our group has previously studied the effects of a typical TMD food, virgin olive oil, on some of these traits [10]. To date, however, the effects of the whole TMD on a complete set of LDL atherogenic properties remain to be fully elucidated.

Thus, the aim of the present study was to assess whether a long-term consumption of a TMD, enriched with virgin olive oil or nuts, could decrease the atherogenicity of LDL particles in humans.

MATERIALS AND METHODS

Study design

Our study population was a random subsample of volunteers (*N*=210) from the PREDIMED Study (*Prevención con Dieta Mediterránea*), a randomized, controlled, large-scale, parallel, multicenter trial that assessed the long-term effects of a TMD on the primary prevention of cardiovascular events in a high cardiovascular risk population [2]. Participants were randomly allocated to: 1) a TMD enriched with virgin olive oil (TMD-VOO, *N*=71); 2) a TMD enriched with nuts (TMD-Nuts, *N*=68); and 3) a low-fat control diet following the indications of the American Heart Association (*N*=71). Volunteers allocated to the TMD interventions were instructed to replace cooking fats with virgin olive oil; increase their consumption of fruit, vegetables, nuts, legumes, fish, and poultry; and decrease their intake of red/processed meat and processed foods. Individuals in the low-fat control diet were taught to decrease their consumption of fatty foods (oils, nuts, butter, meat, fish, and processed foods) and to promote their intake of vegetal foods. In addition, TMD-VOO volunteers received 1 L/week of virgin olive oil, and TMD-Nuts individuals were given 210 g/week of mixed nuts (walnuts, hazelnuts, and almonds) to particularly promote the intake of these food items. A more detailed description of the three dietary interventions is available elsewhere [2]. We studied the effects of a TMD on the characteristics related to the atherogenicity of LDL particles before and after one year of intervention. Local institutional ethic committees approved the protocol of the study, and all volunteers provided a signed informed consent before entering the trial. Further details of the study have been previously published [2]. The PREDIMED Study protocol was registered with the International Standard Randomized Controlled Trial Number ISRCTN35739639 (www.controlled-trials.com).

Biological samples and clinical information

K3-EDTA plasma samples were obtained from blood collected from the participants before and post-intervention. The samples did not suffer any thaw-freeze cycles before our experiments. We isolated LDL particles from a plasma aliquot by means of a

density gradient ultracentrifugation [10]. Samples were stored at -80ºC until required. We also gathered the following information before and after the intervention: 1) the general clinical status of the volunteers (sex, age, body mass index, waist circumference, blood pressure); 2) their adherence to the TMD and their usual diet in the previous year (by a food frequency questionnaire); and 3) their levels of physical activity (through a validated Minnesota Leisure-Time Physical Activity questionnaire) [2].

Biochemical profile

We performed all systemic determinations in plasma samples in an ABX Pentra-400 autoanalyzer (Horiba-ABX, Montpellier, France). We determined the levels of fasting glucose, triglycerides, and total cholesterol by enzymatic methods (ABX Pentra Glucose HK CP, ABX Pentra Triglycerides CP, and ABX Pentra Cholesterol CP, all from Horiba-ABX), the levels of HDL cholesterol by the Accelerator Selective Detergent method (ABX Pentra HDL Direct CP, Horiba-ABX), and the levels of apolipoproteins B (ApoB) (ABX Pentra ApoB, Horiba-ABX) and A-I (ApoA-I) (ABX Pentra ApoA1, Horiba-ABX) by immunoturbidimetry. The inter-assay coefficients of variation (CVs) of these determinations were: 1.91% for fasting glucose, 4.07% for triglycerides, 1.24% for total cholesterol, 1.79% for HDL cholesterol, 1.59% for ApoB, and 1.68% for ApoA-I. We also calculated LDL cholesterol (LDL-C) levels (according to the Friedewald formula whenever triglycerides were <300 mg/dL) and the ApoB/ApoA-I ratio.

LDL resistance against oxidation

We incubated isolated LDL particles with an oxidizing agent ($CuSO_4$) to assess their resistance to accumulate Cu^{2+}-induced conjugated dienes. We dialyzed LDL lipoproteins against PBS and incubated them (final concentration: 10 mg cholesterol/dL) with $CuSO_4$ (final concentration: 5 μM) in 96-well transparent plates at 37ºC in an Infinite M200 reader (Tecan Ltd, Männedorf, Switzerland). We measured

absorbance at 234 nm every 3 minutes for 4 hours to obtain the curves of LDL oxidation. From the curves, we calculated the lag time (the time when maximal oxidation started, in minutes). High LDL lag time values are associated with a greater resistance of LDL particles against oxidation [10]. The inter-assay CV was 12.4%.

Degree of LDL oxidative modifications

We measured the quantity of oxidative modifications in LDL particles (malondialdehyde equivalents) by the thiobarbituric reactive acid species technique in isolated LDL samples [11]. We then divided the malondialdehyde equivalents by the cholesterol content in each LDL sample (see “LDL composition”). The inter-assay CV was 9.21%.

Estimated LDL particle size

From the data of the volunteers’ plasma lipid profile we calculated a surrogate marker for LDL size, the LDL-C/ApoB ratio (unitless). Low ratio values are associated with LDL particles of smaller size [12].

LDL composition

We analyzed the composition of isolated LDL lipoproteins in an ABX Pentra-400 autoanalyzer (Horiba-ABX). We measured the levels of triglycerides (ABX Pentra Triglycerides CP, Horiba-ABX) and cholesterol (Cholesterol-LQ, Spinreact) by enzymatic methods, total protein (ABX Pentra Total Protein CP, Horiba-ABX) by the Biuret reaction, and ApoB (ABX Pentra ApoB, Horiba-ABX) by immunoturbidimetry. The inter-assay CVs of these measurements were: 4.62% for triglycerides, 3.86% for cholesterol, 2.47% for total protein, and 1.59% for ApoB.
From these values, we determined the content of cholesterol and triglycerides in isolated LDL particles (adjusted for the ApoB quantity of the lipoproteins), the triglyceride/cholesterol ratio, and the percentage of LDL proteins other than ApoB, as

follows: (total protein in LDL – ApoB in LDL)/total protein in LDL x100.

LDL cytotoxicity in macrophages

We grew human THP-1 in RPMI-1640 medium (complemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, and 1% sodium pyruvate), refreshed them every 72h, and differentiated them into macrophages (by incubating them with 200 nM phorbol-myristate-acetate –Sigma, Barcelona, Spain–, for 96h). Next, we washed the macrophages and incubated them with isolated LDL particles (concentration: 10 mg/dL cholesterol in LDL particles [10, 13]) or without LDL (as negative control), for 16h. After incubation, we washed the cells and incubated them with 0.5 mg/mL soluble MTT bromide (Thiazolyl Blue Tetrazolium bromide, Sigma), during 4h. Then, we removed the supernatant, washed the cells again, and dissolved the cell content (and the MTT-formazan crystals inside the cells) with DMSO (Sigma), for 1h under stirring. Finally, we measured absorbance at 570 nm in an Infinite M200 reader (Tecan Ltd). If the viability of the cells was high, they would transform the soluble MTT pigment more rapidly into insoluble MTT-formazan crystals, and the absorbance of the DMSO-dissolved cell content would be greater. Therefore, high LDL cytotoxicity would be related to low MTT-absorbance values [13].

To calculate the index of LDL cytotoxicity in macrophages, we subtracted the blank (absorbance of the cells non-treated with MTT) from all absorbance values, and calculated the difference in the MTT-absorbance in the LDL-treated cells versus the untreated cells (the negative control): (MTT-absorbance in LDL-treated cells – MTT-absorbance in untreated cells)/MTT-absorbance in untreated cells*100. The inter-assay CV of the experiment (N=7) was 35.5%.

Data quality control

LDL oxidation and cytotoxicity experiments followed a predefined process to control inter-assay variability. In all these experiments, we analyzed the samples from the

same volunteers in the same analytical run, in duplicate, and we did not allow intra-repetition CVs over 15%. We also included an LDL pool (isolated from a pool of plasma from 20 healthy volunteers) in each experiment. We finally divided the values obtained in the samples by the value of the pool for each parameter, to obtain normalized ratios without units.

Sample size calculation

A sample size of 68 participants per group allowed ≥80% power to detect a significant difference of 0.05 points in LDL lag time values (expressed as normalized units) between pre-and post-intervention values, and of 0.07 points among the three interventions, considering a 2-sided type I error of 0.05, a loss rate of 1%, and the standard deviation of the differences in normalized LDL lag time values (SD=0.144) after an analogous dietary intervention [10].

Statistical analyses

We examined the distribution of continuous variables in normality plots and the Shapiro-Wilk test, and log-transformed the non-normally distributed variables. To find possible differences in the baseline characteristics of our subsample and the whole PREDIMED population, we performed a T-test. To investigate possible differences in baseline values among the three intervention groups, we carried out a chi-square test for categorical variables and a one-way ANOVA for continuous variables.

We studied the differences between pre- and post-intervention values after the three interventions in a paired T-test. We also analyzed the effects of the TMD interventions (relative to the low-fat diet) on the changes in the variables of interest in a multivariate regression analysis (using two dummy variables, one for each intervention group) adjusted for: sex; age; center of origin of the volunteer (*k*-1 dummy variables); baseline value of the variable; and changes in the presence of dyslipidemia, diabetes, hypertension, and tobacco habit (*k*-1 dummy variables) throughout the study.

To detect potential relationships among LDL atherogenic traits, we carried out Spearman's correlation analyses among the baseline values of these determinations. In addition, to assess the relationships among the changes in LDL atherogenic characteristics after the TMD-VOO intervention (the one after which most of the differences occurred), we carried out Spearman's correlation analyses and a principal component analysis among these variables.

We accepted any two-sided *P*-value <0.05 as significant. We performed all the previous analyses in R Software, version 3.0.2 (*R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*).

RESULTS

Participants and dietary adherence

Study design is available in **Supplemental Figure 1**. No differences in the baseline characteristics were found among the three groups in our subsample (**Table 1**). With respect to the whole PREDIMED Study population, our volunteers were on average 1.6 years younger, with 9.2% more males, and 6.9% more dyslipidemic individuals at baseline (*P*<0.05) (**Supplemental Table 1**). We found no differences in energy expenditure in leisure-time physical activity among interventions.

Subjects appeared to be relatively compliant to the diets. The augmented TMD adherence after the TMD-VOO intervention was observed as: 1) increments in the consumption of virgin olive oil, legumes, and fish; and 2) decreases in the intake of red and processed meat, refined olive oil, and precooked meals (*P*<0.05). The augmented TMD adherence after the TMD-Nuts intervention was due to: 1) increases in the intake of nuts, virgin olive oil (less than in the TMD-VOO intervention), and canned and oily fish; and 2) decrements in the consumption of meat, refined olive oil, precooked meals, and industrial confectionery (*P*<0.05). Finally, adherence to the low-fat diet was

reflected as a reduction in the intake of saturated fats, due to decreases in the consumption of whole-fat dairy products, meat (particularly red and processed), processed meals, and industrial confectionery (P<0.05). Total, monounsaturated, and polyunsaturated fat consumption was significantly reduced in the low-fat diet relative to both TMD interventions (**Supplemental Tables 2-3**).

Biochemical profile

We observed a 10.9 mg/dL decrease in the levels of total cholesterol after the low-fat diet (P=0.023 and P=0.007, relative to baseline values and the TMD-VOO intervention). The reduction took place essentially through a 10.5 mg/dL decline in LDL-C levels (P=0.007 and P=0.003, when compared to baseline and the TMD-VOO intervention, respectively) (**Figure 1A-1B**). Despite the changes in LDL-C levels, ApoB concentrations (**Figure 1C-1D**) and the ratio between ApoB and apolipoprotein A-I levels remained unchanged after the three interventions. Finally, there was a significant decrease in remnant cholesterol (-15.1%, **Figure 1E-1F**) and triglyceride concentrations (-2.98%) after the TMD-Nuts intervention when compared to the low-fat diet (P=0.020 and P=0.021, respectively) (**Supplemental Tables 4-5**).

Estimated LDL particle size

The LDL-C/ApoB ratio in plasma diminished after the low-fat diet relative to baseline (-4.47%, P<0.001). In concordance, we observed a significant increase (+3.06%) in estimated LDL particle size after the TMD-VOO intervention relative to the low-fat diet (P=0.021) (**Figure 1G-1H**).

LDL oxidation-related parameters

LDL resistance against oxidation (LDL lag time) increased after both TMD interventions. After the TMD-VOO intervention, LDL lag time increased relative to baseline (+6.77%) and the low-fat diet (+6.46%) (P<0.001 and P=0.007, respectively).

After the TMD-Nuts intervention, it increased significantly only relative to baseline (+6.45%) (P=0.002) (**Figure 2A-2B**).

Degree of LDL oxidative modifications (malondialdehyde equivalents in LDL, adjusted for the content of cholesterol in LDL particles) decreased significantly after the TMD-VOO intervention when compared with the low-fat diet (-36.3%) (P<0.05) (**Figure 2C-2D**).

LDL composition

Cholesterol content in LDL particles increased after the TMD-VOO intervention relative to the low-fat diet (+2.41%) (P=0.013) (**Figure 3A-3B**).

Triglyceride content in LDL lipoproteins and the ratio between triglycerides and cholesterol in isolated LDL particles (data not shown) did not vary significantly after any intervention.

Finally, the content of LDL proteins other than ApoB decreased relative to baseline after the three dietary interventions (-5.06% –P=0.001–, -4.99% –P=0.006–, and -3.99% –P=0.020– for the TMD-VOO, the TMD-Nuts, and the low-fat diet, respectively) (**Figure 3C-3D**). We found no statistically significant differences among the three interventions.

LDL cytotoxicity

After the TMD-VOO intervention, the cytotoxicity of LDL particles in human macrophages lessened relative to baseline (-13.4%, P=0.019) (**Figure 4A-4B**). We found no effects after the TMD-Nuts intervention.

Relationships among LDL atherogenic traits

LDL atherogenic characteristics that reflect limited atherogenic properties (high lag time values, low levels of oxidative modifications, high average estimated LDL particle size, low triglyceride load, and high cholesterol content) were all inter-correlated (P<0.05 in

all cases except the relationship between LDL lag time and the ratio between triglycerides and cholesterol in LDL). Low LDL cytotoxicity in macrophages was also associated with a low degree of LDL oxidative modifications, and triglyceride-poor, protein-poor, cholesterol-rich LDL particles (all $P<0.001$), and with increases in estimated LDL particle size ($P=0.056$) (**Supplemental Table 6**).

Changes in LDL atherogenic traits after the TMD-VOO intervention also correlated amongst each other (**Supplemental Table 7**). First, decreases in LDL oxidation after this intervention were associated with increases in triglyceride-poor, protein-poor, cholesterol-rich LDL particles, and low LDL cytotoxicity in macrophages (all $P<0.001$). Second, increases in cholesterol content and decreases in the relative levels of triglycerides in LDL particles were linked to decreases in LDL cytotoxicity ($P=0.009$ and $P=0.090$, respectively). Finally, as observed in the principal component analysis (**Supplemental Figure 2**): 1) changes in LDL lag time and estimated LDL particle size were inter-related; 2) changes in the degree of LDL oxidative modifications, the triglyceride/cholesterol ratio in LDL particles, and the percentage of LDL proteins other than ApoB were associated, and probably inter-related to changes in LDL cytotoxicity; and 3) all these effects were independent from the changes in LDL-C and ApoB levels.

Values of the comparisons between post- and pre-intervention values, and between the changes in the TMD interventions relative to the low-fat diet for LDL atherogenic traits, are available in **Supplemental Tables 4** and **5**, respectively.

DISCUSSION

Our results indicate that a 1-year intervention with a TMD improves several LDL traits related to its atherogenicity (resistance against oxidation, size, composition, and cytotoxicity) in high cardiovascular risk individuals, particularly when the TMD is enriched with virgin olive oil. To the best of our knowledge, this is the first time that the

effect of a healthy dietary pattern on a complete set of LDL atherogenic properties has been studied in humans.

LDL oxidation is one of the most relevant biochemical events that leads to the formation of an atherosclerotic plaque [14]. Oxidized LDL particles are avidly phagocytized by macrophages which results in their transformation to foam cells [15], and they also induce cytotoxic responses in endothelial cells [16]. Although the causal relationship between LDL oxidation and atherosclerosis is still a controversial topic [17], increased oxidized LDL levels and high susceptibility of LDL lipoproteins to oxidation have been associated with greater cardiovascular risk in some clinical trials [18, 19], but not in an independent manner in others [20]. In our trial, the TMD (especially when enriched with virgin olive oil) augmented LDL resistance against oxidation and decreased the quantity of LDL oxidative modifications. Some of these effects have been previously observed after similar dietary interventions [9, 10]. As a possible explanation, TMD dietary antioxidants may bind to LDL or preserve other dietary antioxidants in the lipoprotein (e.g., vitamin E) in a non-oxidized state, increasing the resistance of the lipoprotein against oxidative attacks [21].

Small LDL particles are also more atherogenic [22]: they remain longer in circulation (they interact more poorly with LDL receptors), are more easily oxidized, and tend to traverse the endothelial barrier more than large ones [23]. Therefore, high concentrations of small LDL lipoproteins have been associated with a greater incidence of coronary heart disease [24]. In our trial, the TMD-VOO intervention increased estimated LDL particle size (measured as the LDL-C/ApoB ratio [12]), in agreement with the effects induced by other similar interventions such as the consumption of virgin olive oil [10] or adherence to a TMD enriched with nuts [8]. The improvement in the general oxidative status after the intervention could contribute to explaining this benefit, since pro-oxidative states are linked to an increased number of small LDL particles in circulation [25].

LDL composition affects the atherogenicity of the particle. On the one hand, cholesterol-poor, triglyceride-rich LDL particles are present in high cardiovascular risk states [26] and have been related to changes in ApoB conformation that hinder its binding to LDL receptors [27]. On the other hand, although 95% of LDL protein is ApoB, LDLs are known to be able to bind some proteins that may be detrimental (apolipoprotein C-III, pro-inflammatory proteins such as serum amyloid A4 and elements of the complement system, and pro-thrombotic proteins such as the fibrinogen α chain). Therefore, an increase in LDL protein content different from ApoB may be considered an indirect sign of a dysfunctional, pro-inflammatory, pro-thrombotic particle [28]. Moreover, the most atherogenic LDL (small, dense, electronegative) is also protein-rich [28]. According to our data, adherence to the TMD-VOO intervention made LDL particles cholesterol-rich (they carried more cholesterol per each ApoB molecule). In addition, the levels of proteins other than ApoB in LDL lipoproteins decreased after both TMD interventions and the low-fat diet. These two changes could have contributed to diminishing LDL atherogenicity.

Atherogenic LDL particles are toxic for some cell types: when macrophages phagocyte modified LDL lipoproteins, the cells begin to release pro-inflammatory signals and finally become foam cells [15]. In the present trial, the TMD-VOO intervention decreased LDL cytotoxicity in human macrophages. In this regard, an *in vitro* treatment with a flavonoid-rich extract has been previously reported to decrease the cytotoxic response induced by oxidized LDL on macrophages [13]. However, this is the first time that an intervention in humans has been able to decrease the *ex vivo* cytotoxicity of LDL particles. The improved oxidative status, estimated size, and composition of LDL lipoproteins after the intervention could help to explain this enhancement [14]. Nevertheless, the relevance of LDL *ex vivo* cytotoxicity in the development of cardiovascular outcomes remains to be elucidated in future trials.

According to our data, all the benefits of the TMD-VOO intervention on LDL atherogenic characteristics seemed inter-related (anti-atherogenic LDL traits were

356 associated among each other at baseline, as well as most changes after the TMD-VOO
357 intervention) and independent from LDL-C or ApoB quantity. This evidence supports
358 the hypothesis that adherence to a TMD (particularly when enriched with virgin olive
359 oil) may lead to the development of a less atherogenic LDL phenotype [29]. Although
360 not directly examined in this study, this phenotype could be partially responsible for
361 some of the cardioprotective benefits of the Mediterranean Diet.
362 Another general comment in this work could be the potentially deleterious effect of the
363 low-fat diet on characteristics beyond the lipid profile. Although this diet was able to
364 decrease the quantity of LDL-C in plasma, it reduced the estimated values of LDL
365 particle size (LDL-C levels decreased whilst ApoB levels did not, possibly leading to an
366 increase in the pro-atherogenic, cholesterol-poor, small LDLs) and also increased
367 remnant cholesterol levels (another lipid parameter associated with greater
368 cardiovascular risk [30]). These detrimental traits may contribute to explaining why
369 TMD is more cardioprotective than a low-fat diet, and could also highlight that,
370 regarding the lipid profile, quality may be more relevant than quantity.
371 Our study has various strengths. First, it presents a randomized design and involves an
372 active comparator (the low fat control intervention). Second, it comprises a large
373 sample size (*N*=210) and a long intervention duration (one year). Finally, it studies
374 comprehensively several LDL atherogenic traits and their interrelationships.
375 Nevertheless, the study also has limitations. The volunteers were elderly people with
376 high cardiovascular risk values; hence the extrapolation of our results to the general
377 population is complex. The results obtained were modest because: 1) the dietary
378 intervention in our trial is based on discreet lifestyle changes; and 2) the low-fat control
379 intervention is a well-known healthy diet. Finally, although the sample selection was
380 random, and the baseline characteristics of the three groups were comparable, they
381 varied modestly from the baseline characteristics of the whole PREDIMED Study
382 population. Differences among the changes observed in our results and other
383 PREDIMED Study sub-samples, particularly relative to lipid profile, could be due to the

longer duration of the intervention in our sub-group, and the varying proportion of patients with dyslipidemia. Nevertheless, to take into consideration all the possible confounders, we included age, sex, center, and changes in classical cardiovascular risk factors as co-variables in our multivariate linear regression analyses.

In conclusion, adherence to a TMD, particularly when enriched with virgin olive oil, decreased LDL atherogenicity (ameliorating LDL characteristics related to oxidation, estimated size, and composition) and LDL *ex vivo* cytotoxicity. These data reinforce the previous evidence regarding the healthy effects of the Mediterranean Diet, since the development of a less atherogenic LDL phenotype could be a possible explanation for some of the cardioprotective benefits of this dietary pattern.

AUTHOR CONTRIBUTIONS

A.H. and M.Fitó designed the experiments. A.H. performed the experimental work, interpreted the data, and drafted the manuscript. R.T. and M.C.L-S. contributed to the experimental development. A.H., O.C., A.G., and M.Fitó contributed in the search of funds and in the critical revision of the manuscript. E.R., X.P., R.E., J.S-S., D.C., F.A., M.A.M-G., M.Fiol, and J.L. contributed with biological samples and in the critical revision of the manuscript.

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CONFLICT OF INTEREST

Authors have no conflict of interest to declare for this research.

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FIGURE LEGENDS

Figure 1. Effects of the Traditional Mediterranean Diet enriched with virgin olive oil (TMD-VOO) or nuts (TMD-Nuts), relative to a low-fat diet, on LDL-C levels (**A-B**), ApoB concentrations (**C-D**), remnant cholesterol levels (**E-F**), and estimated LDL particle size (LDL-C/ApoB ratio) (**G-H**). **A,C,E,G**. Post- vs. pre-intervention changes (mean, 95% CI). **B,D,F,H**. Inter-treatment differences in a multivariate linear regression model adjusted for: age; sex; center of origin of the volunteer; baseline value of the variable; and changes in the presence of dyslipidemia, diabetes, hypertension, and smoking habit throughout the study (adjusted coefficient, 95% CI). *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Figure 2. Effects of the Traditional Mediterranean Diet enriched with virgin olive oil (TMD-VOO) or nuts (TMD-Nuts), relative to a low-fat diet, on the resistance of LDL particles against oxidation (LDL lag time) (**A-B**) and LDL oxidation (**C-D**). **A,C**. Post- vs. pre-intervention changes (mean, 95% CI). **B,D**. Inter-treatment differences in a multivariate linear regression model adjusted for: age; sex; center of origin of the volunteer; baseline value of the variable; and changes in the presence of dyslipidemia, diabetes, hypertension, and smoking habit throughout the study (adjusted coefficient, 95% CI). *: $P<0.05$; **: $P<0.01$.

Figure 3. Effects of the Traditional Mediterranean Diet enriched with virgin olive oil (TMD-VOO) or nuts (TMD-Nuts), relative to a low-fat diet, on the cholesterol content in LDL particles (**A-B**), and the percentage of LDL proteins other than apolipoprotein B (**C-D**). **A,C**. Post- vs. pre-intervention changes (mean, 95% CI). **B,D**. Inter-treatment differences in a multivariate linear regression model adjusted for: age; sex; center of origin of the volunteer; baseline value of the variable; and changes in the presence of

dyslipidemia, diabetes, hypertension, and smoking habit throughout the study (adjusted coefficient, 95% CI). *: $P<0.05$; **: $P<0.01$.

Figure 4. Effects of the Traditional Mediterranean Diet enriched with virgin olive oil (TMD-VOO) or nuts (TMD-Nuts), relative to a low-fat diet, on the cytotoxicity of LDL particles in macrophages (**A-B**). **A**. Post- vs. pre-intervention changes (mean, 95% CI). **B**. Inter-treatment differences in a multivariate linear regression model adjusted for: age; sex; center of origin of the volunteer; baseline value of the variable; and changes in the presence of dyslipidemia, diabetes, hypertension, and smoking habit throughout the study (adjusted coefficient, 95% CI). *: $P<0.05$.

547 **TABLES**

548

549 **Table 1.** Baseline characteristics of the volunteers in the three intervention groups.

550

VARIABLES	TMD-VOO *N*=71	TMD-Nuts *N*=68	Low-fat diet *N*=71	*P*-value
Age (years)	66.5 ± 6.34	65.1 ± 6.85	64.7 ± 6.58	0.270
Sex (% male)	45.1%	61.8%	47.9%	0.111
Body Mass Index (kg/m^2)	30.2 ± 3.96	29.2 ± 3.92	29.7 ± 3.98	0.386
Waist Circumference (cm)	99.8 ± 10.7	102 ± 10.2	101 ± 11.5	0.489
Leisure-time physical activity (MET·min/day)	156 (67.5-247)	169 (59.1-323)	150 (15.5-332)	0.782
Smoking status (% of smokers)	16.9%	11.8%	12.7%	0.642
Type 2 diabetes (% of diabetic patients)	76.1%	76.5%	84.5%	0.380
Hypertension (% of hypertensive patients)	47.9%	55.9%	38.0%	0.107
Dyslipidemia (% of dyslipidemic patients)	83.1%	77.9%	85.9%	0.458
Fasting glucose (mg/dL)	105 (92.5-127)	118 (96.0-140)	105 (94.0-128)	0.470
Triglycerides (mg/dL)	108 (90.7-157)	105 (73.0-147)	115 (97.0-140)	0.610
Total cholesterol (mg/dL)	206 ± 39.1	198 ± 35.9	210 ± 38.4	0.231
HDL cholesterol (mg/dL)	49.8 ± 11.8	49.2 ± 10.8	49.2 ± 10.6	0.932
LDL cholesterol (mg/dL)	129 ± 30.0	125 ± 30.1	135 ± 33.0	0.190
Apolipoprotein B (mg/dL)	104 ± 22.0	97.6 ± 17.1	105 ± 22.7	0.121
Apolipoprotein B/A-I ratio (unitless)	0.78 ± 0.16	0.75 ± 0.16	0.82 ± 0.22	0.123

Variables are expressed as percentages (categorical variables), means ± SD (normally distributed variables) or median (1^{st}-3^{rd} quartile) (non-normally distributed variables). *MET*: metabolic equivalent of task. *TMD-Nuts*: Traditional Mediterranean Diet enriched with mixed nuts. *TMD-VOO*: Traditional Mediterranean Diet enriched with virgin olive oil.