-Mediterranean Diet and telomere length in high cardiovascular risk subjects from the PREDIMED-NAVARRA study

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Abbreviations: BMI, Body mass index; EVOO, extra virgin olive oil; MeDiet, Mediterranean Diet; TL, telomere length; FFQ, food frequency questionnaire.

ABSTRACT

Background & Aims: A healthy lifestyle has been associated with longer telomeres, but whether Mediterranean Diet (MeDiet) affect telomere length (TL) has not been fully elucidated yet. Our aim was to assess the relationship between MeDiet and TL in high cardiovascular risk subjects in the context of a randomized nutritional intervention trial.

Methods: We assessed 520 participants (55-80 years, 55% women) from the PREDIMED-NAVARRA trial. Leukocyte TL was measured by qPCR at baseline and after 5 years of a dietary intervention program where subjects were randomly assigned to a low-fat control diet or to two MeDiets, one supplemented with extra virgin olive oil (MeDiet-EVOO) and the other with mixed nuts (MeDiet-nuts). A validated 14-item questionnaire was used to appraise baseline adherence of participants to the MeDiet.

Results: A greater adherence to MeDiet (14-item score) was associated with longer basal telomeres in women in the baseline cross-sectional analysis, whereas the opposite was observed in men (*P* interaction=0.036). Female subjects who scored 10 points had longer basal telomeres (0.27, 95% CI: 0.03 to 0.52) than women scoring ≤ 6 points at the beginning of the study (-0.46, 95% CI: -0.85 to -0.7) (P=0.003). However, allocation to the MeDiet-nuts group (-0.24, 95%CI: -0.38 to -0.01) was associated with a higher risk of telomere shortening after 5 years of intervention, whereas no differences were found for the MeDiet-EVOO group (0.14, 95%CI: 0.02 to 0.27), in comparison with the Control group (0.07, 95% CI: -0.08 to 0.23) (P=0.003 and P=0.537, respectively).

Conclusion: A greater baseline adherence to a Mediterranean dietary pattern was associated with longer telomeres only in women. No beneficial effect of the intervention

with the MeDiet for the prevention of telomere shortening in comparison with a low-fat diet was observed.

INTRODUCTION

The Mediterranean diet (MeDiet) has been widely considered as a model of healthy eating [1]. The traditional pattern is characterised by the daily use of olive oil as the principal fat; an abundant consumption of fruits and vegetables, nuts, non-refined grains and legumes; moderate to high consumption of fish and poultry; moderate-to-low intake of dairy products mostly from fresh cheese and yogurt; moderate alcohol mostly in the form of red wine in meals, and a less frequent consumption of red meat and meat products [2]. Numerous epidemiological studies have explored the health benefits of the MeDiet and evidence consistently shows that individuals who adhere to this dietary pattern have healthier ageing and a longer life span, including a better cardiovascular risk profile [3, 4].

Telomeres are nucleoprotein structures that protect the end of chromosomes maintaining genome stability [5]. These telomeres, which are tandem TTAGGG repeats of DNA, are also considered biomarkers of aging since they become shorter in each cell division, indicating that the older an individual gets the shorter their telomeres are [6]. However, it has been reported that lifestyle factors such as diet or physical activity could have an important role in modulating telomere shortening [7]. Given that shorter telomeres have been observed in patients with chronic diseases and accelerated telomere erosion is associated to increased risk of developing age-related pathologies [8], it is very important to identify environmental factors that could lessen telomere attrition. Therefore, elucidating whether telomere length (TL) is a potential biomarker for predicting risk of age-related diseases or for evaluating the effects of the diet could be of high interest. However, it is not clear whether TL is itself causal or a biomarker of underlying disease related mechanisms [8].

Chronic oxidative stress and inflammation have been reported as the main underlying mechanisms responsible for telomere shortening [9]. In this context, the health effects of MeDiet seem to support anti-inflammatory and antioxidant properties, among others [10]. Indeed, some works have already shown the beneficial effect of the MeDiet on TL [11-15]. Marin *et al.* [11] reported that a 4-week intervention with a MeDiet (enriched in MUFA by virgin olive oil) prevented telomere shortening of endothelial cells in 20 elderly subjects. The cross-sectional study by Boccardi *et al.* [12] suggested that longer telomeres and a higher telomerase activity was associated with better adherence to a traditional MeDiet in 217 elderly subjects. Similarly, Crous-Bou *et al*. [13] reported that a greater adherence to the MeDiet was associated with longer telomeres in 4676 disease-free women belonging to the Nurses' Health Study. Similarly, Gu *et al*. [14] showed that higher adherence to the MeDiet was associated with longer telomeres among whites but not among African Americans and Hispanics in a third cross-sectional study conducted in 1743 multi-ethnic community residents of New York aged 65 years or older. Moreover, Garcia-Calzón *et al*. [15] also with data of the PREDIMED-NAVARRA study found that higher adherence to the MeDiet pattern contributed to the prevention of telomere shortening after 5 years among Ala carriers of the Pro12Ala polymorphism of *PPARG2* gene. So far, as far as we know, the PREDIMED-NAVARRA study is the only work with a longitudinal design and repeated measurements of telomeres that has been conducted in high cardiovascular risk subjects and with a long follow-up period.

However, more studies are needed to further confirm this remarkable finding. Therefore, the main aim of the present investigation was to assess the association between the Mediterranean dietary pattern and TL in high cardiovascular risk subjects from the PREDIMED-NAVARRA trial. Thus, we hypothesized that a high adherence to

the MeDiet would be related to longer telomeres, both at the beginning of the study and after 5 years of the nutritional intervention.

MATERIAL AND METHODS

Study design

This study has been conducted within the frame of the PREDIMED trial. The design and methods of this trial have been reported in a specific publication [16]. The PREDIMED study is a large, parallel-group, multicentre, randomized, controlled, clinical trial designed to assess the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. Participants were randomly allocated to one of three arms: Mediterranean diet supplemented with extra virgin olive oil (MeDiet-EVOO), Mediterranean diet supplemented with mixed nuts (MeDiet-nuts) or a control group (low-fat diet). Further details are also available at www.predimed.es.

The study population was composed of women (60 to 80 years) or men (55 to 80 years) with no previously documented history of cardiovascular disease, but at high cardiovascular risk. Inclusion criteria were either type-2 diabetes mellitus or at least three of the following major cardiovascular risk factors: current smoking, hypertension, elevated low-density lipoprotein cholesterol, low high-density lipoprotein cholesterol, overweight/obesity or family history of premature coronary heart disease.

All subjects provided informed consent and the protocol was approved by the institutional review boards according to the Principles of Helsinki Declaration. This trial is registered at http://www.controlled-trials.com/ISRCTN35739639.

The present analysis deals with a subsample from one of the eleven recruitment centres (PREDIMED-NAVARRA). The PREDIMED-NAVARRA recruitment centre included

1055 of the 7447 subjects participating in the trial, being the first of the eleven centres to complete the enrolment of participants (June 2003 – May 2005). For this work, 520 participants with available DNA and information at baseline and after 5 years of the nutritional intervention were included (Supplementary figure 1). Unfortunately, we lack of some data and DNA samples after 5 years of several subjects which explains the loss of participants. The lower number of subjects in the control group in comparison with the other groups observed in our sub-study reflects the higher drop out in the control group found in the PREDIMED trial, probably because participants did not receive food incentives.

Dietary assessment

Participants completed a validated 14-item questionnaire to evaluate the adherence to a Mediterranean dietary pattern at baseline [17, 18]. The higher the score is, the higher the adherence to the MeDiet. This questionnaire is less time-demanding, less expensive and requires less collaboration from participants than the usual full-length food frequency questionnaire (FFQ). The 14-item tool was developed in a Spanish case-control study of myocardial infarction, where the best cut-off points for discriminating between cases and controls were selected for each food or food group. With this first step, 9 of the 14 items were obtained. Five additional items that were felt to be especially relevant to assess adherence to the traditional MeDiet were subsequently added. Two of these items used short questions to inquire on food habits and the other three items inquired on frequency of consumption of nuts, soda drinks and a typical Mediterranean sauce (sofrito). This 14-item tool is a key element in the intervention conducted in the PREDIMED trial. It is worth mentioning that within the PREDIMED study a FFQ was also used for evaluating the dietary intake of the participants. The adherence to the

MeDiet at baseline, assessed with the 14-item questionnaire, was independent of group assignment.

The individuals were randomized to three groups of intervention. The two groups allocated MeDiets received intensive education to follow the MeDiet and supplemental foods at no cost. EVOO (1L/week) was provided to the first group and 30 g/day of mixed nuts to the second group. Participants in the control group were advised to follow a low-fat diet according to the American Heart Association's guidelines and also received other gifts for their attendance to the formative sessions. All the groups had a good adherence to the intervention, according to changes in the MeDiet adherence score (14-item), and changes in extra virgin olive and nuts consumption after 5 years (Supplementary table 1).

Telomere length assessment

Genomic DNA was extracted from peripheral blood samples (white blood cells) obtained at baseline and after 5-years follow up for each subject. TL was measured in both DNA samples per subject with a real-time quantitative PCR approach [19, 20]. This method expresses TL as a T/S ratio calculated as $2^{-\Delta CT}$ with the following equation $[2^{CT(telomeres)}/2^{CT(single\ copy\ gene)}] = 2^{-\Delta CT}$. A Ribosomal Protein Large PO (RPLPO) single-copy gene was used as a reference for each sample.

The total reaction volume was 10 μL containing 10 ng of genomic DNA. PCRs for telomere and single copy gene expression were performed on white 384-well plates on an ABI-Applied Biosystems 7900 HT thermal cycler (Applied Biosystems, CA, USA). The final telomere primer concentrations were as follows: for telomere amplification tel1, 675 nmol/L and for tel2, 1350 nmol/L; and for the amplification of the single copy gene RPLPO: hRPLPO1, 800 nmol/L; hRPLPO2, 800 nmol/L. The primer sequences

were tel1 (5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3'), tel2 (5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'), hRPLPO1 (5'-CCCATTCTATCATCAACGGGTACAA -3') and hRPLPO2 (5'-CAGCAAGTGGGAAGGTGTAATCC -3'). All primers were purchased from Sigma-Aldrich, St.Louis, MO, USA. QuantiTect Syber Green PCR kit (Qiagen, Valencia, CA, USA) was used as master mix.

A calibration curve (64-0.25 ng in 2-fold dilutions) was included as a standard for each measurement to control the day-to-day variations. Standard curve with linearity $R^2>$ 0.98 was accepted. For quality control, all samples were run in duplicate and checked for concordance between duplicate values. For obtaining stronger consistency, samples showing a high variation (more than 10%) were rerun and reanalysed. Intra-assay coefficient between duplicates was 3.0% for telomere and 2.6% for the single copy gene, whereas the inter-assay between plates was 0.8% for telomere and 1.3% for the single copy gene. In addition, the simple correlation coefficient for TL measurement between duplicates was 0.893 and between plates was 0.997. Regarding the single copy gene, the correlation coefficient between duplicates was 0.922 and between plates was 0.998.

Confounder assessment

Participants were interviewed at baseline by a dietician, obtaining information about lifestyle, diet and incident diseases. Information about medical conditions or new medical diagnosis of illnesses was collected at baseline. Lifestyle variables such as smoking habit or physical activity (assessed through the validated Minnesota leisure time physical activity questionnaire) [21] were also collected at baseline. Professional nurses measured anthropometric variables following standardized protocols.

Statistical analyses

All statistical tests were two-tailed and a *P* value <0.05 was considered statistically significant. We used STATA® version 12.0 (Stata Corp) for all the analyses.

We calculated z-scores of log transformed leukocyte TL adjusted for age in order to meet the assumption of normality [7, 13]. Briefly, we used generalized linear regression to calculate least-square means of log transformed TL z-score adjusted for age. Z-scores were calculated by standardizing leukocyte TL in comparison with the mean within each individual study, considering the age of the participants. At baseline, means and standard deviations (SD) or percentages for each variable according to the group of intervention were shown, and we assessed the statistical significance of the differences among them with ANCOVA models and χ^2 tests, respectively.

Baseline assessment (14-item questionnaire)

The likelihood ratio test was used to examine the interaction between sex and the adherence to the MeDiet for age-adjusted z-score TL. ANCOVA models and linear trend tests were conducted to assess the association between TL and five categories of baseline adherence to the MeDiet (≤6, 7-8, 9, 10 and ≥11 points of the 14-item questionnaire) [17, 22] in analyses stratified by sex. We also used multivariable-adjusted logistic regression to estimate odds ratios (OR) for having short telomeres (age-adjusted z-score TL ≤ 20th percentile) [23-25] for subjects with higher baseline levels of adherence to the MeDiet (7-8, 9, 10 or ≥11 in the 14-item score) *versus* those with lower adherence (≤6 points, reference category) in separated models for females and males. We also used the 14-item score as a continuous variable evaluating the risk of low TL for each two-point additional increment in this score.

For these models, the following potential confounders measured at baseline were considered: age, body mass index (BMI) (kg/m^2), total energy intake (Kcal/day), physical activity (METS-min per day), smoking status (current, former or never smoking), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous) and intervention group assignment (MeDiet-EVOO, MeDiet-nuts and Control group).

<u>5-year change assessment (groups of intervention)</u>

An ANCOVA model was used to evaluate telomere shortening after 5 years follow-up (z-score of log (T/S ratio) after 5 years - log (T/S ratio) at baseline) according to the three groups of intervention. Furthermore, logistic regression models were performed to assess the risk for telomere shortening (Δ age-adjusted z-score TL $\leq 20^{th}$ percentile).

For assessing changes in TL, the following potential confounders measured at baseline were considered: age, sex, body mass index (BMI) (kg/m^2), total energy intake (Kcal/day), adherence to the MeDiet (points), physical activity (METS-min per day), smoking status (current, former or never smoking), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous) and TL.

RESULTS

A significant inverse association was observed between TL and age at baseline (r=-0.104, P=0.018, data not shown). Table 1 shows baseline characteristics of the 520 participants by group of intervention. No significant differences were found for age, sex, BMI, smoking or the presence of chronic diseases among the three groups. However, subjects in the Control group had lower total energy intake and adherence to the MeDiet, as well as lower physical activity levels than individuals allocated to the MeDiet groups. It should be mentioned that at the beginning of the study none of the

participants was encouraged to stick to a diet. Unexpectedly, longer baseline telomeres were observed in the MeDiet-EVOO group compared to the other subject groups. Since we could only measure TL in a subsample from the PREDIMED-NAVARRA study, the randomization was not able to balance all baseline characteristics, as it would be expected with a sample size that was not very large. Therefore, we adjusted all our estimates for these imbalances.

A higher baseline adherence to the MeDiet (14-item questionnaire) was associated with a greater age-adjusted z-score TL but in women and not in men (P interaction=0.036). We assessed differences in TL across categories of adherence to the MeDiet by sex (Supplementary figure 2). Notably, those women who scored 10 points in the 14-item questionnaire had longer telomeres than females scoring ≤ 6 points after adjusting for potential confounders (P=0.003). No significant differences (P_{ANCOVA}= 0.979, P trend= 0.550) were observed in men, though the slope was similar in magnitude to that of women but it was in the opposite direction.

We also found (only among women) an association between a higher baseline adherence to the MeDiet (14-item questionnaire) and a lower risk of having short telomeres (age-adjusted z-score TL $\leq 20^{th}$ percentile) in women at the beginning of the study (Table 2). Taking as reference $\leq$6 points (OR=1), significantly lower *odds* for short telomeres was found for women scoring $\geq$11 points (OR 0.29, CI: 0.08 to 0.99) in fully-adjusted models.

Analyses were performed to investigate the risk of telomere shortening after 5 years of the randomized-clinical trial according to the three intervention groups (EVOO, mixed nuts and Control). Surprisingly, those subjects allocated to the MeDiet-EVOO group showed no beneficial effect in telomere erosion in comparison with the Control group

after 5 years of the intervention, whereas a detrimental effect in telomere shortening was observed in the MeDiet-nuts group (P_{ANCOVA} <0.001; Figure 1). Taking as reference the Control group (OR=1), the OR for telomere shortening (Δ age-adjusted z-score TL $\leq$ 20th percentile) was 1.28 (95% CI, 0.67 to 2.44) among subjects in the MeDiet-EVOO group and 3.19 (1.73 to 5.90) in those individuals allocated to the MeDiet-nuts nutritional intervention (Table 3). In these longitudinal analyses, no differences by sex were found (Supplementary table 2) and no association between changes in MeDiet (14-item) and telomere shortening according to sex was observed (Supplementary figure 3)Moreover the covariates in these models with their estimates on telomere shortening (Δ age-adjusted z-score TL $\leq$ 20th percentile) are shown in Supplementary table 3. It should be mentioned that a significant inverse correlation (r=0.567, p<0.001) was found between log T/S values of TL timepoint 1 and timepoint 2 (after 5 years). For age-adjusted values, Pearson correlation was r=0.561, p<0.001 (data not shown).

DISCUSSSION

We did find a cross-sectional association between baseline MeDiet adherence and TL in women from the PREDIMED-NAVARRA study. A higher adherence to a Mediterranean dietary pattern was related to longer telomeres in 285 women at high cardiovascular risk. Nevertheless, regarding telomere attrition after 5 years, the intervention with MeDiet was not apparently able to prevent the rate of telomere shortening in comparison with a low-fat diet.

It is shown that dietary components and the overall healthy eating pattern could have a potential influence on TL [26]. Whereas a higher consumption of healthy food has been associated with longer telomeres [27-29], unhealthy dietary habits may have the opposite effect [30]. For example, a high consumption of fruit, vegetables [28, 29]

dietary fibre [27] or antioxidants [20] have been correlated with longer telomeres and on the contrary an increase intake in red meat [30] or alcohol [7] may lead to shortening the telomere. Unfortunately, inconsistent results have been found between TL and specific dietary components. This inconsistency is also present in our results.

In any case, our observed association between baseline adherence to the MeDiet and TL (though it was only apparent among women) is consistent with previous research. Accordingly, a study conducted in 217 Italian elderly subjects found that individuals with a high adherence to the MeDiet, scoring ≥6 points in Trichopoulou score, had not only longer telomeres but also a higher telomerase activity compared to others [12]. The recent work carried out by Gu *et al.* [14] reported that the association between MeDiet and TL varied by ethnicity but it is not modified by sex. Thus, among 506 non-Hispanic whites it seems to be a positive association between MeDiet and TL after controlling for some covariates (β=0.48, *P*=0.050), but this tendency was lost when further adjusting for more potential confounders. In line with our findings, Crous-Bou *et al.* [13] found a relationship between MeDiet and TL in a large sample of women from the Nurses' Health Study, indicating that for each one point change in the Alternate MeDiet score corresponded on average to 1.5 years of aging. Similarly, our study found the beneficial role of the MeDiet on TL in women and not in men. In this context, showing that women have longer telomeres than men [31], and also gender differences between leukocyte TL have been observed for several cell subsets [32]. The latter could be a possible explanation for the observed sex-effect on the association between MeDiet and TL. But, as far as we know, there are no studies assessing the sex effect on the relationship between a dietary pattern and TL. Additionally the assessment of the association within subgroups may lack enough statistical power in our study and a larger sample size would be probably needed to detect a significant relationship. When

analysing key components of the MeDiet separately, such as extra virgin olive oil and nuts, there is not an apparent association between these individual dietary items and TL at baseline according to sex (Supplementary table 4). This suggests that in our population, the MeDiet as a whole is the relevant factor that showed a beneficial cross-sectional association with basal TL in women.

It has been suggested that this positive effect of the MeDiet on TL could be due to a reduction in systemic oxidative stress and inflammation [13], which might modulate telomerase activity, telosome expression and/or DNA oxidation and mechanical fragmentation [33].

Only two observational studies, but no trial, have previously examined the longitudinal association between the MeDiet and TL shortening. Both previous observational studies reported a beneficial effect of the MeDiet on the rate of telomere shortening [11, 15]. The results of our intervention are apparently no consistent with them. But caution is needed when interpreting this discrepancy since in one of the previous studies only 20 elderly subjects were included [11] and the other study only found this association in subjects who had a specific genotype, suggesting that genetic background may modulate the relationship between the MeDiet and TL [15]. It is also possible that, due to its sample size, our trial might not have sufficient statistical power as to detect a small benefit of the MeDiet in the prevention of telomere shortening. In any case, the paradoxical results that we found in the group allocated to MeDiet-nuts were largely unexpected. Due to the scarce literature on the association between the adherence to MeDiet and telomere erosion after a follow-up period, in the present work we wonder whether in the frame of the PREDIMED trial we could shed light into this matter. However, we did not find a beneficial effect of the MeDiet on telomere attrition after 5 years in volunteers allocated to both MeDiets. Unexpectedly though, a detrimental

effect was found in preventing telomere shortening in the MeDiet-nuts groups. Although we do not have any explanation for the latter, it is worth pointing out that all the individuals lived in a MeDiet country with a long and established tradition of following a Mediterranean dietary pattern. Indeed, we assumed that subjects who had a higher adherence to the MeDiet at baseline were those with a lifelong exposure to the traditional Mediterranean dietary pattern. Thus, we thought that lifelong dietary habits can be more important in determining TL than 5 years of dietary intervention, because the effectiveness of such an intervention in the long-term may be affected by suboptimal compliance with the intervention among a subset of participants. In addition, despite the fact that the two MeDiet intervention groups significantly increased the adherence to MeDiet after 5 years reaching 11 points in the 14-item questionnaire, the Control group also increased the adherence to MeDiet according to the 14-item questionnaire (from 8.5 to 9.2 points, $P<0.001$), thus minimising differences between groups. Moreover, the low-fat diet planned in the Control group was based on the American Heart Association guidelines which has been demonstrated to be a healthy diet reducing the risk of developing cardiovascular disease and therefore mortality [34]. Notably, following a low-fat diet has also been associated with longer telomeres [29].

On the other hand, we did not find any sex-specific effect on telomere shortening after 5 years, nor did we find any differences between sexes within the three groups of intervention. Therefore, we could not state that sex affects telomere shortening, but it plays an important role on TL at baseline. A possible explanation for these surprising findings could be that the PREDIMED intervention trial modulated telomere attrition making the differences between men and women smaller after 5 years.

The strengths of our study include the reproduction of real-time conditions with home-prepared food, multiple-adjusted models to minimise potential confounders that could

affect TL and a validated 14-item questionnaire which it is easier for participants to fill in rather than the full-length FFQ. On the other hand, this study has several limitations. The main limitation is the cross-sectional nature of most of our significant findings. Moreover, these potential benefits found in our cross-sectional analyses were only restricted to women. We have controlled the experimental conditions in the measurement of TL to avoid potential errors, and thus we have obtained low CV and higher correlation coefficients between duplicates and plates. Genomic DNA used along this project had been processed following a standardized protocol and aliquoted to preserve its stability; the two DNA samples of each patient (at baseline and after 5 years of recruitment) were run in the same plate; in each 384-well plate, as a standard calibration curve, different amounts of a genomic DNA sample of reference were included to control the plate-to-plate variations; and finally, in a small subset of individuals, we had previously checked that the quantification of telomeres by PCR (T/S ratio) did perfectly correlate with telomeric restriction fragment length determined by Southern blot analysis. The use of a self-reported questionnaire in the dietary assessment may involve a possibility of measurement error leading to misclassifications of exposure. However, a registered dietician helped to collect the dietary information through individual sessions and the 14-item MeDiet questionnaire was previously validated and used in other studies [18]. Therefore, this 14-item questionnaire correlated significantly with the corresponding FFQ PREDIMED score (r = 0.52; intraclass correlation coefficient = 0.51) and in the anticipated directions with the dietary intakes reported on the FFQ. Using Bland Altman's analysis, the average MeDiet score estimate was 105% of the FFQ PREDIMED score estimate. Furthermore, it should be taken into account that a 5-year period in a population aged between 55 to 80 years (when the rate

of telomere attrition is very low), might not be enough to detect any changes in TL [35, 36]. At the same time, the sample size might be too small to find any changes.

In conclusion, a higher baseline adherence to the MeDiet is associated with longer telomeres only in women from the PREDIMED-NAVARRA trial. However, no beneficial effect in lessening telomere erosion after 5 years was observed with a MeDiet in comparison with a low-fat diet.

Statement of authorship

The authors' contributions were as follows: SG contributed to the data collection, performed the experiments and the statistical analyses and wrote the manuscript; CR performed experiments and interpretation of the data. AmM, M.A.M, F.A, J.L and J.A.M were responsible of the follow-up, design, financial management and editing of the manuscript. All the authors actively participated in the manuscript preparation, as well as revise and approved the final manuscript.

Conflict of Interest Statement

None declared.

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FIGURE LEGEND

Figure 1. Multivariable-adjusted differences (95% CI) in changes in telomere length z-score after 5 years, according to the group of intervention. Adjusted for sex, initial z-score telomere length, total energy intake (kcal per day), BMI (kg/m^2), adherence to the MeDiet (points), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous). $P_{ANCOVA} < 0.001$. P=0.003 (MeDiet-Nuts *vs.* Control), P=0.537 (MeDiet-EVOO *vs.* Control)
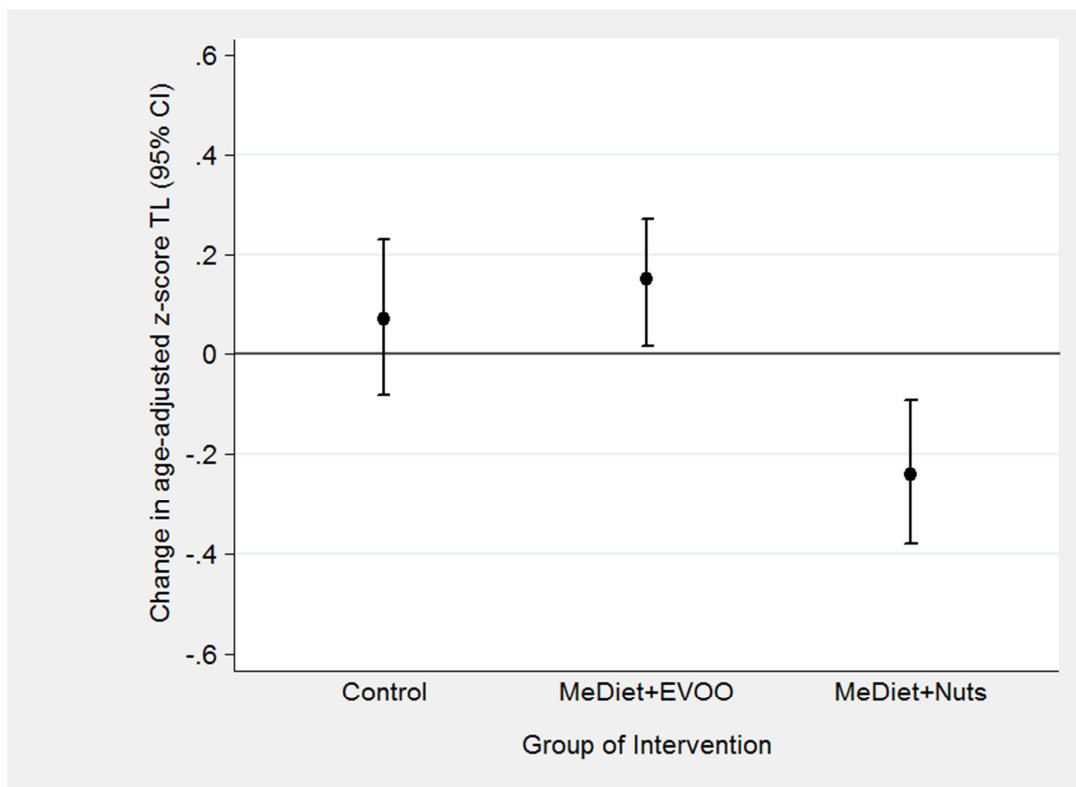
Table 1. Baseline characteristics of the participants according to the group of intervention

	MeDiet- EVOO	MeDiet- Nuts	Control	*P*-value
n	210	170	140	
Age, years	67.0 (5.9)	66.8 (6.1)	67.4 (5.9)	0.611
Sex, % males	42.9	51.2	41.4	0.158
BMI	29.1 (3.3)	29.1 (3.1)	29.2 (3.4)	0.966
Physical activity, METS-min/day	282.9 (193.0)	301.1 (207.0)	239.2 (182.5)	0.018
Energy intake, kcal/day	2269.9 (541.4)	2314.2 (482.5)	2140.8 (524.3)	0.011
Adherence to the MeDiet, points	9.3 (1.8)	9.6 (1.7)	8.5 (1.9)	<0.001
Smoking				0.623
Current smokers, %	12.9	14.1	18.6	
Former smokers, %	22.9	24.1	20.0	
Dyslipidaemia, %	66.7	66.5	65.0	0.944
Hypertension, %	80.0	85.9	85.0	0.272
Diabetes, %	37.1	40.6	31.4	0.247
TL, age-adjusted z-scores	0.4 (1.1)	-0.3 (0.8)	-0.3 (0.7)	<0.001

The table shows means and standard deviations for continuous variables or percentages for categorical variables. Abbreviations: BMI, body mass index; MeDiet, Mediterranean Diet; EVOO, extra virgin olive oil; TL, telomere length.

Table 2. Multivariable-adjusted odds ratios (OR, 95% confidence intervals) for short telomeres (age-adjusted z-score TL $\leq 20^{th}$ percentile) by adherence to the Mediterranean Diet in women and men.

| | Adherence to Mediterranean diet (0 to 14 point score) | | | | | | |
	≤ 6	7-8	9	10	≥ 11	P-trend	For +2 points
Women (n)	**25**	**78**	**57**	**62**	**63**		
Crude	1 (Ref.)	0.68 (0.25 to 1.83)	0.51 (0.17 to 1.48)	0.51 (0.18 to 1.46)	0.26 (0.08 to 0.84)	0.017	0.68 (0.49 to 0.94)
Model 1	1 (Ref.)	0.71 (0.26 to 1.92)	0.55 (0.19 to 1.61)	0.57 (0.20 to 1.66)	0.32 (0.09 to 1.06)	0.059	0.73 (0.52 to 1.02)
Model 2	1 (Ref.)	0.60 (0.21 to 1.69)	0.44 (0.14 to 1.40)	0.51 (0.17 to 1.56)	0.29 (0.08 to 0.99)	0.059	0.71 (0.49 to 1.02)
Men (n)	**19**	**57**	**40**	**57**	**62**		
Crude	1 (Ref.)	2.51 (0.51 to 12.32)	2.83 (0.55 to 14.46)	2.51 (0.51 to 12.32)	1.44 (0.28 to 7.34)	0.877	0.98 (0.70 to 1.36)
Model 1	1 (Ref.)	2.53 (0.51 to 12.45)	2.85 (0.56 to 14.65)	2.51 (0.51 to 12.30)	1.44 (0.28 to 7.35)	0.876	0.98 (0.70 to 1.36)
Model 2	1 (Ref.)	3.17 (0.62 to 16.30)	3.49 (0.65 to 18.75)	3.47 (0.66 to 18.07)	2.13 (0.39 to 11.67)	0.673	1.09 (0.77 to 1.56)

Crude: adjusted for age (z-score TL).

Model 1: adjusted for total energy intake (kcal per day).

Model 2: additionally adjusted for BMI (kg/m^2), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous) and group of intervention.
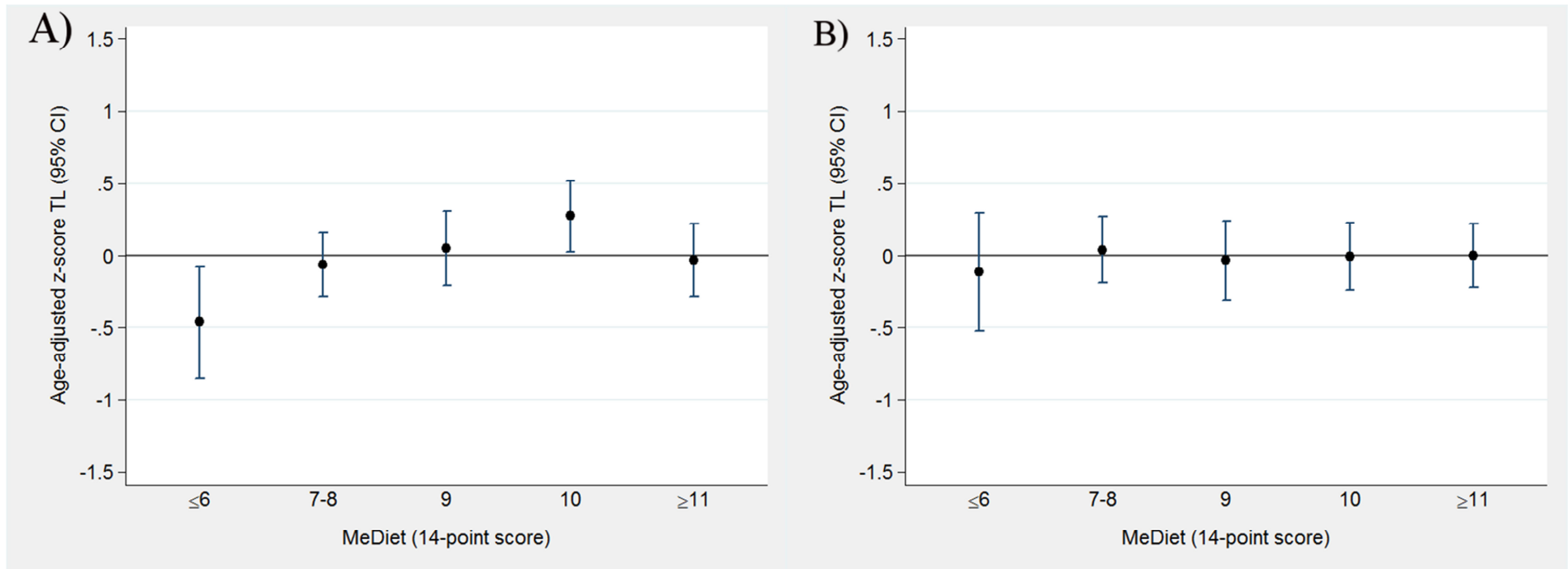
Supplementary figure 1. Baseline age-adjusted z-score TL by baseline adherence to the Mediterranean diet (14-item score) in A) women and B) men after adjusting for total energy intake (kcal per day), BMI (kg/m^2), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous) and group of intervention. A) P_{ANCOVA}= 0.033 (P= 0.019 for the comparison 10 points *vs.* ≤6 points categories after Bonferroni correction), *P* trend= 0.064; B) P_{ANCOVA}= 0.979, *P* trend= 0.550.

Table 3. Risk for telomere shortening (Δ age-adjusted z-score TL ≤ 20th percentile) after 5 years of the nutritional intervention according to the nutritional groups.

	OR for telomere shortening after 5 years follow-up		
	Crude	**Model 1**	**Model 2**
Control	1 (Ref.)	1 (Ref.)	1 (Ref.)
MeDiet-EVOO	0.67 (0.38 to 1.21)	1.23 (0.65 to 2.32)	1.28 (0.67 to 2.44)
MeDiet-Nuts	2.45 (1.44 to 4.16)	2.95 (1.65 to 5.29)	3.19 (1.73 to 5.90)

Model 1: adjusted for sex and initial z-score TL

Model 2: adjusted for sex, initial z-score TL, total energy intake (kcal per day), BMI (kg/m^2), adherence to the MeDiet (points), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), and dyslipidaemia status (dichotomous).

Supplemental material
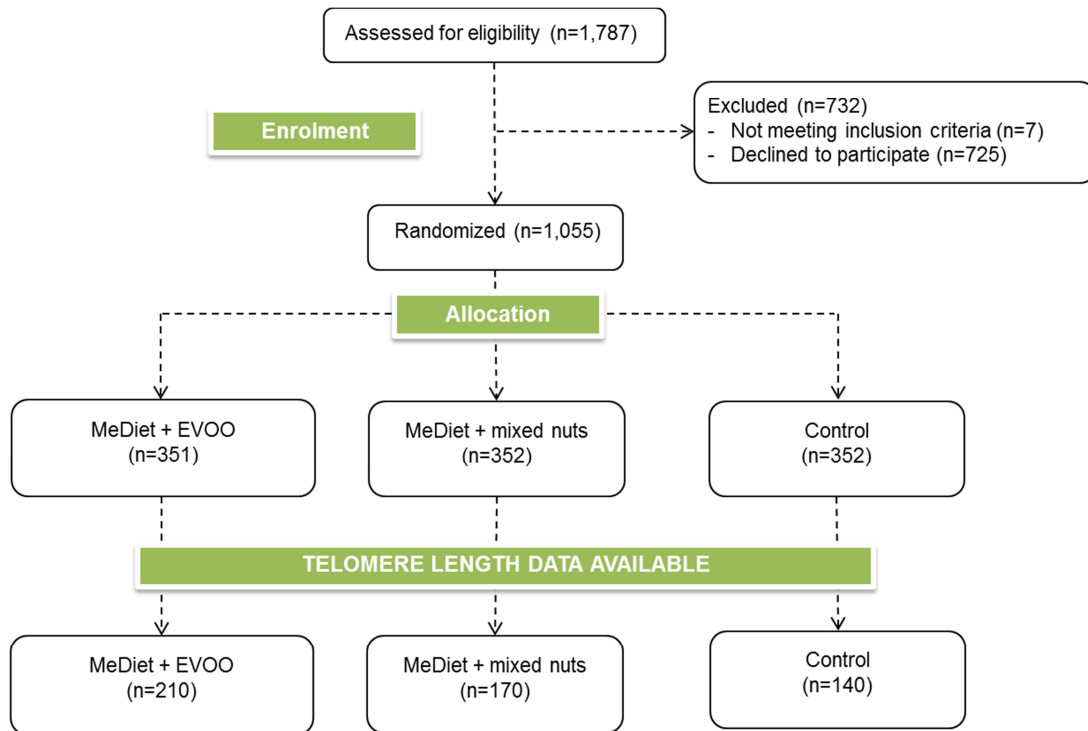
**Supplementary figure 1.** Flow-chart of participants in the PREDIMED-NAVARRA.

Supplementary table 1. Changes in adherence to the Mediterranean dietary pattern (14-item questionnaire) and in the consumption of extra virgin olive oil and nuts after 5 years of the dietary intervention according to the three groups of intervention.

	Between-group relative differences in MeDiet adherence and in the consumption of EVOO or nuts after 5-year follow-up		
	Δ MeDiet adherence (14-item questionnaire)	Δ Extra virgin olive oil (FFQ questionnaire)	Δ Mixed nuts (FFQ questionnaire)
MeDiet-EVOO	1.82 (95%: 1.47 to 2.17)	0 (Ref.)	-5.60 (-8.19 to -3.00)
MeDiet-Nuts	1.89 (95% CI: 1.53 to 2.24)	-5.42 (-9.06 to -1.77)	0 (Ref.)
Control	0 (Ref.)	-17.92 (-21.80 to -14.03)	-14.94 (-17.77 to -12.11)

Adjusted for sex, initial z-score telomere length, total energy intake (kcal per day), BMI (kg/m^2), adherence to the MeDiet (points), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous).
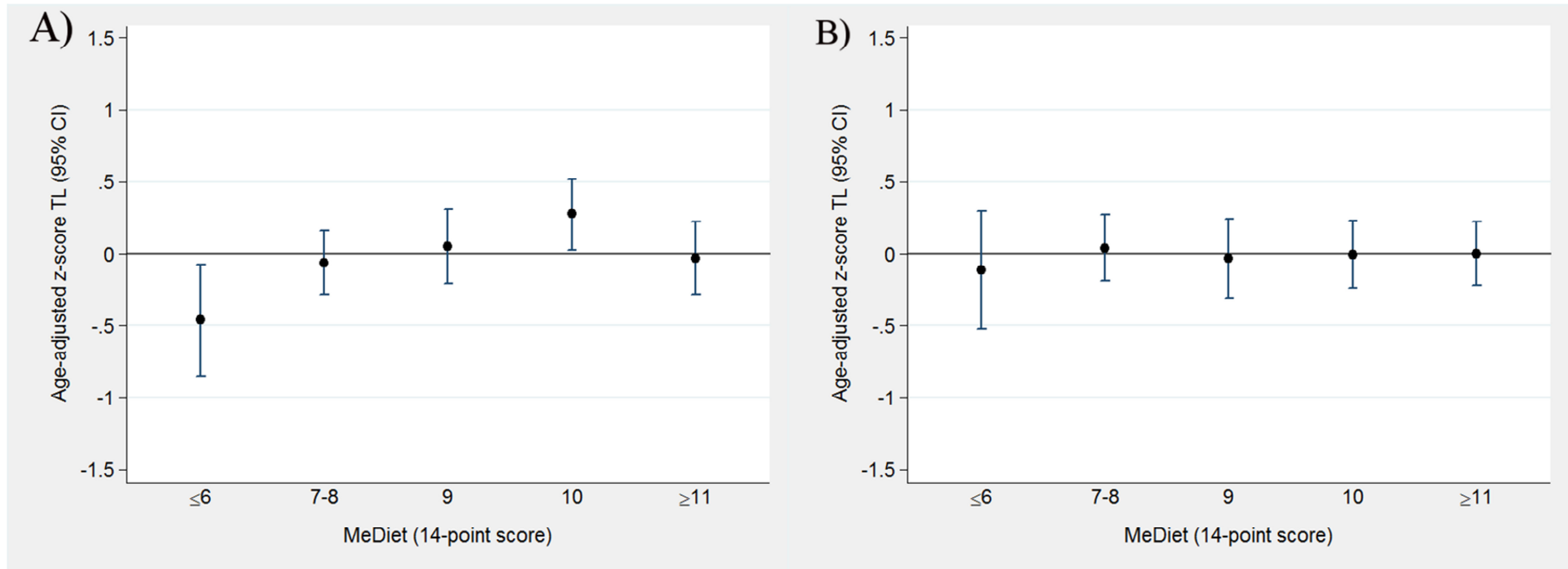
Supplementary figure 2. Baseline age-adjusted z-score TL by baseline adherence to the Mediterranean diet (14-item score) in A) women and B) men after adjusting for total energy intake (kcal per day), BMI (kg/m^2), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous) and group of intervention. P for interaction (sex x MeDiet) =0.036. A) P_{ANCOVA}= 0.033 (P= 0.019 for the comparison 10 points vs. ≤6 points categories after Bonferroni correction), P trend= 0.064; B) P_{ANCOVA}= 0.979, P trend= 0.550.
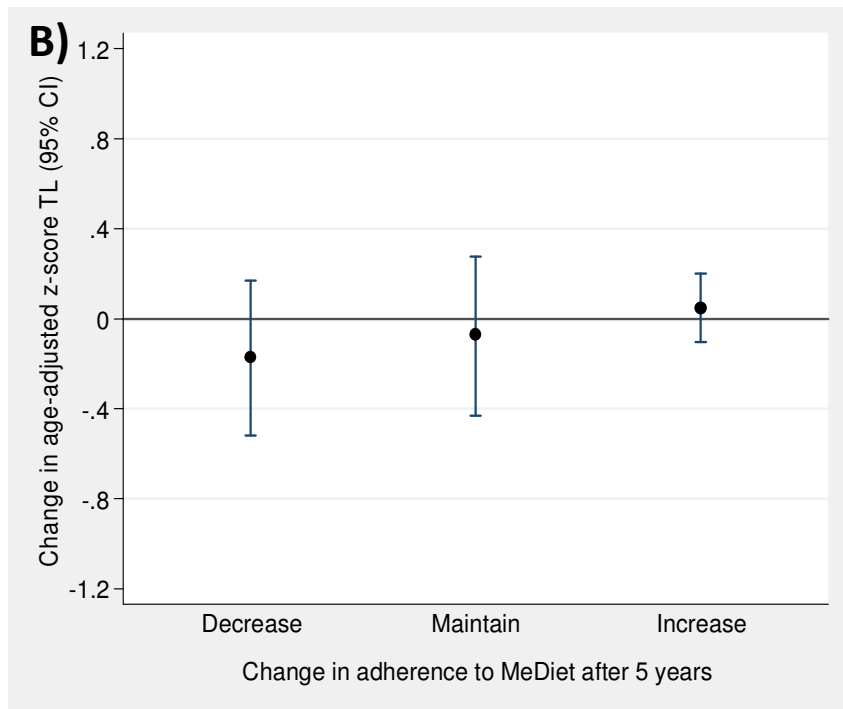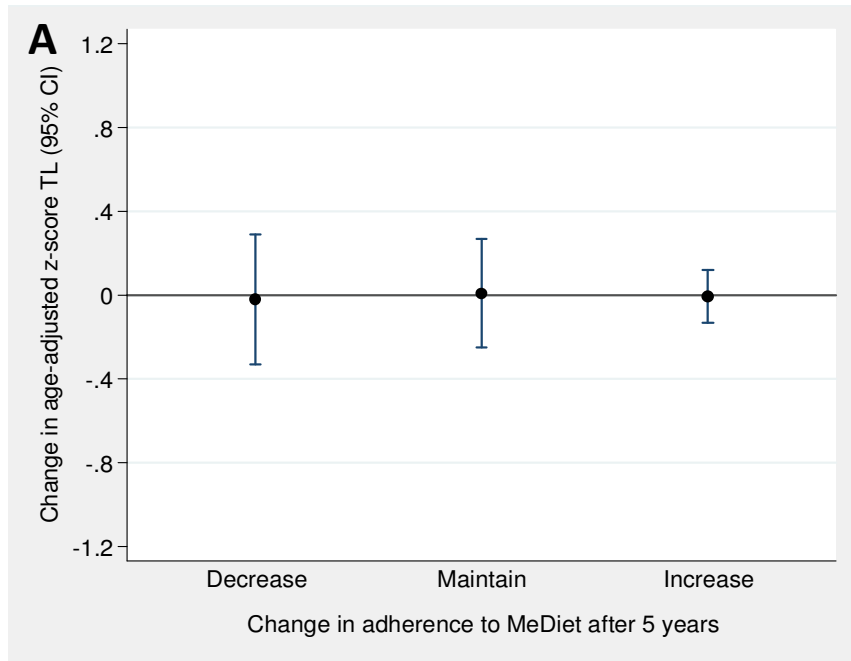
Supplementary figure 3. Multivariable-adjusted differences (95% CI) in changes in telomere length z-score after 5 years, according to the changes in the adherence to the MeDiet in A) women and B) men. Adjusted for sex, initial z-score telomere length, total energy intake (kcal per day), BMI (kg/m2), baseline adherence to the MeDiet (points),

physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous).

Supplementary table 2. Changes in telomere length between men and women after 5 years within each group of intervention.

	Change in age-adjusted z-score TL		P-ANCOVA[1]
	Men	**Women**	
MeDiet-EVOO	-0.09 (0.32 to 0.13)	-0.01 (-0.19 to 0.18)	0.579
MeDiet-Nuts	-0.12 (-0.39 to 0.16)	-0.12 (-0.40 to 0.16)	0.976
Control	0.21 (-0.06 to 0.49)	0.21 (-0.01 to 0.42)	0.970

[1] Adjusted for initial z-score telomere length, total energy intake (kcal per day), BMI (kg/m^2), adherence to the MeDiet (points), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous).

Supplementary table 3. Risk for telomere shortening (Δ age-adjusted z-score TL $\leq$ 20th percentile) after 5 years of the nutritional intervention for the covariates used in the models.

	OR for telomere shortening after 5 years follow-up
	OR (95%CI)
Sex	0.73 (0.37 to 1.45)
Initial z-score TL	0.23 (0.16 to 0.33)
Total energy intake, kcal /day	1.00 (0.99 to 1.00)
BMI, kg/m^2	1.06 (0.98 to 1.14)
Adherence to the MeDiet, points	0.96 (0.84 to 1.09)
Physical activity, METS-min/day	0.99 (0.99 to 1.00)
Smoking	0.91 (0.64 to 1.31)
Diabetes	0.77 (0.45 to 1.32)
Hypertension	1.46 (0.70 to 3.06)
Dyslipidaemia	0.88 (0.53 to 1.48)

OR: odds ratio, CI: confidence interval, TL: telomere length, BMI: body mass index.

Supplementary table 4. Association between basal telomere length and extra virgin olive oil or nuts consumption at baseline in all the population and according to sex.

	Age-adjusted z-score TL at baseline		
	All (n=520)	**Men (n=235)**	**Women (n=285)**
	B (*P*-value)	**B (*P*-value)**	**B (*P*-value)**
Extra virgin olive oil (g/day)	-0.004 (0.230)	-0.003 (0.575)	-0.010 (0.162)
Nuts (g/day)	-0.005 (0.234)	-0.003 (0.636)	-0.010 (0.101)

Adjusted for sex, total energy intake (kcal per day), BMI (kg/m^2), adherence to the MeDiet (points), physical activity (METS-min per day), smoking status (3 categories), diabetes status (dichotomous), hypertensive status (dichotomous), dyslipidaemia status (dichotomous).