

The Pro12Ala polymorphism of the *PPAR* γ 2 gene interacts with a Mediterranean Diet to prevent telomere shortening in the PREDIMED-NAVARRA randomized trial.

García-Calzón: Pro/Ala SNP, Mediterranean diet & telomere length.

Authors: Sonia García-Calzón, MSc ¹, Miguel A Martínez-González, MD, PhD ^{2,3}, Cristina Razquin, PhD ², Dolores Corella, MD, PhD ^{3,4}, Jordi Salas-Salvadó, MD, PhD ^{3,5}, J Alfredo Martínez, MD, PhD^{1,3,6}, Guillermo Zalba, PhD ⁷, Amelia Marti, PhD ^{1,3}.

¹Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.

²Department of Preventive Medicine and Public Health, University of Navarra, Pamplona, Spain.

³ CIBER Fisiopatología de la Obesidad y Nutrición (CIBERObn), Instituto de Salud Carlos III, Madrid, Spain.

⁴Department of Preventive Medicine and Public Health, University of Valencia, Valencia, Spain.

⁵ Human Nutrition Unit, IISPV, Universitat Rovira i Virgili, Reus, Spain.

⁶Centre for Nutrition Research, University of Navarra, Pamplona, Spain.

⁷Department of Biochemistry and Genetics, University of Navarra, Pamplona, Spain.

Corresponding author: Dr. Amelia Marti del Moral (amarti@unav.es). Department of Nutrition, Food Science and Physiology. University of Navarra. C/Irunlarrea s/n, 31008 Pamplona, Navarra, Spain. Telephone: +34 948 425600; Fax: +34 948 425619.

Word count: 6,381

Journal Subject Code: 109, 121, 101.

Abstract

Background: The gene variant Pro/Ala (rs1801282) in the *PPAR γ 2* has been associated with lower cardiovascular risk and greater benefit from lifestyle interventions. This polymorphism also seems to be associated with longer lifespan, but no information on telomere length (TL) is available. Our aim was to study the association between the Ala allele and changes in TL in high cardiovascular risk subjects, and the potential interaction with a Mediterranean Diet (MeDiet) pattern.

Methods and Results: A total of 521 subjects (55-80 years) participating in the Prevención con Dieta Mediterránea (PREDIMED) randomized trial were genotyped. Changes in TL, measured by quantitative real-time PCR, were assessed over 5 years of a nutritional intervention which promoted adherence to the MeDiet. Interestingly, Ala carriers showed lower telomere shortening after 5 years, compared with the Pro/Pro genotype ($P=0.031$). This association was modulated by MeDiet since those Ala carriers who reported better conformity to the MeDiet exhibited increased TL ($P<0.001$). Moreover, a reduction in carbohydrate intake (≤ 9.5 g/d) resulted in increased TL among Ala carriers. Notably, an apparent gene-diet interaction was found through the observed changes in the MUFA+PUFA/Carbohydrates ratio: as this ratio increased, TL lengthening was detected to a greater extent in the Ala carriers compared with the Pro/Pro subjects (P for interaction <0.001).

Conclusions: The Pro12Ala polymorphism is associated with TL homeostasis after 5 years follow-up in subjects at high cardiovascular risk. In addition, a higher adherence to the MeDiet pattern strengthens the prevention of telomere shortening among Ala carriers.

Clinical trial registration: URL: www.controlled-trials.com. Unique Identifier: ISRCTN35739639.

Keywords: telomeres, nutrigenetics, nutrition, gene-diet interaction.

Telomeres are tandem TTAGGG repeats of DNA that, together with associated proteins, protect the ends of chromosomes.¹ Importantly, they become shorter during repeated DNA replication and leukocyte telomere length (TL) has therefore been proposed as a potential biomarker of biological age.² There are a number of endogenous factors that cause telomere shortening, such as inflammation or oxidative stress,³⁻⁵ and many age-related diseases, such as obesity⁶ and cardiovascular disease⁷ are linked to shortened telomeres.^{8,9} Several studies indicate that patients with early-onset coronary heart disease; hypertension; and diabetes mellitus complications have lower TL, whilst increased left ventricular hypertrophy or ventricular mass is associated with higher TL.¹⁰ Moreover, telomere attrition is reported to be accelerated in endothelial cells in the regions affected by atherosclerosis^{11, 12} and in coronary endothelial cells it may contribute to coronary endothelial dysfunction and the progression of cardiovascular disease in humans.¹³ In this context, cardiovascular risk factors can be causes of oxidative stress and endothelial injury, leading to telomere shortening.

There is evidence that TL is modified by the environment (poor diet, smoking, psychosocial factors)⁶ and also by non-environmental influences such as genetic¹⁴ or epigenetic factors¹⁵. In healthy individuals, the estimated heritability of TL ranges from 0.36 to 0.84.¹⁶ The impact of telomere pathway gene variants on TL has been widely studied, especially for single nucleotide polymorphisms (SNPs) encoding telomerase subunits.^{17,18} However, other GWAS studies have identified SNPs associated with TL which do not contain candidate genes of telomere biology, but rather genes related to several cancers and other diseases.^{19, 20} Recently, Saxena et al.²¹ showed that variants in casein kinase II (*CSNK2A2*) are associated with leukocyte TL in a Punjabi Sikh Diabetic Cohort.

The presence of the Ala allele in the peroxisome proliferator-activated receptor gamma 2 (*PPAR*γ2) results in a moderate reduction in transcriptional activity of the *PPAR*γ2 in adipose tissue, thus improving insulin sensitivity and lipid and glucose homeostasis.^{22, 23} This gene variant seems to regulate inflammation by controlling downstream inflammatory molecules,²⁴ and it may decrease resistance to oxidative stress.²⁵ Moreover, several studies have reported that

carriers of the Ala allele are considerably better protected against cardiovascular disease^{26, 27}, and the effectiveness of primary prevention of cardiovascular disease by a lifestyle intervention even increased among them.^{28, 29}

Interestingly, it has been suggested that the presence of the Ala allele could be associated with longer lifespan in humans.^{30, 31} However, no further studies have examined the association of the Pro12Ala polymorphism and TL in high cardiovascular risk subjects after a Mediterranean diet (MeDiet) intervention. We hypothesized that subjects carrying the Ala allele of the *PPAR* γ 2 gene would have lower telomere attrition after 5 years of intervention with a Mediterranean dietary pattern. Thus, our aim was to assess the relationship between the Ala allele of the *PPAR* γ 2 gene and changes in TL in high cardiovascular risk subjects, whilst they received a nutritional intervention with a Mediterranean dietary pattern.

Methods

Study design

The PREDIMED study was a large, parallel-group, multicenter, randomized and controlled clinical trial, designed to assess the effects of the MeDiet on the primary prevention of cardiovascular disease. All subjects provided informed consent and the protocol was approved by the Institutional Review Boards at all study locations, according to the principles of the Helsinki Declaration. The trial is registered at <http://www.controlled-trials.com/ISRCTN35739639>. Participants were randomly assigned one of three different dietary interventions: MeDiet supplemented with extra virgin olive oil (EVOO), MeDiet supplemented with mixed nuts, or a control group (low-fat diet). The design and methods of this trial have been reported in specific publications^{32, 33} and further details are also available at www.predimed.es.

Participants were women (60 to 80 years old) or men (55 to 80 years old) without a 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/obese, and family history of premature coronary heart disease.

This study was designed to assess TL in a subset of participants from one of the eleven recruitment centers (PREDIMED-NAVARRA). The PREDIMED-NAVARRA recruitment center was the first of the eleven centers to complete the enrolment of participants, comprising 1055 of the 7447 subjects taking part in the trial. Individuals from this center were recruited between June 2003-May 2005, whilst in the rest of the centers it took place between October 2003 and March 2009. The intervention was stopped in 2011 due to early evidence of benefit, according to a joint decision of the Data and Safety Monitoring Board and the Steering Committee of the study.

From the PREDIMED-NAVARRA total sample, participants with either baseline or 5-year missing data or missing DNA samples were excluded from the TL analysis. Therefore, 521 subjects were included in this study. They did not differ from the total cohort (n=1,055) in terms of their main characteristics. Among them, 211 participants were allocated to the MeDiet supplemented with EVOO group, 170 to the MeDiet supplemented with mixed nuts group and 140 to the Control group (Fig. 1).

Genotyping

DNA was extracted from overnight fasting venous blood samples using a commercial kit (Master Pure™; Epicentre, Madison, WI, USA). All participants were genotyped for the Pro12Ala polymorphism of the *PPARγ2* gene (rs1801282) using the PCR-restriction fragment length polymorphism (RFLP) method as previously described.³⁴

Telomere length assessment

Telomeres were measured at two points: at baseline and after 5 years of the nutritional intervention using a real-time quantitative polymerase chain reaction (qPCR), as described by Cawthon.³⁵ This method displays concentrations of telomere repeat copy number (T) and single-copy gene (Ribosomal Protein Large PO) copy number (S), as a reference for each sample.

White 384-well plates were used to perform PCRs on an ABI-Applied Biosystems 7900 HT thermal cycler (Applied Biosystems, CA, USA). The total reaction volume was 10 μL containing 10 ng of genomic DNA, and quantiTect Syber Green PCR kit (Qiagen, Valencia, CA, USA) was used as master mix. 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 (Sigma-Aldrich, St.Louis, MO, USA) were tel1 (5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3'), tel2 (5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'), hRPLPO1 (5'-CCCATCTATCATCAACGGGTACAA -3') and hRPLPO2 (5'-

CAGCAAGTGGGAAGGTGTAATCC -3'). This procedure normalizes T to S by taking the ratio (T/S ratio) for each sample. The T/S ratio was calculated as follows [$2^{\text{CT}(\text{telomeres})}/2^{\text{CT}(\text{single copy gene})}] = 2^{-\Delta\text{CT}}$, since the amount of the PCR product approximately doubles in each cycle of the PCR.³⁵

A calibration curve of the same DNA reference sample (64-0.25 ng in 2-fold dilutions) was included for each measurement as a standard, 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 concordance between duplicate values was checked. In order to ensure consistency, samples showing a high variation (more than 10%) were rerun and reanalyzed. The intra-assay coefficient of variation between duplicates for telomere was 3.0% and for the single copy gene it was 2.6%. The inter-assay coefficient of variation between plates for telomere was 0.8% and for the single copy gene it was 1.3%. The small coefficients of variation support the reliability of this procedure.

Dietary assessment

Dietary habits were evaluated using a semi-quantitative 137-item Food frequency Questionnaire previously validated in Spain.³⁶ Frequency of consumption was registered in nine categories that ranged from 'never or almost never' to '6 times/day'. In addition, a validated 14-item questionnaire was used in this study to appraise adherence of participants to the MeDiet.³⁷

The two groups which were allocated MeDiets received intensive education to be able to follow the MeDiet, and supplemental foods at no cost. EVOO (1 L/week) was provided to the first group and 30 g/day of mixed nuts (15 g walnuts, 7.5 g hazelnuts and 7.5 g almonds) to the second group. In the control group, participants did not receive education on the MeDiet, but were advised to reduce all types of fat and were given written recommendations according to the American Heart Association guidelines. Each participant received a personal interview with a trained dietician and a group session conducted by the same dietician every 3 months during

the 5 years. No energy restrictions were prescribed for any intervention group. Other specific details of the intervention protocol have been published elsewhere.³³

Confounders assessment

The baseline interview included information about medical, socio-demographic and lifestyle variables. Physical activity was assessed through a validated physical activity questionnaire.^{38,39} Anthropometric data were obtained by trained dietitians using standardized methods⁴⁰ and were measured in each of the yearly follow-up visits. Educational attainment was used as the indicator of socioeconomic status, since it does not change much over a lifetime, and – either directly or indirectly – it affects an individual's adoption of health behaviors and their outcomes.

Statistical analysis

Hardy-Weinberg equilibrium was assessed using the χ^2 test and the allele frequency was determined by direct counting. Participants' baseline characteristics were compared according to the Pro12Ala *PPAR γ 2* polymorphism (dominant models). All analyses were carried out with the dominant genetic model due to the small sample of Ala/Ala homozygotes. At baseline, we calculated means and standard deviations (SD) or percentages for each variable across the two genotypes and assessed the statistical significance of the differences among them with the unpaired t-test and χ^2 tests, respectively.

To determine modulation by dietary components, changes in macronutrients after the 5-year nutritional intervention were dichotomized based on individual's median levels, with the exception of the 14-item questionnaire that was divided into tertiles. The ratio (MUFA+PUFA)/CHO was also evaluated as a continuous variable. Indicated interactions between the gene variant (dichotomous) and all the dietary components were tested for TL changes using the likelihood ratio tests. When the dietary component was a dichotomous variable the interaction test had 1 degree of freedom, whereas the interaction test had 2 degrees of freedom in the case of a dietary component that was categorized into three groups, without

assuming any linear effect. Changes in TL and dietary habits were defined as the difference between the two points in time (baseline and 5-year follow-up).

The comparison between-genotype differences in TL within each dietary category was assessed using ANCOVA techniques after adjusting for the following potential confounders measured at baseline: age, sex, body mass index (BMI) (kg/m^2), TL, smoking habit (current smoker or never smoker), physical activity (METS-min/day), total energy intake (Kcal/day) and the score obtained from the 14-item MeDiet tool. In additional analyses, the model was also adjusted for each initial dietary component as indicated in the Results section.

We achieved a statistical power higher than 80% (α -level=0.05 and β -level=0.20), with a sample size of 521 and a prevalence of 13% in Ala allele carriers, when detecting significant mean differences of 40 arbitrary units in TL between both genetic variants, after adjusting for potential confounders. All *P* values presented are two-tailed and statistical significance was defined at $P < 0.05$. Data analyses were performed using STATA version 12.0 (StataCorp, College Station, TX, USA).

Results

The mean age of participants was: 67.0 years (DE: ± 6.0), and 54.7% of them were women. The overall frequencies of the Pro/Pro, Pro/Ala and Ala/Ala genotypes were 86.7%, 13.1% and 0.2%, respectively. The minor allele frequency of Ala allele was 0.067, which is similar to 0.076 in HapMap-CEU (European) subjects as recorded in the NCBI SNP database.⁴¹ The genotype frequency did not deviate from the Hardy-Weinberg equilibrium expectations ($P=0.344$). All analyses were undertaken with a dominant genetic model due to the small number of Ala/Ala homozygotes.

Baseline characteristics of the 521 participants according to the Pro12Ala polymorphism are presented in Table 1. Most of them were overweight or obese (92%), non-smokers, and more than three-quarters had hypertension. No significant differences were observed for the Pro12Ala polymorphism in this population. Specifically, no differences were observed for adherence to MeDiet with the 14-item questionnaire, within the Pro12Ala gene variant ($p=0.616$), meaning that they have a similar adherence to Mediterranean diet at enrolment. However it should be highlighted that carriers of the Ala allele tended to have higher TL ($p=0.095$).

As expected, TL was negatively associated with age in this population both at baseline ($\rho=-0.104$, $p=0.018$) and after the 5-years nutritional intervention ($\rho=-0.124$, $p=0.004$) (data not shown). Remarkably, the 5-year MeDiet intervention did prevent telomere shortening and even increased TL among Ala carriers, whilst this effect was not observed in Pro/Pro homozygotes after adjusting for potential confounders ($P_{\text{ANCOVA}}=0.031$) (Fig. 2).

We assessed the effects of changes in the dietary pattern, as categorical factors, after the intervention on telomere lengthening linked to the *PPAR γ 2* Pro/Ala gene variant, using multivariable-adjusted analysis (Table 2). Interestingly, an increased TL was detected when subjects reported better conformity to the MeDiet (14-item score) after the intervention, but only in subjects carrying the Ala allele of the *PPAR γ 2* gene ($P_{\text{ANCOVA}}<0.001$). The interaction for modifications in TL between changes in adherence to MeDiet and the *PPAR γ 2* gene was also

statistically significant (P for interaction= 0.016). Moreover, the presence of the Ala allele increased TL in subjects with a greater reduction in carbohydrates (CHO) intake (≤ 9.5 g/d) after 5 years in comparison with Pro/Pro participants ($P_{\text{ANCOVA}}= 0.006$). Notably, there was evidence of statistical interaction (P for interaction= 0.007). Similar results were observed when individuals with the Pro/Ala genotype reduced glycemic load intake (≤ 5) ($P_{\text{ANCOVA}}= 0.004$; P for interaction= 0.004).

As the nutritional intervention promoted increased intake of unsaturated fats (MUFA+PUFA) at the expense of reduced CHO intake, we assessed whether changes in the ratio unsaturated fat:CHO modulated the relationship between Pro12Ala polymorphism and changes in TL. We found a modulation of the genetic predisposition to TL changes by the unsaturated fat:CHO ratio (Fig. 3). The prediction plot showed a gene-nutrient interaction: as the unsaturated fat:CHO ratio increased, TL was predicted to increase only in the Ala carriers and not in the Pro/Pro homozygotes (P for interaction < 0.001).

When considering the items included in the 14-item MeDiet score separately, we found that higher consumptions of virgin olive oil, vegetables, fruits and the use of *sofrito* sauce during follow-up, were significantly associated with increased TL, but again, this association was only present in the Ala carriers; whereas a lower consumption of red meat, butter/margarine/cream, soda drinks, commercial sweets and confectionery led to decreased telomere attrition, also only among these subjects (Supplemental table 1). Contrary to expectations, subjects carrying the Ala allele consuming less wine, legumes, fish/seafood and tree nuts exhibited an increase in TL after adjusting for potential confounders.

Analysis performed on an intention-to-treat basis showed no differences in TL changes after 5 years in any of the intervention groups (EVOO, Nuts and Control) after splitting the sample according to the Pro/Pro and Pro/Ala genotypes (Table 3). However, when distributing subjects according to the intervention group and changes in adherence to MeDiet, only Ala carriers with

higher scores in the MeDiet screener and allocated to one intervention group, either EVOO or Nuts, prevented telomere shortening (Fig. 4).

Discussion

To our knowledge this is the first study to report an association between the common genetic variant at the *PPAR γ 2* locus, rs1801282, and TL, in which the Ala allele prevented telomere shortening associated with age after a 5-year nutritional intervention in high cardiovascular risk subjects. In addition, it has been shown that a Mediterranean dietary pattern interacts with the Ala allele, resulting in higher TL.

This Ala variant has been associated with lower cardiovascular disease risk in some studies.^{27, 28} Regieli *et al.*²⁷ showed that Ala allele carriers had less widespread coronary artery disease and were protected against 10-year cardiovascular morbidity and mortality, with a hazard ratio of 0.24 (0.08-0.74, $P= 0.013$) for vascular death, per each added copy of Ala allele. It is also reported that lifestyle interventions for cardiovascular prevention are more effective in carriers of the Ala allele of the *PPAR γ 2* gene than in those with the Pro/Pro genotype.^{28, 29} Since shorter telomeres have been found in subjects with cardiovascular disease,⁷ it could be suggested that the Pro/Ala genotype may potentially protect against telomere attrition, as observed in our population at high cardiovascular risk.

Of particular interest is the reported relationship between the presence of the Ala allele and longevity, as TL is considered a biomarker for age.² Work conducted by Luo *et al.*²⁵ showed that *PPAR γ 2* heterozygous mice exhibited improvement in insulin sensitivity and increased resistance to oxidative stress, underlying an extended lifespan. Another study indicated that Pro/Ala mice seem to be leaner, with improved insulin sensitivity and plasma lipid profiles, and have longer lifespans.⁴² Notably, this evidence has been supported in a human study performed on a group of centenarian men who had increased frequency of the Pro/Ala genotype.³⁰ In contrast, however, a recent study proposed the opposite, having found a high frequency of the Ala allele in men younger than 90 years old.³¹ Our findings give valuable information in longitudinal terms, by suggesting *PPAR γ 2* as a possible longevity candidate gene, since the Ala

allele was associated with telomere lengthening in multiple-adjusted models after 5 years of a nutritional intervention.

The response of subjects to dietary components might be different depending on the genotype. In the population studied, we found a consistent gene-diet interaction between the Pro/Ala gene variant and adherence to MeDiet for changes in TL after the intervention. There are few examples in the literature on the subject of the influence of a MeDiet on maintaining TL. There are two potential mechanisms that account for changes in TL: one may occur through telomerase-mediated elongation, and the other by mechanisms resulting in "pseudo-telomeric lengthening", as might result from changes in cell type distribution.⁴³ Boccardi *et al.*⁴⁴ showed that people with the highest adherence to a MeDiet have longer telomeres and higher telomerase activity levels in peripheral white blood cells, independently of multiple confounding variables. This positive effect of the MeDiet could be direct, through some specific nutrients that may stimulate telomerase activity, or indirect, through the global effect of the diet on the modulation of inflammation and oxidative status.⁴⁴

With regard to this, oxidative stress and inflammation might be proposed as the underlying causes of accelerated telomere shortening.⁵ Interestingly, the Ala allele has been associated with lower levels of inflammation and oxidative stress^{24,25} On the other hand, activation of *PPAR γ 2* can inhibit telomerase activity,⁴⁵ elucidating a potential relationship between this gene and TL. Since, the Pro12Ala variant is associated with lower *PPAR γ 2* activity,²³ it may be hypothesized that this effect of the Ala allele is mediated by telomerase activity. Nevertheless, we could not rule out inflammation and oxidative stress as other mechanisms implicated in the change. Our findings need to be further explored in other populations to better understand this biological mechanism.

Surprisingly, no significant interaction was observed between the intervention groups established in the PREDIMED study (on an intention-to-treat basis) and the polymorphism for changes in TL. Nevertheless, when analyzing the adherence to the MeDiet within each group,

we observed a significantly more increased TL in Ala carriers than in Pro/Pro homozygotes when the compliance to MeDiet was high, both in the EVOO and in the nuts group, but not in the control group. A possible explanation could be that these two groups presented a higher adherence to MeDiet as they received intensive education to follow MeDiet and supplemental foods at no cost. Thus, it should be highlighted that the MeDiet pattern itself had the positive, principal effect on the association between the Pro/Ala genotype and TL changes.

Several limitations should be acknowledged. Firstly, that TL has several weaknesses given the extent of inter-individual variation at a given chronological age.^{46, 47} Moreover, there are some methodological problems due to TL measurement techniques. For this reason, we looked after the experimental conditions carefully, in order to avoid potential errors in measurement.⁴⁸

Genomic DNA was processed following a standardized protocol and kept to preserve its stability, and the two DNA samples per participant (at baseline and after 5 years of recruitment) were run on the same plate. Secondly, the lack of several biological measurements, such as telomerase activity and others related to inflammation and oxidative stress. Nor do we have information on the epigenetics profile status of participants.

Another weakness of this study was the self-reported dietary consumption using questionnaires that might have led to misclassifications of exposure. Our participants lived in a MeDiet country and had high cardiovascular risk, which may also limit the generalizability of our findings. On the other hand, the prospective nature of our study, with a long follow-up period, enabled us to measure TL at two points in the context of a MeDiet intervention and thus makes this research particularly robust. Other strengths include: the randomized design, the high compliance of the participants to the intervention and the reproduction of real-time conditions with home-prepared foods, as in usual clinical practice.

In summary, our results are in line with previous finding on the great beneficial effect of a lifestyle intervention on Ala carriers who are high cardiovascular risk subjects. Specifically, we demonstrated an increased TL in Ala carriers after 5 years of a MeDiet intervention, suggesting

that the MeDiet pattern appears to have a beneficial effect in modulating the association between the polymorphism and changes in TL. Understanding the underlying mechanism of this gene-diet interaction may help to improve personalized dietary recommendations to prevent cardiovascular risk factors. However, to date, this is apparently the only study to report these results, and therefore further investigation is warranted to confirm our findings.

Acknowledgments

We thank all the participants, the personnel of the primary care centres of Navarra, and other investigators of the PREDIMED group: Estruch R, Covas MI, Fiol M, Ros E, Aros F, Lamuela-Raventós RM, Gomez-Gracia E, Ruiz-Gutierrez V, Warnberg J, Saez G, Lapetra J, Serra-Majem L, Pinto L, Tur JA, Mitjavila MT, Portillo MP, Basora J, Fernandez-Crehuet J, Muñoz MA, Tello S. We gratefully acknowledge technical assistance by Idoia Rodríguez.

Funding sources

This research was funded by grants from Línea Especial, Nutrición, Obesidad y Salud of the University of Navarra (LE/97), the Spanish Government (FIS-ISCI: PI051579, PI050976, PI070240, PI081943, PI1002293, RTIC 06/0045, CIBERobn, CNIC/06, SAF-2010-20367) and the Government of Navarra (PI41/2005, PI79/2006, PI36/2008, PI54/2009). The FPU fellowship to Sonia García-Calzón from the Spanish Ministry is gratefully acknowledged.

Disclosures

None.

References

1. Blackburn EH. Telomeres and telomerase: The means to the end (Nobel lecture). *Angew Chem Int Ed Engl.* 2010;49:7405-7421.
2. Sanders JL, Newman AB. Telomere length in epidemiology: A biomarker of aging, age-related disease, both, or neither? *Epidemiol Rev.* 2013.
3. Babizhayev MA, Savel'yeva EL, Moskvina SN, Yegorov YE. Telomere length is a biomarker of cumulative oxidative stress, biologic age, and an independent predictor of survival and therapeutic treatment requirement associated with smoking behavior. *Am J Ther.* 2011;18:e209-226.
4. Kiecolt-Glaser JK, Epel ES, Belury MA, Andridge R, Lin J, Glaser R, et al. Omega-3 fatty acids, oxidative stress, and leukocyte telomere length: A randomized controlled trial. *Brain Behav Immun.* 2013;28:16-24.
5. Shiels PG, McGlynn LM, MacIntyre A, Johnson PC, Batty GD, Burns H, et al. Accelerated telomere attrition is associated with relative household income, diet and inflammation in the PSOBID cohort. *PLoS One.* 2011;6:e22521.
6. Garcia-Calzon S, Gea A, Razquin C, Corella D, Lamuela-Raventos RM, Martinez JA, et al. Longitudinal association of telomere length and obesity indices in an intervention study with a mediterranean diet: The PREDIMED-NAVARRA trial. *Int J Obes (Lond).* 2014;38:177-182.
7. De Meyer T, Rietzschel ER, De Buyzere ML, Van Criekinge W, Bekaert S. Telomere length and cardiovascular aging: The means to the ends? *Ageing Res Rev.* 2011;10:297-303.
8. Fyhrquist F, Saijonmaa O. Telomere length and cardiovascular aging. *Ann Med.* 2012;44 Suppl 1:S138-142.
9. Shammass MA. Telomeres, lifestyle, cancer, and aging. *Curr Opin Clin Nutr Metab Care.* 2011;14:28-34.

10. Nilsson PM, Tufvesson H, Leosdottir M, Melander O. Telomeres and cardiovascular disease risk: An update 2013. *Transl Res.* 2013;162:371-380.
11. Chang E, Harley CB. Telomere length and replicative aging in human vascular tissues. *Proc Natl Acad Sci U S A.* 1995;92:11190-11194.
12. Okuda K, Khan MY, Skurnick J, Kimura M, Aviv H, Aviv A. Telomere attrition of the human abdominal aorta: Relationships with age and atherosclerosis. *Atherosclerosis.* 2000;152:391-398.
13. Ogami M, Ikura Y, Ohsawa M, Matsuo T, Kayo S, Yoshimi N, et al. Telomere shortening in human coronary artery diseases. *Arterioscler Thromb Vasc Biol.* 2004;24:546-550.
14. Andrew T, Aviv A, Falchi M, Surdulescu GL, Gardner JP, Lu X, et al. Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. *Am J Hum Genet.* 2006;78:480-486.
15. Tollefsbol TO. Dietary epigenetics in cancer and aging. *Cancer Treat Res.* 2014;159:257-267.
16. Holohan B, Wright WE, Shay JW. Cell biology of disease: Telomeropathies: An emerging spectrum disorder. *J Cell Biol.* 2014;205:289-299.
17. Codd V, Mangino M, van der Harst P, Braund PS, Kaiser M, Beveridge AJ, et al. Common variants near TERC are associated with mean telomere length. *Nat Genet.* 2010;42:197-199.
18. Bojesen SE, Pooley KA, Johnatty SE, Beesley J, Michailidou K, Tyrer JP, et al. Multiple independent variants at the tert locus are associated with telomere length and risks of breast and ovarian cancer. *Nat Genet.* 2013;45:371-384, 384e371-372.
19. Lee JH, Cheng R, Honig LS, Feitosa M, Kammerer CM, Kang MS, et al. Genome wide association and linkage analyses identified three loci-4q25, 17q23.2, and 10q11.21-associated

- with variation in leukocyte telomere length: The long life family study. *Front Genet.* 2013;4:310.
20. Codd V, Nelson CP, Albrecht E, Mangino M, Deelen J, Buxton JL, et al. Identification of seven loci affecting mean telomere length and their association with disease. *Nat Genet.* 2013;45:422-427, 427e421-422.
21. Saxena R, Bjornes A, Prescott J, Dib P, Natt P, Lane J, et al. Genome-wide association study identifies variants in casein kinase ii (CSNK2A2) to be associated with leukocyte telomere length in a PUNJABI SIKH diabetic cohort. *Circ Cardiovasc Genet.* 2014;7:287-295.
22. Gonzalez Sanchez JL, Serrano Rios M, Fernandez Perez C, Laakso M, Martinez Larrad MT. Effect of the Pro12Ala polymorphism of the peroxisome proliferator-activated receptor gamma-2 gene on adiposity, insulin sensitivity and lipid profile in the Spanish population. *Eur J Endocrinol.* 2002;147:495-501.
23. Gouda HN, Sagoo GS, Harding AH, Yates J, Sandhu MS, Higgins JP. The association between the peroxisome proliferator-activated receptor-gamma2 (PPARG2) Pro12Ala gene variant and type 2 diabetes mellitus: A huge review and meta-analysis. *Am J Epidemiol.* 2010;171:645-655.
24. Yao Q, Nordfors L, Axelsson J, Heimburger O, Qureshi AR, Barany P, et al. Peroxisome proliferator-activated receptor gamma polymorphisms affect systemic inflammation and survival in end-stage renal disease patients starting renal replacement therapy. *Atherosclerosis.* 2005;182:105-111.
25. Luo W, Cao J, Li J, He W. Adipose tissue-specific PPARgamma deficiency increases resistance to oxidative stress. *Exp Gerontol.* 2008;43:154-163.
26. Doney AS, Fischer B, Cecil JE, Boylan K, McGuigan FE, Ralston SH, et al. Association of the Pro12Ala and c1431t variants of PPARG and their haplotypes with susceptibility to type 2 diabetes. *Diabetologia.* 2004;47:555-558.

27. Regieli JJ, Jukema JW, Doevendans PA, Zwinderman AH, van der Graaf Y, Kastelein JJ, et al. PPAR gamma variant influences angiographic outcome and 10-year cardiovascular risk in male symptomatic coronary artery disease patients. *Diabetes Care*. 2009;32:839-844.
28. Rittig K, Thamer C, Machicao F, Rietig R, Stefan N, Fritsche A, et al. The Pro12Ala polymorphism in PPARG2 increases the effectiveness of primary prevention of cardiovascular disease by a lifestyle intervention. *Diabetologia*. 2007;50:1345-1347.
29. Razquin C, Alfredo Martinez J, Martinez-Gonzalez MA, Corella D, Santos JM, Marti A. The Mediterranean diet protects against waist circumference enlargement in 12Ala carriers for the PPARgamma gene: 2 years' follow-up of 774 subjects at high cardiovascular risk. *Br J Nutr*. 2009;102:672-679.
30. Barbieri M, Bonafe M, Rizzo MR, Ragno E, Olivieri F, Marchegiani F, et al. Gender specific association of genetic variation in peroxisome proliferator-activated receptor (PPAR)gamma-2 with longevity. *Exp Gerontol*. 2004;39:1095-1100.
31. Corbo RM, Pinto A, Scacchi R. Gender-specific association between FSHR and PPARG common variants and human longevity. *Rejuvenation Res*. 2013;16:21-27.
32. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med*. 2013;368:1279-1290.
33. Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ros E, Covas MI, Fiol M, et al. Cohort profile: Design and methods of the PREDIMED study. *Int J Epidemiol*. 2012;41:377-385.
34. Ochoa MC, Marti A, Azcona C, Chueca M, Oyarzabal M, Pelach R, et al. Gene-gene interaction between PPAR gamma 2 and ADR beta 3 increases obesity risk in children and adolescents. *Int J Obes Relat Metab Disord*. 2004;28 Suppl 3:S37-41.
35. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res*. 2002;30:e47.

36. Martin-Moreno JM, Boyle P, Gorgojo L, Maisonneuve P, Fernandez-Rodriguez JC, Salvini S, et al. Development and validation of a food frequency questionnaire in Spain. *Int J Epidemiol.* 1993;22:512-519.
37. Martinez-Gonzalez MA, Garcia-Arellano A, Toledo E, Salas-Salvado J, Buil-Cosiales P, Corella D, et al. A 14-item Mediterranean diet assessment tool and obesity indexes among high-risk subjects: The PREDIMED trial. *PLoS One.* 2012;7:e43134.
38. Elosua R, Marrugat J, Molina L, Pons S, Pujol E. Validation of the minnesota leisure time physical activity questionnaire in Spanish men. The marathom investigators. *Am J Epidemiol.* 1994;139:1197-1209.
39. Martinez-Gonzalez MA, Lopez-Fontana C, Varo JJ, Sanchez-Villegas A, Martinez JA. Validation of the Spanish version of the physical activity questionnaire used in the nurses' health study and the health professionals' follow-up study. *Public Health Nutr.* 2005;8:920-927.
40. Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ruiz-Gutierrez V, Covas MI, et al. Effects of a Mediterranean-style diet on cardiovascular risk factors: A randomized trial. *Ann Intern Med.* 2006;145:1-11.
41. NCBI dbSNP [database]. Accessed February 14, at: <http://www.ncbi.nlm.nih.gov/snp>.
42. Heikkinen S, Argmann C, Feige JN, Koutnikova H, Champy MF, Dali-Youcef N, et al. The Pro12Ala PPARgamma2 variant determines metabolism at the gene-environment interface. *Cell Metab.* 2009;9:88-98.
43. Epel E. How "reversible" is telomeric aging? *Cancer Prev Res (Phila).* 2012;5:1163-1168.
44. Boccardi V, Esposito A, Rizzo MR, Marfella R, Barbieri M, Paolisso G. Mediterranean diet, telomere maintenance and health status among elderly. *PLoS One.* 2013;8:e62781.
45. Lehrke M, Lazar MA. The many faces of PPARgamma. *Cell.* 2005;123:993-999.

46. Shiels PG. Improving precision in investigating aging: Why telomeres can cause problems. *J Gerontol A Biol Sci Med Sci.* 2010;65:789-791.
47. Gingell-Littlejohn M, McGuinness D, McGlynn LM, Kingsmore D, Stevenson KS, Koppelstaetter C, et al. Pre-transplant CDKN2A expression in kidney biopsies predicts renal function and is a future component of donor scoring criteria. *PLoS One.* 2013;8:e68133.
48. Steenstrup T, Hjelmborg JV, Kark JD, Christensen K, Aviv A. The telomere lengthening conundrum--artifact or biology? *Nucleic Acids Res.* 2013;41:e131.

Table 1. Baseline characteristics according to the *PPAR γ 2* genotype among all the participants.

	Pro/Pro	Ala carriers	P value
n	451	70	
Male/female, %	46/54	36/64	0.106
Age, y	66.9 (5.9)	68.1 (5.7)	0.101
Socioeconomic status			0.395
Low level, %	75.8	71.1	
High level, %	24.2	29.0	
BMI, kg/m ²	29.2 (3.2)	28.9 (3.2)	0.482
Waist, cm	95.3 (10.1)	94.0 (7.9)	0.313
Physical activity, METs-min per day	276.2 (197.3)	283.9 (189.2)	0.763
Adherence to the MeDiet, points	9.1 (1.9)	9.3 (1.7)	0.616
Dietary intake			
Energy intake, Kcal/day	2258.2 (526.8)	2210.5 (530.5)	0.485
Carbohydrates, % energy	40.0	41.2	0.150
Fat, % energy	40.6	40.0	0.460
Protein, % energy	15.9	16.1	0.560
Glycemic load	125.0 (43.3)	124.8 (49.2)	0.977
Intervention groups			0.732
MeDiet+olive oil, %	39.9	44.9	
MeDiet+nuts, %	32.8	30.4	
Control, %	27.3	24.6	
Current smokers, %	15.3	11.6	0.407
Diabetes, %	36.1	39.1	0.633
Hypertension, %	83.6	81.2	0.619
Dyslipidemia, %	65.0	73.9	0.136
Telomere length, T/S ratio	258.3 (453.8)	368.3 (781.9)	0.095

* Values are means and standard deviations (SD) for continuous variables or percentages for categorical variables.

† BMI, body mass index; MeDiet, Mediterranean Diet.

Table 2. Interactions between *PPAR γ 2* and changes in adherence to MeDiet, CHO intake, glycemic load and ratio (MUFA+PUFA)/ CHO in association with changes in telomere length after 5 years of the nutritional intervention.

Δ Telomere length				
	Pro/Pro	Ala carriers	P value[#]	P for interaction
Δ Adherence MeDiet, score points				0.016
Low < 1 (n=240)	-57.2 (-88.0 to -26.3)	-98.1 (-176.3 to -20.0)	0.342	
Medium 2-3 (n=180)	-40.8 (-109.6 to 27.8)	141.0 (-40.9 to 323.0)	0.070	
High > 4 (n=101)	-121.9 (-176.5 to -67.3)	160.4 (9.5 to 311.3)	<0.001	
Δ CHO intake, g/d[‡]				0.007
Below median: \leq 9.5 (n=260)	-76.6 (-128.7 to -24.6)	131.2 (-6.8 to 269.1)	0.006	
Above median: > 9.5 (n=261)	-47.0 (-74.7 to -19.3)	-86.8 (-157.3 to -16.3)	0.307	
Δ Glycemic load, units[§]				0.004
Below median: \leq 5.0 (n=260)	-80.4 (-132.0 to -28.9)	144.4 (-0.7 to 289.4)	0.004	
Above median: > 5.0 (n=261)	-44.8 (-73.3 to -16.3)	-68.1 (-136.9 to 0.6)	0.541	
Δ (MUFA+PUFA)/ Δ CHO				0.092
Below median: \leq 0.03 (n=260)	-39.1 (-69.4 to -8.8)	-33.7 (-106.7 to 39.3)	0.894	
Above median: > 0.03 (n=261)	-84.8 (-134.8 to -34.8)	92.0 (-48.9 to 232.9)	0.022	

*Adjusted for age, sex, basal BMI, basal telomere length, physical activity (METS-min per day), total energy intake (Kcal per day), total 14-point score at baseline and smoking status (dichotomous).

† MeDiet, Mediterranean Diet; CHO, carbohydrates; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

‡ Additionally adjusted for basal CHO intake.

§ Additionally adjusted for basal Glycemic load.

|| Additionally adjusted for basal ratio (MUFA+PUFA)/CHO.

P-values are for the comparison between-genotype differences in telomere length within each dietary category after our corresponding adjustment for potential confounders.

Table 3. Changes in telomere length after 5 years of the nutritional intervention associated with Pro12Ala polymorphism according to groups of intervention.

Δ Telomere length				
	Pro/Pro	Ala carriers	P value[†]	P for interaction
Group of intervention				0.737
Control	7.5 (-17.7 to 32.8)	39.1 (-31.0 to 109.1)	0.412	
Extra virgin olive oil	-137.5 (-199.7 to -75.2)	-52.6 (-204.7 to 99.4)	0.315	
Nuts	-26.2 (-68.9 to 16.6)	93.2 (-22.5 to 208.9)	0.061	

* Adjusted for age, sex, basal BMI, basal telomere length, physical activity (METS-min per day), total energy intake (Kcal per day), total 14-point score at baseline and smoking status (dichotomous).

[†] P-values are for the comparison between-genotype differences in telomere length within each dietary category after our corresponding adjustment for potential confounders

Figures legends

Figure 1. Flowchart of participants in a subsample of the PREDIMED-NAVARRA trial.

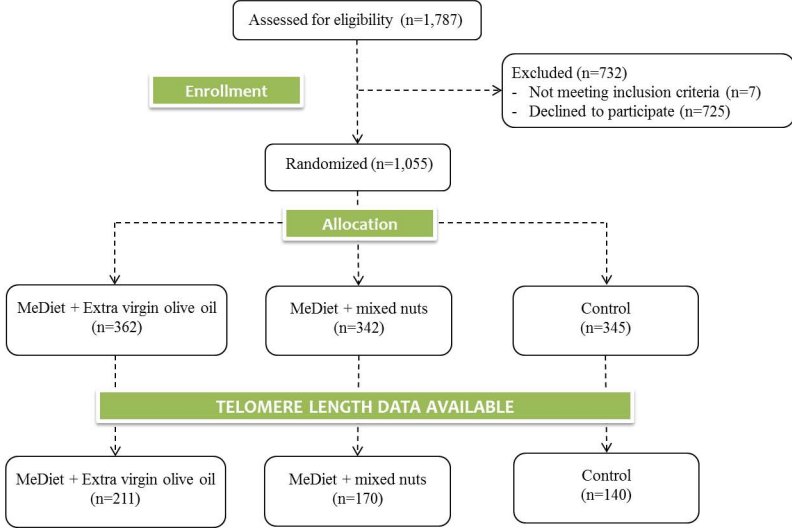
MeDiet: Mediterranean Diet.

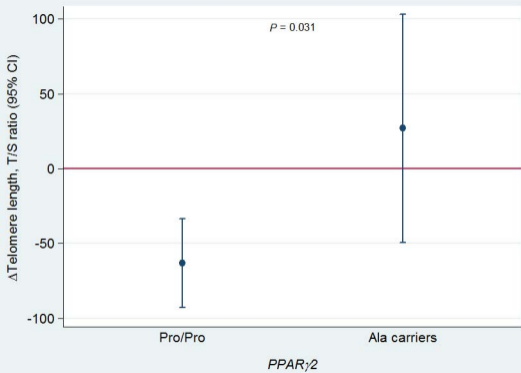
Figure 2. Effect of the rs1801282 polymorphism of the *PPAR* γ 2 gene on changes in telomere length after 5 years of a nutritional intervention in the frame of the PREDIMED-NAVARRA trial (n=521). Multivariate adjusted means for telomere length changes depending on the polymorphism, adjusted for age, sex, baseline telomere length, BMI (kg/m²), physical activity (METS-min per day), total energy intake (Kcal per day), total 14-point score at baseline and smoking status (dichotomous).

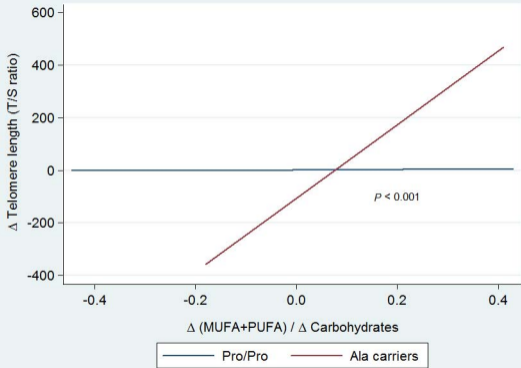
Figure 3. Interaction between the rs1801282 polymorphism of the *PPAR* γ 2 gene with changes in the ratio (MUFA+PUFA)/Carbohydrates in determining changes in telomere length after 5 years of the nutritional intervention in the frame of the PREDIMED-NAVARRA trial (n=521).

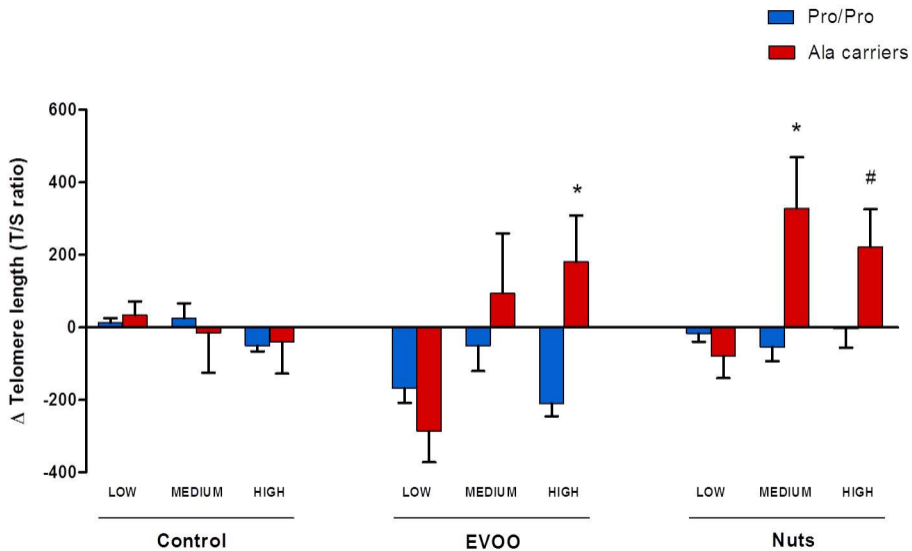
P for interaction was adjusted for age, sex, baseline telomere length, baseline ratio MUFA+PUFA/carbohydrates, BMI (kg/m²), physical activity (METS-min per day), total energy intake (Kcal per day), total 14-point score at baseline and smoking status (dichotomous). The two following equations correspond to both regression lines, Pro/Pro genotype (blue line): $y = 2.83 + 6.52 x$; Ala carriers (red line): $y = -106.76 + 1398 x$.

Figure 4. Changes in telomere length after 5 years of the nutritional intervention associated with Pro12Ala polymorphism, according to the group of intervention and tertiles of adherence to the Mediterranean Diet (14-item questionnaire) at follow-up. Means and SD for telomere length are adjusted for age, sex, basal telomere length, BMI (kg/m²), physical activity (METS-min per day), total energy intake (Kcal per day), total 14-point score at baseline and smoking status (dichotomous). * $P < 0.025$, # $P = 0.070$ are *P* values comparing Ala carriers vs. Pro/Pro subjects within the three groups of intervention.









SUPPLEMENTAL MATERIAL

Supplemental table 1. Changes in telomere length after the nutritional intervention associated with the Pro12Ala polymorphism according to the fulfilment of each item included in the 14-point screener of adherence to the Mediterranean diet at year 5.

Δ Telomere length					
Adherence to MeDiet at follow-up	%	Pro/Pro	Ala carriers	P value	P for interaction
1. Use of olive oil as main culinary lipid	Yes (98.8)	-65.3 (-95.2 to -35.5)	28.6 (-48.9 to 106.2)	0.027	0.209
	No (1.2)	-	-	-	
2. Olive oil >4 tablespoons	Yes (84.8)	-78.3 (-112.7 to -44.0)	28.9 (-59.0 to 116.8)	0.027	0.243
	No (15.2)	19.6 (-18.2 to 57.4)	-26.3 (-132.9 to 80.2)	0.430	
3. Vegetables ≥2 servings/d	Yes (72.3)	-82.6 (-121.0 to -44.1)	47.2 (-50.7 to 145.1)	0.016	0.124
	No (27.7)	-15.2 (-53.0 to 22.5)	-42.6 (-147.3 to 62.0)	0.632	
4. Fruits ≥3 servings/d	Yes (74.8)	-55.0 (-92.7 to -17.4)	57.9 (-40.5 to 156.3)	0.037	0.376
	No (25.2)	-89.7 (-125.3 to -54.1)	-64.6 (-155.9 to 26.5)	0.619	
5. Red/processed meats <1/d	Yes (98.8)	-62.7 (-92.7 to -32.6)	24.3 (-52.9 to 101.5)	0.041	-
	No (1.2)	-	-	-	
6. Butter, cream, margarine <1/d	Yes (97.5)	-61.2 (-91.5 to -30.8)	30.0 (-48.2 to 108.2)	0.034	0.538
	No (2.5)	-151.8 (-207.8 to -95.8)	-336.4 (-563.3 to -109.4)	0.227	
7. Soda drinks <1/d	Yes (98.3)	-61.1 (-91.2 to -31.0)	25.1 (-52.1 to 102.3)	0.042	-
	No (1.7)	-	-	-	
8. Wine glasses ≥7/wk	Yes (36.9)	-62.9 (-108.1 to -17.7)	-39.4 (-162.9 to 84.0)	0.729	0.443
	No (63.1)	-62.9 (-102.1 to -23.8)	52.7 (-47.0 to 152.4)	0.035	
9. Legumes ≥3/wk	Yes (25.6)	-29.9 (-71.7 to 11.9)	-146.9 (-257.7 to -36.2)	0.056	0.007
	No (74.4)	-74.9 (-111.8 to -38.0)	80.4 (-15.1 to 175.9)	0.003	

10. Fish/seafood ≥ 3/wk	Yes (70.2)	-78.7 (-110.8 to -46.6)	-53.9 (-138.9 to 31.2)	0.593	0.014
	No (29.8)	-32.0 (-94.6 to 30.7)	216.1 (55.2 to 377.0)	0.006	
11. Commercial sweets and confectionary ≤ 2/wk	Yes (88.8)	-66.9 (-99.1 to -34.7)	28.4 (-55.3 to 112.1)	0.038	0.681
	No (11.2)	-37.2 (-103.0 to 28.7)	-1.5 (-169.8 to 166.9)	0.702	
12. Tree nuts ≥ 3/wk	Yes (61.0)	-83.4 (-118.9 to -47.8)	-25.3 (-123.6 to 72.9)	0.278	0.302
	No (39.0)	-33.9 (-83.0 to 15.2)	97.4 (-20.1 to 215.0)	0.046	
13. Poultry more than red meats	Yes (86.5)	-61.1 (-93.7 to -28.4)	24.9 (-63.3 to 113.1)	0.074	0.679
	No (13.5)	-84.3 (-150.0 to -18.7)	36.1 (-105.2 to 177.74)	0.142	
14. Use of <i>sofrito</i> sauce ≥ 2/wk	Yes (88.8)	-69.4 (-102.6 to -36.3)	33.0 (-52.4 to 118.4)	0.029	0.329
	No (11.2)	-9.2 (-41.0 to 22.6)	-111.2 (-211.1 to -11.3)	0.072	

* Adjusted for age, sex, basal BMI, basal telomere length, physical activity (METS-min per day), total energy intake (Kcal per day), smoking status (dichotomous) and the total 14-point score at baseline.

† MeDiet, Mediterranean Diet