Mediterranean diet and non enzymatic antioxidant capacity in the PREDIMED study: Evidence for a mechanism of antioxidant tuning

R. Zamora-Rosa,b,1, M. Serafinic,1, R. Estruchd,e, R.M. Lamuela-Raventós a,d, M.A. Martı´ nez-González d,f, J. Salas-Salvadó d,g, M. Fiol d,h, J. Lapetrad,i, F. Arós d,j, M.I. Covasd,k, C. Andres-Lacuevaa,l,*, on behalf of the PREDIMED Study Investigators

a Nutrition and Food Science Department, XaRTA INSA, Pharmacy School, University of Barcelona, Av/Joan XXIII s/n, 08028 Barcelona, Spain
b Unit of Nutrition, Environment and Cancer, Cancer Epidemiology Research Programme, Catalunian Institute of Oncology (ICO-IDIBELL), Barcelona, Spain
c Antioxidant Research Laboratory at the Unit of Human Nutrition, National Institute for Food and Nutrition Research, Rome, Italy
d CIBER 08/08, Fisiopatologı´ a de la Obesidad y Nutricio´ n, and RD06/0045/1003, Alimentacio´ n Saludable, Instituto de Salud Carlos III, Spain
e Internal Medicine Department, Hospital Clı´ nic, Institut d'Investigacio´ Biomédica August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain
f Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona, Spain
g Human Nutrition Unit, Hospital Universitari Sant Joan, IISPV, Universitat Rovira i Virgili, Reus, Spain
h Department of Cardiology, Hospital Son Dureta, Palma de Mallorca, Spain
i Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla, Spain
j Department of Cardiology, Hospital Txagorritxu, Vitoria, Spain
k Cardiovascular Risk and Nutrition Research Group (ULEC-CARIN), Institut Municipal d’Investigació Medica (IMIM-Hospital del Mar), Barcelona, Spain
l Ingenio-CONSOLIDER Program, FUN-C-Food, CSD2007-063, Spain

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Abbreviations: NEAC, non enzymatic antioxidant capacity; TRAP, total radical-trapping antioxidant parameter; FRAP, ferric reducing antioxidant potential; PREDIMED, Prevencio´n con Dieta Mediterra´nea; RONS, reactive oxygen nitrogen species; MED, Mediterranean diet; CVD, cardiovascular diseases; VOO, virgin olive oil.

* Corresponding author. Tel.: +34 93 403 48 40; fax: +34 93 403 59 31.
E-mail address: candres@ub.edu (C. Andres-Lacueva).
1 Both authors equally contributed to this research.

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Introduction

The antioxidant network of the body, due to its wide range of redox potential and localization, allows an efficient protection against reactive oxygen nitrogen species (RONS). The multi-functional properties of the antioxidant network highlight the crucial importance of the dynamic interactions among the components of the network in protecting body fluids from oxidative stress [1]. Non enzymatic antioxidant capacity (NEAC) takes into account the antioxidant activity of single compounds present in food or biological samples as well as their potential synergistic and redox interactions [1]. However, despite NEAC methodology has been widely used to investigate the role of diet in modulating antioxidant function in humans, criticisms have been raised on the real ability of NEAC to be a proper biomarker of antioxidant function in vivo [2,3].

Nowadays, there is a controversy over the effect of antioxidants on health, particularly the use of antioxidant supplements. On the one hand, large epidemiologic studies have shown that a greater adherence to the Mediterranean (MED) diet is associated with elevated NEAC levels [4,5]. Different studies have shown the ability of diet to modulate plasma NEAC following acute consumption of plant foods in humans [1,6–8]. However, when long-term intervention studies are critically evaluated the picture is more complex and results are not fully consistent or homogenous [2]. It has been recently suggested that the efficiency of dietary antioxidants in modulating NEAC in long-term trial depends on the "healthy status" of subjects, with a higher effect in subjects having some risk factors at baseline (i.e. smoking) or some prevalent diseases [2]. Healthy subjects might have a low responsiveness to antioxidant supplementation due to the lack of oxidative stress conditions in which a higher intake of redox molecules is required. In diseases with oxidative stress, dietary or blood NEAC levels have been inversely associated with incidence of cardiovascular disease (CVD) [9], diabetes [10], and gastric [11] or rectal [12] cancer. On the other hand, several meta-analyses collecting randomized, placebo-controlled intervention studies have found a negative effect of galenic antioxidant supplementation on overall mortality [13,14] as well as on cardiovascular diseases [15], type 2 diabetes [16] and cancer [17–19]. Taken together, all this evidence highlights the complexity of the interactions between exogenous and endogenous antioxidants: physiological diversity in the absorption and disposal of antioxidants and the existence of homeostatic mechanisms of regulation might affect the ability of exogenous antioxidants to modulate redox defenses in vivo and play a role in the prevention of oxidative stress-related diseases [20]. We hypothesize that antioxidant status is regulated by endogenous mechanism of control designed to avoid redox overloading, maintaining a physiological homeostasis. In a previous study in the PREDIMED cohort, a MED diet increased the plasma NEAC levels after 3 years of intervention, but it did not assess the potential modification effect by the baseline NEAC levels [5]. Therefore, we investigated whether there is an effect of MED dietary pattern supplemented with either virgin olive oil or nuts (vs. a control low-fat diet) on plasma NEAC after 1 year of intervention, and if it is related to baseline NEAC levels or not.

Methods

Study design

The PREDIMED (Prevención con Dleta MEDiterránea) Study is a large, parallel-group, multicenter, randomized,
controlled clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. The design and methodology of the PREDIMED study have been described in detail elsewhere (www.predimed.org and www.predimed.es) [21,22]. The recruitment of the trial was completed between 2003 and 2009 and included 7447 participants at high risk for CVD who were randomly allocated to three intervention groups: 1) Mediterranean diet supplemented with virgin olive oil (MED + VOO), 2) Mediterranean diet supplemented with mixed nuts (MED + nuts), and 3) advice on a control low-fat diet. Eligible participants were community-dwelling men 55–80 years of age and women 60–80 years of age without prior CVD and with type 2 diabetes or at least three or more of the following CVD risk factors: current smoking, hypertension (blood pressure > 140/90 mm Hg or treatment with antihypertensive drugs), LDL cholesterol ≥ 160 mg/dL (or treatment with hypolipidemic drugs), low HDL cholesterol (< 40 mg/dL), body mass index (BMI) ≥ 25 kg/m², or family history of premature CVD. The Institutional Review Boards of all participant centers approved the study protocol, and the study has been registered in the Current Controlled Trials, London (ISRCTN 35739639).

The population sample consisted of 564 participants (288 women and 276 men) recruited between October-2003 and July-2004, and randomly selected within those who had been one year in the intervention program.

### Measurements

At baseline, all participants completed a validated semi-quantitative food frequency questionnaire [23], the validated Spanish version [24] of the Minnesota Leisure Time Physical Activity Questionnaire, and a questionnaire about education, lifestyle, history of illnesses and medication use. Trained personnel measured weight and height and obtained samples of fasting blood. All examinations were repeated at 1 year of intervention. Energy and nutrient intake were calculated from Spanish food composition tables [25]. Dietary FRAP and TRAP intake were estimated using a specific database [26].

Samples of EDTA plasma were coded, shipped to a central laboratory and stored at −80 °C until assay. Plasma NEAC was analyzed through the assessment of the FRAP assay [27] and the TRAP assay [28] indicator of the ability of plasma to reduce iron and to scavenge peroxyl radicals, respectively. The intra- and inter-assay coefficients of variation of TRAP and FRAP assays did not exceed 5%.

### Statistical analysis

Normality tests were applied using the Kolmogorov–Smirnov criterion. Mean (and standard deviation) or percentage was used to describe the baseline characteristics. Statistical differences were assessed by using one-way ANOVA and Pearson’s chi-square test for continuous and categorical variables, respectively. We examined 1-year changes in dietary and laboratory variables in response to each intervention group using paired t-tests. We compared changes in plasma TRAP and FRAP levels according to intervention-specific NEAC quartiles at baseline and intervention groups with repeated-measures ANOVA and post hoc Bonferroni tests. The intervention-specific TRAP quartiles at baseline were for MED + VOO (Q1 < 1270.4; Q2: 1270.4–1393.5; Q3: 1393.6–1529.2; Q4 > 1529.2), for MED + nuts (Q1 < 1282.4; Q2: 1282.4–1407.2; Q3: 1407.3–1521.5; Q4 > 1521.5) and for control low-fat diet (Q1 < 1261.8; Q2: 1261.8–1413.0; Q3: 1413.1–1531.6; Q4 > 1531.6). The intervention-specific FRAP quartiles at baseline were for MED + VOO (Q1 < 842.3; Q2: 842.3–954.8; Q3: 954.9–1089.5; Q4 > 1089.5), for MED + nuts (Q1 < 829.2; Q2: 829.2–932.4; Q3: 932.5–1086.4; Q4 > 1086.4) and for control low-fat diet (Q1 < 871.7; Q2: 871.7–966.2; Q3: 966.3–1069.0; Q4 > 1069.0).

Multiple linear regression models were applied to evaluate the association between plasma NEAC levels and intervention groups with and without taking into account intervention-specific NEAC quartiles at baseline, after controlling for age and sex. To calculate P values for trends across quartiles, participants were assigned a score ranging from 1 to 4 according to their quartile of plasma NEAC level at baseline and this variable was entered as a continuous term in the linear regression models. The results from the regression models are presented as beta-coefficients and 95% CI of the coefficient. Interaction between intervention-specific NEAC quartiles at baseline and intervention groups in relation to change of NEAC levels after 1 y of intervention were evaluated using a likelihood ratio test based on the models with and without an interaction term. The results were not significant for both TRAP (P for interaction 0.506) and FRAP (P for interaction 0.137). We also calculated the $R^2$ in order to find how well each fitted model predicts the dependent variables.

All statistical tests were 2-tailed, and the significance level was 0.05. All analyses were carried out by using SPSS statistical software version 19.0 (SPSS Inc., Chicago, USA).

### Results

Baseline characteristics of the study population by intervention group are presented in Table 1. Participants in the different intervention groups had similar sex (approximately 50% of women), age (65 years), and prevalence of CVD risk factors. Plasma levels of FRAP at baseline were non significantly higher in the individuals of the control low-fat diet (P = 0.095), for this reason the quartiles of FRAP and TRAP were considered as intervention group-specific. Table 2 shows the dietary changes recorded at 1-year of follow-up according to intervention group, using paired t-tests. Mediterranean diet score and dietary NEAC, particularly FRAP, increased in the two MED groups and remained unchanged in the control low-fat diet group. Both MED groups increased the consumption of plant-based foods, such as vegetables, fruits, nuts, legumes and olive oil, and consequently fat, fiber and NEAC. Participants who followed the control low-fat diet decreased their consumption of pastries, cakes or sweets, meat or meat products, and fish or seafood. Thus, they significantly reduced their intake of total protein, fat and cholesterol.

Plasma levels of FRAP [mean difference, 72.0 μmol/L (95% CI, 34.2–109.9); P < 0.001] and TRAP [45.0 μmol/L (0.9–89.1); P = 0.046] increased significantly after one year of intervention with MED + nuts. An increase in FRAP...
was also shown in individuals with MED + VOO [48.9 μmol/L (24.3–73.5); P < 0.001] and changes in TRAP levels were borderline significant [30.36 μmol/L (−4.3 to 63.0); P < 0.08]. No changes were observed in the control low-fat diet group in FRAP and TRAP (Fig. 1).

The effect of supplementation differed significantly on the basis of starting quartile of plasma NEAC levels (Fig. 2). In all three interventions, participants in the lowest baseline quartile of plasma NEAC levels showed a significant increase in both TRAP and FRAP levels. Only participants, who initially were in the second TRAP quartile, showed an increase in TRAP levels, after the MED + VOO intervention, whereas no significant effect on NEAC was observed for participants who initially were in the third TRAP baseline quartile. A significant decrease in plasma TRAP levels was shown in subjects allocated at the top quartile for both MED + VOO and control low-fat diet. For the three interventions, subjects in the second baseline quartile of FRAP showed a significant increase in their FRAP levels. Participants at the 3rd baseline quartile (MED + nuts only) increased significantly FRAP levels whereas no changes were observed for the other groups. No changes were observed for subjects at highest quartile of FRAP levels after both MED interventions, whereas a significantly decrease was shown after 1-year of intervention with the control low-fat diet.

The adjusted mean differences of plasma NEAC levels after 1-year of dietary intervention are shown in Table 3. After taking into account the potential confounding effect of age and sex, a significant difference in the increase in both plasma TRAP and FRAP levels was observed between both MED interventions and the control low-fat diet. After the inclusion of NEAC baseline quartiles, the aforementioned relationships were confirmed, and a significant inverse trend was observed between changes of plasma NEAC levels after 1-year of intervention and NEAC baseline quartiles.
quartiles. The explanatory ability of NEAC baseline quartiles on plasma NEAC level variation was 21.7% and 9.7% (i.e. adjusted $R^2$) for plasma TRAP and FRAP levels, respectively.

Discussion

For the first time, we provide evidence for the existence of tuning mechanisms of plasma antioxidant network finalized to maintain a dynamic homeostasis. Our results suggest the efficacy of 1-year dietary antioxidant supplementation with foods (for both MED + VOO and MED + nuts diets). This could be explained by the specific requirement of the organism for antioxidants. Participants with the lowest plasma FRAP level at baseline significantly increased their levels after any intervention (117.6, 134.0, 76.7 μmol/L, after MED + VOO, MED + nuts and control low-fat diet, respectively), contrarily respect to people in the fourth quartile (−17.4, −23.3, −102.3 μmol/L, after MED + VOO, MED + nuts and control low-fat diet, respectively). Similar results occurred with TRAP levels. The effect was evident for both MED groups supplemented with VOO or nuts but also for the control low-fat diet in which no specific antioxidant-rich foods have been provided. However, it is important to highlight the fact that a low-fat diet is healthier than the diet followed by the subjects before the enrollment in the trial. Low-fat diet, through a reduction of foods rich in fat and energy, reduces the induction of post-prandial oxidative and inflammatory stress phenomenon, translating in different responses according to starting level of NEAC. However, when mean levels of NEAC were calculated, control low-fat group after 1-year did not show any significant increase (−2.7 and 13.9 μmol/L for TRAP and FRAP, respectively).

Reduction of NEAC levels in subjects in the highest quartile, suggests the necessity of the body to trigger constraint mechanisms avoiding antioxidants overloading to maintain levels into physiological range. Antioxidant supplementation is not needed, even it may be harmful [13–19], in subjects with sufficiently high baseline plasma concentrations.

Table 2  Changes in energy, nutrient and dietary total antioxidant capacity intake, food items and MED diet score after 1-year of follow-up according to intervention groups, using paired t-tests.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean changes from baseline at 1-year (95% CI)</th>
<th>MED + VOO (n = 190)</th>
<th>MED + nuts (n = 190)</th>
<th>Control low-fat diet (n = 184)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-Unit MED diet score</td>
<td></td>
<td>2.4 (1.4–3.4)</td>
<td>1.7 (0.7–3.0)</td>
<td>0.3 (−0.4 to 1.1)</td>
</tr>
<tr>
<td>Dietary TRAP (mmol/day)</td>
<td></td>
<td>0.4 (−0.1 to 0.8)</td>
<td>0.4 (−0.1 to 0.8)</td>
<td>−0.1 (−0.5 to 0.4)</td>
</tr>
<tr>
<td>Dietary FRAP (mmol/day)</td>
<td></td>
<td>1.6 (0.4–2.8)</td>
<td>4.0 (2.9–5.1)</td>
<td>−0.5 (−1.7 to 0.6)</td>
</tr>
<tr>
<td>Vegetables (g/day)</td>
<td></td>
<td>43.6 (22.1–65.1)</td>
<td>46.6 (30.3–62.9)</td>
<td>−1.0 (−21.6 to 19.5)</td>
</tr>
<tr>
<td>Fruits (g/day)</td>
<td></td>
<td>58.5 (20.5–96.4)</td>
<td>62.9 (37.4–88.5)</td>
<td>23.6 (−4.2 to 51.4)</td>
</tr>
<tr>
<td>Total nuts (g/day)</td>
<td></td>
<td>5.2 (3.1–7.4)</td>
<td>22.2 (19.3–25.1)</td>
<td>−1.8 (−3.7 to 0.1)</td>
</tr>
<tr>
<td>Legumes (g/day)</td>
<td></td>
<td>5.5 (3.4–7.6)</td>
<td>5.2 (3.9–6.5)</td>
<td>0.4 (−1.6 to 2.4)</td>
</tr>
<tr>
<td>Total olive oil (g/day)</td>
<td></td>
<td>12.1 (9.6–14.6)</td>
<td>8.6 (6.3–11.0)</td>
<td>−1.0 (−3.8 to 1.8)</td>
</tr>
<tr>
<td>Cereals (g/day)</td>
<td>−27.2 (−45.3 to −9.1)</td>
<td>−6.4 (−20.2 to 7.5)</td>
<td>−12.4 (−30.7 to 5.9)</td>
<td></td>
</tr>
<tr>
<td>Dairy products (g/day)</td>
<td></td>
<td>39.9 (7.4–72.2)</td>
<td>−17.8 (−47.1 to 11.5)</td>
<td>0.5 (−28.7 to 29.7)</td>
</tr>
<tr>
<td>Meat or meat products (g/day)</td>
<td>−6.7 (−15.4 to 2.2)</td>
<td>−9.6 (−17.3 to −1.9)</td>
<td>−9.7 (−17.2 to −2.2)</td>
<td></td>
</tr>
<tr>
<td>Fish or seafood (g/day)</td>
<td>8.9 (1.8–16.0)</td>
<td>3.5 (−1.1 to 8.1)</td>
<td>−8.1 (−14.5 to −1.7)</td>
<td></td>
</tr>
<tr>
<td>Pastry, cakes or sweets (g/day)</td>
<td>−4.2 (−8.5 to 0.1)</td>
<td>−8.4 (−13.6 to −3.1)</td>
<td>−5.0 (−9.8 to −0.2)</td>
<td></td>
</tr>
<tr>
<td>Wine (g/day)</td>
<td>6.6 (−8.1 to 21.3)</td>
<td>2.6 (−14.8 to 20.0)</td>
<td>3.5 (−8.5 to 15.5)</td>
<td></td>
</tr>
<tr>
<td>Alcohol (g/day)</td>
<td>−0.4 (−2.2 to 1.5)</td>
<td>−1.3 (−3.4 to 0.8)</td>
<td>−0.7 (−2.4 to 1.0)</td>
<td></td>
</tr>
<tr>
<td>Total energy (kcal/day)</td>
<td>40.3 (−51.0 to 131.7)</td>
<td>82.5 (20.9–144.0)</td>
<td>−158.9 (−238.1 to −79.8)</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g/day)</td>
<td>−9.0 (−22.2 to 4.2)</td>
<td>2.1 (−6.9 to 11.2)</td>
<td>−8.9 (−20.5 to 2.7)</td>
<td></td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>2.9 (−0.5 to 6.3)</td>
<td>2.8 (0.5–5.1)</td>
<td>−4.5 (−7.5 to −1.4)</td>
<td></td>
</tr>
<tr>
<td>Fat (g/day)</td>
<td>7.5 (3.1–11.9)</td>
<td>7.9 (4.1–11.8)</td>
<td>−11.2 (−15.2 to −7.1)</td>
<td></td>
</tr>
<tr>
<td>Fiber (g/day)</td>
<td>2.0 (0.7–3.3)</td>
<td>4.8 (3.7–5.9)</td>
<td>−0.4 (−1.7 to 0.9)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/day)</td>
<td>−0.2 (−17.2 to 16.7)</td>
<td>−30.2 (−42.5 to −17.9)</td>
<td>−29.2 (−48.8 to −9.6)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: TRAP, total radical-trapping antioxidant parameter; FRAP, ferric reducing antioxidant potential; MED Mediterranean diet; VOO, virgin olive oil.
levels of NEAC within physiological ranges. The mechanisms through which physiological levels are reached are still unclear, and it might involve the balance of both dietary and endogenous antioxidants. Indirect evidence to support our findings came from the SUVIMAX (Supplementation en Vitamines et en Minéraux Antioxydants) study, where a cocktail of antioxidants and minerals was used at nutritional dosages for 8 years. In this study, supplementation was effective only in men, who showed lower baseline levels of β-carotene and vitamin E compared to women, and this translated into a mean 30% reduced cancer incidence [29]. Moreover, Serafini’s group recently systematically reviewed intervention studies involving plant foods and plasma NEAC, showing that efficiency of supplementation with antioxidant-rich foods leads to a higher response, in terms of increase in NEAC, in subjects at high risk of CVD and suffering from some diseases compared to “healthy” controls [2]. These results suggest that individuals with oxidative stress would benefit more of a possible exogenous antioxidant supplementation. In an interesting review, Elayed summarized some experiments of vitamin E supplementation demonstrating that vitamin E was more likely to be mobilized in a variety of situations associated with high oxidative stress (such as photochemical-oxidant air pollutants, tobacco smoking, paraquat toxicity, burn injury, and excessive physical activity) compared to normal conditions [30]. However, in clinical trials with healthy subjects, antioxidant supplements increased the risk of overall mortality [13,14], cardiovascular diseases [15], type 2 diabetes [16] and cancer [17–19].

Our study supports that long-term interventions with MED diet supplemented with nuts or VOO increases plasma NEAC, but not after the control low-fat diet [4,5]. MED diet is characterized by a high consumption of fruits, vegetables, unrefined cereals, legumes, nuts and seeds, olive oil, moderate wine consumption and lower intake in animal products. Several studies also suggest an increase in plasma NEAC after chronic supplementation with plant foods, especially fruits and vegetables [20], but it is unclear which molecules are responsible for this effect. In the last two decades, phenolic compounds have highlighted as partially responsible of these antioxidant effects, although a recent review concludes that the direct antioxidant effect of phenolic compounds in vivo is still to be clarified [31]. Other molecules present in plant foods that may increase plasma NEAC are vitamins, such as vitamin E, ascorbic acid and carotenoids [1,32].

Our study has some limitations. The participants were older subjects at high risk for CVD, thus the results cannot be directly extrapolated to the general population. Another limitation is that our results could be partially due to the statistical phenomenon called regression toward the mean. It means that if a variable is extreme on its first measurement, it will tend to be closer to the average on a second measurement. This phenomenon is usually solved using a control group, but in the current study, our control group is the low-fat diet intervention which was healthier than the usual food pattern. For this reason, the plasma NEAC levels were likely increased in the first quartile. However, people in the fourth quartile, even having healthier diets, decreased plasma NEAC levels, especially after low-fat intervention which is the less effective diet against NEAC. Although we have to be cautious in drawing conclusions due to regression toward the mean, our results are plausible with the hypothesis of modulation mechanisms of antioxidant network [1].

In conclusion, we provided evidence that, MED diet increases plasma NEAC levels after 1 year of intervention in subjects at high risk for cardiovascular disease. Furthermore, efficiency of dietary supplementation with antioxidants may be related to baseline levels of plasma NEAC. A better understanding of the mechanism regulating the requirement of the body for exogenous antioxidants is needed in order to optimize strategies for oxidative stress prevention.
**Conflict of interest**

The authors are not aware of any conflict of interest.

**Other predimed study investigators**

School of Pharmacy, University of Barcelona: Rafael Llorach, Montserrat Rabassa, Alex Medina-Remón; Internal Medicine Department, Hospital Clinic, Barcelona: Emilio Sacanella, Ferran Masanés, Rosa Casas, Concha Viñas; University of Navarra-Osasunbidea Primary Care Division: Vicente Extremera, Concepción Arroyo, Luisa García Pérez, Pilar Bulí-Cosiales; Human Nutrition Unit, School of Medicine, Universitat Rovira i Virgili, Reus: Monica Bullo, Nancy Pilar Buil-Cosiales; Human Nutrition Unit, School of Pharmacy, University of Barcelona: Rafael Llorach, Montserrat Rabassa, Alex Medina-Remón; Internal Medicine Department, Hospital Clinic, Barcelona: Emilio Sacanella, Ferran Masanés, Rosa Casas, Concha Viñas; University of Navarra-Osasunbidea Primary Care Division: Vicente Extremera, Concepción Arroyo, Luisa García Pérez, Pilar Bulí-Cosiales; Human Nutrition Unit, School of Medicine, Universitat Rovira i Virgili, Reus: Monica Bullo, Nancy Pilar Buil-Cosiales; Cardiovascular Risk and Nutrition Research Group, Institut Mar d’Investigacions Mèdiques (IMIM), Barcelona: Montse Fitó, Rafael de la Torre, Helmut Calvo, Francisco José García, Pilar Román.

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