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Mediterranean diet supplemented with coenzyme Q10 induces postprandial changes in p53 in response to oxidative DNA damage in elderly subjects

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Abstract Coenzyme Q10 (CoQ) is a powerful antioxidant that reduces oxidative stress. We explored whether the quality of dietary fat alters postprandial oxidative DNA damage and whether supplementation with CoQ improves antioxidant capacity by modifying the activation/stabilization of p53 in elderly subjects. In this crossover study, 20 subjects were randomly assigned to receive three isocaloric diets during 4 weeks each: (1) Mediterranean diet (Med diet), (2) Mediterranean diet supplemented with CoQ (Med+CoQ diet), and (3) saturated fatty acid-rich diet (SFA diet). Levels of mRNAs were determined for p53, p21, p53R2, and mdm2. Protein levels of p53, phosphorylated p53 (Ser20), and monoubiquitinated p53 were also measured, both in cytoplasm and nucleus. The extent of DNA damage was measured as plasma 8-OHdG. SFA diet displayed higher postprandial 8-OHdG concentrations, p53 mRNA and monoubiquitinated p53, and lower postprandial Mdm2 mRNA levels compared with Med and Med +CoQ diets ($p<0.05$). Moreover, Med+CoQ diet induced a postprandial decrease of cytoplasmatic

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p53, nuclear p-p53 (Ser20), and nuclear and cytoplasmatic monoubiquitinated p53 protein ($p<0.05$). In conclusion, Med+CoQ diet improves oxidative DNA damage in elderly subjects and reduces processes of cellular oxidation. Our results suggest a starting point for the prevention of oxidative processes associated with aging.

Keywords Aging · Coenzyme Q10 · p53 · Oxidative stress · DNA damage

Abbreviations

Apo	Apolipoprotein
BMI	Body mass index
CoQ	Coenzyme Q10
HDL-C	HDL cholesterol
LDL	Low-density lipoprotein
Med diet	Mediterranean diet
Med+CoQ diet	Mediterranean diet supplemented with CoQ
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SFA diet	Saturated fatty acid-rich diet

Introduction

Oxidative stress defines an imbalance between formation of reactive oxygen species (ROS) and antioxidative defense mechanisms (Sen 1996) and can be directly involved in degenerative processes (Lambeth et al. 2000). The oxidative-stress hypothesis of aging postulates that deleterious effects of ROS are responsible for the functional deterioration associated with aging and age-related diseases, such as Alzheimer, Parkinson, cancer, diabetes, and cardiovascular diseases. The p53 tumor suppressor is a transcription factor which mediates the cellular response to DNA damage. In addition to its roles in cell cycle control and in the maintenance of genomic stability, p53 has antioxidant functions protecting the genome from oxidation produced by ROS (Appella and Anderson 2001; Sablina et al. 2005).

Exaggerated postprandial spikes in glucose and lipids generate excess ROS that can trigger a biochemical cascade resulting in inflammation, endothelial dysfunction, and sympathetic hyperactivity (Weissman et al. 2006; Bonora et al. 2006). Dietary and lifestyle factors play a central role in the etiology of postprandial dysmetabolism (O'Keefe and Bell 2007). In Western societies, high-calorie meals rich in processed foods and drinks, usually rich in saturated fatty acids (SFA) can lead to exaggerated postprandial elevations in blood glucose and triglycerides, and overwhelms the metabolic capabilities of the mitochondria creating free radicals (Monnier et al. 2006). The postprandial oxidant stress acutely triggers atherogenic changes, including increases in low-density lipoprotein (LDL) oxidation, sympathetic tone, vasoconstriction, and thrombogenicity. The Mediterranean diet (Med diet), which is rich in minimally processed natural foods, has been associated with improved cardiovascular health and longevity (Lichtenstein et al. 2006; Fito et al. 2007). Specifically, diets that include large amounts of fresh unprocessed plants, with moderate levels of lean protein and beneficial fats (such as omega-3 and monounsaturated fats) and that are rich in antioxidants substantially improve postprandial dysmetabolism (Lichtenstein et al. 2006; Jimenez-Gomez et al. 2010).

Coenzyme Q_{10} (2,3-dimethoxy-5-methy-6-decaprenyl-1,4-benzoquinone, CoQ) has been proposed as an additional source of antioxidant because of its key role in mitochondrial bioenergetics and its widely studied antioxidant capacity under lipophilic conditions (Turunen et al. 2004). Around 50% of ubiquinone is obtained through fat ingestion and the other 50% through endogenous synthesis (Turunen et al. 2004). CoQ protects phospholipids, mitochondrial membrane proteins, and DNA from oxidative damage accompanying lipid peroxidation (Forsmark-Andree and Ernster 1994). Indeed, supplementation with exogenous CoQ has been shown to lead to an increase in the CoQ content of LDL, and a decrease of their peroxidizability (Stocker et al. 1991; Thomas et al. 1996). Studies in animal models have shown the protective effect of CoQ when supplemented in the diet (Bello et al. 2005; Santos-Gonzalez et al. 2007), in which supplementation attenuated oxidative alterations, with lower levels of DNA damage (Quiles et al. 2004). However, to date there are no human studies, especially cross-randomized controlled trials, aimed to determine the antioxidant effects of CoQ during the postprandial phase associated with aging.

On this basis, our aim was to determine whether diets with different fat quality influence postprandial

oxidative DNA damage and whether supplementation with CoQ to a Med diet improves antioxidant capacity by modifying the activation/stabilization of p53 in elderly subjects.

Experimental procedures

Participants and recruitment

Volunteers were recruited using various methods including use of general practitioner databases, and poster and newspaper advertisements. A total of 63 persons were contacted among those willing to enter the study. We calculated sample size for p-p53 nuclear values with an alpha risk of 0.05 and power of 0.85, and based on these premises it takes at least 17 patients.

All participants underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrolment and gave their informed consent before joining the study. Inclusion and exclusion criteria were fulfilled by 20 patients (age ≥65 years; 10 men and 10 women). Clinical inclusion criteria were age ≥65 years, body mass index (BMI) 20–40 kg/m^2, total cholesterol concentration equal to or <8.0 mmol/L, and non-smokers. The clinical exclusion criteria were age <65 years, diabetes or other endocrine disorders, chronic inflammatory conditions, kidney or liver dysfunction, iron deficiency anemia (hemoglobin <12 g/dL men, <11 g/dL women), prescribed hypolipidemic and anti-inflammatory medication, fatty acid supplements including fish oil, consumers of high doses of antioxidant vitamins (A, C, E, and β-carotene), highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week, weight change equal or >3 kg within the last 3 months, smokers, and with alcohol or drug abuse history (based on clinical judgment). The study protocol was approved by the Human Investigation Review Committee of the Reina Sofia University Hospital, according to institutional and Good Clinical Practice guidelines.

Study design

Participants were randomly assigned to receive, in a crossover design, three isocaloric diets for 4-week periods each (Fig. 1). The three diets were as follows:

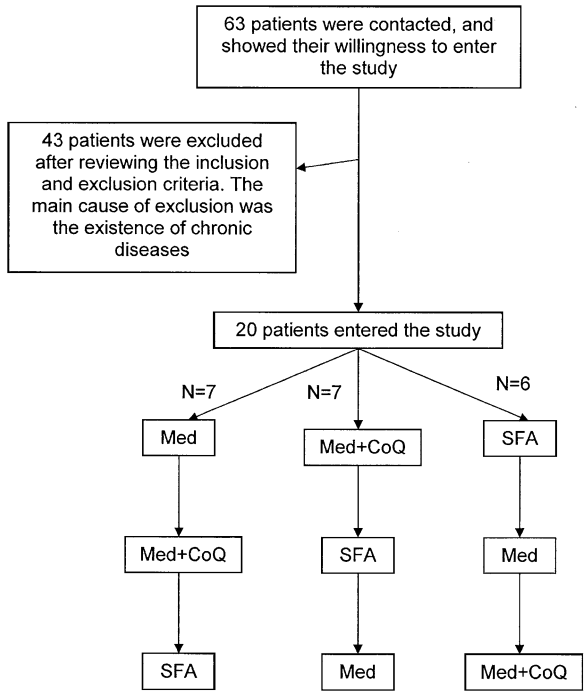

Fig. 1 Flow chart of subjects who were recruited at the beginning of the study, the number of subjects who were excluded, and the number of subjects who participated in each 4-week feeding trial. *Med diet* Mediterranean diet, *Med+CoQ diet* Mediterranean diet supplemented with CoQ, *SFA diet* saturated fatty acid-rich diet

(1) Med+CoQ diet (200 mg/day in capsules), containing 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat [24% monounsaturated fatty acid (MUFA; provided by virgin olive oil), 10% saturated fatty acid, and 4% polyunsaturated fatty acid (PUFA)]; (2) Med diet not supplemented with CoQ (Med diet), with the same composition of the first diet, but supplemented by placebo capsules; and (3) Western diet rich in saturated fat (SFA diet), with 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat [12% MUFA, 22% SFA, and 4% PUFA].

The cholesterol intake was kept constant (<300 mg/day) during the three periods. Both the CoQ and the placebo capsules were specially produced by the same company (Kaneka Corporation, Osaka, Japan) and were identical in weight and external aspect. Patients taking capsules were unaware whether they were in the Med+CoQ or Med dietary period (Table 1). The composition of the experimental diets was calculated by using the US Department of Agriculture

Table 1 Composition of diet at end of intervention period, alongside dietary targets

	Diet		
	Med	Med+CoQ	SFA
Target, MJ/day			
%E from fat	38	38	38
%E from SFA	<10	<10	22
%E from MUFA	24	24	12
%E from PUFA	4	4	4
End of intervention			
n	20	20	20
Energy, MJ/day	8.22±0.35	8.16±0.32	8.21±0.31
%E from fat	39.11±2.02	39.73±2.65	40.38±2.03
%E from SFA	8.78±0.61[a]	9.04±0.71[a]	20.75±0.87[b]
%E from MUFA	24.24±0.92[a]	24.36±1.47[a]	13.46±0.71[b]
%E from PUFA	4.15±0.23[a]	4.28±0.29[a]	3.92±0.25[b]
%E from CHO	44.64±1.86	44.31±2.29	43.64±1.73
%E from protein	16.33±0.52	16.07±0.64	16.16±0.53
Total α-tocopherol, mg/day	18.91±0.73[a]	18.95±0.84[a]	6.97±0.76[b]
Ascorbic acid, mg/day	187.54±5.83	181.17±6.12	195.62±6.86
β-carotene, mg/day	3.11±0.92	3.13±0.66	3.21±0.23
Total fiber, g/day	27.93±2.22	27.17±2.38	27.42±2.34
Cholesterol, mg/day	345.71±35.93	345.86±36.92	378.18±59.29

Values are means ± SE. Means in a row with superscripts without a common letter differ, $p<0.05$

CHO carbohydrate

(Human Nutrition Information Service 1987) food tables and Spanish food-composition tables for local foodstuffs (Varela 1980).

Before the start of the intervention period, volunteers completed a 3-day weighed food diary and an extensive Food Frequency Questionnaires, which allowed identification of foods to be modified. At the start of the intervention period each patient was provided with a handbook for the diet to which they had been randomized. Advice was given on foods to choose and those to avoid if eating outside the home, they were also instructed to write down in the diary about any menu eaten out of home and call the monitoring study nurse reporting such event. At baseline, volunteers were provided with a supply of study foods to last for 2 weeks, and they collected additional study foods every fortnight or when required. At these times, a 24-h recall of the previous day's food intake and a short food-use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A points system was used to assess the number of food exchanges achieved in the 24-h recall and additional advice was given if either the 24-h recall or food-use questionnaire showed inadequate intake of food-exchange options. Volunteers were asked to complete the 3-day weighed food diaries at baseline, week 2, and week 4. Weighed food intake over two weekdays and one weekend day was obtained using scales provided by the investigators. Fat foods were administered by dietitians in the intervention study. A dietary analysis program Dietsource version 2.0 was used.

At the end of the dietary intervention period and after a 12-h fast, at time 0, the subjects were given a fatty breakfast with the same fat composition as consumed in each of the diets. Patients presented at the clinical centers at 8-h following a 12-h fast and abstained from alcohol intake during the preceding 7 days. After canulation of a blood vessel, a fasting blood sample was taken before the test meal, which was then ingested within 20 min under supervision. The test meal reflected fatty acid composition of each subject's chronic dietary intervention. Subsequent blood samples were drawn at 2 and 4 h. Test meals provided an equal amount of fat (0.7 g/kg body weight), cholesterol (5 mg/kg of body weight), and vitamin A (60,000 IU/m^2 body surface area). The test

meal provided 65% of energy as fat, 10% as protein, and 25% as carbohydrates. The composition of the breakfasts was as follow: Med+CoQ (400 mg in capsules) breakfast (12% SFA, 43% MUFA, and 10% PUFA); Med with placebo capsules breakfast (12% SFA, 43% MUFA, and 10% PUFA); and SFA-rich breakfast (38% SFA, 21% MUFA, and 6% PUFA).

Biochemical determinations

Plasma samples

Samples were collected in tubes containing 1 g EDTA/L and then stored in containers with ice and kept in the dark. Particular care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from whole blood by low-speed centrifugation at 1,500×*g* for 15 min at 4°C within 1 h of extraction.

Lipid analysis

Concentrations of the different lipid variables were analyzed with a modular autoanalyzer (DDPPII Hitachi; Roche, Basel, Switzerland) with the use of Boehringer-Mannheim reagents. Total cholesterol (TC) and triglycerides (TG) in plasma and lipoprotein fractions were assayed by means of enzymatic procedures. Apolipoprotein (Apo) A-I and Apo B were determined by immunoturbidimetry. High-density lipoprotein-cholesterol (HDL-C) was measured by analyzing the supernatant obtained following precipitation of plasma aliquot with dextran sulfate-Mg^{2+}. LDL-C levels were estimated using the Friedewald formula based on TC, TG, and HDL-C concentrations. Plasma glucose concentrations were measured with an Architect-CG16000 analyzer (Abbott Diagnostics, Tokyo, Japan) by the exoquinase method. Plasma insulin concentrations were measured by chemoluminesence with an Architect-I2000 analyzer (Abbott Diagnostics, Tokyo, Japan). High sensitivity C-reactive protein (hsCRP) concentrations were measured according to Rifai et al. (1999).

Coenzyme Q_{10} determination

Levels of CoQ_{10} were carried out in plasma samples according to the method described by Santos-Gonzalez et al. (2007). The quantification of these substances was performed by reversed-phase HPLC. Separation was performed at 1 mL/min in a C18 column (5 μm particles, 25×0.45 cm) and with a mobile phase that consisted of a mixture of methanol and *n*-propanol (1:1) containing lithium perchlorate (2.12 g/L). Monitoring was carried out with a Coulochem II electrochemical detector (ESA, Chelmsford, MA, USA) fitted with a Model 5010 analytical cell with the electrodes set at potentials of −500 mV and +300 mV. CoQ was detected from the signal obtained at the second electrode. Eluted compounds were quantified by integration of peak areas and comparison with an internal CoQ_{10} standard (Sigma Aldrich, Madrid, Spain).

Isolation of PBMCs

Mononuclear cells were isolated from 20 mL of venous blood in tubes containing 1 mg/mL of EDTA. The blood samples were diluted 1:1 in PBS, and cells were separated in Ficoll gradient by centrifugation at 800×*g* for 25 min at 20°C. The cells were collected and washed with cold PBS two times and finally resuspended in buffer A. This buffer contained 10 mM HEPES, 15 mM KCl, 2 mM MgCl2, and 1 mM EDTA and, at the time of use, 1 mM PMSF and 1 mM DTT were added. The cells thus obtained were stored at −80°C for further analysis.

Protein extraction

The cells were thawed on ice and buffer A was supplemented with 5 μg Aprotinin, 10 μg Leupeptin, and 0.8% Nonidet NP-40. Cells were incubated on ice for 5 min, subjected to gentle agitation for 20 s in the vortex, and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant containing cytoplasmic proteins was distributed in aliquots that were stored at −80°C. The pellet was treated with 100 μL of lysis buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 20 μg Aprotinin, and 40 μg Leupeptin). The sample was incubated on ice for 20 min with periodic mixing with a vortex by stirring for 30 s every 5 min of incubation. Cells were then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant thus obtained, containing the nuclear proteins, was distributed in aliquots, and stored at −80°C. The extracted proteins were quantified using

the method of Bradford (1976) (Bio Rad Protein Assay).

Western blot

Electrophoretic separation was carried out with 50 μg of protein for both cytoplasmic and nuclear fractions. After separation in SDS-PAGE gels (11% polyacrylamide) proteins were transferred to nitrocellulose membranes (BioTrace NT Membrane; PALL Gelman Laboratory). The following proteins were detected using their corresponding antibodies: p53 (D-11: mouse monoclonal, Santa Cruz Biotechnology, inc.); p-p53 (hSer 20-R: rabbit polyclonal, Santa Cruz Biotechnology, inc.); actin (C-2: mouse monoclonal, Santa Cruz Biotechnology, inc.). After incubation with these primary antibodies, samples were incubated with respective secondary antibodies (goat anti-mouse or anti-rabbit HRP-conjugate, Santa Cruz Biotechonology, inc.). The development process was carried out with ECL Plus Western Blotting Detection System (Amersham™) and used for autoradiography Hyperfilm MP high-performance autoradiography film (Amersham™).

Quantification of protein bands

The proteins were identified in the autoradiography by its position relative to molecular weight markers: 53 kDa for p53 and p-p53, 61.6 kDa for monoubiquitinated p53 (53 kDa of the p53+8.6 kDa of ubiquitin), and 40 kDa for actin. The relative amount of each was quantified by densitometry using the software WIN1D.

8-OHdG determination

The concentration of 8-OHdG was determined in plasma samples using an ELISA kit (Japan Institute for the Control of Aging, Fukuroi, Japan).

RNA extraction

Total RNA from PBMCs was extracted using the trizol method according to the recommendations of the manufacturer (Tri Reagent®, Sigma, St Louis, MO, USA) and quantified in a NanoDrop 1000A Spectrophotometer. RNA integrity was verified on agarose gel electrophoresis and stored at −80°C. Next, since PCR can detect even a single molecule of DNA, RNA samples were digested with DNAse I (AMPD-1 KT, Sigma) before RT-PCR.

qRT-PCR analysis of gene expression

PCR reactions were performed using an iQ™5 Multicolor Real-Time PCR Detection System (Bio Rad) and the iQ SYBR Green Supermix (Bio Rad) commercial kit in a final volume of 20 μl with 10 pmol of each primer. Each reaction was performed with 1 μl of a 1:5 (*v*/*v*) dilution of the first cDNA strand, synthesized from 1 μg of total RNA using the commercial kit iScript cDNA Synthesis Kit (Bio Rad) according to the manufacturer's instruction. The reaction mixture was incubated at 96°C for 3 min, followed by 40 cycles of 1 min at 96°C, 30 s at 60°C, and 20 s at 72°C. Primers used were: p53-forward (5′-TAACAGTTCCTGCATGGGCG-3′), p53-reverse (5′-AGGACAGGCACAAACACGCACC-3′); MDM2-forward (5′-TGCAAAGAAGCTAAAGAAAAGGAATA-3′), MDM2-reverse (5′-AGACAGGTCAACTAGGGGAAATAAG-3′); p21-forward (5′-TCCAAACGCCGGCTGATCTTC-3′), p21-reverse (5′-GAGCGGGCCTTTGAGGCCCTC-3′); p53R2-forward (5′-TCCAATGAACTCATCAGCAGAGATG-3′), p53R2-reverse (5′-GAAACTCGTTTCTCAAAGAAATTTGT-3′); and RPL13-forward (5′-CCTGGAGGAGAAGAGGAAAGAGA-3′), RPL13-reverse (5′-TTGAGGACCTCTGTGTATTTGTCAA-3′). The specificity of PCR amplifications was checked by a melting curve program (60–95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement) and analyzed by electrophoresis on a 1.6% agarose gel, TBE 1×. The expression values were obtained as relative expression of the target gene versus the constitutively expressed RPL13a gene.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, Illinois) was used for the statistical comparisons. The Kolmogorov–Smirnov test did not show a significant departure from normality in the distribution of variance values. In order to evaluate data variation, Student's *t* test and an analysis of variance (ANOVA) for repeated measures was performed, followed by Bonferroni's correction for multiple comparisons. We studied the statistical effects of the type of fat meal ingested,

independent of time (represented by $p1$), the effect of time (represented by $p2$), and the interaction of both factors, indicative of the degree of the postprandial response in each group of subjects with each fat meal (represented by $p3$). Differences were considered to be significant when $p<0.05$. All data presented in text and tables are expressed as means ± standard error of the mean (±SEM).

Results

Baseline characteristics of participants in the study Anthropometric and biochemical characteristics of the subjects are presented in Table 2. Height, waist circumference, triglycerides, and ApoB plasma levels were higher in males than in females (Table 2). Dietary compliance was good, with close attainment of the dietary intervention targets (Table 1).We did not find any other differences by gender.

Characteristics of participants across the study At the end of each period of dietary intervention, fasting plasma concentrations of TC ($p<0.001$), LDL-C ($p=0.013$), ApoB ($p=0.017$), and ApoA-I ($p=0.002$) were higher after participants consumed the SFA diet than when they consumed the others diets (Table 3).

Coenzyme Q determination We observed higher fasting plasma CoQ concentration ($p<0.001$) after the intake of the Mediterranean diet supplemented with CoQ (Med+CoQ diet) compared with the Mediterranean and saturated fatty acid-rich diets (Med and SFA diets; Fig. 2a). An increase in postprandial (2 h) plasma CoQ levels was observed after consumption of the Med+CoQ diet compared with the Med and SFA diets ($p=0.018$).

DNA damage (8-OHdG) The long-term consumption of the Med and Med+CoQ diets decreased 8-OHdG plasma concentrations compared with the SFA diet ($p<0.0001$). Furthermore, 8-OHdG levels were lower after consumption of the Med+CoQ compared with the Med diet ($p<0.001$). In addition, during the postprandial period, a lower concentration of 8-OHdG was observed after the intake of the Med +CoQ as compared to the SFA diet ($p=0.026$; Fig. 2b).

Cytoplasmic and nuclear p53 in PBMCs p53 become upregulated upon DNA damage. After Med+CoQ diet, a decrease in postprandial levels of cytoplasmic p53 was observed ($p<0.05$) as compared to the other diets. In contrast, no significant differences were observed in nuclear p53 levels after intake of these diets both in fasting and during the postprandial period (Fig. 2c, d).

Cytoplasmic and nuclear p-p53 (Ser20) in PBMCs After DNA damage, a phosphorylating stimulus is produced for p53 in Ser20 which leads to p53 activation and its binding to target gene promoters on DNA. The

Table 2 Baseline characteristics of the participants in the study

	Men ($n=10$)	Women ($n=10$)	p Value
Age (years)	65.82±0.91	68.44±1.36	0.208
Weight (kg)	87.73±4.72	78.44±3.19	0.232
Height (cm)	166.65±1.43[a]	154.8±1.41	<0.001
BMI (kg/m^2)	31.25±1.57	32.52±1.33	0.614
Waist circumference (cm)	109.68±2.92[a]	92.41±1.84	0.001
Plasma TC (mmol/L)	4.95±0.21	4.52±0.16	0.141
Plasma TG (mmol/L)	1.25±0.11[a]	0.87±0.13	0.013
Plasma HDL-C (mmol/L)	1.26±0.05	1.42±0.07	0.137
Plasma LDL-C (mmol/L)	3.06±0.12	2.70±0.11	0.107
Plasma Apo B (g/L)	0.90±0.04[a]	0.76±0.02	0.036
Plasma Apo A-I (g/L)	1.43±0.03	1.52±0.05	0.301
Plasma glucose (mmol/L)	5.77±0.23	5.07±0.14	0.064
Plasma insulin (pmol/L)	80.72±8.68	56.75±4.45	0.078
Plasma hsCRP (mg/L)	4.36±1.35	4.37±1.01	0.996

Values are means ± SE; $n=20$

[a] Different from women, $p<0.05$

Table 3 Lipid parameters, glucose, insulin, and hsCRP for each intervention period[a]

	Med diet	Med+CoQ diet	SFA diet	*p* Value
TC (mmol/L)	4.65±0.13[a]	4.73±0.14[a]	5.21±0.17[b]	<0.001
TG (mmol/L)	1.10±0.01	1.15±0.12	1.16±0.09	0.675
HDL-C (mmol/L)	1.31±0.06	1.34±0.06	1.40±0.07	0.069
LDL-C (mmol/L)	2.80±0.12[a]	2.82±0.06[a]	3.19±0.17[b]	0.013
Apo B (mmol/L)	0.83±0.04[a]	0.85±0.04[a]	0.91±0.05[b]	0.017
Apo A-I (mmol/L)	1.44±0.05[a]	1.47±0.05[a]	1.56±0.06[b]	0.002
Glucose (mmol/L)	4.97±0.12	5.06±0.17	5.15±0.22	0.440
Insulin (mU/L)	10.33±2.39	14.40±5.02	8.63±1.39	0.470
hsCRP (mg/L)	3.01±0.83	3.60±1.39	3.60±1.06	0.240

All values are ±SE; $n=20$

TC total cholesterol, *TG* triglycerides, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *hsCRP* high sensitivity C reactive protein

[a] Main effect of diet by repeated measures ANOVA. Different lowercase letters are significantly different

Med+CoQ diet induced a significant decrease of nuclear p-p53 (Ser20) postprandial levels ($p=0.0013$). No significant differences were observed between the two other remaining diets (Fig. 3a). Moreover, no significant differences were observed after analysis of cytoplasmic p-p53 (Ser20) levels after consumption of the three diets (Fig. 3b).

Cytoplasmic and nuclear monoubiquitinated p53 in PBMCs Monoubiquitination of p53 is a post-translational modification which mediates the transfer of p53 from the nucleus to the cytoplasm and participates in its stabilization or degradation. A decrease in nuclear monoubiquitinated p53 postprandial levels after intake of Med+CoQ diet was observed compared to the other two diets ($p<0.05$; Fig. 3c). In the case of Med and Med+CoQ diets, cytoplasmatic monoubiquitinated p53 postprandial levels decreased as compared to SFA ($p=0.046$ Med +Q vs SFA diet, $p=0.043$ Med vs SFA diet; Fig. 3d).

Relative gene expression of p53 in PBMCs p53 gene expression is self-regulated. Therefore, when DNA damage appears and p53 protein stabilization is produced, an upregulation of p53 expression is observed. Analysis of p53 mRNA levels showed a postprandial increase after 2 h following the SFA diet as compared to Med diet ($p=0.047$). This postprandial increase was not observed after consumption of the two other diets (Fig. 4a).

Relative gene expression of mdm2 in PBMCs Mdm2 is an ubiquitin RING E3 ligase which specifically ubiquitinates p53 and is involved in its stabilization, degradation, and transfer. Additionally, the mdm2 gene is controlled by p53 at transcriptional level. Med diet induced an increase in mdm2 mRNA levels as compared to SFA diet ($p=0.014$). The Med diet induced higher mdm2 mRNA levels at fasting than did the Med+CoQ ($p=0.008$) and SFA diets ($p=0.041$). After 4 h, postprandial expression levels were higher with Med than with SFA diet ($p=0.025$; Fig. 4b).

Relative gene expression of p21 in PBMCs We did not observe any significant differences in p21 mRNA levels among the three diets both in fasting and during the postprandial period (Fig. 4c).

Relative gene expression of p53R2 in PBMCs p53R2 is the subunit 2 of p53-induced ribonucleotide reductase which is involved in DNA repair. Analyzed levels of mRNA p53R2 did not show any significant differences among the three diets (Fig. 4d).

Correlation analysis We observed a positive correlation between 8-OHdG plasma concentration with p53 mRNA levels at postprandial period (2 h; $p<0.05$; Fig. 5a). There was also a positive correlation between p53 protein levels in nucleus with p53

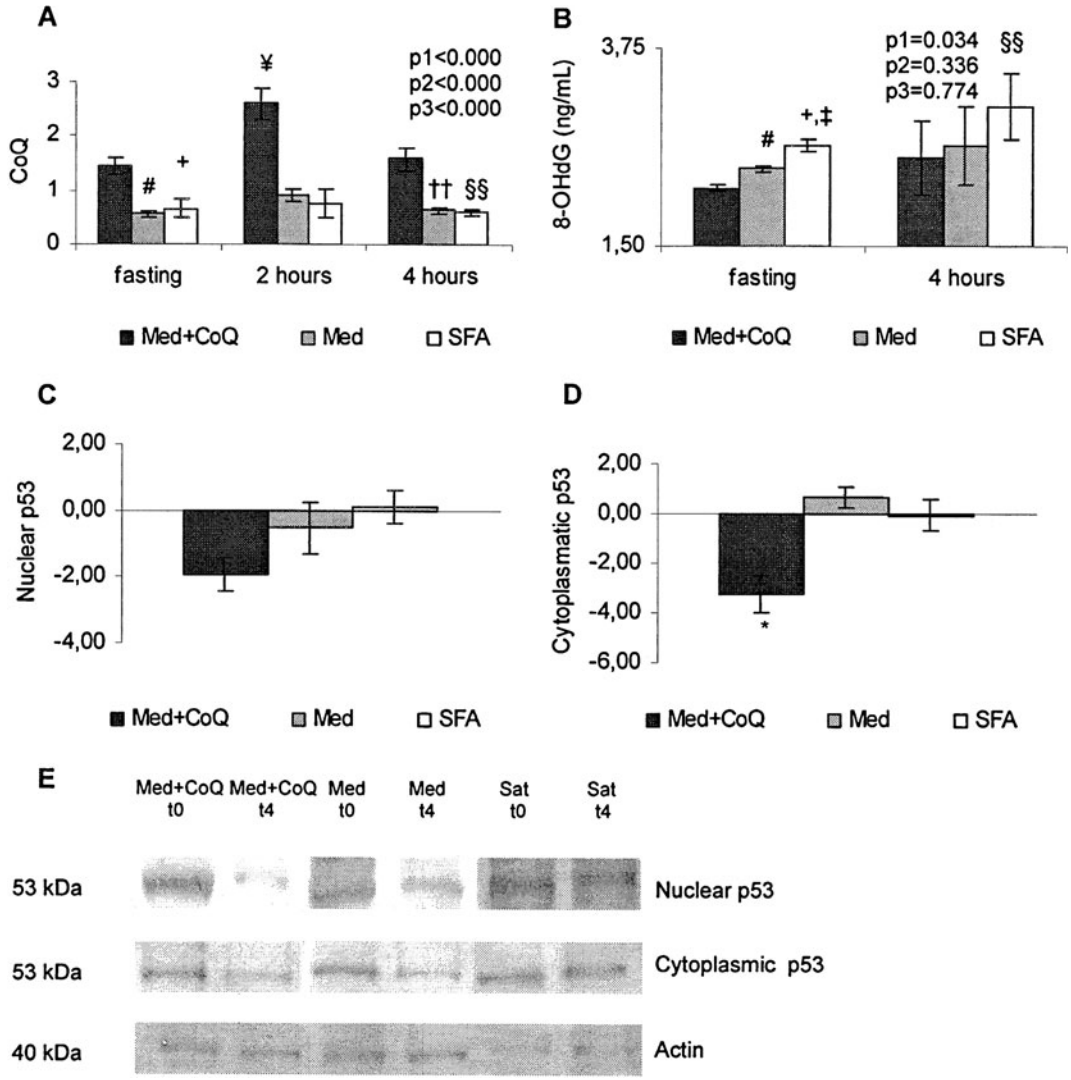

Fig. 2 Fasting and postprandial plasma levels of CoQ (**a**) and 8-OHdG (**b**) and nuclear p53 (**c**) and cytoplasmic p53 (**d**) in peripheral mononuclear cells of healthy elderly subjects at the end of the three diets. Representative immunoblot of nuclear and cytoplasmatic p53, and actin. Depicted bands are different parts of the same blot stained for each antigen (**e**). Results are means ± SEM, $n=20$. Results were analyzed using ANOVA for repeated measures. $^{\#}p<0.001$ Med diet vs Med+CoQ diet at fasting; $^{+}p<0.001$ Med+CoQ diet vs SFA diet at fasting; $^{\ddagger}p<0.001$ Med diet vs SFA diet at fasting; $^{\S}p<0.05$ Mediterranean diet vs SFA-rich diet; $^{\S\S}p<0.05$ Med+CoQ diet vs SFA diet; $^{\dagger\dagger}p<0.05$ Med+CoQ diet vs Med diet; $^{*}p<0.05$ decreased postprandial of Med+CoQ diet (fasting vs 4 h). $^{¥}p<0.05$ increased postprandial of Med+CoQ diet (fasting vs 2 h). *P1* diet effect, *P2* time effect, *P3* diet by time interaction, *Med+CoQ* Mediterranean diet supplemented with CoQ, *Med* Mediterranean diet, *SFA* saturated fatty acid-rich diet

mRNA at fasting ($p<0.01$; Fig. 5b), and postprandial period (4 h; $p<0.01$). In addition, we found a negative correlation between mdm2 mRNA levels and nuclear p53 protein at fasting ($p<0.001$; Fig. 5c), and postprandial period (4 h; $p<0.01$). Furthermore, a negative correlation was found between mdm2 mRNA levels and nuclear monoubiquitinated p53 protein levels at fasting ($p<0.01$; Fig. 5d) and postprandial period (4 h; $p<0.05$). In cytoplasm, we also observed a negative correlation between mdm2 mRNA levels and monoubiquitinated p53 protein levels at fasting ($p<0.001$) and at postprandial period (4 h; $p<0.01$). Since mdm2 is a negative regulator of p53, we analyzed the correla-

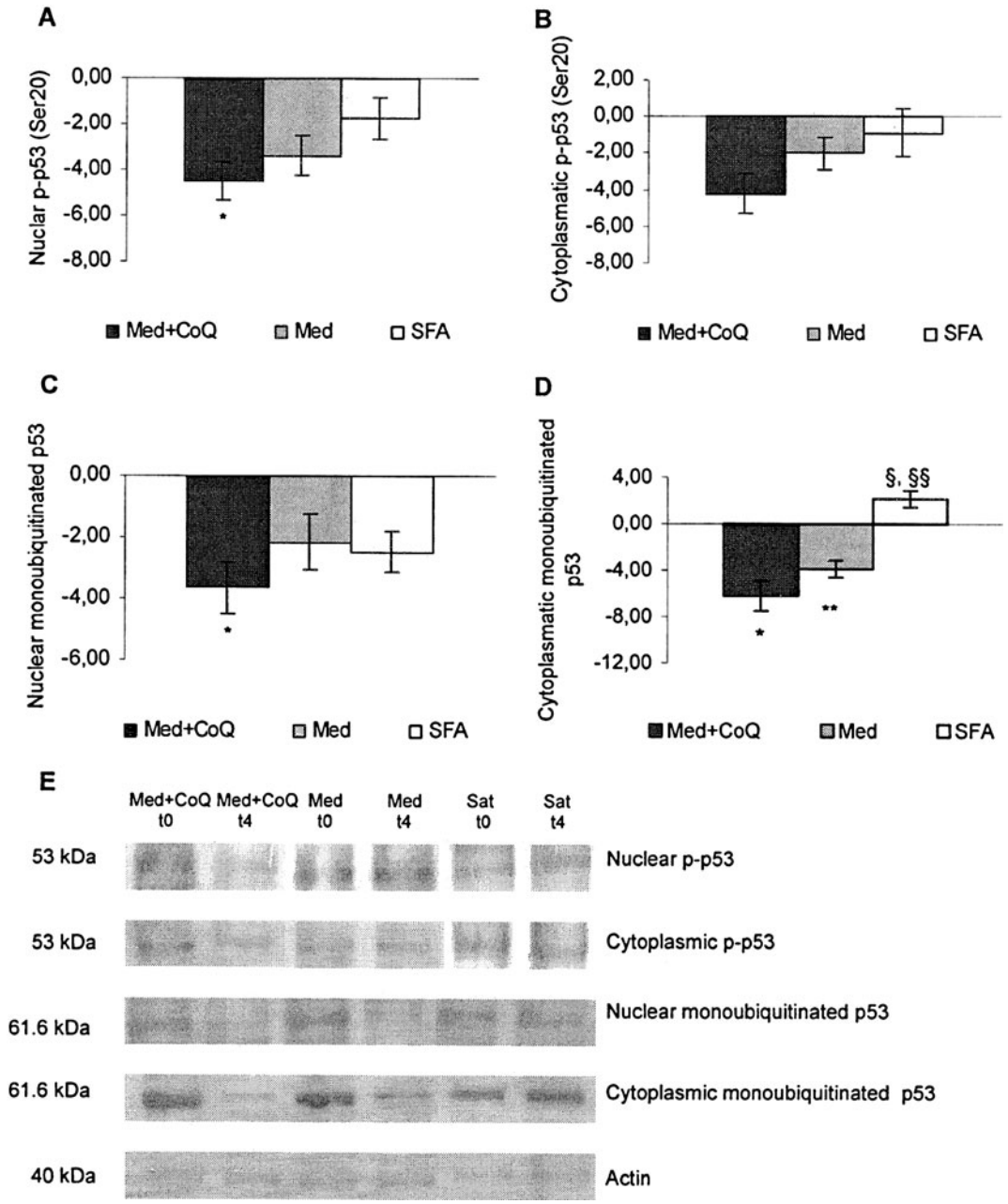

Fig. 3 Fasting and postprandial nuclear phosphorylated p53 (Ser20) (**a**), cytoplasmic phosphorylated p53 (Ser20) (**b**), nuclear monoubiquitinated p53 (**c**), and cytoplasmic monoubiquitinated p53 (**d**) in peripheral mononuclear cells of healthy elderly subject at the end of the three diets. Representative immunoblots of nuclear and cytoplasmic p-p53, nuclear and cytoplasmic monoubiquitinated p53, and actin. Depicted bands are different parts of the same blot stained for each antigen (**e**). Results are mean ± SEM, $n=20$. Results were analyzed using ANOVA for repeated measures. *$p<0.05$ decreased postprandial Med+CoQ diet (fasting vs 4 h); $^{\S\S}p<0.05$ Med+CoQ diet vs SFA diet; $^{\ddagger}p<0.05$ Med diet vs SFA diet; **$p<0.05$ decreased postprandial of Med diet (fasting vs 4 h). *Med +CoQ* Mediterranean diet supplemented with CoQ, *Med* Mediterranean diet, *SFA* saturated fatty acid-rich diet

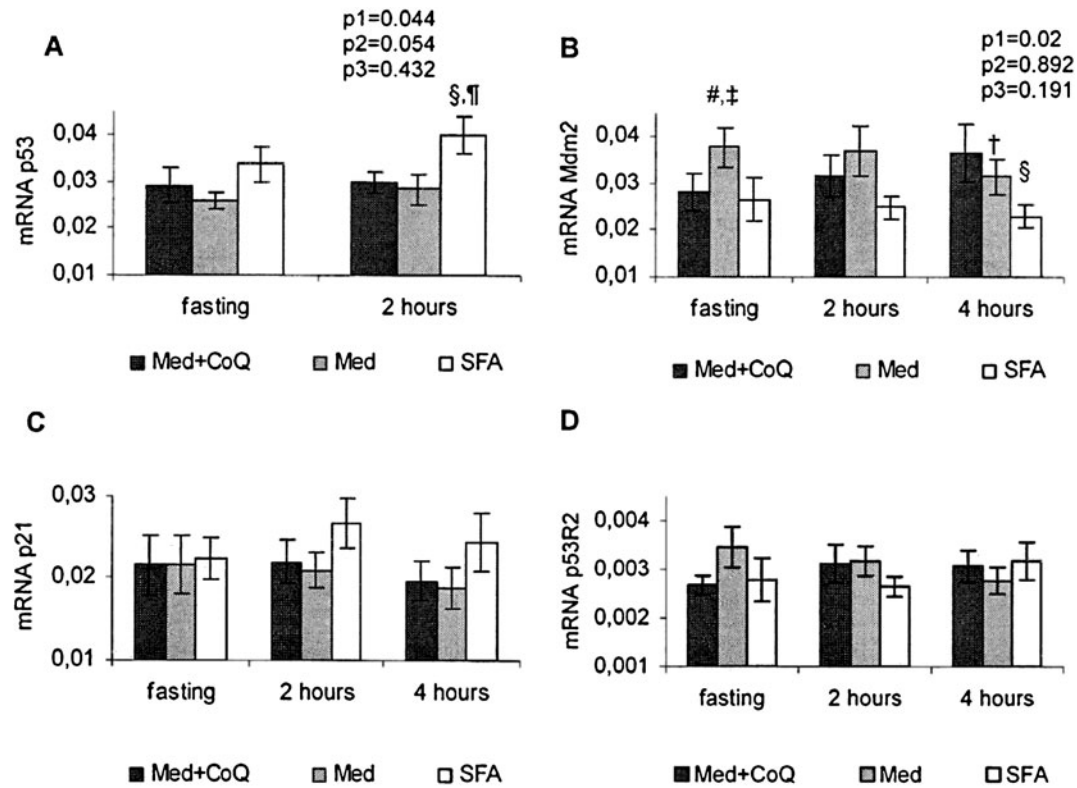

Fig. 4 Fasting and postprandial p53 mRNA (**a**), mdm2 mRNA (**b**), p21 mRNA (**c**), and p53R2 mRNA (**d**) in peripheral mononuclear cells of healthy elderly subject at the end of the three diets. Results are means ± SEM, $n=20$. Results were analyzed using ANOVA for repeated measures. $^{\S}p<0.05$ Med diet vs SFA diet; $^{\P}p<0.05$ postprandial increase with SFA diet. $^{\#}p<0.01$ Med+CoQ diet vs Med diet at fasting. $^{+}p<0.05$ Med diet vs SFA diet at fasting. $^{\dagger}p<0.05$ Med diet vs SFA diet at postprandial (4 h). *P1* diet effect, *P2* time effect, *P3* diet by time interaction, *Med+CoQ* Mediterranean diet supplemented with CoQ, *Med* Mediterranean diet, *SFA* saturated fatty acid-rich diet

tion between the mRNA levels of these two genes and we observed a negative correlation during fasting ($p<0.01$), and postprandial ($p<0.05$) period (2 and 4 h; Fig. 5e). However, since gene expression regulation for mdm2 is carried out by p53 protein as transcription factor, we found a positive correlation between nuclear p-p53 (Ser20) protein levels and mdm2 mRNA levels during the postprandial period (4 h; $p<0.05$; Fig. 5f).

Discussion

Our present study supports the notion that the Med diet leads to lower DNA damage in PBMCs. In addition, the exogenous CoQ supplementation in synergy with a Med diet reduced the activation and stabilization of p53 in response to the DNA damage produced by oxidative stress during the postprandial period in an elderly population.

The concept claimed by Zilversmit (1979) that atherogenesis is a postprandial phenomenon is gaining relevance. Humans spend the majority of time in a non-fasting postprandial state, with a continual fluctuation in the degree of lipemia throughout the day. In line with this notion, oxidative stress has received considerable attention over the past several years in the fasting state; however, there is a paucity of data on postprandial oxidative stress. The fact that oxidative stress is related with the fatty acid composition of biological membranes and fluids provides a very interesting approach to this problem. In fact, the intake of a particular type of dietary fat affects directly the fatty acid and antioxidant profiles of the body and, indirectly, the susceptibility of the organism to suffer oxidation (Huertas et al. 1991; Mataix et

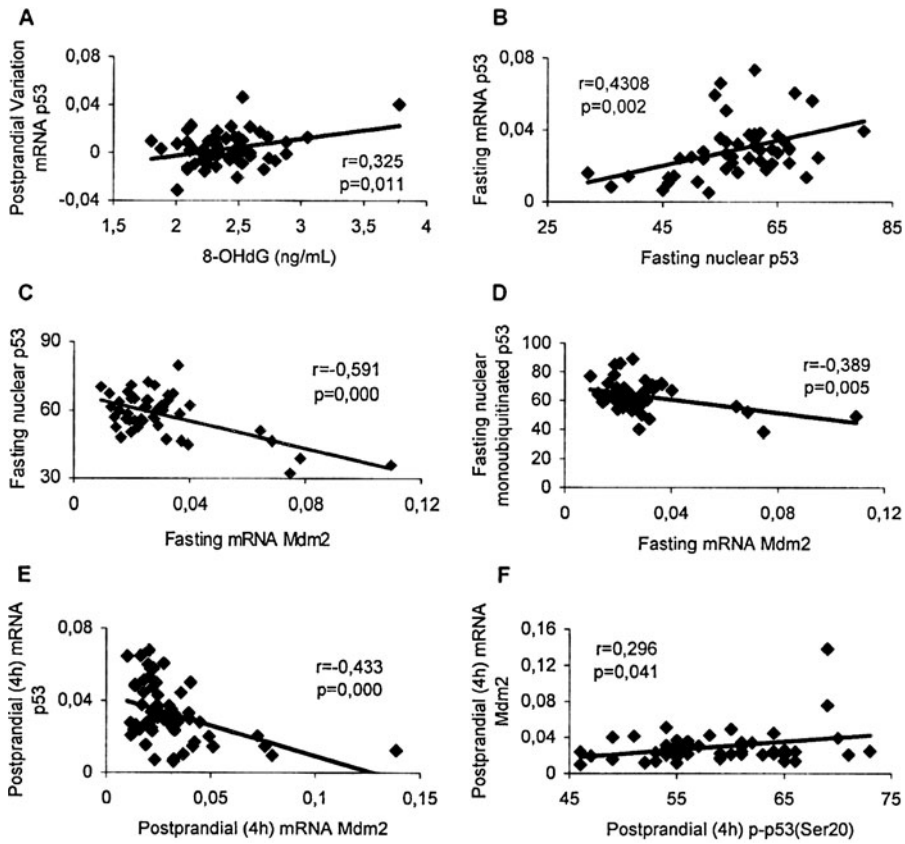

Fig. 5 Correlation between plasma 8-OHdG concentration and the increase of mRNA p53 levels at 2 h of postprandial period (**a**). Correlation between nuclear p53 levels and mRNA p53 at fasting (**b**). Correlation between mRNA Mdm2 and nuclear p53 levels at fasting (**c**). Correlation between mRNA Mdm2 and nuclear monoubiquitinylated p53 levels at fasting (**d**). Correlation between mRNA Mdm2 and mRNA p53 at postprandial period (4 h) (**e**). Correlation between nuclear phosphorylated p53 (Ser20) levels and mRNA Mdm2 at postprandial period (4 h) (**f**)

al. 1998; Quiles et al. 1999; Ramirez-Tortosa et al. 1999; Ochoa-Herrera et al. 2001; Quiles et al. 2002). In the same population of this study, we previously demonstrated the effect of Med+CoQ diet improves the postprandial oxidative stress (Yubero-Serrano et al. 2010). However, few studies explored the effects that diet has on DNA damage during the postprandial state, or the effect of CoQ supplements in humans. Previous evidence showed that DNA damage plays a crucial role in the mechanisms of atherosclerosis (Bennett 2001), as well as other diseases related to aging. In the same line, studies carried out on rats fed with diets rich in polyunsaturated fatty acids demonstrated that CoQ supplements have a protective effect against oxidative damage (Thomas et al. 1996; Santos-Gonzalez et al. 2007). Thus, our findings demonstrate for the first time that the long-term consumption of the Med+CoQ diet induced less DNA damage in elderly subjects, with an intermediate effect for the Med diet, both diets producing less damage than the SFA diet. However, during the postprandial period these differences were only observed between the Med+CoQ and the SFA diets, which suggest that the Med diet has only a long-term, rather than an acute effect.

In the absence of DNA-damaging stimuli, p53 is maintained at low levels in cells through a fast mechanism of labeling for degradation (Ko and Prives 1996). Activation of p53 consists mainly of a rapid stabilization of the protein, which decreases its degradation rate and thereby allows the raising of cellular levels of p53. Most of the p53 is degraded via the ubiquitin-dependent proteasome pathway, and thus it is interesting to determine how the p53 activation signals (DNA damage) affect this process of degradation. The phosphorylation of p53 in Ser20 activates p53 as a transcription factor and mediates its cellular stabilization in response to DNA damage (Appella and Anderson 2001; Chehab et al. 1999; Ashcroft et al. 2000). Thus, we have observed that during the postprandial period, the intake of the Med +CoQ diet induced a decrease in p53 protein phosphorylated at Ser20, probably due to a decrease in the signals that trigger this phosphorylation—especially DNA damage—as it can be corroborated by the reduced levels of 8-OHdG observed in this

study. The Ser20 residue maps to the domain of p53 that contacts Mdm2 (Kussie et al. 1996). Thus, its phosphorylation inhibits the binding between p53 and Mdm2. Mdm2 is a protein with E3 ligase activity whose function is to ubiquitinate p53, and serves to degrade the protein when not needed (Chehab et al. 1999; Brooks and Gu 2004).

The role of Mdm2 in the ubiquitination of p53 is extremely complex (Li et al. 2003), as it can carry out the poly- or monoubiquitination of p53 in the nucleus. Recent data suggest that monoubiquitination is a critical nuclear export signal for p53 (Stommel and Wahl 2005), while polyubiquitination causes it to degrade rapidly in the nuclear proteasomes. At low levels, Mdm2 catalyzes the monoubiquitination of p53, which is in fact exported to the cytoplasm for degradation and/or later modifications. However, when Mdm2 levels are high, p53 is rapidly polyubiquitinated and degraded in the nucleus. During the early stages of DNA damage, low levels of Mdm2 can be found, allowing for the stabilization of p53 and the concomitant activation of genes involved in DNA repair. However, when the damage is being repaired, the levels of Mdm2 rise so that p53 is polyubiquitinated and taken away quickly for degradation (Li et al. 2003). The expression of the Mdm2 gene is regulated in turn by p53 in a negative feedback loop (Stommel and Wahl 2005). During the postprandial study, we observed lower levels of Mdm2 expression in those subjects who followed the SFA diet compared with the Med and Med+CoQ diets. These results are consistent with the lower levels of DNA damage observed for the two latter diets, as well as with the decrease in the amount of p53 protein in its active form in the nucleus, and its monoubiquitinated forms, both in the nucleus and cytoplasm. These findings could be explained by the fact that the increased expression of Mdm2 in the Med and Med +CoQ diets leads to the degradation of p53 by polyubiquitination directly in the nucleus. However, during the postprandial period of the SFA diet, lower levels of expression of Mdm2 may have favored p53 monoubiquitination in the nucleus, which activates p53 transport into the cytoplasm. In fact, a postprandial rise in cytoplasmic monoubiquitinated p53 diet was observed for the SFA diet in our study. This phenomenon, together with the fact that there were no observed decreases of p53 either in the cytoplasm or in the nucleus (through stabilization), could be due to the fact that the damage caused by oxidative stress is higher in the SFA diet than in the Med+CoQ diet. In addition, the existing regulation between p53 and Mdm2 is also performed at the protein level. The Mdm2 protein is regulated directly by DNA damage and in turn regulates the stabilization of p53 protein. However, the results obtained at the level of gene expression in this sense are so outstandingly clear that allow us to offer a plausible physiological explanation for them, without taking into account the levels of Mdm2 protein.

The present study has the advantage of a randomized crossover design in which all the participants have experienced the three diet periods, each individual acting as his/her own control and strengthening the fact that the effects observed are due to the influence of the type of diet. We acknowledge that our study has certain limitations, since ensuring adherence to dietary instructions is difficult in a feeding trial. However, adherence to the recommended dietary patterns was satisfactory, as can be judged by the measurements of compliance.

In summary, the Med+CoQ diet induced a decrease in the concentrations of p53 in the cytoplasm, phosphorylated p53 at Ser20 in the nucleus and monoubiquitinated p53 both in the cytoplasm and the nucleus. p53 reduces its active form in the nucleus due to a lack of activation and stabilization, so it is rapidly degraded through polyubiquitination. This degradation is reflected in the reduction of monoubiquitinated p53 in the nucleus, and therefore there is a lower transport to the cytoplasm, so that the p53 values in this form are also reduced in this compartment.

In conclusion, our results support that consumption of a Med diet protects DNA from oxidative damage and that this protection is enhanced by supplementing with CoQ, thus reducing the activation of p53. In contrast, consumption of the SFA diet induces increased oxidative stress and stabilization of p53. These data may provide a starting point for the prevention of the oxidative stress generated during the aging process by demonstrating the benefits of the consumption of a Med diet supplemented with CoQ. This model could provide a suitable therapy for processes that lead to a rise in oxidative stress, such as cardiovascular or neurodegenerative diseases and aging.

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