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Original article:

ARYLESTERASE ACTIVITY IS ASSOCIATED WITH ANTIOXIDANT INTAKE AND *PARAOXONASE-1* (*PON1*) GENE METHYLATION IN METABOLIC SYNDROME PATIENTS FOLLOWING AN ENERGY RESTRICTED DIET

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ABSTRACT

The arylesterase (ARE) activity linked to the *paraoxonase-1* (*PON1*) gene is known to protect lipoproteins from oxidation and provide defense against metabolic syndrome (MetS) and cardiovascular diseases. The epigenetic regulation of enzymatic activities is gaining importance nowadays. This research aimed to assess the potential relationships between the ARE activity with the methylation levels of the *PON1* gene transcriptional regulatory region, anthropometrics, biochemical markers and antioxidant dietary components. Forty-seven subjects (47 ± 10 y.o; BMI 36.2 ± 3.8 kg/m^2; 46.8 % female) with MetS features, who followed a six-month energy-restricted dietary weight-loss intervention, were included in this study (*www.clinicaltrials.gov; NCT01087086).* Anthropometric, biochemical, enzymatic and dietary data were assessed using validated procedures. *PON1* transcriptional regulatory region methylation was analyzed by a microarray technical approach. Volunteers reduced ARE activity in parallel with body weight ($p = 0.005$), BMI ($p = 0.006$), total fat mass ($p = 0.020$), diastolic blood pressure ($p = 0.018$), mean blood pressure ($p = 0.022$) and triglycerides ($p = 0.014$). Methylation levels of some CpG sites of the *PON1* gene correlated negatively with ARE activity ($p < 0.05$). Interestingly, dietary vitamin C ($p = 0.001$), tocopherols ($p = 0.009$) and lycopene ($p = 0.038$) were positively associated with ARE activity and showed an inverse correlation ($p = 0.004$, $p = 0.029$ and $p = 0.021$, respectively) with the methylation of some selected CpG sites of the *PON1* gene. In conclusion, ARE activity decreased in parallel with MetS-related markers associated to the energy restriction, while dietary antioxidants might enhance the ARE activity by lowering the PON1 gene methylation in patients with MetS features.

Keywords: DNA methylation, ARE, PON1 gene, obesity, metabolic syndrome, energy restriction, antioxidants

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INTRODUCTION

Paraoxonase-1 (PON1) is a calcium-dependent glycoprotein hepatically synthesized that belongs to the paraoxonase family (Precourt et al., 2011). This enzyme is related to high density lipoprotein-cholesterol (HDL-c) and has been described to protect lipoproteins, particularly low density lipoprotein-cholesterol (LDL-c), from oxidation (Kim et al., 2013; Kumar et al., 2013). Thus, some of the antiatherogenic properties of HDL-c are attributed to PON1 functions (Cohen et al., 2012). The precise mechanisms by which the PON1 acts remains unclear, but several *in vitro* activities have been attributed to this enzyme (Nus et al., 2008): paraoxonase (hydrolysis of organophosphates), lactonase (hydrolysis of lactones), and arylesterase or ARE (hydrolysis of aromatic carboxylic acid esters).

Obesity and metabolic syndrome (MetS) features are main risk factors for the development of atherosclerosis and cardiovascular diseases (CVD) onset (Garg et al., 2014). Due to the increasing prevalence of obese people, these accompanying diseases are considered a major concern for public health authorities and many scientific efforts are being carried out to detect, treat and prevent them. Hypocaloric diets are one of the most common treatments employed to combat MetS and related diseases (Straznicky et al., 2010). However, more investigation is needed to understand the PON1 activity role as part of these nutritional strategies. In this context, some research have been performed focusing on the relationship between the PON1 activity levels and the MetS/ obesity states (Ferretti et al., 2012; Koncsos et al., 2011; Kota et al., 2013; Tabur et al., 2010) while other authors have investigated the influence of some specific dietary factors on the activity levels of this enzyme (Canales et al., 2011; Jarvik et al., 2002; Vazquez-Velasco et al., 2011). But the studies carried out are scarce and reported controversial results.

Epigenetics is defined as heritable changes in gene expression that cannot be explained by changes in DNA sequence (Christensen et al., 2011). Among the different possible epigenetic modifications, DNA methylation is probably the most widely studied (Milagro et al., 2011). In this context, dietary factors, specially antioxidants, have become agents of strong interest in the field of epigenetics due to their prominent role as potent modulators of epigenome-regulated gene expression through regulation of DNA methylation (Bartels, 2007; Malireddy et al., 2012; Milagro et al., 2009).

Thus, within this scenario, the current study aimed to assess the potential relationships between the PON1 ARE activity and anthropometric and biochemical markers, dietary antioxidants intake, and the cytosine methylation levels of *PON1* gene transcriptional regulatory region, in volunteers with MetS symptoms after following an energy-restricted dietary program.

MATERIALS AND METHODS

Subjects and study protocol

The current analysis was an ancillary study conducted within the RESMENA (Metabolic Syndrome Reduction in Navarra) project, a randomized controlled trial (Zulet et al., 2011) where a subsample of 47 obese adults (47 ± 10 y.o; BMI 36.2 ± 3.8 kg/m^2; 46.8 % female) who presented MetS features was selected. The study lasted a total of 6 months divided in two sequential stages: an initial 8-week nutritional-learning intervention period, during which nutritional assessment was carried out for the participants every 15 days (Lopez-Legarrea et al., 2013) and a 4-month self-control period, during which the participants followed the previously acquired dietary habits (de la Iglesia et al., 2013).

The study was approved by the Ethics Committee of the University of Navarra (065/2009) and appropriately registered at www.clinicaltrials.gov (NCT01087086). Consequently, all the participants gave written informed consent for participation in agreement with the Declaration of Hel-

sinki. More details about the procedures and protocols have been previously reported (Zulet et al., 2011).

Anthropometric, body composition, blood pressure and dietary intake assessment

Anthropometric measurements were conducted in fasting conditions according to previously described procedures (Zulet et al., 2011). Body mass index (BMI) was calculated as the body weight divided by height squared (kg/m^2). Body composition analyses were carried out by Dual Energy X-ray Absorptiometry (DXA) following validated protocols as reported elsewhere (Zulet et al., 2011). Systolic (SBP) and diastolic (DBP) blood pressures were measured following standardized World Health Organization criteria (Whitworth et al., 2004). Mean blood pressure (MBP) was calculated as: [(DPB x 2) + SBP]/3 as advised elsewhere (Shapiro et al., 2010).

Information about dietary intake was collected using a 48 h weighed food record and analysed using the DIAL (Alce Ingeniería) software (http://www.alceingenieria.net/nutricion) as previously reported (Perez-Cornago et al., 2013).

Biochemical assessments

Venous blood samples were drawn after a 12 h overnight fast by venipuncture. The EDTA–plasma and serum samples as well as WBC were separated from whole blood by centrifugation at 3,500 rpm, 5 °C, 15 min (Model 5804R, Eppendorf, Germany), and were frozen immediately at -80 °C until assay (WBC in buffy-coat).

Plasma concentrations of triglycerides (TG), total cholesterol (TC), HDL-c (Wako Chemicals, GmbH, Nuiss, Germany) and glucose (Horiba ABX Diagnostics, Montpellier, France) were measured by specific colorimetric assays, using an automated analyzer system Pentra C-200 (HORIBA ABX, Madrid, Spain). LDL-c levels were calculated using the Friedewald formula: LDL-c = TC - HDL-c – TG/5 (Friedewald et al., 1972). Apolipoprotein B (Apo B) was measured with a specific kit (Tina-quant Apolipoprotein B ver.2, Mannheim, Germany) using a Model 904 Modular Roche/ Hitachi autoanalyser (Roche Diagnostics, Tokio, Japan).

Plasma concentrations of ARE activity were measured with simulated body fluid (SBF) as buffer and phenylacetate as substrate at pH 7.34–7.4 and 37 °C, as published elsewhere (Nus et al., 2006). Reaction rates of ARE were followed at 270 nm in thermostatically controlled 10-mm Lightpath quartz cuvettes using a Shimadzu UV-2401PC spectrophotometer (Tokio, Japan). The final reaction volume in the cuvettes was 2.0 mL, and the total time was 3 min. One unit of ARE activity was defined as the mmol phenol formed from phenyl acetate per min.

DNA isolation and DNA methylation study

Genomic DNA from WBC was extracted using the Master Pure kit (Epicenter, Madison, WI, USA), whose quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). A total of 500 ng of DNA was modified by using EZ-96 DNA Methylation Kit (Zymo Research Corporation, USA) according to the manufacturer's instructions, converting thus cytosine into uracil. Array-based specific DNA methylation analysis was performed with the Infinium Human Methylation 450K bead chip technology (Illumina, USA). Bisulfite-treated genomic DNA was whole-genome amplified, hybridized to HumanMethylation450 BeadChips (Illumina, USA) and scanned using the Illumina iScanSQ platform (Mansego et al., 2013). The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, USA). Eight Cytosine-phosphate-guanine (CpG) sites of the *PON1* gene that codes for the PON1 enzyme were selected. CpG sites located in the transcriptional regulatory region (promoter, 5'-untranslated region and exon 1) were included (Figure 1). Refer-

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ence names and characteristics of the selected CpG sites are shown in Table 1.

Statistical analyses

Results are shown as mean value ± standard deviation. Variable distribution was determined by the Shapiro-Wilk test and no normal variables (glucose, HDL-c, LDL-c, TG, ARE and ARE/HDL-c) were logarithmically transformed for statistical purposes. Differences between the beginning and the end of the complete study were analyzed by paired Student *t*-test. Pearson correlations adjusted for age and sex were fitted to evaluate the potential associations of PON1 ARE activity and anthropometric, body composition, blood pressure and biochemical variables, and also to assess the relationships between *PON1* transcriptional regulatory region methylation and ARE activity and specific dietary factors. Pearson correlations adjusted for sex and age were also used to study the association between the ARE activity and the dietary factors intake at the end of the study. Moreover, multiple testing correction (Benjamini–Hochberg) analyses were performed when appropriate. The SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) for Windows XP (Microsoft, USA) was used for statistical analyses. Globally, $p < 0.050$ was considered as statistically significant.

RESULTS

Information about selected anthropometric and biochemical measurements was recorded at baseline and at day 180 (Table 2). After the 6-month-long trial, participants presented significantly lower ($p < 0.001$) values concerning anthropometric and body composition variables, such as body weight, BMI, waist circumference, WHR, total fat mass and android fat mass. Volunteers also significantly reduced the SBP, DBP and MBP values as well as the plasma glucose

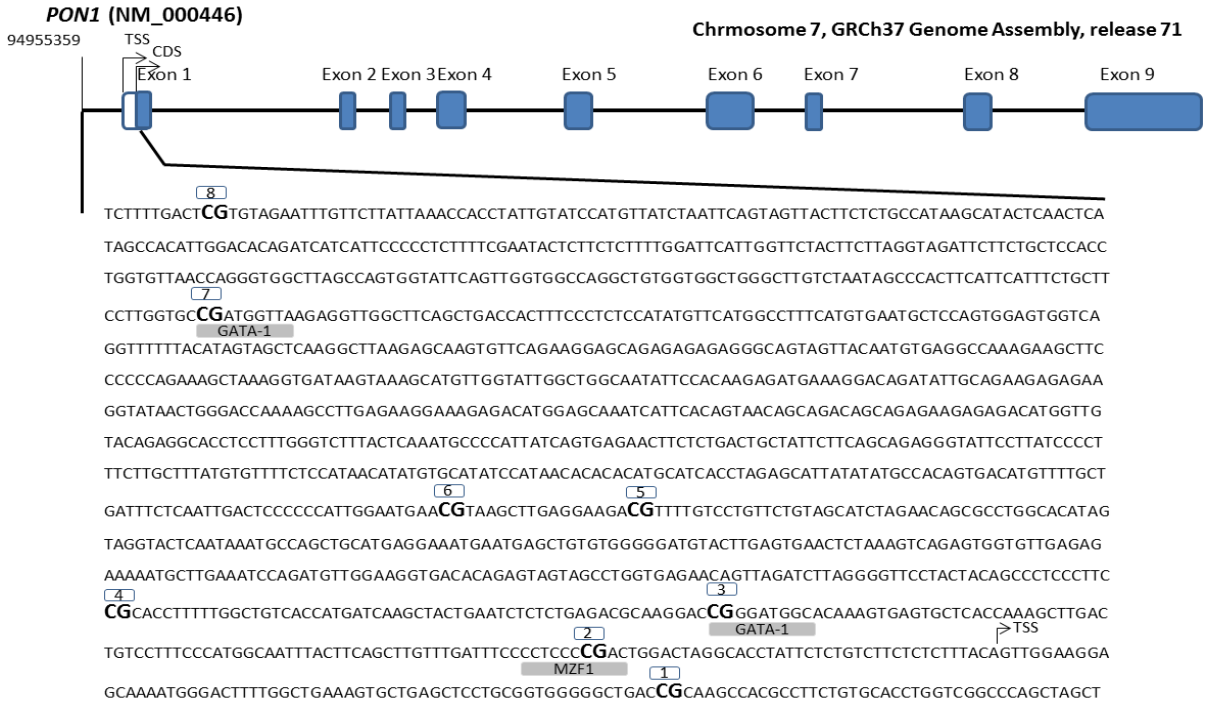

Figure 1: Genomic localization and nucleotide sequence of 8 CpG sites covered by the Illumina probe for the study of DNA methylation levels of *PON1* promoter (from - 1330 to +104 pb). Transcription Start Site (TSS).

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Table 1: Information of the selected CpG sites for each *PON1* gene

CpG ID[1]	Illumina ID	CHR position[2]	Reference[3]
1	cg17330251	7: 94953956	c.+63
2	cg01874867	7: 94954059	c.-41
3	cg20119798	7: 94954144	c.-126
4	cg04871131	7: 94954202	c.-184
5	cg23055772	7: 94954438	c.-420
6	cg07809369	7: 94954455	c.-437
7	cg17020263	7: 94955053	c.-1035
8	cg15887283	7: 94955348	c.-1330

1: Studied CpG identifier

2: Genome assembly: GRCh37, Ensemble release 73.37

3: It begins in the first nucleotide of exon 1

Table 2: Changes in anthropometric, body composition, blood pressure, biochemical parameters and arylesterase (ARE) activity after 6 month-study (N = 47)

Variable	Baseline	Day 180	p
Body weight (kg)	103.5±18.7	94.8±20.1	< 0.001
BMI (kg/m^2)	36.0±4.1	32.9±4.6	< 0.001
Waist circumference (cm)	112.7±12.9	104.7±14.8	< 0.001
WHR	0.97±0.10	0.94±0.10	< 0.001
Total fat mass (kg)	43.0±9.6	36.0±10.8	< 0.001
Android fat mass (kg)	4.75±1.37	3.75±1.31	< 0.001
SBP (mmHg)	151.2±17.2	134.4±13.2	< 0.001
DBP (mmHg)	85.6±8.9	77.7±10.5	< 0.001
MBP (mmHg)	107.4±11.1	96.6±10.5	< 0.001
Glucose$^{\#}$ (mmol/L)	6.77±1.90	6.16±1.41	< 0.001
TC (mmol/L)	5.61±1.34	5.50±1.14	0.584
HDL-c$^{\#}$ (mmol/L)	1.13±0.28	1.22±0.32	0.011
LDL-c$^{\#}$ (mmol/L)	3.50±1.19	4.27±0.98	< 0.001
ApoB (g/L)	0.94±0.27	0.88±0.21	<0.001
TG$^{\#}$ (mmol/L)	2.12±1.12	1.75±1.19	0.001*
ARE$^{\#}$ (IU/L)	483.4±241.0	461.5±217.1	0.222
ARE/HDL-c$^{\#}$ (IU/mmol)	436.2±189.1	393.1±174.4	0.003

Data are mean ± SD. p values from Student t test. $^{\#}$Log transformed variables. BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MDP, mean blood pressure; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, triglycerides; Apo B, apolipoprotein B; ARE, arylesterase.

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($p < 0.001$), Apo B ($p < 0.001$), TG ($p = 0.001$). On the other hand, LDL-c ($p < 0.001$) and HDL-c ($p = 0.011$) values were significantly higher at the end of the study.

Plasma TC and ARE activity tended to be reduced, although did not reach significance, while the ARE/HDL-c ratio was significantly decreased ($p = 0.003$).

Pearson correlation analyses adjusted for sex and age were performed to assess the possible associations between the PON1 ARE activity and the anthropometric, body composition, blood pressure and biochemical variables. The PON1 ARE activity levels significantly correlated with TC ($r = 0.362$, $p = 0.016$), HDL-c ($r = 0.346$, $p = 0.021$) and Apo B ($r = 0.446$, $p = 0.002$) concentrations, at baseline. Moreover, significant positive associations were found between changes in ARE activity and variation of body weight, BMI, total fat mass, DBP, MBP and plasma TG levels (Table 3). The association with waist circumference and SBP resulted in a trend towards significance. Some of these relationships should be considered as explorative since after applying a multiple comparison correction, the statistical significance was toned down. Interestingly, in the case of BMI variation, which is one of the main variables, remained statistically associated with the change of PON1 ARE activity.

At the end of the intervention, the PON1 ARE activity measurements showed positive correlations with the intake of the selected antioxidants vitamin C ($p = 0.001$), total tocopherols ($p = 0.009$) and lycopene ($p = 0.038$) recorded by the 48h weighed food record (Figure 2). Interestingly, when assessing the association between the selected CpG sites methylation of the *PON1* gene and the related ARE activity at baseline (Table 4), a significant inverse correlation was found concerning the *PON1* CpG 1, CpG 2, CpG 3 and CpG 4 sites with the enzymatic ARE activity. Moreover, these associations remained significant after applying the Benjamini-Hochberg test for multiple comparisons.

Table 3: Pearson correlation analyses between arylesterase (ARE) activity variation and changes in selected anthropometric, body composition, blood pressure and biochemical variables.

Variable	r	p
Δ **Body weight (kg)**	0.473	0.005
Δ **BMI (kg/m^2)**	0.459	0.006*
Δ **Waist circumference (cm)**	0.336	0.052
Δ **WHR**	0.131	0.459
Δ **Total fat mass (kg)**	0.396	0.020
Δ **Android fat mass (kg)**	0.020	0.910
Δ **SBP (mmHg)**	0.323	0.062
Δ **DBP (mmHg)**	0.402	0.018
Δ **MBP (mmHg)**	0.392	0.022
Δ **Glucose$^{\#}$ (mmol/L)**	0.123	0.487
Δ **TC (mmol/L)**	0.216	0.221
Δ **HDL-c$^{\#}$ (mmol/L)**	0.201	0.254
Δ **LDL-c$^{\#}$ (mmol/L)**	0.000	1.000
Δ **Apo B (g/L)**	0.200	0.249
Δ **TG$^{\#}$ (mmol/L)**	0.419	0.014

r and p values from Pearson correlations adjusted for sex and age. $^{\#}$Log transformed variables. BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Apo B, apolipoprotein B; TG, triacilglycerides. *P-value < 0.05 after correcting for Benjamini–Hochberg multiple comparisons.

Table 4: Pearson correlation analyses between arylesterase (ARE) activity and the selected CpG sites methylation (%) of PON1 gene at baseline

CpG site	r	p
CpG 1	-0.490	0.003*
CpG 2	-0.606	< 0.001*
CpG 3	-0.503	0.002*
CpG 4	-0.434	0.009*
CpG 5	-0.013	0.939
CpG 6	-0.326	0.056
CpG 7	-0.235	0.174
CpG 8	-0.159	0.361

*P-value < 0.05 after correcting for Benjamini–Hochberg multiple comparisons.

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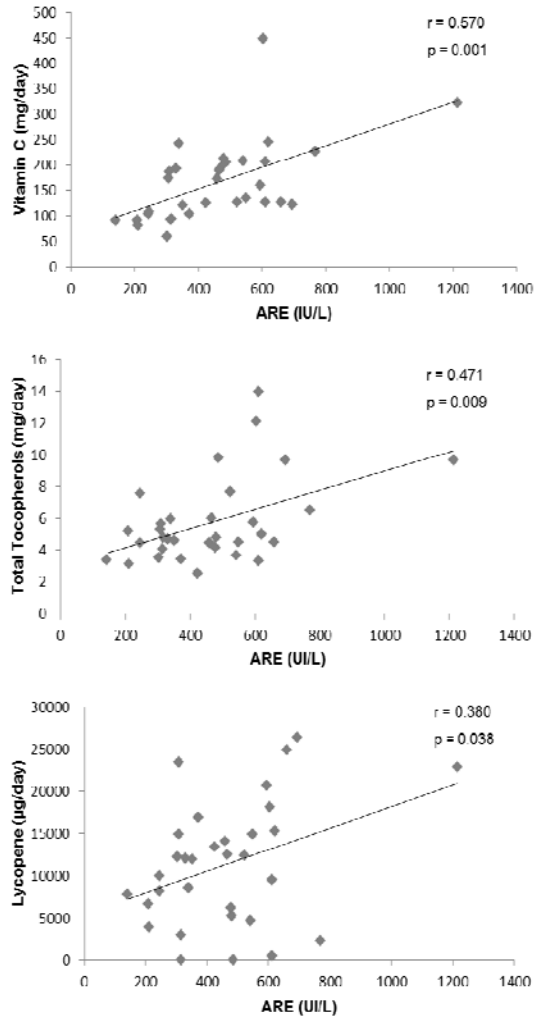

Figure 2: Associations between antioxidant dietary components intake and arylesterase (ARE) activity

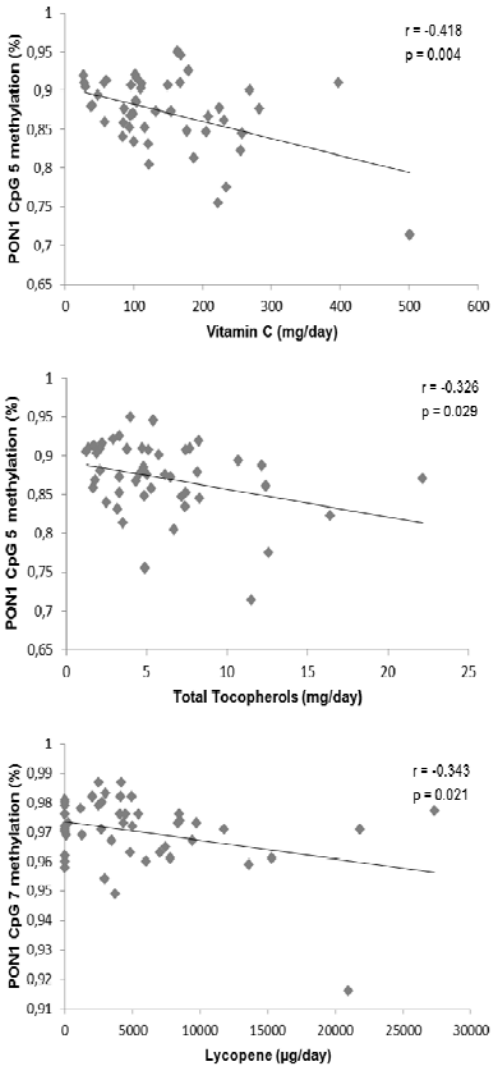

Figure 3: Statistically significant correlations between antioxidant dietary components intake and methylation (%) of *PON1* gene CpG sites at baseline

Finally, the analysis of the possible relationships between the antioxidant dietary intake and the percentage of methylation of the different *PON1* gene CpG sites, revealed a correlation between the selected antioxidants: vitamin C, total tocopherols and lycopene and the selected CpG sites at baseline (Figure 3).

DISCUSSION

The assessment of processes associated to weight loss may contribute to a better understanding of the metabolic and genetic machinery, which will benefit the implementation of personalized dietary treatments.

Indeed, as reported in other studies concerning hypocaloric diets (Straznicky et al., 2010), the energy-restricted intervention proved to be effective in improving MetS abnormalities except for LDL-c, which was higher at the end of the study. This outcome agrees with Clifton et al. who described that in some cases, LDL-c may increase despite weight loss (Clifton et al., 2007). Nevertheless, Apo B, which has been considered a better predictor of CVD onset than any other lipid measurement (McQueen et al., 2008), evidenced statistically significant decreased levels.

Concerning PON1 ARE activity, it seems logical the positive correlation found with the HDL-c levels at baseline since it is an HDL-c related enzyme, as other studies have previously reported (Aksoy et al., 2009; Mascarenhas-Melo et al., 2013; Tabur et al., 2010). The positive correlation with LDL-c and TC also agree with previous studies (Aksoy et al., 2009; Younis et al., 2013).

The PON1 ARE activity was reduced in parallel to some anthropometric (body weight, BMI), body composition (total fat mass), blood pressure and biochemical biomarkers (TG) related to the MetS. Although there is scarce research about the relationship of PON1 activity and obesity or MetS, the studies carried out point to relate obese/MetS status to lower levels of ARE activity (Ferretti et al., 2012; Kota et al., 2013). Nevertheless, when it comes to clinical trials, in accordance to our results, a significant decrease of ARE activity and serum PON1 protein levels after a weight loss intervention, as well as a significant association between reduced PON1 and reductions in body fat has been reported (Rector et al., 2007). Similarly, it has been shown that significant depletions of all metabolic outcomes in addition to a significant reduction of PON1 activity correlated with BMI reduction, after a low calorie diet intervention (Kotani et al., 2009). In this last study, a significant association of PON1 activity changes and LDL-c depletion was also observed; however, no significant relationships were found in the present experimental trial. On the other hand, to our knowledge, this is the first research that has found an association of ARE activity and TG changes after a dietary intervention. With regards to the significant association of PON1 activity depletion and reduction of blood pressure levels, it has been reported significantly higher ARE levels in hypertensive than in normotensive children (Akis et al., 2009), while in other studies carried out in different adult populations no association was found (Tabur et al., 2010; Usta et al., 2011). However, the studies mentioned above were cross-sectional studies, while the present work is a clinical intervention. Taking into account the previous issues and the lack of research in the area, the findings observed in the present study may indicate that reductions on PON1 general activity might be indicative of the improvement in the overall MetS/obese related features, in patients with MetS manifestations under an energy-restricted programme.

The activity of PON1 is under genetic and environmental regulation (Jarvik et al., 2002). Within the environmental factors that may alter PON1 activity, dietary antioxidants are of major importance, since PON1 is an oxidative stress related enzyme (de la Iglesia et al., 2014; Kheir-Eldin et al., 2008). Among the different antioxidants that can be incorporated in the diet, the antioxidant capacity of vitamins C and E and lycopene is well-established (Chen et al., 2013; Mustacich et al., 2007; Story et al., 2010). The positive relationships between the ARE activity and the selected antioxidant components reported in the present work may be explained by the capacity of the dietary antioxidants to scavenge free-oxygen radical products that may depress PON1 activity, as previous studies have suggested (Jarvik et al., 2002; Tsakiris et al., 2009).

DNA methylation is one of the major epigenetic mechanisms considered to regulate gene expression, together with histone modifications and noncoding RNA activity (Portela et al., 2010). Methylation of the CpG-rich region (CpG island) overlapping a gene's promoter is a generally accepted mechanism for silencing expression (Vanderkraats et al., 2013). Our findings confirm this outcome since four CpG sites and the average methylation of all the CpG sites studied inversely correlated with the ARE activity.

About the possible mechanisms by which the dietary antioxidants exert their effects, modulation of gene expression through regulation of DNA methylation is

one of the main studied mechanisms (Bartels, 2007; Malireddy et al., 2012; Milagro et al., 2009). In the present work, inverse correlations between dietary vitamin C, total tocopherols and lycopene with different CpG sites methylation was observed, which may influence in the ARE activity as other studies have suggested (Jarvik et al., 2002; Tsakiris et al., 2009).

This work has the limitations that we have not determined *PON1* methylation levels at the end of the intervention and we have not measured expression levels. However, we found that CpG2 and CpG7 sites match a core-binding consensus motif for the GATA binding protein 1 (globin transcription factor 1) and CpG2 site matches for myeloid zinc finger 1 (MZF1), which are known transcriptional regulators for several pathways (Gaboli et al., 2001; Zheng et al., 2010). Furthermore, some statistical associations concerning changes in PON1 ARE activity and anthropometric and biochemical measurements variations were lost after the application of a multiple comparison correction test.

In conclusion, the present study reports that ARE activity decreased in parallel with MetS-related markers, while dietary antioxidants intake may enhance the ARE activity by lowering the *PON1* gene methylation in patients with MetS features, under an energy-restricted intervention. These results suggest that further investigations into PON1 activity might be a good target to better understand the metabolic effects of the dietary intervention studies, in which epigenetic processes may be involved.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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