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ORIGINAL CONTRIBUTION

Tachyphylaxis effects on postprandial oxidative stress and mitochondrial-related gene expression in overweight subjects after a period of energy restriction

Ana B. Crujeiras · Dolores Parra · Estíbaliz Goyenechea · Itziar Abete · J. Alfredo Martínez

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Abstract

Background Postprandially induced oxidative stress can cause damage to mitochondrial components and initiate cellular degradative processes; which are related to obesity comorbidities.

Aim of the study This trial sought to determine whether weight loss induced by caloric restriction provides antioxidant protection to reduce the postprandial response of mitochondrial function and oxidative stress markers.

Methods A group of overweight/obese volunteers ($n = 17$; 39 ±7 years, 32.5 ± 4.8 kg/m^2) followed an 8-week hypocaloric diet. Volunteers provided blood samples at fasting and 2-h after a test drink (CHO: 95% E, PROT: 5% E and containing antioxidants) and these were examined for postprandial oxidative stress responses, before and after the nutritional intervention. The expression of four mitochondrial-related genes, COX15, NDUFS2, MGST2 and TNF-alfa, was measured in peripheral blood mononuclear cells (PBMC) by quantitative RT-PCR. Lipid peroxidation and nitrosative stress biomarkers, total antioxidant capacity (AOP), uric acid and glutathione peroxidase were also determined.

Results Before nutritional treatment, the test drink induced a postprandial increase in lipid peroxidation and nitrosative stress biomarkers with a concomitant increase in the AOP. The increase in postprandial oxidative stress biomarkers was accompanied by a decrease in PBMC COX15 mRNA levels. Interestingly, after the weight loss period (−5.8 ± 2.3%), the postprandial-induced changes were lower than at the beginning of the study and involved oxidative stress biomarkers and the COX15 and MGST2 transcripts. This finding suggests the occurrence of a tachyphylactic process.

Conclusions We demonstrate for the first time that the well-known effect of energy restriction on oxidative stress is accompanied by a tolerance mechanism on the postprandial oxidative stress response and mitochondrial function-related genes. Indeed, the COX15 and MGST2 gene expression assays in PBMC emerged as valuable nutrigenomic biomarkers of the oxidative response under energy-restriction conditions.

Keywords Caloric restriction · Cytochrome *c* oxidase · COX15 · Oxidative stress · Glutathione S-transferase · PBMC gene expression

Abbreviations

AOP	Serum total antioxidant capacity
BMI	Body mass index (kg/m^2)
COX15	Cytochrome *c* oxidase assembly protein
CR	Caloric restriction
GPx	Glutathione peroxidase
MDA	Malondialdehyde
MGST2	Microsomal glutathione S-transferase
NDUFS2	NADH-Coenzyme Q reductase
NO	Nitric oxide
NT	Nitrotyrosine
Ox-LDL	Oxidized-LDL
PBMC	Peripheral blood mononuclear cell
TNFAIP8L1	Tumor necrosis factor alfa-related gen

A. B. Crujeiras · D. Parra · E. Goyenechea · I. Abete · J. A. Martínez (✉)
Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra, C/ Irunlarrea 1, 31008 Pamplona, Spain
e-mail: jalfmtz@unav.es

A. B. Crujeiras
e-mail: anabelencrujeiras@hotmail.com

UA	Uric acid
UV	Ultraviolet visible

Introduction

Obesity is a complex multifactorial disease, in which genetic and environmental-related factors are involved [21]. Among the causative agents, inadequate eating habits in genetically predisposed subjects are an important contributor to the development of obesity, such habits have been repeatedly linked to a number of metabolic impairments and associated with oxidative stress disturbances such as atherosclerosis and insulin resistance [13].

In this context, defective cellular energy metabolism has been suggested as a relevant cause of obesity [22, 33]. This impairment appears to be originated, in some cases, by increased levels of free radicals, which cause damage to mitochondrial components and initiate cellular degradative processes [6].

Chronic hypernutrition occurring in obesity could be a key cause of the increase in the risk of oxidative damage, which could trigger mitochondrial dysfunction [34]. Indeed, a recent study demonstrates that mitochondrial dysfunction is a complication of hyperglycemia- and hyperlipidemia-induced reactive oxygen species (ROS) production in skeletal muscle, which induces a decrease in cytochrome *c* oxidase (COX)-related genes mRNA levels [5].

Reducing these undesirable oxidative processes could be a part of the strategy for the prevention of diseases such as obesity and associated comorbidities [17]. Thus, supplementation with antioxidants may reduce some risks associated with such postprandial effects [29, 35]. Moreover, caloric restriction has been shown to be involved in slowing the progression of a variety of diseases [12], likely acting by decreasing oxidative stress [8].

Therefore, the aim of this study was to investigate, apparently for the first time, if weight loss induced by caloric restriction could specifically influence postprandial free radical production in healthy obese subjects by measuring specific markers of oxidative stress as well as the gene expression of selected proteins such as COX15, NDUFS2, MGST2 and TNFAIP8L1 involved in mitochondrial-related oxidative processes.

Materials and methods

Subjects

Caucasian overweight/obese subjects with a mean age of 39 ± 7 years and a mean body mass index (BMI) of 32.5 ± 4.8 kg/m^2, were recruited through local advertisements. The participants in this translational research ($n = 17$; 10 men and 7 women) were in apparent good health as determined by medical history, physical examination, and routine laboratory tests. They reported neither consumption of supplemental vitamin or minerals nor regular prescription of medications during the previous 3 months, and changes in body weight were less than ±3 kg during this time. A written informed acceptance to participate in the trial was obtained before the start of the study. This consent, as well as the study protocol, was previously approved by the Ethical Committee of the University Clinic of Navarra (Ref. 54/2006), in agreement with the Helsinki Declaration.

Study design

The trial was a controlled nutritional intervention supervised by trained dieticians from the Department of Nutrition and Food Sciences, Physiology and Toxicology of the University of Navarra. The acute experimental protocol was performed at rest after an overnight fast. Obese volunteers were given a test drink (200 mL) in the form of commercial orange juice (Kasfruit, Vitoria, Spain) supplying 84 kcal with 95% energy from carbohydrates and 5% energy from proteins of which 90% corresponded to leucine. The test drink was selected based on the carbohydrate and protein properties to enhance ROS production [27, 28, 32].

The day after the acute intake experiment, the subjects began the 8-week energy-restricted dietary treatment to lose weight. After the energy-restricted intervention treatment to lower weight, the experimental protocol was repeated, so the volunteers consumed a test drink with the same composition as at the beginning. Before and after the nutritional intervention, selected anthropometric measurements were made.

Blood samples were drawn at fasting and 2 h after the test drink intake to determine changes in postprandial oxidative stress before and after the weight loss process. The EDTA-plasma and serum of volunteers were separated from whole blood, peripheral blood mononuclear cells (PBMC) were extracted as described below and the samples were immediately frozen at −80°C until assay.

Weight loss intervention protocol

Weight loss was induced by means of a hypocaloric diet devised to produce a 30% caloric restriction with respect to the subject's baseline energy expenditure, which was individually measured by indirect calorimetry (Deltratac, Datex Ohmeda, Finland), following conventional protocols described elsewhere [18] and adjusting for the physical

activity level according to WHO criteria [30]. The macronutrient distribution of the hypocaloric diet was 18% energy as proteins, 52% energy as carbohydrates and 30% energy as lipids and was prescribed according to a food exchange system, in which the menu plans were individually designed as described elsewhere [1]. Dietary compliance was assessed through 3-day weighted food records [1, 7, 11], calculating the energy and nutrient intake by using Medisystem software (Sanocare Human Systems LS, Spain) based on recognized Spanish food composition tables.

Biochemical parameters assessment

Plasma levels of glucose, total cholesterol and triacylglycerol were measured by specific colorimetric assays (Horiba ABX Diagnostics, Montpellier, France) using an automatized system (COBAS MIRA, Roche, Basel, Switzerland), while circulating insulin was determined by using a commercially available ELISA kit (Mercodia, AB, Uppsala, Sweden). The HOMA index (HOMA-IR) was calculated according to Mathews et al. [24] in order to assess insulin resistance.

Measurement of oxidative stress markers

Analyses of antioxidant status in blood

Total antioxidant capacity (AOP) and malondialdehyde (MDA) were evaluated in serum by colorimetric assay kits (OXIS International, OR, USA). Uric acid (UA) was measured by a specific colorimetric assay (Horiba ABX Diagnostics) using an automatized system (COBAS MIRA). Plasma levels of circulating oxidized LDL (ox-LDL) were assessed using an ELISA kit (Mercodia, AB). Plasma nitrotyrosine (NT) and glutathione peroxidase (GPx) values were determined by commercially available ELISA kits, respectively (HyCult Biotechnology b.v., Uden, The Netherlands and OXIS International). Nitric oxide (NO) levels were evaluated with a nitrate/nitrite colorimetric assay kit (Oxford Biomedical Research, MI, USA). Absorbance was spectrophotometrically read at appropriate wavelengths using the Multiskan Spectrum (Thermo Electron Corporation, Vantaa, Finland).

Gene expression assessment

The gene expression quantification was performed as previously described [9]. PBMC were isolated by differential centrifugation using Polymorphprep medium (Axis Shield PoC AS, Oslo, Norway). The extraction of total RNA from the PBMC was based on the Trizol reagent method according to the manufacturer's instructions (Invitrogen, CA, USA). Quantitative real-time PCR was performed using an ABI PRISM 7000 HT Sequence Detection System as described by the provider (Applied Biosystems, CA, USA).

The genes to be explored were selected from microarray analyses on PBMC after the caloric restriction carried out in our laboratory [10] due to their putative relationships with free radicals production and modulation.

Statistical analysis

The sample size for this translational investigation was calculated by the equation published by Mera et al. [25], taking into account reported values for the MDA standard deviation [3]. This condition required seven subjects as the minimum sample size of participants that completed the nutritional intervention. The sample size recruited in this study was twenty-three subjects; seventeen obese volunteers (10 men, 7 women) completed both baseline and after weight loss testing and were included in the analyses. Gene expression parameters were analyzed in 14 volunteers due to insufficient blood sampling. Statistical analysis was initially performed in men and women, separately. Then, given that no differences were found, the data were pooled.

The normal distribution was explored through the Kolmogorov-Smirnov and the Shapiro Wilk tests. The paired student test was applied to detect differences before and after the intake and before and after the hypocaloric treatment to evaluate global nutritional status variables. Since oxidative stress markers, including gene expression were not normally distributed, the Wilcoxon signed ranks test was applied. Sequential comparisons of nitrotyrosine levels were made with log-transformed values [4]. Postprandial changes from fasting were calculated as the difference between 2 h after the intake and fasting measures to determine whether the postprandial measurements at baseline and at endpoint of the nutritional intervention were different. Sequential comparisons of postprandial changes in the oxidative stress biomarkers before and after nutritional treatment were performed with data normalized to a baseline of 100% and endpoint values expressed as percentage of baseline because these biomarkers changes varied markedly from one person to another. Untransformed data on circulating oxidative stress markers are reported as the mean ± standard deviation. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantitation method [20, 26] according to the manufacturer's guidelines (Applied Biosystems) reporting data as the mean (SEM). The Spearman coefficient was used to evaluate the potential association between gene expression and AOP. A P value ≤ 0.05 was considered statistically significant and a p value ≤ 0.1 as a trend for significance [36]. Statistical analysis was performed by

SPSS 15.0 software (SPSS Inc, IL, USA) for Windows XP (Microsoft, WA, USA).

Results

Biochemical and oxidative stress changes

The 8-week caloric restriction (CR) was associated with a reduction of $-5.8 \pm 2.3\%$ ($-5.46 \pm 2.08\%$ for men vs. $-6.43 \pm 2.64\%$ women; $p = 0.475$) in body weight, a decrease in BMI and other variables related to nutritional status (Table 1). As expected, these responses were accompanied by a decrease in total plasma cholesterol and insulin fasting levels (Table 2), although no relevant changes in plasma glucose and triacylglycerol concentrations as compared with baseline were detected (Table 2). When the postprandial changes were evaluated after CR, differences in such biochemical variables were not found (Table 2).

As for the oxidative stress markers after the nutritional treatment, MDA and ox-LDL fasting plasma levels fell with no statistical changes in NT, when compared with baseline point (Table 3). Values of NO and AOP significantly increased with no changes in UA, while GPx protein levels fell significantly (Table 3). At the beginning of the intervention study, the acute intake of the test drink induced increases of different degree in circulating MDA ($p = 0.044$), ox-LDL ($p = 0.287$), and NT ($p = 0.022$) when compared with the fasting state (Table 3). Also, an increase in NO ($p = 0.019$) and AOP ($p = 0.001$), and a trend for decrease in uric acid ($p = 0.068$) with no changes in GPx protein levels ($p = 0.382$) were observed (Table 3). After the caloric restriction period, only the postprandial increase in MDA was maintained. Interestingly, the postprandial changes from fasting after CR in MDA ($-2 \pm 25\%$), NT ($-52 \pm 104\%$) and NO ($-14 \pm 19\%$) were statistically lower than those before CR, while no statistically differences were found in ox-LDL ($0.62 \pm 29\%$), AOP ($-15 \pm 41\%$), UA ($-2 \pm 21\%$) and GPx ($34 \pm 297\%$) after treatment (Table 3).

Table 1 Phenotypical changes in global nutritional status at fasting state in response to a 8-weeks caloric restriction (CR) period prescribed to lose weight ($n = 17$)

	Before CR, day 0	After CR, day 56	p value
Body weight (kg)	95.4 ± 17.9	90.1 ± 17.9	<0.001
Body mass index (kg/m^2)	32.5 ± 4.8	30.6 ± 4.7	<0.001
Body fat mass (kg)	34.6 ± 12.3	32.1 ± 11.9	<0.001
Free fat mass (kg)	60.8 ± 12.4	58.0 ± 12.4	<0.001
Waist-to-height ratio	0.58 ± 0.06	0.57 ± 0.07	0.005
HOMA-IR	2.17 ± 1.31	1.68 ± 0.97	0.025

Oxidative stress-related gene expression changes

After a caloric restriction period to lose weight, no relevant changes were found in fasting mRNA levels of COX15, MGST2, TNFAIP8L1, while, the NDUFS2 transcripts at fasting slightly increased with a trend for the significance (fold change 2.92(0.30); $p = 0.096$) after dieting.

Interestingly, at the beginning, the test drink intake induced a significant decrease in COX15 mRNA levels (Fig. 1a), with no statistical changes in MGST2 (Fig. 1b), when compared with fasting state, while TNFAIP8L1 tended to diminish (Fig. 1c), and NDUFS2 showed no marked changes (Fig. 1d). These postprandial changes in the COX15 transcripts were not observed after the caloric restriction period (Fig. 1a), while MGST2 mRNA levels increased dramatically (Fig. 1b).

Indeed, the postprandial fold change in gene expression after the weight loss tended to be higher in COX15 (5.18(3.26); $p = 0.084$) and TNFAIP8L1 (2.16(0.45); $p = 0.056$). It was statistically higher in MGST2 (2.64(0.76); $p = 0.010$) than before the nutritional

Table 2 Effects of the short-term (2 h) test drink intake on metabolic variable before and after the weight loss induced by caloric restriction ($n = 17$)

	Before CR (day 0)		After CR (day 56)		CR effects on fasting values	CR effects on postprandial changes (%)
	Fasting	2 h-postprandial	Fasting	2 h-postprandial	p value[a]	p value[b]
Glucose (mM)	5.08 ± 0.39	4.76 ± 0.29*	5.01 ± 0.24	4.71 ± 0.19*	0.509	0.583
Insulin (mU/L)	9.25 ± 5.23	8.68 ± 5.39	7.36 ± 4.10	6.95 ± 4.23	0.018	0.527
Total cholesterol (mM)	5.32 ± 0.96	5.61 ± 1.05	4.74 ± 0.99	5.07 ± 1.07*	0.002	0.992
Triacylglycerol (mM)	1.16 ± 0.35	1.23 ± 0.40	1.11 ± 0.42	1.10 ± 0.30	0.525	0.398

* Denotes differences statistically significant in blood biomarkers ($p < 0.05$) when comparing 2 h-postprandial with fasting state

[a] Statistical differences in fasting levels of biomarkers when comparing before and after caloric restriction

[b] Statistical differences in postprandial changes (2 h postprandial vs. fasting) before and after caloric restriction

Table 3 Effect on 2 h after postprandial oxidative/nitrosative stress blood markers of an 8-weeks calorie-restriction intervention (CR) designed to lose weight ($n = 17$)

	Before CR (day 0)		After CR (day 56)		CR effects on fasting values	CR effects on postprandial changes (%)
	Fasting	2 h-postprandial	Fasting	2 h-postprandial	p value[a]	p value[b]
MDA (μM)	1.70 ± 0.32	1.85 ± 0.28*	1.58 ± 0.34	1.71 ± 0.29*	0.015	0.031
Ox-LDL (U/L)	101 ± 35	104 ± 32	91 ± 27	96 ± 33	0.044	0.796
NT (mM)	5.07 ± 5.95	5.45 ± 5.33*	3.92 ± 3.87	4.18 ± 4.03	0.758	0.039
NO (μM)	108 ± 29	116 ± 33*	131 ± 34	121 ± 37	0.009	0.013
AOP (mM)	0.58 ± 0.18	0.71 ± 0.26*	0.66 ± 0.23	0.66 ± 0.22	0.047	0.102
UA (mM)	0.29 ± 0.06	0.27 ± 0.06#	0.28 ± 0.07	0.27 ± 0.07	0.326	0.501
GPx (ng/mL)	108 ± 19	101 ± 29	92 ± 26	94 ± 36	0.009	0.173

MDA malondialdehyde, *ox-LDL* oxidized low density lipoprotein, *NT* nitrotyrosine, *NO* nitric oxide, *AOP* total antioxidant capacity, *UA* uric acid, *GPx* glutathione peroxidase, *CR* caloric restriction

* Denotes differences statistically significant ($p < 0.05$) and # denotes trend for significance ($p < 0.1$) in blood biomarkers, when comparing 2 h-postprandial with fasting state

[a] Statistical differences in fasting levels of biomarkers before and after caloric restriction values

[b] Statistical differences in postprandial changes (2 h postprandial vs. fasting) before and after caloric restriction

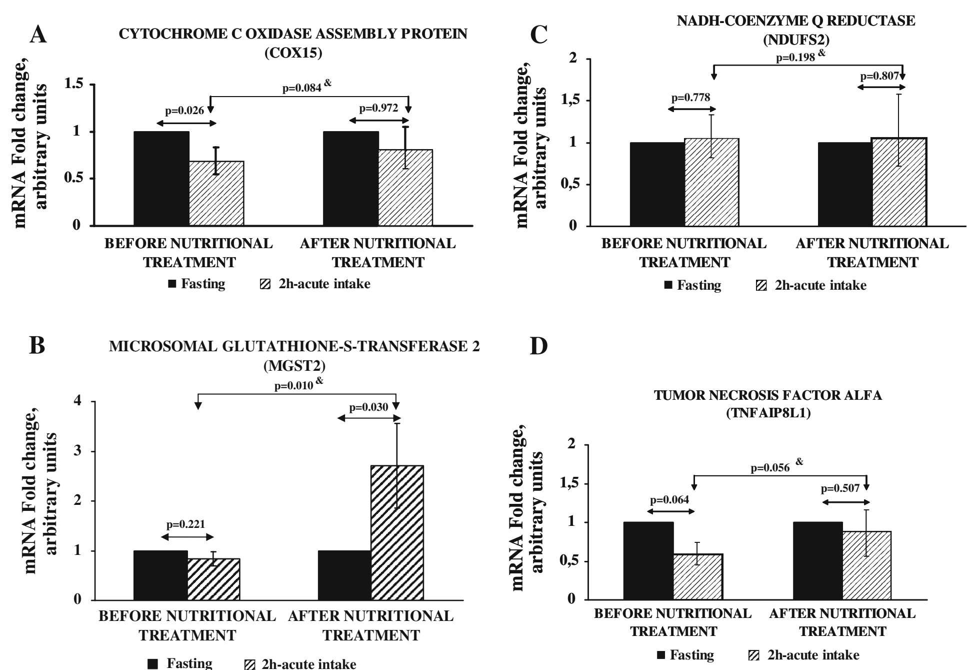

Fig. 1 Fold change of each subject with regard to their fasting mRNA levels ($2^{-\Delta\Delta Ct}$) in PBMC (mean and SEM as range; $n = 14$) concerning (1A) COX15 (Cytochrome c oxidase assembly protein 15), (1B) MGST2 (microsomal glutathione S-transferase), (1C) NDUFS2 (NADH-Coenzyme Q reductase) and (1D) TNFAIP8L1 (tumor necrosis factor alfa-related gene). &Statistical differences in the fold change induced by test drink in gene expression after the nutritional intervention as compared with the fold change 2 h after the intake at baseline

intervention. While no changes were found in NDUFS2 (3.05(1.06); $p = 0.198$). Moreover, the baseline COX15 and MGST2 mRNA levels were directly associated with baseline AOP circulating levels (Fig. 2).

Discussion

The relationship between an excess in body weight and oxidative stress could be partially explained by the overeating characteristically found in obesity, since that inflammatory and postprandial oxidative stress is greater and more prolonged in obese than in normal subjects. As a result, the reduction of undesirable postprandial oxidative stress processes could form part of the strategy for prevention of diseases such as obesity and its associated comorbidities [31].

In the current study, weight loss decreased lipid peroxidation and nitrosative stress markers and raised the antioxidant defence, as has been found in previous studies

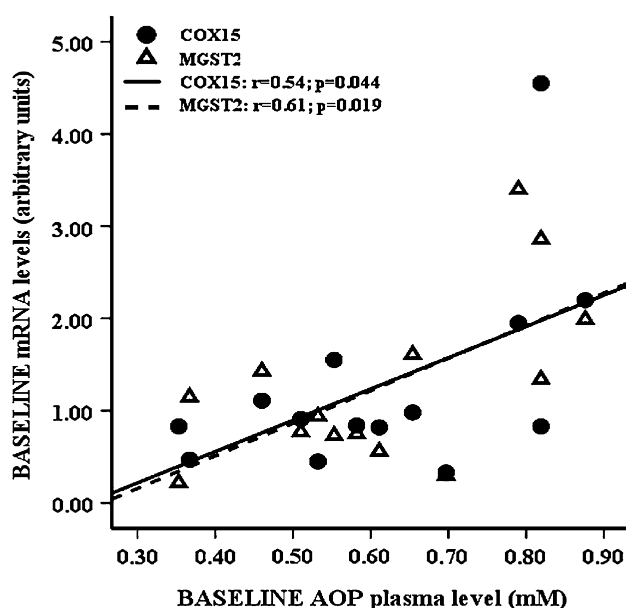

Fig. 2 Association between total antioxidant capacity (AOP) and mRNA levels of COX15 (Cytochrome c oxidase assembly protein 15) and MGST2 (microsomal glutathione S-transferase) at baseline ($n = 14$)

[7, 11, 14, 15]. In consequence, the postprandial changes from fasting in the studied markers, in both circulating levels and gene expression in PBMC, after the weight loss period were less marked than those at the beginning of the study. This suggests the existence of a tachyphylactic effect on the antioxidant defences after the weight loss treatment, which attenuates the postprandial oxidative response. In other words, some kind of tolerance or accommodation is accompanying the effects of a hypoenergetic diet on the oxidative stress response to an acute intake of a test drink containing antioxidants and a macronutrient mixture. Indeed, a major finding of this research was that the improvement in the antioxidant status induced by the energy restriction tended to tone down the postprandial reduction in COX15 gene expression induced by the nutrient intake, while a marked increase in the MGST2 postprandial gene expression was produced in relation to fasting. COX15 is an essential protein for the biogenesis of COX [16], which is an important marker of aerobic metabolism, whereas MGST2 is an important factor against oxidative injury [19]. Reinforcing the link of these genes with oxidative stress, COX15 and MGST2 gene expression was directly associated with AOP at baseline.

It has been suggested that the hyperglycemia- and hyperlipidemia-induced ROS generation mediated by the downregulation of pathways involved in the mitochondrial respiratory chain appears to be an initial key event triggering high-fat diet induced insulin resistance [5, 23]. Therefore, this downregulation of mitochondrial function appears to be prevented by caloric restriction devised to lose weight, while a postprandial up-regulation of an antioxidant enzyme-related gene is induced.

Taking these data together, this study showed that in obese subjects a hypocaloric diet to lose weight per se seems to be an effective strategy to minimize the oxidative processes related to obesity and its comorbidities. Thus, the novelty of this research relies on the fact that a process of tachyphylaxis concerning oxidative stress is occurring after an energy-restricted regime has been followed. Moreover, COX15 and MGST2 gene expression in PBMC could constitute novel markers for the application of nutrigenomic studies to personalize dietary treatment in obesity [2, 10].

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