Nutrition 32 (2016) 48–55

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

Expression of inflammation-related miRNAs in white blood cells from subjects with metabolic syndrome after 8 wk of following a Mediterranean diet–based weight loss program

José Luiz Marques-Rocha B.Sc. [a,b,c], Fermin I. Milagro Ph.D. [b,c,d], Maria Luisa Mansego Ph.D. [b,c], Maria Angeles Zulet Ph.D. [b,c,d], Josefina Bressan Ph.D. [a], J. Alfredo Martínez M.D., Ph.D. [b,c,d,*]

[a] Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil
[b] Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain
[c] Center for Nutrition Research, University of Navarra, Pamplona, Spain
[d] CIBERobn, Physiopathology of Obesity, Carlos III Institute, Madrid, Spain

ARTICLE INFO

Article history:
Received 15 May 2015
Accepted 18 June 2015

Keywords:
microRNA
miR-130a
Inflammation
miR-132-3p
Let-7b
miR-155

ABSTRACT

Objectives: The aim of this study was to evaluate the influence of a dietary strategy for weight loss (the RESMENA [reduction of metabolic syndrome in Navarra, Spain] diet) on the expression of inflammation-related microRNAS (miRNAs) and genes in white blood cells (WBC) from individuals with metabolic syndrome (MetS).

Methods: The clinical, anthropometric, and biochemical characteristics of 40 individuals with MetS (20 men and 20 women; age: 48.84 ± 10.02 y; body mass index: 35.41 ± 4.42 kg/m^2) were evaluated before and after an 8-wk hypocaloric diet based on the Mediterranean dietary pattern. Nutrient intake was assessed with a food frequency questionnaire and 48-h weighed food records. Total RNA was isolated from WBC and the expression of some inflammation-related miRNAs and mRNAs (*IL-6, TNF-α, ICAM-1, IL-18, SERPINE1, VCAM-1, GAPDH*) was assessed by quantitative polymerase chain reaction.

Results: The RESMENA nutritional intervention improved most anthropometric and biochemical features. The expression of miR-155-3p was decreased in WBC, whereas Let-7b was strongly upregulated as a consequence of the dietary treatment. However, they were not correlated with the expression of the proinflammatory genes in the same cells. The changes in the expression of let-7b, miR-125b, miR-130a, miR-132-3p, and miR-422b were significantly associated with changes in diet quality when assessed by the Healthy Eating Index. Moreover, low consumption of lipids and saturated fat (g/d) were associated with higher expression of let-7b after the nutritional intervention.

Conclusions: The Mediterranean-based nutritional intervention was able to induce changes in the expression of let-7b and miR-155-3p in WBC from patients with MetS after 8 wk. Moreover, the quality of the diet has an important effect on the miRNAs expression changes. These results should be highlighted because these miRNAs have been associated with inflammatory gene regulation and important human diseases.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. JLM-R and MLM contributed to the analysis and the writing of the manuscript. JB was involved in the fieldwork as well as in the critical reading of the manuscript. MAZ, JAM, and FIM were responsible for general coordination, follow-up, design, financial management, and the editing of the manuscript. All of the authors actively participated in manuscript preparation, and they all read and approved the final manuscript. The authors have declared that no competing interests exist.

* Corresponding author. Tel.: +34 94 842 5600; fax: +34 94 842 5619.
E-mail address: jalfmtz@unav.es (J. A. Martínez).

http://dx.doi.org/10.1016/j.nut.2015.06.008
0899-9007/

Introduction

Metabolic syndrome (MetS) is a complex disorder defined by a cluster of interconnected cardiometabolic alterations, including hypertension, insulin resistance, dyslipidemia, and abdominal obesity [1]. Individuals exhibiting combinations of these metabolic disturbances have a substantial synergistic cardiovascular risk greater than the sum associated with each abnormality [2]. Furthermore, the pathologic enlargement of the adipose tissue in obesity leads to an elevated production of proinflammatory mediators [3]. These altered signals mediate multiple processes, which predispose to diabetes mellitus, hepatic steatosis, atherosclerosis, plaque rupture, and atherothrombosis [1]. However, to date, the available information is controversial and does not necessarily imply a causal role neither putatively involve epigenetic mechanisms.

Data obtained from functional genomic methodologies indicate that several hundred genes participate in the inflammatory response and that their coordinated expression is tightly controlled [4]. Hence, microRNAs (miRNAs) are small noncoding RNAs that have the ability to control multiple genes, establishing an orchestrated network governing remote processes through intertwined pathways [5]. Several studies have highlighted the significance of miRNAs in maintaining metabolic homeostasis, and the regulation of these miRNAs could serve as a potential therapeutic target for metabolic disorders [6]. For example, the inflammatory toll-like receptor/nuclear factor-κB–related miR-181a is downregulated in monocytes of obese patients, which is associated with a higher number of MetS components and with coronary artery disease [7]. In this sense, miRNA expression contributes to regulate body weight, sometimes in relation to inflammation regulation [8]. Thus, some of these transcripts could be used as prognostic biomarkers of response to hypocaloric diets.

The aim of this study was to evaluate the effect of a weight loss strategy based on the Mediterranean dietary pattern (the RESMENA [reduction of metabolic syndrome in Navarra, Spain] diet) on anthropometric measurements, biochemical markers, and the expression of some selected inflammation-related genes and miRNAs in white blood cells (WBC) of individuals with MetS. We hypothesized that a hypocaloric pattern, as well as the improvement of the overall diet quality components designed to reduce MetS features, could have a positive effect on the expression of genes and miRNAs related to the inflammatory state. We sought to further explore the possible epigenetic underlying mechanisms and the putative interactions with the diet.

Methods

Study population

Forty white individuals (20 men and 20 women) with a body mass index (BMI) of 35.41 ± 4.42 kg/m^2, aged 48 ± 10 y, and diagnosed with MetS according to the International Diabetes Federation cutoffs [9] were enrolled in this study. The inclusion and exclusion criteria have been previously reported [10], but it should be pointed out that individuals with presence of psychiatric disturbances, eating disorders, chronic diseases related to the metabolism of nutrients, major body weight changes in the previous 3 mo, and difficulties in changing food habits were excluded. Volunteers were recruited through local newspaper advertisements and the database of Department of Nutrition, Food Science and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, Spain. Calculations were based on findings of previous studies [10,11]. The study protocol was performed in accordance with the ethical guidelines of the Declaration of Helsinki, and was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009). All individuals provided written informed consent also approved by the same research ethics committee.

Study protocol

The research is based on a subsample of the RESMENA-S study [10], a controlled intervention study that aimed to reduce MetS features based on energy restriction over 6 mo [10,11] and with components of the Mediterranean diet to specifically combat MetS features. The complete project is registered at www.clinicaltrials.gov (NCT01087086) and can be accessed elsewhere [10]. The diet of the RESMENA study prescribed an energy restriction of 30% applied to the total energy requirements of each patient. Resting metabolic rate was calculated using the Harris–Benedict equation, in which the Wilkens-adjusted weight was applied [12]. The physical activity factor was considered to calculate the total energy requirements according to the Food and Nutrition Board, National Research Council: Recommended Dietary Allowances [13]. Fasting blood samples, habitual dietary intake, and body composition were measured at baseline and at the end point of the 8-wk intervention period following standardized protocols, as published elsewhere [10,12].

Diet and dietary assessments

Participants were provided a 7-d menu plan as previously described [14]. The plan was composed of seven meals per day, including breakfast, lunch, dinner, two snacks in the morning, and two more snacks in the afternoon. Some characteristics of the RESMENA diet were a moderately high protein intake (24.6% ± 2.8%), higher daily average intake (seven meals daily), and increased total antioxidant capacity (TAC) than the usual recommendations. Moreover, the dietary advice included a cholesterol content <300 mg and focused on low glycemic index and glycemic load (GL) carbohydrate meals [10,12].

Dietary intake was assessed with a semiquantitative 136-item food frequency questionnaire previously validated in Spain for energy and nutrient intake [14]. A 48-h weighed food record was required at the beginning and at the end of the study. Diet composition was analyzed using the DIAL software (Alce Ingenieria, Madrid, Spain). The amount of eicosapentaenoic fatty acid and docosahexaenoic fatty acid was obtained through the DIAL software to estimate ω-3 fatty acid intake. The Healthy Eating Index (HEI) was calculated using the DIAL software as described elsewhere [15]. The program gives different values between 0 and 100 considering the servings per day of cereals, vegetables, fruits, dairy products, and meat. Also, this score takes into account the percentage of energy provided by total and saturated fats, the amount of cholesterol and sodium daily, and the variety of the diet. The final value was classified into five categories: >80 points indicates “excellent diet”; 71 to 80 points, a “very good diet”; 61 to 70 points, a “good diet”; 51 to 60, an “acceptable diet”; and 0 to 50 points, an “inadequate diet.” Dietary TAC was calculated using a list that takes into account raw or cooked food preparations. The dial software provides a list of the total antioxidant content (mmol/100 g) of >3100 foods, beverages, spices, herbs, and supplements used worldwide. The TAC value corresponding to the different scheduled/ingested servings per day was calculated [16]. Finally, GL was obtained from the updated international database published online by the Human Nutrition Unit, School of Molecular Biosciences, University of Sydney [17].

Anthropometric, clinical, and biochemical assessments

Anthropometric measurements (body weight, height, waist and hip circumferences) were carried out with the individuals in their underwear using validated processes [10]. Body fat was measured by dual energy x-ray absorptiometry (Lunar iDXA, software version 6.0, Madison, WI, USA) as described elsewhere [10]. Blood pressure was recorded with a standard mercury sphygmomanometer (Minimus II, Riester, Junginger, Germany). Measurements were taken three times after a 5-min resting period, following World Health Organization criteria [18].

Venous blood samples were drawn by venipuncture after a 12-h overnight fast. The ethylenediamine tetraacetic acid plasma and serum samples and WBCs were separated from whole blood by centrifugation at 3500 g at 5°C for 15 min (Model 5804 R, Eppendorf, Germany) and were frozen immediately at −80°C until assay (WBC in buffy coat). Glucose, total cholesterol (TC), high-density lipoprotein cholesterol, triacylglycerols, and acid uric serum concentrations were measured in an autoanalyzer Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits. Insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Insulin resistance was estimated by the homeostasis model assessment (HOMA) index {HOMA-IR = [glucose (mmol/L) × insulin (μU/mL)]/22.5} [19]. Low-density lipoprotein cholesterol (LDL-C) levels were calculated with the Friedewald formula: LDL-C = TC−high-density lipoprotein cholesterol−very-low-density lipoprotein [20]. Plasma malondialdehyde (MDA) was calorimetrically determined with a commercial kit (BIOXYTECH LPO-586, Oxis Research, Portland, OR, USA). Plasma-oxidized LDL-C levels were measured using a capture-ELISA kit from Mercodia (Uppsala, Sweden). Plasma concentrations of plasminogen activator inhibitor (PAI)-1 (BioVendor, Germany), interleukin (IL)-6 (R&D Systems, Minneapolis, MN), tumor

necrosis factor (TNF)-α (R&D Systems) and high-sensitivity C-reactive protein (Demeditec, Germany) were measured using ELISA kits and an automated analyzer system (Triturus, Grifols, Barcelona, Spain). In our laboratory, the inter- and intra-assay variability were <10% for all analytical determinations.

RNA extraction, miRNA, and mRNA quantitative PCR

At baseline and at the end of the study, total RNA was extracted from WBC using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined at 260/280 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

For the miRNA analysis, 20 ng of total RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies, Foster City, CA, USA) according to the manufacturer's protocol and the miRNA-specific reverse-transcription primers provided with the TaqMan MicroRNA Assay (Life Technologies), as shown in Table 1. The miRNA-specific cDNA was amplified in triplicate with the TaqMan Universal polymerase chain reaction (PCR) master mix and the respective specific probe provided in the TaqMan MicroRNA Assay (Life Technologies).

To perform the mRNAs analyses, 2 μg of total RNA was reverse-transcribed using the high-capacity complementary DNA (cDNA) reverse transcription (RT) kit (Life Technologies) according to the manufacturer's protocol. cDNA was amplified in triplicate with the TaqMan Universal PCR master mix and the respective specific probe provided in the TaqMan Gene Expression Assays (Life Technologies; Table 1). The analyzed miRNAs and genes were selected on the basis of previous studies strongly supporting their possible involvement in inflammatory pathways related to MetS features.

miRNA and mRNA levels were normalized to the endogenous controls, *RNU48* and *GAPDH* (glycerol-3-phosphate dehydrogenase), respectively. The ΔΔCT method was used for quantification and the fold changes are reported as $2^{-\Delta\Delta CT}$ [21]. All quantitative real-time RT-PCR measurements were performed using a 7900 HT Fast Real-Time PCR system (Life Technologies).

Statistical analyses

A group size of 40 was estimated to be necessary to obtain a significant ($P < 0.05$) difference in the reduction of waist circumference of 4.3 ± 6.8 cm with a power of 80% [10]. Only those individuals who completed the study were analyzed. The results are expressed as mean ± SD. Normality distributions of the measured variables were determined according to the Shapiro–Wilk test. Differences between the beginning and the end of the intervention period were analyzed by a paired *t* test. Spearman correlation and linear regression analyses were applied to assess the potential relationships and associations among diet, biochemical, and anthropometrical components with miRNA and mRNA variation. Analyses were carried out using SPSS 15.1 software for Windows (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Table 1
References of the specific PCR primers/probes used for the measurement of the miRNAs and mRNAs

miRNAs	TaqMan MicroRNA Assay reference
Let-7b	002619
miR-125b	000449
miR-130a	000454
miR-132-3p	000457
miR-146a	000468
miR-155-3p	002287
miR-223-5p	002098
miR-422b	001314
miR-4772- p	464414
RUN18	001006
Genes (mRNAs)	**TaqMan assay reference**
IL-6	Hs00985639_m1
TNF-α	Hs01113624_g1
ICAM-1	Hs00164932_m1
IL-18	Hs01038788_m1
SERPINE1	Hs01126606_m1
VCAM-1	Hs01003372_m1
GAPDH	Hs02758991_g1

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; IL, interleukin; miR, microRNA; PCR, polymerase chain reaction; SERPINE, serine protease inhibitor, member E; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule

Results

All clinical, laboratory, and dietary characteristics of study participants at the beginning and at the end of the dietary intervention are shown in Table 2. After 8 wk, the RESMENA diet improved most anthropometric and biochemical markers included in this study (Table 2). There was a significant reduction of all variables related to body weight, such as BMI, waist circumference, and fat mass ($P < 0.05$). There was also a significant reduction of TC, triacylglycerols, glucose, and insulin levels and, consequently, of the HOMA index ($P < 0.05$). Moreover, the circulating levels of MDA and PAI-1 were lower when compared with the levels at baseline ($P < 0.05$).

Table 2
Anthropometric, biochemical, and dietary characteristics of the participants at baseline and after 8 wk on the RESMENA Diet

Variables	Baseline	8 wk	*P* value*
	Mean ± SD	Mean ± SD	
Anthropometric measurements (N = 40)			
Body weight (kg)	99.7 ± 16.5	92.6 ± 15.8	**<0.01**
BMI (kg/m^2)	35.4 ± 4.4	32.8 ± 4.2	**<0.01**
Waist circumference (cm)	110.9 ± 12.1	103.2 ± 10.1	**<0.01**
Waist/hip ratio	0.96 ± 0.10	0.92 ± 0.09	**<0.01**
DXA total fat mass (%)	42.6 ± 6.0	40.1 ± 6.6	**<0.01**
DXA gynoid fat mass (kg)	7.96 ± 1.74	7.33 ± 1.66	**<0.01**
DXA android fat mass (kg)	3.93 ± 0.94	3.72 ± 0.87	**0.02**
DXA truncal fat mass (kg)	26.73 ± 5.18	25.92 ± 5.03	**<0.01**
Blood pressure (N = 40)			
SBP (mm Hg)	147.39 ± 20.87	134.10 ± 16.07	**<0.01**
DBP (mm Hg)	84.43 ± 9.01	76.69 ± 9.07	**<0.01**
Biochemical parameters (N = 40)			
Total cholesterol (mg/dL)	219 ± 45	202 ± 45	**<0.01**
HDL-C (mg/dL)	43 ± 10	41 ± 9	0.06
LDL-C (mg/dL)	137 ± 39	131 ± 39	0.29
Ox-LDL (U/L)	44.3 ± 18.5	40.2 ± 13.9	0.11
TG (mg/dL)	196 ± 124	150 ± 100	**<0.01**
Glucose (mg/dL)	124 ± 38	109 ± 25	**<0.01**
Insulin (μU/mL)	14.18 ± 8.12	8.95 ± 5.97	**<0.01**
HOMA index	4.46 ± 3.01	2.49 ± 1.82	**<0.01**
Uric acid (mg/dL)	5.82 ± 1.38	5.94 ± 1.06	0.49
Inflammatory markers (N = 40)			
MDA (μM)	0.84 ± 0.36	0.74 ± 0.28	**<0.01**
CRP (mg/L)	3.30 ± 3.45	3.53 ± 5.75	0.71
IL-6 (pg/mL)	2.63 ± 1.73	2.76 ± 1.58	0.53
PAI-1 (pg/mL)	157 ± 127	144 ± 152	**<0.01**
TNF-α (pg/mL)	0.69 ± 0.50	0.88 ± 0.94	0.99
Dietary characteristics (n = 35)			
Energy intake (kcal/d)	2255 ± 287	1353 ± 559	**<0.01**
Meal frequency (meals/d)	5.35 ± 1.30	6.47 ± 1.08	**<0.01**
Proteins (% TCV/d)	17.1 ± 2.8	24.5 ± 2.8	**<0.01**
Lipids (% TCV/d)	44.4 ± 6.0	37.8 ± 7.0	**<0.01**
CHO (% TCV/d)	37.9 ± 5.9	37.4 ± 6.4	0.64
Fiber (g/d)	18.3 ± 10.2	20.2 ± 8.5	0.07
GI (U/d)	663 ± 217	332 ± 124	**<0.01**
GL (U/d)	112 ± 44	53 ± 26	**<0.01**
Saturated fatty acids (g/d)	34.3 ± 13.2	16.1 ± 5.7	**<0.01**
ω-3 fatty acids (g/d)	0.33 ± 0.16	0.34 ± 0.04	0.06
TAC (mmol/d)	8.32 ± 4.74	13.89 ± 5.21	**<0.01**
Healthy Eating Index (U)	55.9 ± 11.8	71.4 ± 10.2	**<0.01**

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; DXA, dual-energy x-ray absorptiometry; GL, glycemic load; HDL-C, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; GI, glycemic index; LDL-C, low-density lipoprotein cholesterol; MDA: malondialdehyde; ox-LDL, oxidized low-density lipoprotein cholesterol; RESMENA, reduction of metabolic syndrome in Navarra, Spain; SBP, systolic blood pressure; TG, triacylglycerols; TCV, total caloric value

Bolded *P* values indicate statistically significant (less than 0.05).

* *P* value: comparison by paired samples statistics from baseline (Student's *t* test).

The dietary characteristics of the participants changed in accordance with the recommendations provided by the RESMENA diet. There was a lower intake of energy, lipids, and saturated fatty acids ($P < 0.05$). On the other hand, meal frequency (meals/day), protein intake, and TAC of the diet were higher ($P < 0.05$) at the end of the nutritional intervention (Table 2). These changes subsequently improved ($P < 0.05$) the HEI.

The analysis of the expression of proinflammatory genes in WBC showed no relevant changes ($P > 0.05$) as a result of the 8-wk nutritional intervention (Table 3). However, the expression of miR-155-3p decreased ($P = 0.007$), whereas let-7b was strongly upregulated ($P = 0.002$) as a consequence of the dietary intervention (Table 3). However, they were not correlated with the expression of the proinflammatory genes analyzed. Interestingly, the expression of let-7b was highly correlated ($P < 0.05$) with the other miRNAs analyzed, both at baseline (miR-130a, miR-132-3p, miR-146a, miR-155-3p, miR-223-5p, miR-422b, and miR-4772-3p) or after the RESMENA diet (miR-125b, miR-130a, miR-146a, miR-223-5p, miR-422b, and miR-4772-3p; Table 4).

The changes (8 wk versus baseline) in the miRNA expression levels were statistically associated with the changes in the diet quality, when assessed by the HEI. The improvement of HEI positively correlated with the changes in the expression of some miRNAs (Table 5) such as let-7b ($r^2 = 0.478$; $P = 0.008$), miR-125b ($r^2 = 0.359$; $P = 0.047$), miR-130a ($r^2 = 0.546$; $P = 0.001$), miR-132-3p ($r^2 = 0.551$; $P = 0.001$), and miR-422b ($r^2 = 0.374$; $P = 0.042$). Some of these significant correlations were found even after adjustment for energy restriction, sex, and age: let-7b ($\beta = 0.425$; $P = 0.035$), miR-130a ($\beta = 0.469$; $P = 0.009$), and miR-132-3p ($\beta = 0.543$; $P = 0.003$; Table 6). Furthermore, the miR-155 expression was associated with changes in the HEI and weight loss in a regression model adjusted for sex and age ($\beta = 0.577$; $P = 0.014$ and $\beta = 0.740$; $P = 0.003$, respectively; Table 6).

Table 3
Relative expression (fold change) of some miRNAs and genes (mRNAs) at baseline and after 8 wk on the RESMENA diet

Relative expression	Baseline	8-wk	*P* value*
(N = 40)	Mean ± SD	Mean ± SD	
miRNAs†			
Let-7b	1.04 ± 0.63	1.51 ± 0.90	<**0.01**‡
miR-125b	1.22 ± 0.84	1.33 ± 0.72	0.70
miR-130a	1.29 ± 0.75	1.52 ± 0.86	0.76
miR-132-3p	0.54 ± 0.48	0.51 ± 0.25	0.20
miR-146a	0.57 ± 0.23	0.58 ± 0.25	0.83
miR-155-3p	0.89 ± 0.49	0.59 ± 0.27	<**0.01**‡
miR-223-5p	0.82 ± 0.50	0.89 ± 0.47	0.26
miR-422b	0.70 ± 0.43	0.74 ± 0.36	0.13
miR-4772-3p	2.60 ± 0.59	2.78 ± 0.27	0.56
mRNA†			
IL-6	0.62 ± 0.47	1.05 ± 0.86	0.20
TNF-α	1.01 ± 0.86	1.53 ± 1.25	0.08
ICAM-1	2.21 ± 1.79	1.92 ± 1.64	0.66
IL-18	2.30 ± 2.16	1.54 ± 1.32	0.21
SERPINE1	5.59 ± 4.15	4.74 ± 2.96	0.91
VCAM-1	5.21 ± 4.62	3.02 ± 2.32	0.30

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; ICAM, intercellular adhesion molecule; PCR, polymerase chain reaction; RESMENA, reduction of metabolic syndrome in Navarra, Spain; SERPINE, serine protease inhibitor, member E; VCAM, vascular cell adhesion molecule

Bolded *P* values indicate statistically significant (less than 0.05).

* *P* value: comparison by paired samples after log transformation (Student's *t* test).

† miRNA and mRNA expression were measured by quantitative real-time PCR and normalized by *RNU48* and *GAPDH*, respectively.

‡ Different from baseline.

Table 4
Spearman correlations between the relative expression of Let-7b and other miRNAs* analyzed at baseline and after 8 wk on the RESMENA diet

Let-7b (N = 40)*	Baseline		8 wk	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
miR-125b	0.290	0.09	0.360	**0.02**†
miR-130a	0.702	**<0.01**†	0.636	**<0.01**†
miR-132-3p	0.002	0.99	0.025	0.88
miR-146a	0.049	0.77	0.425	**<0.01**†
miR-155-3p	−0.435	**0.03**†	−0.159	0.41
miR-223-5p	0.605	**<0.01**†	0.674	**<0.01**†
miR-422b	0.441	**<0.01**†	0.404	**<0.01**†
miR-4772-3p	0.380	**0.02**†	0.632	**<0.01**†

PCR, polymerase chain reaction; RESMENA, reduction of metabolic syndrome in Navarra, Spain

Bolded *P* values indicate statistically significant (less than 0.05).

* miRNAs were measured by quantitative real-time PCR and normalized by *RNU48*.

† $P < 0.05$.

Individually analyzing the components of HEI, it was evident that intakes of lipids and saturated fatty acids (g/d) were negatively associated with the expression of let-7b ($r^2 = -0.443$; $P = 0.014$ and $r^2 = -0.432$; $P = 0.017$, respectively; Fig. 1), suggesting that the improvement in HEI, in part as consequence of the low consumption of lipids and saturated fat intake (g/d), could be implicated in the increased expression of let-7 b in WBC.

Discussion

In the present study, the effects of a novel dietary strategy on the expression levels of miRNAs and genes related to the inflammatory process in WBC were reported. To our knowledge, this is a pioneer study in patients with MetS evaluating the effects of an energy-restricted intervention based on Mediterranean diet principles, including a modified macronutrient distribution and increased meal frequency, as well as the

Table 5
Spearman correlations between changes in the healthy eating index and changes in the relative expression of the miRNAs and genes analyzed*

Δ healthy eating Index ($n = 35$)	Correlation coefficient	*P* value
Δ Let-7b	0.478	**<0.01**†
Δ miR-125b	0.359	**0.04**†
Δ miR-130a	0.546	**<0.01**†
Δ miR-132-3p	0.551	**<0.01**†
Δ miR-146a	−0.026	0.88
Δ miR-155- p	0.414	0.12
Δ miR-223-5p	0.023	0.90
Δ miR-422b	0.374	**0.04**†
Δ miR-4772-3p	−0.238	0.20
Δ IL-6	−0.258	0.16
Δ TNF-α	−0.059	0.75
Δ ICAM-1	0.058	0.74
Δ IL-18	0.153	0.41
Δ SERPINE1	0.020	0.91
Δ VCAM-1	−0.049	0.81

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; IL, interleukin; PCR, polymerase chain reaction; SERPINE, serine protease inhibitor member; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule

Bolded *P* values indicate statistically significant (less than 0.05).

* Changes (8 wk vs baseline) in miRNA and gene expression were measured by quantitative real-time PCR and normalized by *RNU48* and *GAPDH*, respectively.

† $P < 0.05$.

Table 6
Regression coefficients between changes in the healthy eating index and changes in the relative expression of some miRNAs analyzed (N = 35)*

Linear regression models	ANOVA test	β-coefficient	*P* value
Δ miR-130a			
Δ HEI	0.024†	0.469	**0.009**†
Energy restriction		0.039	0.858
Age		−0.028	0.866
Sex		0.337	0.053
Δ miR-132-3p			
Δ HEI	0.025†	0.543	**0.003**†
Energy restriction		0.234	0.170
Age		−0.038	0.817
Sex		0.133	0.432
Δ Let-7b			
Δ HEI	0.025†	0.410	**0.024**†
Energy restriction		−0.191	0.276
Δ miR-155			
Δ HEI	0.007†	0.577	**0.014**†
Weight loss		0.740	**0.003**†
Age		−0.313	0.094
Sex		−0.020	0.922

ANOVA, analysis of variance; HEI, Healthy Eating Index; PCR, polymerase chain reaction
Bolded *P* values indicate statistically significant (less than 0.05).
* Changes (8 wk vs baseline) in miRNA expression were measured by quantitative real-time PCR and normalized by *RNU48*.
† $P < 0.05$.

presence of bioactive ingredients, such as fiber and beneficial fatty acids, and controlling glycemic index and GL, dietary TAC, and HEI score.

Metabolic diseases are characterized by the failure of regulatory genes or enzymes to effectively orchestrate specific pathways involved in the control of many biological processes [2]. In addition to the classical regulators of metabolic homeostasis, recent discoveries have evidenced the putative remarkable role of miRNAs in the post-transcriptional regulation of a number of genes, and their involvement in many pathologic states, such as the MetS features [22]. However, results from human studies are still unclear and more research is needed to find links between miRNAs and the pathways involved.

The main results of the current study were the expression changes of miRNA-155 and let-7b, especially the latter. Let-7 family was, after lin-4, the second miRNAs found and has nine members, namely, let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, and miR-98. All members are believed to exert similar functions because they share a common seed region (nucleotides 2–8), which mediates miRNA interaction with target mRNAs [23]. Previous studies have identified let-7 as a tumor suppressor, which is downregulated or even lost in many human cancers [24], regulating multiple cellular processes including cell division and DNA repair pathways. More recently, the role of let-7 in the physiopathology of some diseases has received significant attention. Furthermore, aberrant let-7 expression has been associated with a variety of human diseases including cardiovascular events, liver fibrosis, and lung disorders. Moreover, it has been demonstrated that let-7b regulates both atherogenic and adipogenic phenomena. Biostatistical tests and network analyses suggest that let-7b could act as a global synergistic key mediator controlling hundreds of protein-coding genes [25]. Interestingly, let-7b expression had a high correlation with all of the miRNAs analyzed at baseline or at the end of the period studied. A recent work suggested that let-7b could be a strong candidate as a master regulator of products on coding and non-coding genes related with ovarian carcinoma [25]. Other publications have provided new insights into the protective properties of let-7b in preventing the endothelial dysfunction associated with cardiovascular disease [26]. Circulating let-7b levels were lower in patients with acute myocardial infarction than in healthy adults [27]. Additionally, overexpression of let-7b promoted nitric oxide production [26], which is considered a key factor in vascular protective actions including vasodilatation. Although this study did not imply a direct action, we found that blood pressure was lower at the end of the dietary intervention period.

Global knockdown of the let-7 family with an antimiR was sufficient to prevent and treat impaired glucose tolerance in mice with diet-induced obesity, at least in part by improving insulin sensitivity in liver and muscle [23]. In the same study, antimiR treatment of mice on a high-fat diet also resulted in increased lean and muscle mass, but not increased fat mass, and prevented ectopic fat deposition in the liver. These findings demonstrate that let-7 regulates multiple aspects of glucose metabolism and suggests antimiR-induced let-7 knockdown as a potential treatment for type 2 diabetes.

In this trial, the expression of miR-155 was lower at the end of the nutritional intervention and was associated with the quality of the diet and with the weight loss by regression analysis. These results suggest that miR-155 could be a prognostic marker of obesity, as advanced by previous studies. In this context, some authors have shown that the expression of miR-155 was higher in diet-induced obese rats than in control animals [28]. In the same study, a 30% calorie restriction led to significant reduction in the expression of miR-155. Recent findings identified miR-155 as a central apigenin-regulated miRNA in inflammation and provided evidence of the underlying mechanism by which apigenin and diets rich in apigenin, a flavonoid abundant in parsley and celery, contribute to restore homeostasis [29]. Additionally, quercetin decreased lipopolysaccharide (LPS)-induced expression of miR-155 in macrophages [30], highlighting the benefits of dietary interventions as a strategy to restore proper immune function in vivo.

Overexpression of miR-155 has been reported in lung, breast, and colon tumors [31] and has been positively associated with intestinal inflammation and ulcerative colitis [32]. Furthermore, in vivo studies have found that transgenic mice overexpressing miR-155 in B-cell lineage (Eμ-miR-155) produced more TNF-α when challenged with LPS and were hypersensitive to LPS/D-galactosamine–induced septic shock with respect to their normal counterparts [33]. Interestingly, miR-155 targets the lipid phosphatase SHIP1 [34], an important signal for macrophage activation. The exposure of cultured macrophages to LPS leads to upregulation of miR-155, which targets the CCAAT/enhancer binding protein β (*C/EBPβ*) mRNA, implicated in the regulation of proinflammatory cytokines during macrophage activation and the acute-phase response [35].

To evaluate the quality of the diet, some indices or scores have been developed, such as the HEI, the Alternate HEI, or the Diet Quality Index and derivatives [16]. Most of these tools take into consideration the Mediterranean diet guidelines, widely recognized as a healthy pattern [36]. They consist of a single score that results from computing different components, such as foods, food groups, or a combination of foods and nutrients. In this context, the HEI score was selected because it takes into account macro- and micronutrient intake, as well as food variety. The improvement of HEI score due to the 8-wk RESMENA intervention was related to changes in the expression of let-7b, miR-125, miR-130, miR-132, and miR-422. Additionally, the consumption of total and saturated fat inversely correlated with the HEI and

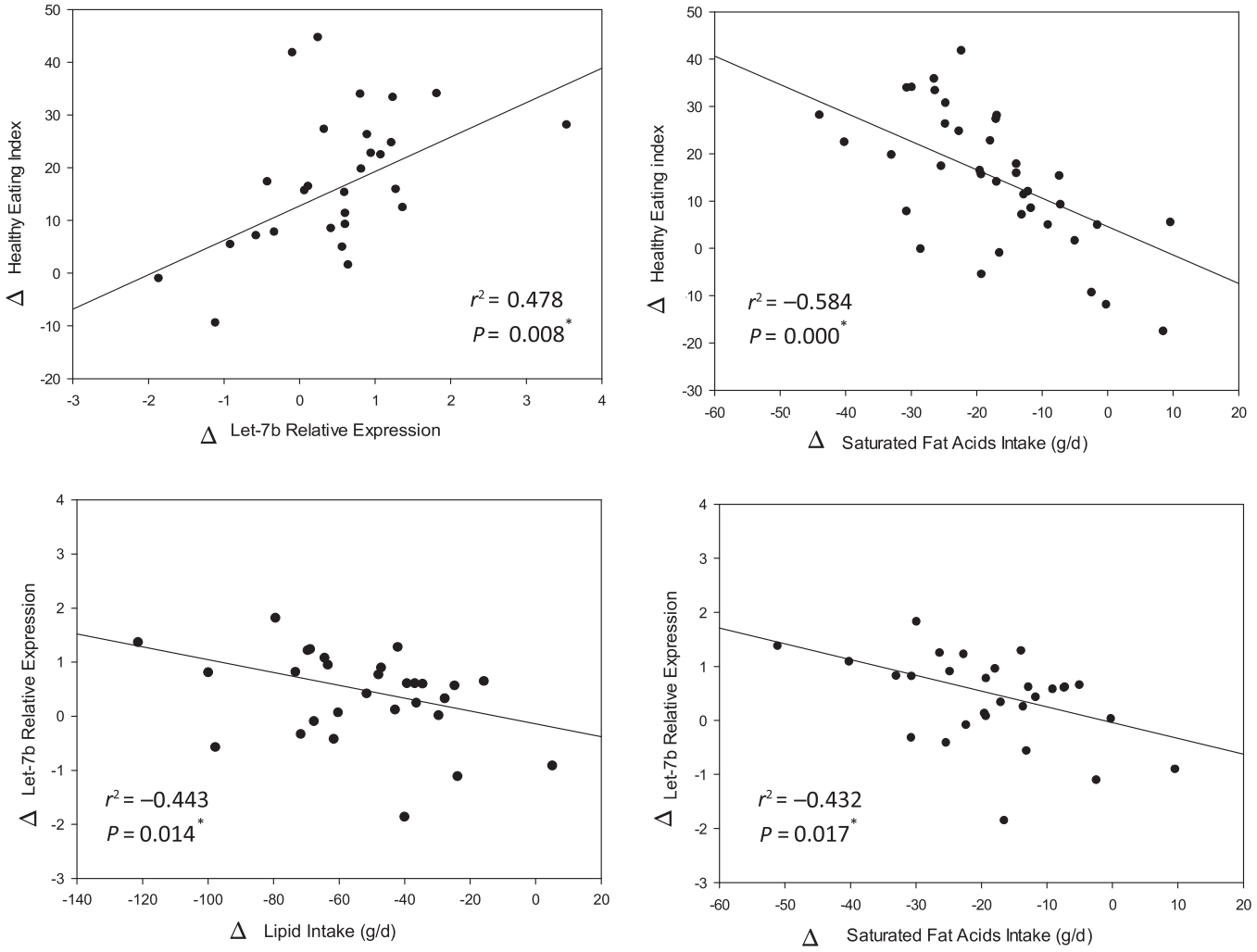

Fig. 1. Correlations between the changes in the Healthy Eating Index (HEI) and the relative expression of let-7b and changes in the intake of lipids and saturated fat (g/d). Spearman correlations; $^{*}P < 0.05$; Changes (8 wk vs baseline); n = 39 to HEI and n = 30 to let-7b relative expression analysis.

let-7b expression. Our regression analyses also indicated that the expression of these miRNAs was more dependent on the quality of the diet than on the weight loss achieved or the energy restriction. To our knowledge, this was the first time that these associations were described in the scientific literature. It has been previously mentioned that the diet may influence the expression of some miRNAs, which has been considered useful in the assessment of nutritional status in dietary intervention studies. For example, miR-125b has been reported to regulate in a negative manner the expression of the vitamin D receptor (*VDR*) [37]. This functional link between *VDR* expression and miR-125b might explain why *VDR* expression is upregulated, whereas miR-125b is downregulated in distinct types of cancer as the genomic locus that contains miR-125b is deleted in several cancers [37]. On the other hand, miR-125b has been reported to post-transcriptionally regulate vitamin D_3 hydroxylase (*CYP24*), an enzyme that catalyzes the inactivation of vitamin D; the low expression of miR-125b in cancer tissues may be a possible mechanism for explaining the high *CYP24* expression in cancer tissues [38]. The influence of nutrients on the expression of other miRNAs is still unclear, being a new promising area of nutrigenomic research.

In this study, MDA and PAI-1 levels decreased significantly after the intervention. High levels of plasma MDA, a biomarker of lipid peroxidation, have been associated with type 2 diabetes [39], whereas calorie-restricted dietary strategies have been previously associated with lower MDA levels [40]. In this sense, a recent publication revealed that Let-7b overexpression inhibited reactive oxygen species production [26]. In this context, it can be hypothesized that the increased expression of let-7 at the end of the intervention may contribute to decrease reactive oxygen species production and lipid peroxidation.

PAI-1, encoded by the *SERPINE1* gene, is the principal inhibitor of tissue plasminogen activator and urokinase, and therefore is an inhibitor of fibrinolysis [41]. This serine protease is produced by vascular endothelium, liver, monocytes/macrophages, platelets, and adipose tissue [42]. High plasma levels of PAI-1 have been associated with higher risk for cardiovascular diseases [43]. Furthermore, PAI-1–dependent mechanisms are also implicated in the pathogenesis of obesity, insulin resistance, and type 2 diabetes [44]. In fact, increased PAI-1 levels can be considered a component of MetS. Clinical studies have demonstrated a strong correlation between PAI-1 and BMI and fibrinolysis. As elevated levels of inflammatory cytokines could increase PAI-1

expression, the link between obesity and elevated PAI-1 levels could be the low-grade inflammatory state [45]. Although the RESMENA dietary strategy was successful for weight loss and the improvement of most of the anthropometric and biochemical parameters, including PAI-1, the circulating levels of other inflammatory biomarkers, such as CRP, TNF-α, and IL-6, were not reduced.

The study has some limitations. First, gene expression varies depending on the tissue or cell type [22], and it is possible that specific miRNAs are not widely expressed in human WBC. Second, this study specifically aimed to evaluate the association between the variables included and conclusions on causality cannot be measured. Also, the number of participants in this study is not very high, but it may be proposed that type 2 errors were overcome because important statistical differences were found. In the analysis of before and after responses in a single group, confounding by measured or unmeasured factors that are potentially time varying across the time periods compared remains a critical threat. However, we used statistical methods adjusting for measured confounding factors and quantifying the potential effect of unmeasured factors on effect estimates. Finally, despite limitations of structured questionnaires of this type, the advantage of food frequency questionnaire is its feasibility for establishing long-term habitual dietary intake [46].

Conclusion

The results of this study provide evidence that the quality of a Mediterranean-based nutritional intervention was related to changes in the expression of let-7b and miR-155-3p in WBC from patients with MetS after 8 wk. These results should be highlighted because these miRNAs have been associated with important human diseases linked to MetS, such as cancer, atherogenic and adipogenic processes, and other inflammatory conditions.

Acknowledgments

The authors acknowledge the volunteers of this study and the excellent technical assistance of Ana Lorente and the dietitians Rocio de la Iglesia, Aurora Perez-Cornago, and Patricia Lopez-Legarrea. They also acknowledge the Spanish Ministry of Economy and Competitiveness (AGL2013-45554-R project), the CAPES Foundation (Ministry of Education of Brazil, Brasília–DF 70040-020, Brazil) for providing JLM-R with a research grant (process n° 6409-13-0), Instituto de Salud Carlos III (CIBERobn) and CNPq Foundation (Brazil) for financial support.

References

[1] Kaur J. A comprehensive review on metabolic syndrome. Cardiol Res Pract 2014;2014:943162.
[2] Conen D, Rexrode KM, Creager MA, Ridker PM, Pradhan AD. Metabolic syndrome, inflammation, and risk of symptomatic peripheral artery disease in women: a prospective study. Circulation 2009;120:1041–7.
[3] Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 2004;110:1564–71.
[4] Jolanta Jura and Aleksander Koj (2011). Regulatory Mechanisms Controlling Inflammation and Synthesis of Acute Phase Proteins, Acute Phase Proteins - Regulation and Functions of Acute Phase Proteins, Prof. Francisco Veas (Ed.), ISBN: 978-953-307-252-4, InTech, DOI: 10.5772/18207. Available at: http://www.intechopen.com/books/acute-phase-proteins-regulation-and-functions-of-acute-phase-proteins/regulatory-mechanisms-controlling-inflammation-and-synthesis-of-acute-phase-proteins. Accessed May 25, 2015.
[5] Aranda JF, Madrigal-Matute J, Rotllan N, Fernandez-Hernando C. MicroRNA modulation of lipid metabolism and oxidative stress in cardiometabolic diseases. Free Radic Biol Med 2013;64:31–9.
[6] Fasanaro P, D'Alessandra Y, Magenta A, Pompilio G, Capogrossi MC. MicroRNAs: promising biomarkers and therapeutic targets of acute myocardial ischemia. Curr Vasc Pharmacol 2015;13:305–15.
[7] Hulsmans M, Sinnaeve P, Van der Schueren B, Mathieu C, Janssens S, Holvoet P. Decreased miR-181 a expression in monocytes of obese patients is associated with the occurrence of metabolic syndrome and coronary artery disease. J Clin Endocrinol Metab 2012;97:E1213–8.
[8] Hulsmans M, Holvoet P. MicroRNAs as early biomarkers in obesity and related metabolic and cardiovascular diseases. Curr Pharm Des 2013;19:5704–17.
[9] Alberti KG, Zimmet P, Shaw J. The metabolic syndrome—a new worldwide definition. Lancet 2005;366:1059–62.
[10] Zulet MA, Bondia-Pons I, Abete I, de la Iglesia R, Lopez-Legarrea P, Forga L, et al. The reduction of the metabolyc syndrome in Navarra-Spain study: a multidisciplinary strategy based on chrononutrition and nutritional education, together with dietetic and psychological control. Nutr Hosp 2011;26:16–26.
[11] de la Iglesia R, Lopez-Legarrea P, Abete I, Bondia-Pons I, Navas-Carretero S, Forga L, et al. A new dietary strategy for long-term treatment of the metabolic syndrome is compared with the American Heart Association guidelines: the MEtabolic Syndrome REduction in NAvarra project. Br J Nutr 2014;111:643–52.
[12] Lopez-Legarrea P, de la Iglesia R, Abete I, Bondia-Pons I, Navas-Carretero S, Forga L, et al. Short-term role of the dietary total antioxidant capacity in two hypocaloric regimes on obese with metabolic syndrome symptoms: the RESMENA randomized controlled trial. Nutr Metab 2013;10:22.
[13] Food and Nutrition Board, National Research Council. Recommended dietary allowances. 10th ed. Washington, DC: The National Academy Press; 1989.
[14] Hermsdorff HH, Zulet MA, Abete I, Martinez JA. Discriminated benefits of a Mediterranean dietary pattern within a hypocaloric diet program on plasma RBP4 concentrations and other inflammatory markers in obese subjects. Endocrine 2009;36:445–51.
[15] Basiotis PP, Gerrior SA, Juan WY, Lino M. The Healthy Eating Index: 1999–2000. Washington DC: US Department of Agriculture, Center for Nutrition Policy and Promotion; 2002.
[16] Puchau B, Zulet MA, de Echavarri AG, Hermsdorff HH, Martinez JA. Dietary total antioxidant capacity: a novel indicator of diet quality in healthy young adults. J Am Coll Nutr 2009;28:648–56.
[17] Foster-Powell K, Brand-Miller JC. International table of glycemic index and glycemic load values: 2002. Am J Clin Nutr 2002;76:5–56.
[18] Whitworth JA, Chalmers J. World health organisation-international society of hypertension hypertension guidelines. Clin Exp Hypertens 2004;26:747–52.
[19] Aller EE, Abete I, Astrup A, Martinez JA, van Baak MA. Starches, sugars and obesity. Nutrients 2011;3:341–69.
[20] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
[21] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2) method. Methods 2001;25:402–8.
[22] Milagro FI, Miranda J, Portillo MP, Fernandez-Quintela A, Campion J, Martinez JA. High-throughput sequencing of microRNAs in peripheral blood mononuclear cells: identification of potential weight loss biomarkers. PLoS One 2013;8:e54319.
[23] Frost RJ, Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A 2011;108:21075–80.
[24] Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME. The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer 2010;17:F19–36.
[25] Tang Z, Ow GS, Thiery JP, Ivshina AV, Kuznetsov VA. Meta-analysis of transcriptome reveals let-7 b as an unfavorable prognostic biomarker and predicts molecular and clinical subclasses in high-grade serous ovarian carcinoma. Int J Cancer 2014;134:306–18.
[26] Bao MH, Feng X, Zhang YW, Lou XY, Cheng Y, Zhou HH. Let-7 in cardiovascular diseases, heart development and cardiovascular differentiation from stem cells. Int J Mol Sci 2013;14:23086–102.
[27] Long G, Wang F, Duan Q, Yang S, Chen F, Gong W, et al. Circulating miR-30 a, miR-195 and let-7 b associated with acute myocardial infarction. PLoS One 2012;7:e50926.
[28] Olivo-Marston SE, Hursting SD, Perkins SN, Schetter A, Khan M, Croce C, et al. Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microRNA expression. PLoS One 2014;9:e94765.
[29] Arango D, Diosa-Toro M, Rojas-Hernandez LS, Cooperstone JL, Schwartz SJ, Mo X, et al. Dietary apigenin reduces LPS-induced expression of miR-155 restoring immune balance during inflammation. Mol Nutr Food Res 2015;59:763–72.
[30] Boesch-Saadatmandi C, Loboda A, Wagner AE, Stachurska A, Jozkowicz A, Dulak J, et al. Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155. J Nutr Biochem 2011;22:293–9.

[31] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006;9:189–98.
[32] Takagi T, Naito Y, Mizushima K, Hirata I, Yagi N, Tomatsuri N, et al. Increased expression of microRNA in the inflamed colonic mucosa of patients with active ulcerative colitis. J Gastroenterol Hepatol 2010;25(Suppl 1):S129–33.
[33] Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125 b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 2007;179:5082–9.
[34] O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci U S A 2009;106:7113–8.
[35] Worm J, Stenvang J, Petri A, Frederiksen KS, Obad S, Elmen J, et al. Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. Nucleic Acids Res 2009;37:5784–92.
[36] Salas-Salvado J, Bullo M, Babio N, Martinez-Gonzalez MA, Ibarrola-Jurado N, Basora J, et al. Reduction in the incidence of type 2 diabetes with the Mediterranean diet: results of the PREDIMED-Reus nutrition intervention randomized trial. Diabetes Care 2011;34:14–9.
[37] Mohri T, Nakajima M, Takagi S, Komagata S, Yokoi T. MicroRNA regulates human vitamin D receptor. Int J Cancer 2009;125:1328–33.
[38] Komagata S, Nakajima M, Takagi S, Mohri T, Taniya T, Yokoi T. Human CYP24 catalyzing the inactivation of calcitriol is post-transcriptionally regulated by miR-125 b. Mol Pharmacol 2009;76:702–9.
[39] Kaefer M, De Carvalho JA, Piva SJ, da Silva DB, Becker AM, Sangoi MB, et al. Plasma malondialdehyde levels and risk factors for the development of chronic complications in type 2 diabetic patients on insulin therapy. Clin Lab 2012;58:973–8.
[40] Crujeiras AB, Parra D, Goyenechea E, Abete I, Gonzalez-Muniesa P, Martinez JA. Energy restriction in obese subjects impact differently two mitochondrial function markers. J Physiol Biochem 2008;64:211–9.
[41] Declerck PJ, Gils A. Three decades of research on plasminogen activator inhibitor-1: a multifaceted serpin. Semin Thromb Hemost 2013;39:356–64.
[42] Huang J, Sabater-Lleal M, Asselbergs FW, Tregouet D, Shin SY, Ding J, et al. Genome-wide association study for circulating levels of PAI-1 provides novel insights into its regulation. Blood 2012;120:4873–81.
[43] Kohler HP, Grant PJ. Plasminogen-activator inhibitor type 1 and coronary artery disease. N Engl J Med 2000;342:1792–801.
[44] Festa A, Williams K, Tracy RP, Wagenknecht LE, Haffner SM. Progression of plasminogen activator inhibitor-1 and fibrinogen levels in relation to incident type 2 diabetes. Circulation 2006;113:1753–9.
[45] Aso Y. Plasminogen activator inhibitor -1 in vascular inflammation and thrombosis. Front Biosci 2007;12:2957–66.
[46] Shaneshin M, Jessri M, Rashidkhani B. Validity of energy intake reports in relation to dietary patterns. J Health Popul Nutr 2014;32:36–45.