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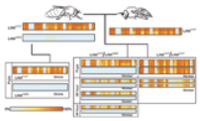

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RESEARCH PAPER

LINE-1 methylation is positively associated with healthier lifestyle but inversely related to body fat mass in healthy young individuals

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ABSTRACT

With the goal of investigating if epigenetic biomarkers from white blood cells (WBC) are associated with dietary, anthropometric, metabolic, inflammatory and oxidative stress parameters in young and apparently healthy individuals. We evaluated 156 individuals (91 women, 65 men; age: 23.1± 3.5 years; body mass index: 22.0±2.9 kg/m^2) for anthropometric, biochemical and clinical markers, including some components of the antioxidant defense system and inflammatory response. DNA methylation of *LINE-1*, *TNF-α* and *IL-6* and the expression of some genes related to the inflammatory process were analyzed in WBC. Adiposity was lower among individuals with higher *LINE-1* methylation. On the contrary, body fat-free mass was higher among those with higher *LINE-1* methylation. Individuals with higher *LINE-1* methylation had higher daily intakes of calories, iron and riboflavin. However, those individuals who presented lower percentages of *LINE-1* methylation reported higher intakes of copper, niacin and thiamin. Interestingly, the group with higher *LINE-1* methylation had a lower percentage of current smokers and more individuals practicing sports. On the other hand, *TNF-α* methylation percentage was negatively associated with waist girth, waist-to-hip ratio and waist-to-stature ratio. Plasma *TNF-α* levels were lower in those individuals with higher *TNF-α* methylation. This study suggests that higher levels of *LINE-1* and *TNF-α* methylation are associated with better indicators of adiposity status in healthy young individuals. In addition, energy and micronutrient intake, as well as a healthy lifestyle, may have a role in the regulation of DNA methylation in WBC and the subsequent metabolic changes may affect epigenetic biomarkers.

Abbreviations: BMI, Body mass index; CI, Confidence interval; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL-c, High-density lipoprotein; IL, Interleukin; LDL-c, Low-density lipoprotein; LINE-1, Long interspersed nucleotide element-1; MET, Metabolic equivalent; MS-HRM, Methylation-sensitive high resolution melting; OS, Oxidative stress; OR, Odds ratio; ox-LDL, Oxidized Low-density lipoprotein; PBMC, Peripheral blood mononuclear cells; ROS, Oxygen-reactive species; TAC, Total antioxidant capacity; TC/HDL-c, Castelli index; TC, Total cholesterol; TNF-α, Tumor necrosis factor α; UFV, Federal University of Viçosa; UTR, Untranslated region; VLDL, Very Low-density lipoprotein; WBC, White blood cell

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Introduction

Lifestyle and nutrients may induce transient or permanent alterations in the epigenetic marks that regulate the expression of genes involved in metabolic processes and networks, which could be one of the factors leading to chronic diseases.[1] The most common epigenetic modification is DNA methylation. Approximately 50% of the human genome is composed of repetitive sequences such as Long Interspersed Nuclear Elements (LINE), which are the most common repetitive elements of interspersed DNA repeats.[2] These elements are usually methylated, and their transcription and retrotransposition are suppressed by a variety of control mechanisms including methylation and non-coding RNA.[3] Because of its high genome dissemination, *LINE-1* methylation status has been proposed as a surrogate marker for estimating global DNA methylation level.[2]

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A global decrease in the methylation of peripheral blood DNA was found to be an independent risk factor for many cancers[4] and for developmental, autoimmune, and other chronic diseases.[5] LINE-1 methylation levels in peripheral blood mononuclear cells (PBMCs) have been reported to predict response to a dietary weight-loss intervention[6] and have been associated with serum glucose levels.[7] The methylation levels of individual genes, such as those of tumor necrosis factor α (*TNF-α*), have been also proposed as biomarkers of response to a hypocaloric diet.[8] Methylation status may, therefore, serve as a biomarker for early diagnostics, prediction of prognosis, and response to treatments. Moreover, some dietary factors are able to alter the percentage of methylation, both at *LINE-1* and in proinflammatory genes. For example, energy restriction has been reported to decrease *IL-6* methylation levels in buffy coat DNA[7] and *TNF-α* methylation has been associated with n-6 polyunsaturated fatty acids (PUFAs) intake.[9] However, the etiopathogenic mechanisms remain poorly understood. Thus, the objective of this study was to explore the relation between DNA methylation levels of *LINE-1, TNF-α* and *IL-6* in white blood cells (WBC) and anthropometric, biochemical, clinical, dietary, inflammatory and oxidative stress parameters in young and apparently healthy adults.

Material and methods

Subjects

One hundred 5six healthy subjects were recruited to participate in the study (91 women and 65 men; age: 23.1 ± 3.5 y and BMI: 22.0 ± 2.9 kg/m^2). Most of the study population self-reported to be white (n = 131) followed by black (n = 16) and others (n = 9). Initial screening excluded subjects with evidence of any metabolic disease, chronic inflammation, hydric balance disorders, changes in body composition and problems in nutrient absorption or metabolism. Other exclusion criteria were drug or nutritional treatment that affects energy balance, dietary intake, lipid profile, insulin levels or glucose metabolism, contraceptive use up to 2 months before participation in the study and weight loss diet follow-up or unstable weight in the past 6 months. In agreement with the principles of the Helsinki Declaration and following a clear explanation of the study protocol, each participant signed a written informed consent form. The study was approved by the Human Research Ethics Committee of the Federal University of Viçosa, Brazil (protocol number 019/2011).

Anthropometric and body composition assessments

Height was measured with a stadiometer (Seca 206 model, Hamburg, Germany) to the nearest 0.1 cm. Body weight was measured to the nearest 0.1 kg by using an electronic microdigital scale (Tanita TBF-300 A model, Tokyo, Japan). Body mass index (BMI) was calculated by the quotient between body weight and square height (kg/m^2). Waist and hip perimeters were measured with an inelastic and flexible tape to the nearest 0.1 m. Triceps, biceps, subscapular and suprailiac skinfold thicknesses were measured to the nearest 1 mm by using a skinfold caliper (Lange caliper, Cambridge Scientific Industries Inc., Maryland, USA). The sum of skinfold thickness was calculated. Total body fat percentage was measured to the nearest 0.1 % using a body composition analyzer (Biodynamics 310 model, Washington, USA). Body fat mass and body fat-free mass were estimated using the same body composition analyzer. Truncal fat percentage was computed as the sum of subscapular and suprailiac skinfold thicknesses divided by the sum of 4 skinfold measurements.[10] Finally, truncal adiposity index was calculated by the ratio of subscapular to triceps skinfold thickness.[11]

Blood pressure assessment

Systolic and diastolic blood pressures were measured with a mercury sphygmomanometer (BIC, SP, Brazil) following World Health Organization criteria.[12]

Dietary intake and lifestyle assessments

A 72-hour food record was used to collect information about energy and nutrient intake. A booklet was given to the participants to record everything they ate or drank over a period of 3 non-consecutive days, including a weekend day. Dietary intake was computed using specific software (DietPro®, version 5.0, AS Systems).

Covariates about diet and lifestyle, such as vitamin supplementation, smoking status (smokers or non-smokers), number of cigarettes per day, regular physical activity (yes or no) and volume of physical activity, were also collected. To quantify the volume of physical activity, an activity metabolic equivalent (MET) was used.[13] This index represents the ratio between energy expenditure during each specific activity and resting metabolic rate. METs were computed by a multiple of resting metabolic rate (MET score) to each activity. The MET scores were provided by Compendium of Physical Activities, a coding scheme that classifies specific physical activity by rate

of energy expenditure.[14] METs were calculated by multiplying time spent on each activity by a specific MET score to that activity. The scores were then summed over all activities to obtain a mean value of overall week, expressed in hours per day.

Analyses of biological samples

Blood samples were drawn by vein puncture after a 12-hour overnight fast. Ethylenediaminetetraacetic acid (EDTA) plasma, heparin plasma and serum samples were separated from whole blood by centrifugation at 3500 rpm at 5°C for 15 min (Eppendorf AG, 5804 R model, Hamburg, Germany) while erythrocytes were separated from whole blood by centrifugation at 3000 rpm at 5°C for 10 min. All samples were immediately stored at −80°C until assay.

Lipid and glucose profile

Serum glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-c), and triacylglycerol concentrations were assessed in an automated biochemical analyzer (BS-200, Shenzhen Mindray Bio-medical Electronics Co., Nanshan, China) using specific colorimetric kits (Bioclin, Quibasa, Minas Gerais, Brazil). Low-density lipoprotein cholesterol (LDL-c) data were calculated by the Friedewald equation as previously validated.[15] The total cholesterol-to-HDL-c ratio was also assessed.[16] Plasma insulin concentrations (sensitivity 2 μU/mL) were measured by an enzyme-linked immunosorbent assay (ELISA) kit as described by the supplier (Linco Research, St. Charles, USA). The homeostasis model assessment of insulin resistance (HOMA-IR), calculated as fasting glucose (nmol/L) x fasting insulin (μU/mL)/22.5,[17] was used to estimate insulin resistance.

Antioxidant markers

Plasma total antioxidant capacity (TAC) was assessed by a colorimetric assay, which relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyoglobin (Cayman Chemical, Ann Arbor, MI, USA). Plasma oxidized-LDL (ox-LDL) concentrations were determined by ELISA (Mercodia, Uppsala, Sweden). Glutathione peroxidase (GPx) activity [nmol/(mL/min)] was measured in erythrocytes using a commercially available kit as described by the supplier (Cayman Chemical, Cat. 703102). Uric acid and ceruloplasmin concentrations were assessed with an automated biochemical analyzer (BS-200, Shenzhen Mindray Bio-medical Electronics Co., China) using specific commercially available kits (Bioclin).

Inflammatory markers

Plasma IL-6, TNF-α and C-reactive protein (CRP) levels were determined by using commercial ELISA kits from Cayman Chemical. Adiponectin levels were also assessed by ELISA (SPIBIO, Montigny le Bretonneux, France). Serum complement factor-3 (C3) was quantified with an automated biochemical analyzer (model BS-200) using a specific colorimetric kit (Bioclin).

Trace elements in nails

Nail samples were collected at the time of interview and stored at room temperature in clean polypropylene bags. Fingernail and toenail samples were treated with sub boiling nitric acid in a high-pressure Teflon digestion vessel using a microwave digestion system (Ethos Plus, Millestone, Sorisole, Italy). A Perkin Elmer Analyst 800 atomic absorption spectrometer (Norwalk, CT, USA), equipped with transverse-heated graphite atomizer, Zeeman background corrector and AS-800 autosampler, was used for measuring selenium at 196.0 nm with a spectral band width of 2.0 nm.[18] An electrodeless discharge lamp (Perkin Elmer) was used as a light source operated at 280 mA. Pyrolytic coated graphite tubes with end caps supplied by Perkin Elmer were used. Zinc and copper concentrations in digested acid solutions were analyzed by flame atomic absorption spectrophotometry. Zinc and copper hollow cathode lamps provided resonance lines of 213.9 and 324.8 and were operated both at 15 mA with a slit width seat at 0.7 nm.

RNA extraction and real time quantitative PCR

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). Total RNA (2 μg) was reverse-transcribed using the SYBR® Green RT-PCR Reagents Kit (Cat. 4306736, Life Technologies, Waltham, MA, USA) according to the manufacturer's protocol. cDNA was amplified in triplicate with SYBR® Green PCR Master Mix (Cat. No. 4309155) and the respective specifics primers (see Supplementary Table 1). The analyzed genes were selected on the basis of previous studies supporting their possible involvement in inflammatory pathways related to metabolic syndrome. mRNA levels were normalized to the endogenous control glycerol-3-phosphate dehydrogenase (GAPDH). The ΔΔCt (crossing threshold) method was used for quantification (ABI) and the fold changes reported as $2^{-\Delta\Delta Ct}$.[9] All quantitative real-time RT-PCR measurements were performed using a 7900 HT Fast Real-Time PCR system (Life Technologies).

DNA isolation and methylated DNA standards

DNA was isolated from the WBC using the Master Pure DNA Purification Kit for Blood Version II (Epicenter, Madison, WI, USA) according to the instructions provided by the manufacturer. Purified DNA was stored at −20°C until use. Purified DNA was quantified by Pico-Green dsDNA Quantitation Reagent (Invitrogen). Cells-to-CpG™ Methylated gDNA Control Kit (Life Technologies) and DNA from placenta cells (D3160, Sigma Aldrich, St. Louis, MO, USA) were used as methylated and non-methylated DNA standards, respectively. To generate a range of methylated and unmethylated DNA standards, the 2 standard DNA controls were mixed in 0, 20, 40, 60, 80, and 100% methylated to unmethylated template ratios. One microgram of each standard and sample DNA was bisulfite-converted (BSC) by using the Epitect Fast Bisulfite Conversion Kit (Qiagen, Venlo, Limburg, The Netherlands) according to the manufacturer's instructions, thus converting non-methylated cytosines into uracil. All bisulfite-converted DNAs were diluted to 5 ng/μL for use in PCR.

Methylation-sensitive high resolution melting (MS-HRM) analysis

Specific primers against the completely methylated sense strand sequence were designed according to the recommendations of Wojdacz and Dobrovic (2007)[19] in order to minimize PCR bias. The promoter region of the consensus *LINE-1* sequence (GenBank: X58075) was used to design primer sets with the Primer3 website (http://frodo.wi.mit.edu/primer3/). Thus, the primers for analyzing *LINE-1* methylation were: forward, 5′-GCGAGGTATTGTTTTATTTGGGA-3′; reverse, 5′-CGCCGTTTCTTAAACC-3′. They screened 8 CpGs in an amplicon length of 141 bp and were first employed by Tse et al.[20] The primers for analyzing *IL-6* methylation were: forward, 5′-TTATGTAGGAAAGAGAATTTGGTTTAG-3′ and reverse, 5′-AAAAAATAA AATCATCCATTCTTCAC-3′. They covered 5 CpGs in an amplicon length of 181 bp as mentioned elsewhere.[7] The primers for *TNF-α* were: forward, 5′-TTTTGGAAAGGATATTATGAGTATTGA-3′ and reverse, 5′-CTAAAACCCTA AAA CCCCCCTAT-3′. They covered 4 CpGs in an amplicon length of 99 bp as mentioned elsewhere.[9] Genomic sequence and CpGs sites covered by the MS-HRM primers studying *TNF-α* and *IL-6* methylation are shown in Figs. S1 and S2. PCR amplification of the DNA was carried out using a 7900 HT Fast Real-Time PCR System (Life Technologies) equipped with the SDS Software (Version 2.4.1, Life Technologies). PCR was performed in a 10-μL reaction volume, and 5 ng of bisulfite-converted DNA templates for *LINE-1* assay were added to each well, which contained 1×MeltDoctor™ HRM Master Mix (HRM) (Life Technologies) and 0.2 μM each primer. The cycling protocol conditions included a single enzyme activation step of 10 minutes at 95°C followed by 40 cycles of the following steps: denaturation 95°C, 15 seconds, and annealing 60°C, 1 minute. The MS-HRM step was performed after 40 cycles of amplification and the MS-HRM analysis was initiated by denaturing all products at 95°C for 1 minute, followed by annealing at 55°C for 1 minute. Samples were slowly warmed to 95°C at 0.1°C/second. The High ReSolution Melt Software v2.0 (Life Technologies) was employed for end-product analysis. This algorithm allowed the raw melt curves to be normalized for fluorescence intensity, and a temperature shift was applied to align the normalized melt curves, which facilitated the analysis of samples with varying Ct values. A difference curve was then derived from the first derivative of the melt curves. Data for the difference melt curves were exported to Excel (Office 2007; Microsoft Corp., Redmond, WA) for further analyses. Graphs were plotted and inverted vertically. Both peak-height and area-under-the-curve from the normalized, temperature-shifted, difference curves were used to generate a standard curve and determine the degree of methylation of each DNA sample. All participant DNA samples were analyzed on a 384-well plate, which included a no-template control (NTC) and a set of reference methylation standards. Reference methylation standard curves and experimental samples were tested in triplicate.

Statistical analysis

The Kolmogorov-Smirnov normality test was used to determine variable distribution. Accordingly, the parametric Student *t* test or nonparametric Mann-Whitney *U* test was performed to detect differences between subjects with higher and lower DNA methylation percentage than the median value. Dichotomous variables were analyzed by X^2 test. Contrasts and Tukey's post-hoc tests for one-way ANOVA were performed to analyze differences among tertiles and quartiles. *P* for trend was calculated. The Spearman correlation coefficients were used to screen the statistical associations between DNA methylation and interest variables. Linear regression model, used to identify the predictors of DNA methylation, was adjusted for covariates such as calories, sex, age, smoking status and regular physical activity, when they showed significant effect. Nutrients from the diet were adjusted by total energy intake by using the residual method.[21] Results are presented as mean ± SD (standard deviation). Confidence intervals (95% CIs) were used to describe linear regression coefficients (β). $P < 0.05$ was considered statistically significant.

Statistical analyses were performed by using SAS software system version 8.0 for Windows (SAS Institute Inc., Cary, NC 27513, USA). GraphPad Prism® version 6.0 C (La Jolla, CA, USA) was used to show graphically the results.

Results

Baseline anthropometric, clinical and biochemical measurements from participants are presented in Supplementary Table 2. The study population was considered healthy and some expected differences in anthropometric variables (i.e., body weight, BMI, waist circumference, truncal fat percentage, etc.) were found between genders.

LINE-1 methylation levels were significantly lower in women (about 7.3% less) ($P < 0.01$). In addition, *LINE-1* methylation were positively associated with body weight ($r = 0.296$; $P = 0.032$; $n = 120$). These results were confirmed when the subjects were divided according to the median of body weight (61.0 kg), even when analyses were made separately by gender ($P < 0.05$ for both). More detailed analyses showed that indicators of adiposity, such as skinfolds and total body fat, were lower among individuals with higher *LINE-1* methylation ($P < 0.05$ for all) (Table 1). On the contrary, body fat-free mass was higher (P for trend $= 0.016$) among those with higher *LINE-1* methylation. These individuals also exhibited lower plasma levels of ceruloplasmin (P for trend $= 0.002$) and higher IL-6 in plasma (P for trend $= 0.006$), as shown in Table 1.

Calorie (kcal) intake was higher (P for trend $= 0.04$) among individuals with higher *LINE-1* methylation even after adjusting for body weight (Table 2). A similar result was observed for daily iron intake (mg) even after adjusting by calories and body weight ($P < 0.05$). It is worth emphasizing that iron intake explained, through the r values, about 8.4% of *LINE-1* methylation, even after adjusting by energy, gender and smoking (Fig. 1). In the same way, higher daily riboflavin (B2 vitamin) intake was found in those individuals with bigger values to LINE-1 methylation ($P < 0.05$ inter quartiles). However, those individuals who presented lowers percentages of LINE-1 methylation reported eating higher amounts of copper, niacin (B3 vitamin) and thiamin (B1 vitamin) (P for trend <0.05 for all). Interestingly, the group with higher *LINE-1* methylation (<83.02%) had a lower percentage of current smokers ($P = 0.012$) and more individuals practicing sports ($P < 0.05$) (Table 3). These results are confirmed with the lower number of cigarettes smoked per day ($P = 0.041$) and higher physical activity per day ($P = 0.047$) in those subjects with bigger values to LINE-1 methylation. These results suggest that high *LINE-1* methylation could be associated to a healthier lifestyle.

LINE-1 methylation positively associated with *TNF-α* and *IL-6* methylation percentage not only when linear regression was adjusted by gender, smoking and age ($r = 0.204$; $P = 0.048$ and $r = 0.332$; $P = 0.003$, respectively) (Fig. 2), but also when the subjects were divided according to the median of *LINE-1* methylation (83.02%; $P<0.05$ for both). Results from logistic regression analysis (odds ratio-OR) confirmed that individuals with higher *LINE-1* methylation levels (third tertile, >83.1 %) were more likely to have higher percentage of *IL-6* methylation (3.08 times) than those with lower methylation levels (first tertile, <77.10 %; IC: 1.13−8.36; $P = 0.013$).

Individuals whose *TNF-α* methylation percentage was above the tertile 3 (T3) showed lowers values of waist girth, waist-to-hip ratio, waist-to-stature ratio and selenium in nails ($P < 0.05$) (Table 3). Moreover, they presented lower *TNF-α* expression in WBC (P for trend $= 0.041$), suggesting that the hypermethylation of this gene was associated with inhibition of its transcription (Table 5 and Fig. 3A). It should be noted that the plasma levels of *TNF-α* were also lower in those individuals with greater methylation for this gene (P for trend $= 0.048$). On the other hand, these individuals presented higher expression of *IL-18* and plasma values of IL-6 ($P < 0.05$ for all) (Table 3). As expected, the individuals with higher *TNF-α* methylation showed also higher methylation of *IL-6* and *LINE-1* (Table 5).

No differences were found between the groups whose *IL-6* methylation levels were above and below the median (≤ or >56.57 %). However, when only men were taken into account, the levels of selenium in nails and mRNA levels of *IL-6* and *TNF-α* were lower in those individuals with higher *IL-6* methylation values ($P < 0.05$ for all). On the other hand, a negative association was found between *IL-6* methylation in white blood cells and relative expression (mRNA) of *IL-6* in the same cells (Fig. 3B).

Discussion

There is growing evidence of the involvement of epigenetic mechanisms in disease onset, including obesity and type 2 diabetes.[1] A decrease in global DNA methylation is associated with increased genomic instability and chromosomal rearrangements,[22] a common biological mechanism in several diseases including cancer. Reduced DNA methylation in WBCs may be an indicator of systemic hypomethylation and of cumulative environmental

Table 1. Anthropometric, clinical and biochemical data (mean ± SD) of all individuals (n=120) categorized by quartiles of *LINE-1* methylation (%).

Variables	*Q1*< 77.01%	*Q2* 77.02 to 83.01%	*Q3* 83.02 to 89.05%	*Q4* >89.06%	*P*-for linear trend
Age (y)	23.2 (3.2)	24.5 (4.1)	23.0 (3.8)	22.8 (3.0)	0.280
BMI (kg/m^2)	21.9 (2.0)	22.6 (3.3)	22.0 (2.7)	22.4 (3.1)	0.732
Waist perimeter (cm)	77.7 (8.2)	80.5 (8.9)	78.0 (7.5)	78.9 (9.4)	0.532
Hip perimeter (cm)	98.0 (4.9)	**97.5 (6.5)**[a]	**95.8 (5.6)**[a]	**93.1 (7.5)**	**0.013**
Waist-to-hip ratio	0.79 (0.06)	0.82 (0.06)	0.81 (0.06)	**0.84 (0.06)**[a]	**0.018**
Total body fat - BIA (%)	23.6 (6.0)	22.6 (7.0)[b]	**20.5 (6.7)**[b]	**18.2 (7.8)**	**<0.001**
Body fat mass - BIA(kg)	18.6 (5.8)	16.9 (7.0)	14.6 (4.3)	14.0 (5.2)	0.080
Truncal fat (%)	56.7 (5.7)	59.6 (7.3)	59.0 (7.2)	60.9 (6.4)	0.052
Sum of 4 STs (mm)	68.9 (17.7)	67.6 (24.8)	**60.0 (20.4)**	**56.1 (30.3)**	**0.050**
Body free fat mass - BIA(kg)	43.8 (8.4)	**47.0 (11.7)**	**48.3 (9.9)**	**51.3 (10.1)**	**0.016**
Systolic blood pressure (mmHg)	10.7 (0.8)	11.0 (0.6)	10.9 (1.0)	**11.4 (1.0)**	**0.038**
Diastolic blood pressure (mmHg)	7.3 (0.6)	7.3 (0.5)	7.3 (0.7)	7.5 (0.8)	0.521
Glucose (mg/dL)	91.0 (8.0)	91.8 (7.0)	90.7 (6.8)	90.5 (6.9)	0.362
Insulin (μU/mL)	15.8 (2.8)	17.2 (4.7)	16.2 (3.9)	13.4 (2.4)	0.423
HOMA-IR	3.63 (0.67)	4.06 (1.09)	3.87 (1.09)	3.14 (0.48)	0.215
Total cholesterol (mg/dL)	162.4 (29.6)	162.7 (21.6)	158.7 (35.8)	151.1 (32.8)	0.426
HDL-c (mg/dL)	48.8 (7.6)	45.6 (11.2)	43.7 (10.1)	42.4 (10.3)	0.087
LDL-c (mg/dL)	91.8 (23.5)	91.7 (18.0)	96.2 (30.1)	91.6 (26.5)	0.890
VLDL-c (mg/dL)	20.5 (7.8)	20.7 (9.1)	20.1 (7.3)	17.0 (5.8)	0.218
Triacylglycerol (mg/dL)	102.7 (39.4)	108.6 (52.3)	100.9 (36.5)	85.4 (29.1)	0.063
Total cholesterol-to-HDL-c ratio	3.31 (0.54)	3.60 (0.80)	3.74 (0.86)	3.56 (0.69)	0.971
CRP-hs	2.00 (2.21)	1.81 (1.58)	1.74 (2.64)	0.99 (1.37)	0.224
IL-6 (pg/mL)	15.8 (14.8)	21.6 (22.5)	**19.8 (15.5)**	**35.5 (21.0)**	**0.006**
TNF-α (pg/mL)	4.13 (1.96)	4.45 (1.58)	4.21 (2.01)	5.05 (2.75)	0.331
C3 complement	110.3 (24.6)	110.8 (25.3)	114.3 (25.6)	110.3 (21.0)	0.904
Ceruloplasmin (mg/dL)	40.37 (8.40)	36.87 (7.91)	**36.50 (7.82)**	**32.51 (5.98)**	**0.002**
Adiponectin	32.7 (10.3)	32.4 (22.9)	22.1 (9.0)	23.1 (8.8)	0.294
GPx activity (nmol/[ml/min])	576 (310)	540 (267)	542 (279)	643 (251)	0.580
Total antioxidant capacity (mM)	1.50 (0.80)	1.76 (0.94)	1.46 (0.72)	1.89 (1.07)	0.196
Ox-LDL (U/L)	62.7 (28.0)	67.7 (22.1)	80.7 (31.8)	76.2 (32.4)	0.081
Selenium (ng/g of nail)	0.41 (0.07)	0.40 (0.08)	0.39 (0.09)	0.35 (0.06)	0.109
Copper (ng/g of nail)	6.97 (4.10)	6.42 (2.90)	7.39 (5.70)	7.04 (4.50)	0.894
Zinc (ng/g of nail)	124.8 (25.0)	114.8 (16.8)	146.0 (99.0)	128.4 (71.7)	0.376

BIA: Electrical bioimpedance; HOMA-IR: Homeostatic model assessment; CRP-hs: High-sensitivity C-reactive protein; GPx: Glutathione peroxidase; Ox-LDL: Oxidized low-density lipoprotein; ST: Skinfold thickness. One-way ANOVA test with linear contrast analysis were performed.
[a]different from Q1.
[b]different from all quartiles.

impacts. Conversely, an increase in DNA methylation levels of repetitive elements may have a protective effect against genomic instability and unwanted chromosomal rearrangements.[23] However, there are many gaps about the determinants of DNA methylation levels in healthy people prior to disease onset, and there is great interest in finding new, early biomarkers for disease risk reduction and health promotion.

Our findings support previous results showing that women have significantly lower levels of *LINE-1* methylation.[24-26] Lower levels of global methylation in women may be due to different levels of dietary folate or other

Table 2. Daily nutrient intake (mean ± SD) for all individuals categorized according to the quartiles of *LINE-1* methylation (%) (n=115).

Variables	*Q1* < 77.01%	*Q2* 77.02 to 83.01%	*Q3* 83.02 to 89.05%	*Q4* >89.06%	*P*-for linear trend
Energy (kcal/BW)	39.26 (9.09)	38.52 (8.80)	**44.62 (9.81)**[a,b]	**47.69 (13.28)**[a,b]	**0.004**
Carbohydrate*	339.7 (54.3)	344.1 (36.8)	350.0 (55.2)	348.1 (46.6)	0.880
Protein (g)*	106.7 (21.6)	98.6 (14.9)	103.3 (19.6)	102.5 (17.3)	0.671
Lipid (g)*	99.2 (15.6)	94.9 (12.3)	100.1 (20.8)	96.0 (14.9)	0.492
Alcohol (g)*	59.67 (86.27)	44.32 (77.34)	**5.34 (40.32)**[a,b]	**1.29 (53.01)**[a,b]	**0.050**
Iron (mg)*	64.32 (13.27)	64.47 (9.84)	**74.31 (19.69)**[a,b]	**83.88 (25.03)**[a,b]	**0.022**
Cupper (mg)*	4.47 (5.53)	3.90 (5.73)	**0.93 (5.84)**[a,b]	**0.23 (7.64)**[a,b]	**0.001**
Niacin (mg)*	32.50 (27.12)	32.19 (26.54)	**14.70 (27.47)**[a,b]	**4.08 (28.04)**[a,b]	**0.001**
Riboflavin (mg)*	1.36 (0.57)	**1.50 (0.51)**[a]	**1.56 (0.52)**[a]	**1.80 (0.72)**[a,b]	**0.050**
Thiamin (mg)*	4.95 (5.50)	**4.61 (5.52)**[a,b]	**1.29 (5.14)**[a,b]	**0.46 (6.45)**[a,b]	**<0.001**

BW: body weight.
One-way ANOVA test with linear contrast analysis were performed.
[a]different from Q1.
[b]different from all quartiles.
*Arbitrary values after adjustment for the calories using the residual method.[21]

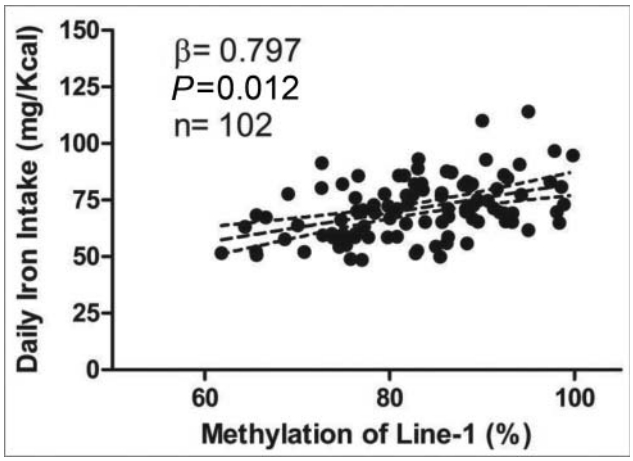

Figure 1. Linear regression model showing association between daily iron intake (refined by calories - residue method)[21] and *LINE-1* methylation (%) after adjustment for smoking and gender. The dotted lines represent the confidence interval limit (95% CI).

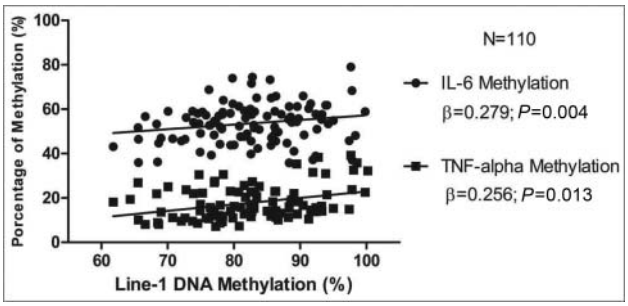

Figure 2. Linear regression model (r) showing association among *LINE-1* methylation (in %) and *IL-6* (r=0.204; p=0.048) and *TNF-α* (r=0.332; p=0.003) methylation percentage after adjusting by gender and smoking.

one-carbon nutrients in men and women.[27] Women may also have a higher folate requirement than men because of regular loss of red blood cells through menstruation, but it can not explain the gender-specific difference in global methylation in postmenopausal women.[27,28] There is still discussion about hormonal factors and the importance of DNA methylation for X-chromosome inactivation in women.[24,29] Further studies are needed to decipher the relationship between gender and *LINE-1* methylation.

The results from this study are also in line with other studies that reported association between *LINE-1* methylation and smoking status.[25] Cigarette smoke is considered one of the most powerful environmental modifiers of DNA methylation.[30,31] For example, an experimental study showed that cigarette smoke could induce DNA demethylation in repeat elements such as *LINE-1*.[32] The specific mechanisms of how cigarette smoke may alter DNA methylation are becoming better understood and may be reviewed elsewhere.[33] Our findings suggest that the toxic effects of tobacco could be at least partly mediated by modulation of the epigenetic landscape. On the other hand, higher *LINE-1* methylation has been previously described in individuals with higher exercise levels,[34,35] suggesting that an increase in *LINE-1* methylation might be associated with healthy lifestyle habits. As epigenetic marks are potentially reversible, this result may have public health implications and merits further investigation.

Methylation of DNA is a biochemical process in which a methyl group is added to DNA nucleotides. Several nutrients act as key enzyme cofactors and play essential roles in methyl group metabolism and DNA methylation in particular, being riboflavin, vitamin B12 and folate the major determinants of one-carbon metabolism.[36] In this sense, our results confirm the importance of riboflavin in DNA methylation process.

Iron intake was positively associated with *LINE-1* methylation level even after adjustment for gender, calories and body weight. A study with 892 individuals also showed a similar association between iron intake and *LINE-1* methylation in leukocytes.[25] Iron, together with 2-oxoglutarate and oxygen, is an essential cofactor for the 10–11 translocation (TET) family of proteins that hydroxylates 5-methylcytosine to 5-hydroxymethylcytosine and further oxidizes to 5-carboxylcytosine and 5-formylcytosine, which have all been suggested to be precursors for both active and passive DNA demethylation.[37] The mechanisms are not well understood, but these data reinforce the role of dietary iron on the methylation status.

A recent trial explored the associations between changes in lifestyle modifications, such as diet, and global epigenetic biomarkers in blood of overweight female breast cancer survivors. After a weight loss intervention consisting on dietary and physical activity changes, *LINE-1* methylation levels were significantly elevated as compared to baseline.[38] Particularly, a 10 % increase in the frequency of fruit

Table 3. Lifestyle features (mean ± SD) for all individuals categorized according to the median of *LINE-1* methylation (%).

Lifestyle features	*LINE-1*≤83.03% (n=54)	*LINE-1*> 83.03% (n=53)	*P* Value
Vitamin supplementation users (%)[a]	6.66	5.10	0.569
Current smokers (%)[a]	20.75	5.50	**0.012**
Smoking (cigarettes/day)[b]	3.6 ± 4.8	0.9 ± 1.7	**0.041**
Regular practice of sport (%)[a]	61.96	79.62	0.050
MET (h/day)[b]	30.3 ± 9.0	39.2 ± 10.0	**0.047**

MET, activity metabolic equivalent;
[a]Chi-Square Test for dichotomous variables was performed.
[b]Not normal distribution. Analyzed by Mann-Whitney U test.

Table 4. Anthropometric, clinical and biochemical data (mean ± SD) of all individuals (n=107) categorized by tertiles of *TNF-α* methylation (%).

Variables	T1 <13.41%	T2 13.41 to 20.45 %	T3 >20.45%	*P*-for linear trend
Age (y)	22.8 (3.5)	23.9 (3.6)	23.5 (3.0)	0.845
BMI (kg/m^2)	21.8 (2.4)	22.2 (2.7)	21.8 (2.9)	0.806
Waist perimeter (cm)	79.7 (7.7)	79.5 (8.3)	**74.9 (7.9)**[a,b]	**0.012**
Hip perimeter (cm)	95.2 (6.0)	95.7 (6.6)	94.5 (7.1)	0.344
Waist-to-hip ratio	0.84 (0.06)	0.83 (0.05)	**0.79 (0.05)**[a,b]	**0.007**
Waist-to-stature ratio	0.47 (0.04)	0.46 (0.04)	**0.44 (0.05)**[a]	**0.034**
Total body fat - BIA (%)[a]	23.5 (6.17)	23.8 (6.16)	24.0 (6.39)	0.578
Body fat mass - BIA(kg)	14.3 (4.2)	15.0 (4.3)	15.0 (5.8)	0.716
Truncal fat (%)	57.1 (6.1)	59.1 (7.0)	58.2 (6.6)	0.966
Sum of 4 STs (mm)[a]	59.3 (20.4)	63.3 (24.8)	60.4 (25.2)	0.567
Body free fat mass - BIA(kg)	47.5 (10.1)	48.2 (10.6)	45.7 (10.0)	0.772
Systolic blood pressure (mmHg)	10.9 (0.91)	10.7 (0.93)	11.1 (0.89)	0.346
Diastolic blood pressure (mmHg)	7.2 (0.67)	7.3 (0.65)	7.4 (0.66)	0.953
Glucose (mg/dL)[a]	90.6 (7.0)	90.5 (6.7)	91.0 (6.2)	0.709
Insulin (μU/mL)	16.0 (3.9)	14.7 (2.6)	15.5 (4.1)	0.621
HOMA-IR [a]	3.71 (1.05)	3.43 (0.62)	3.62 (0.88)	0.845
Total cholesterol (mg/dL)	165.6 (31.9)	159.9 (24.5)	152.7 (29.6)	0.673
HDL-c (mg/dL)[a]	47.8 (10.6)	44.7 (10.0)	45.9 (10.16)	0.667
LDL-c (mg/dL)[a]	97.8 (24.8)	94.3 (22.1)	**85.8 (19.9)**[a]	**0.050**
VLDL-c (mg/dL)	20.3 (8.5)	20.1 (7.1)	19.6 (8.3)	0.498
Triacylglycerol (mg/dL)	101.7 (42.6)	100.5 (35.8)	98.3 (41.8)	0.671
Total cholesterol-to-HDL-c ratio[a]	3.51 (0.53)	3.62 (0.88)	3.34 (0.65)	0.217
CRP-hs	2.02 (2.67)	1.20 (1.39)	1.30 (1.10)	0.186
IL-6 (pg/mL)	16.39 (14.14)	15.86 (14.17)	**28.5 (22.4)**[a,b]	**0.002**
TNF-α (pg/mL)[a]	5.97 (2.10)	4.60 (1.79)	**4.09 (2.43)**[a]	**0.048**
C3 complement[a]	111.6 (28.5)	113.6 (20.5)	112.2 (22.3)	0.416
Ceruloplasmin (mg/dL)[a]	37.7 (8.2)	35.7 (6.8)	37.3 (9.2)	0.927
Adiponectin	28.5 (11.9)	31.5 (21.8)	32.1 (16.2)	0.845
GPx activity (nmol/[ml/min])[a]	492 (213)	560 (318)	636 (269)	0.667
Total antioxidant capacity (mM)[a]	1.58 (0.79)	1.64 (0.73)	1.75 (0.99)	0.671
Ox-LDL (U/L)[a]	66.26 (27.56)	69.70 (35.45)	73.92 (21.37)	0.498
Selenium (ng/g of nail)	0.40 (0.08)	0.41 (0.09)	**0.36 (0.06)**[a]	**0.017**
Copper (ng/g of nail)	8.02 (6.00)	6.69 (3.69)	6.20 (4.14)	0.416
Zinc (ng/g of nail)	118.8 (25.2)	133.9 (77.1)	122.0 (25.5)	0.268

BIA: Electrical bioimpedance; HOMA-IR: Homeostatic model assessment; CRP-hs: high-sensitivity C-reactive protein; GPx: Glutathione peroxidase; Ox-LDL: Oxidized low-density lipoprotein; ST: Skinfold thickness. One-way ANOVA test with linear contrast analysis were performed.
[a]different from T1.
[b]different from all tertiles.

consumption was associated with an increase in *LINE-1* methylation levels of 0.42 %. In a study with 177 young healthy women, those whose consumption of fruit was below the median value (<201 g/day) were 3.7 times more likely to display *LINE-1* hypomethylation than women whose consumption was above the median (OR 3.7; 95% CI 1.4–9.5).[39]

Several studies have examined the association between BMI and *LINE-1* methylation levels with conflicting results.[40] Elevated BMI has been shown to be associated with lower *LINE-1* methylation in some investigations,[41,42] but, in other study, participants with BMI ≥40 kg/m^2 had higher *LINE-1* methylation levels than those with BMI ≤25.[43] On the other hand,

Table 5. Relative expression and percentage of methylation data (mean ± SD) for all participants (n=105) categorized by median of *TNF-α* methylation (%).

Variables	T1 <13.41%	T2 13.41 to 20.45 %	T3 >20.45%	*P*-for linear trend
Relative expression				
IL-6[*]	1.09 (0.67)	1.01 (0.65)	0.98 (0.59)	0.731
IL-18[*]	1.42 (0.60)	**1.95 (0.86)**[a]	**1.97 (0.98)**[a,b]	**0.005**
ICAM-1[*]	0.66 (0.34)	0.71 (0.30)	0.76 (0.38)	0.483
RIL-1[*]	0.61 (0.35)	0.70 (0.36)	0.59 (0.37)	0.381
TNF-α[*]	1.18 (0.44)	1.10 (0.46)	**0.93 (0.39)**[a]	**0.041**
Percentage of methylation				
IL-6 (%)	51.19 (7.44)	54.31 (10.07)	**57.69 (10.78)**[a]	**0.015**
LINE-1 (%)	81.07 (7.81)	84.61 (9.02)	**86.05 (8.59)**[a]	0.053

[*]AU: arbitrary units. One-way ANOVA test with linear contrast analysis were performed.
[a]different from T1.
[b]different from all tertiles.

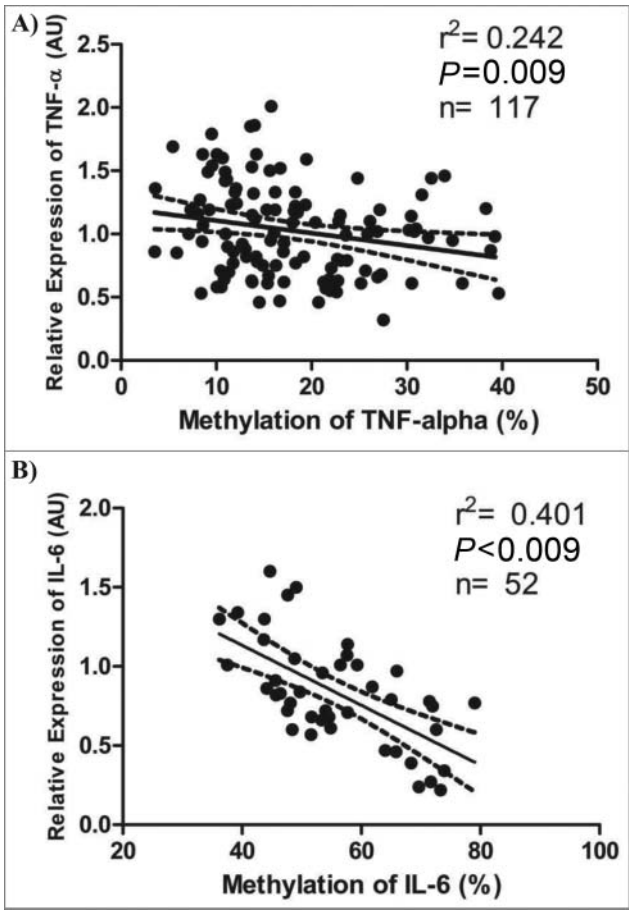

Figure 3. Associations between relative expression and methylation levels of *TNF-α* and *IL-6*. A) Pearson's correlation (r^2) between relative expression in arbitrary units (AU) and methylation levels (%) of *TNF-α*. B) Pearson's correlation between relative expression (AU) and methylation levels (%) of *IL-6* (only in men). The dotted lines represent the confidence rating limit (IC-95%).

some studies found no associations between BMI and *LINE-1* methylation.[44,45] Although the current study did not find an association between BMI and *LINE-1* status, body weight and *LINE-1* were positively associated. A recent report from our group has shown that *LINE-1* methylation was a biomarker of weight loss in obese subjects.[6] In a recent randomized, crossover study of a 6-month weight loss intervention, over a 12-month period, changes in body fat percentage were positively associated with *LINE-1* methylation (β=0.19, p=0.001).[38] In that study, *LINE-1* methylation was statistically significantly elevated at 6 and 12 months compared to baseline. Moreover, analysis of rectal biopsies free of colorectal disease obtained from 185 individuals showed that higher waist and hip perimeters were significantly associated with lower methylation of *LINE-1*.[26] Interestingly the present study found that central obesity and fat mass percent were significant predictors for low *LINE-1* methylation levels, although no associations remained after adjusting for gender and smoking.

In the present research, *TNF-α* methylation analysis demonstrates a negative association with central adiposity. In this sense, a cross-sectional study with 40 normal-weight young women showed that those with higher truncal fat (≥52.3%) presented lower methylation of the *TNF-α* gene promoter than those with lower truncal adiposity.[9] In two other studies, authors concluded that *TNF-α* methylation levels could be used as epigenetic biomarker concerning the response to a low-calorie diet.[8,46] Indeed, methylation profile could help predict susceptibility to weight loss as well as some obesity-related comorbidities, such as hypertension or type 2 diabetes.[47] These results are in line with the findings from the present work, suggesting an important relationship between epigenetic mechanisms and body composition.

Recent studies also reported a good correlation between leukocyte DNA methylation level and degree of inflammation, suggesting that cytosine methylation may represent a novel mechanism underlying the association between obesity, inflammation and disease risk.[48,49] In this context, our study observed that the methylation levels of both, *TNF-α* and *IL-6*, negatively correlated with the mRNA levels of both cytokines in white blood cells. However, it is important to note that a relationship between *IL-6* mRNA and plasma levels was not found, which can be explained by this cytokine not only being released to plasma by white blood cells, but also by muscle, adipose tissue and other organs. It is important to highlight that the present study was conducted in young, apparently healthy individuals, with no physiopathological conditions. Thus, it is possible to consider this negative association as a compensatory mechanism, with the purpose of restoring homeostatic balance.

This study presents some limitations that should be considered. Firstly, the dietary assessment software did not provide important information in order to discuss the data deeply. We only examined consumption of specific nutrients, which does not account for the combinations and interactions of multiple nutrients in the human diet. Moreover, random measurement error in ascertaining dietary intake with the food frequency questionnaire could have resulted in some misclassification of intake. Secondly, some individuals were not included in the present analyses due to lack of methylation data. However, there was no relevant difference in selected characteristics (except age and gender) between those with and without those data. To minimize the potential bias, all the models were adjusted for age, gender and smoking status when necessary. Thirdly, we did not account for the proportion of white blood cell subtypes from the buffy coat in the analyses. DNA methylation is tissue- and cell-specific, and it could depend on the cell type distribution. In this sense, there is some evidence that DNA

methylation is inversely related to the proportion of lymphocytes.[44] Finally, the cross-sectional design of this research does not allow determination of causality and the associations observed should be checked carefully. Therefore, type I/II errors cannot be ruled out, nor can the involvement of other mechanisms in the regulation of methylation status. Future studies are required to replicate and extend these findings to different populations.

As a conclusion, our findings contribute to the growing evidence that the health impacts of changes in dietary habits and other lifestyle factors may be mediated through epigenetic modifications. Moreover, our work provides preliminary evidence for the use of WBC DNA methylation biomarkers to monitor lifestyle interventions trials and identify early biomarkers of metabolic complications.

Conclusions

This study suggests that higher *LINE-1* and *TNF-α* methylation could be associated with some indicators of adiposity, especially body fat, waist girth and waist-to-hip ratio. In addition, high *LINE-1* methylation levels are directly associated with some dietary factors (calories, iron) but inversely associated with total fat mass. Moreover, high *LINE-1* methylation is associated with a healthier lifestyle (physical activity and not smoking) and might be an early indicator of resistance to win adiposity even when eating more food.

Authors' Contributions

Contributors JLM-R: field work, data collection, analysis, and writing of the manuscript; FIM and MLM: design, analysis, and editing of the manuscript; DMM: design, field work, and data collection; JB: project leader, scientific interpretation, financial management, and editing of the manuscript; JAM: design, field work, scientific interpretation, financial management, and editing of the manuscript. All authors read and approved the final manuscript.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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