DNA methylation of leptin before and after weight loss intervention program among sample of Egyptian obese females: Epigenetic approach in obesity

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Background/aim
Obesity phenotype results from interactions between epigenotype and genotype. DNA methylation constitutes the most relevant epigenetic mechanism that regulates gene expression in human cells. Leptin (LEP) has a promoter region that presents CpG (CG dinucleotide repeats) islands which may undergo a methylation process. The DNA methylation process could be influenced by nutrition and alter adipocyte LEP gene expression. This study aimed to investigate whether the LEP gene promoter could be epigenetically modified by dietary weight reduction intervention and whether these changes affect its circulating level which may be involved in regulating the expression of the LEP gene in obesity.
Patient and methods
A total of 82 obese females were prescribed 3 months weight loss program. Fifty-eight women with a mean age of 41.62±10.70 years and a mean baseline BMI of 38.32+ 4.01 kg/m^2 completed this longitudinal follow-up intervention study. Epigenetic mark, baseline and endpoint anthropometric, and laboratory parameters were assessed. Therefore, the methylation of the promoters of the LEP gene, using methylation-specific PCR, and measuring LEP levels before and after the dietary intervention program, and after bisulfite modification were investigated.
Results
After successful weight loss in obese females, Leptin receptor (*LEPR*) promoter methylation patterns had significantly higher values, and serum LEP levels had significantly lower values compared with before the dietary intervention program ($P<0.05$). Among the obese females with BMI equal to or more than 40 kg/m^{2}the most increased variable was LEP promoter methylation (↑10.2%), while among the obese females with BMI less than 40 kg/m^2, it increased by ↑1.7%. Among both groups of obesity, the most decreased variable was LEP (↓94.7% and ↓89.4, respectively).
Conclusion
These results support the assumption that in obesity, there is a link between *LEPR* methylation and LEP blood levels. The epigenetic profile of LEP gene could be affected by serum LEP via feedback regulation.

Keywords:
diet intervention, leptin levels, leptin promoter methylation, obesity

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Introduction

The increased obesity rates in recent decades could not be only explained by genetics. Environmental changes as lifestyle and nutrition may have a role in the observed tendency. These changes can alter gene expression while not affecting the DNA sequence, a phenomenon known as *Epigenetics* [1]. Actually, the specific phenotype is the consequence of complicated interactions between genotype, epigenotype, and environment. A link between obesity, and improper epigenetic programming has been suggested [2]. *Conrad Waddington* was the first to introduce the *epigenetics* concept in 1942, referring to chemical modifications that modulate how the body reads the DNA [3].

DNA methylation constitutes the most relevant epigenetic mechanism that regulates gene expression in human cells. DNA methylation is the most relevant epigenetic change that has been studied mainly because it is chemically highly stable and hence, measurable in a range of archiving cells and tissues. DNA methylation is distributed within the genome, with certain alterations of the methylation pattern according to the nature and function of the cell. The principal target of methylation in humans is cytosines in the CG dinucleotide, called CpG sites. CpG (CG dinucleotide repeats) islands are regions of high-density CpG sites that are present in gene-promoter regions or nearby. These are remarkable features of vertebrate DNA methylation. Nearly 29 000 islands of CpG in the human genome are already identified [4,5]. The 5 –methyl cytosine is the pre-dominant base undergoing modification regarding this mechanism, most often when present in the dinucleotide sequence 5mCpG. It blocks the binding of transcription factors to their target sites; thus inhibiting transcription [6].

DNA methylation which regulates gene expression in human cells is affected by environmental factors, such as nutrition; that can have profound effects on the acquired genetic changes in the expression of specific genes such as Leptin (LEP) [7]. Accordingly, DNA patterns of methylation, environmentally modified (by factors such as nutrition), are assumed to contribute to the pathophysiology of the current obesity pandemic [8].

Concerning LEP, a 16-kDa hormone that is synthesized and then secreted from specialized white adipocytes, its concentration affects weight adjustment and eating behavior, and it is also a key modulator of a variety of functions. Therefore, LEP expression regulation is of utmost importance for the fine-tuning of vital functions. The mechanisms by which LEP expression is switched on and off are still to be investigated [9]. Some studies suggested a constraining effect on gene expression exerted by DNA methylation in most cases and displayed the altered expression of this adipokine (LEP) in obese subjects [10,11].

Dietary factors are presumed to affect epigenetic events, contributing to altering the expression of the LEP gene and hence modifying obesity risk; in this context, the CpG islands seem to be methylated differentially and specifically in different tissues, and in humans, the process of methylation is thought of as 'dynamic'. However, no direct dietary effects on methylation of LEP promoter have been well clarified until now. That is why an investigation of LEP promoter ability to be epigenetically modified by dietary intervention was carried out by the present study that also tried to find out if these modifications, could relate to changes in the LEP circulating levels; being a possible mechanism for regulation of gene expression of LEP in obesity.

Patient and methods

Patients

This cross-sectional study included 82 obese Egyptian females with ages between 25 and 60 years. Only 58 women (with a mean age of 41.62±10.70 years) completed this follow-up interventional study.

Study design

Being originally obese with a mean baseline BMI of 38.32± 4.01 kg/m^2, the participants followed a hypocaloric high-fiber regimen and physical exercise, and they received a daily probiotic supplement for 3 months as well. For every participating female, anthropometry, laboratory investigations, analysis of microbiota, and percent of DNA methylation of LEP promoter were done before and after 3 months of weight loss program {hypo caloric high fiber regimen (as prebiotic), physical exercise and probiotic supplement intervention}.

Inclusion and exclusion criteria

The main inclusion criteria were preintervention grades of obesity (BMI $\geq$30.0 kg/m^2). Participants with a history of previous bariatric surgery, current substance abuse, intellectual disability, and any neurological disorder, disorders that may affect gut microbiota (metabolic, gastrointestinal, and autoimmune diseases as well as medications, especially recent antibiotic use) were all excluded.

Ethical approval

The present protocol of this study was conformed to the 1975 Declaration of Helsinki ethical guidelines, and approved by the Ethics Committee of National Research Centre, Egypt, with approval number 19/236. A written informed consent was obtained from each participant before their inclusion in the study and after being informed about the purpose of the study.

Methods

Anthropometric measurements

Body weight and height as well as waist circumference were measured, according to recommendations of the 'International Biological Program' [12]. Body weight

(Wt.) to the nearest 0.01 kg was measured using a Seca scale balance, with the female barefoot and with as minimal clothes as possible. Body height (Ht) to the nearest 0.1 cm was measured using Holtain Anthropometer. At the midpoint between the lower curvature of the 10th rib (last fixed rib) and the superior border of the iliac crest, Waist circumference (WC) was measured, while the participant was in an upright position and her arms were alongside the body, with close feet, and relaxed abdomen. BMI was calculated as follows: [BMI = weight (in kilograms) divided by the square meter of height]. The participating subjects were all chosen as obese (their BMI $\geq 30\,g/m^2$). They were classified into two groups: those with BMI less than $40\,kg/m^2$, and others with BMI equal or more than $40\,kg/m^2$.

Laboratory investigations and blood sampling

Venous blood samples were drawn from the participating females in the morning. Laboratory tests were applied on stored sera (at −70°C until used) to assess Short Chain Fatty Acids (SCFA, DNA methylation of LEP promoter, and serum LEP level. All were held in the laboratory of Medical Excellence Research Center MERC; in National Research Centre NRC, Egypt.

SCFA in serum were assessed using (ELISA) kits (Enzyme-Linked Immunosorbent Assay); Catalog Number: MBS7269061 according to the method of den Besten *et al.* [13]. The human LEP assay was performed on serum by the ELISA method, using BioLegend Inc. kits (San Diego, California, USA), following the method of Considine *et al.* [14].

Microbiota analysis

To determine the effects of the program of weight loss including the eating plan, and the probiotic supplements on gut microbiota of the participants, stool samples were taken before and after the intervention, with analysis of gene sequence being done, together with comparing gut microbiota for individual variations. The proportion of Bifidobacteria and Lactobacillus strains; as well as the ratio between Firmicutes and Bacteroidetes (F/B ratio); in the stool of all women were assessed using the real-time Polymerase Chain Reaction (PCR).

Stool samples

The stool samples was collected and frozen by defecation in a clean container. stool samples were frozen at −20 °. The probes and primers used to detect Lactobacillus spp; Bifidobacterium spp., Bacteroidetes spp., and Firmicutes spp. were based on gene sequences of 16 S rRNA obtained via the *Entrez program* from the National Center for Biotechnology Information databases [15].

DNA extraction

Extraction of DNA was done according to the manufacturer's instructions of the Minikit of QIAamp DNA Stool (Qiagen) for extracting DNA from fresh or frozen stool samples (one gram of stool). Quantification of bacteria by real-time polymerase chain reaction (PCR) was done.

DNA methylation of leptin promoter

DNA extraction

(1) A 5 ml peripheral blood samples with EDTA were withdrawn and used for Genomic DNA extraction using Zymo DNA Mini Kit (Zymo Research, CA, USA).
(2) An ND-1000 spectrophotometer (NanoDrop Technologies) was used to determine the quantity and quality of purified DNA.

Bisulfite modification

Based on the principle that bisulfite can convert unmethylated cytosine residues into uracil, while methylated cytosine residues stay unaffected, modification of bisulfite was performed. After bisulfite conversion, unmethylated and methylated cytosines could be determined by MSP (methylation-specific PCR).

Bisulfite treatment of DNA was done using a Zymo methylation Gold kit (Zymo Research, CA, USA) according to instructions of the manufacturer.

Methylation specific qPCR

The MSP was assessed as nested PCR with 2 ug of bisulfite-treated DNA in the PCR first round. The bisulfite-treated DNA 1 μL was used for two separate nested PCR with 10 μM of each primer specific for unmethylated and methylated sequences.

Amplification, detection, and after-amplification was performed according to the protocol of the Light Cycler R 480 real-time PCR.

Intervention phase

All the recruited women were provided with the plan of weight loss program, including diet, probiotic supplement, and physical exercise and for three months:

(a) A diet plan was followed; with the guidance of a nutrition consultant; by using different regimens

aimed at correcting the wrong eating habits and to supplying patients with deficient nutrients (hypo caloric high fiber regimen). Dietary therapy was done in the form of different dietary regimens providing the required nutrients and was followed up to assess the impact of a dietary behavioral modification on reaching the ideal weight for age and sex, with nutritional education and behavior modification being done.

(b) Performing an aerobic exercise with a healthy diet after initial assessment, designing the program (with special programs according to age group), patient education, and everyday classes of fitness (provided by video).

(c) Probiotic supplementation as prescribed, 100 gm of an oral dietary supplement (Yogurt containing probiotic strains [10^8/g]), once per day for three months. Strains of probiotics were obtained from a probiotic capsule 'GNC ultra probiotic complex 100' containing a mix of probiotic strains; isolated by the National Research Centre (NRC) probiotics lab.

Statistical analysis

Data analysis was done using the Statistical Package for Social Sciences (SPSS/Windows Version 18, SPSS Inc., Chicago, IL, USA). Data normality was tested by the Kolmogorov–Smirnov test. Data were normally distributed; so, parametric tests were used. The parametric data results were expressed as mean ± SD. Analysis of the different parametric variables of the pre and post-intervention was done as well as comparing those using paired t-tests. Using the EXCL program, the percentage of changes in the various variables between the pre and post-intervention phases were calculated and drawn. The pre and post intervention values for each case were subtracted as (pre – post), then the resulted values were divided by the pre-value and multiplied by 100. Significant differences were considered at P value < 0.05.

Results

After 3 months of following the weight loss intervention program; both obese female groups under study (BMI$<$40 kg/m^2,first group and BMI $\geq$ 40 kg/m^{2}second group) recorded highly significant decreased serum levels of LEP (P=0.000) accompanied by increased DNA methylation% of LEP promoter (P=0.000 and 0.006, respectively). Concerning the anthropometric parameters, body weight and BMI had significantly recorded decreased values for the first group and highly significant statistical decreases for the second group but an insignificant difference in WC was found in the two groups under study. Serum levels of SCFA were significantly reduced (P=0.027 and P=0.000, respectively) in both groups after intervention. They had also highly significantly higher fecal values of Lactobacillus, Bifidobacterium, and Bacteroidetes. While fecal values of Firmicutes and Firmicutes/Bacteroidetes Ratio were significantly lowered in the first group (P=0.020 and 0.012), they were highly significantly increased (P=0.000) in the second group (Tables 1 and 2).

Figure 1 showed the percentage changes of serum LEP and the LEP promoter methylation between pre and post follow-up intervention. Serum LEP showed a highly significant marked decrease after the

Table 1 Comparisons between pre and post follow-up among obese females with BMI less than 40 kg/m^2

	Females with BMI less than 40 kg/m^2(N=39)		
Variables	Pre Mean±SD	Post Mean±SD	P value
Anthropometry:			
Weight (kg)	84.14±11.81	83.25±10.29	0.030*
BMI (kg/m^2)	36.21±2.99	35.85±2.33	0.039*
WC (cm)	105.32±5.09	105.38±11.86	0.967
Lab			
SCFA (μmol/L)	11.31±12.04	6.86±2.16	0.027*
Leptin (ng/mL)	18064.10±7849.56	1918.97±1297.72	0.000**
Leptin methylation %	0.41±0.25	2.13±1.69	0.000**
Microbiota:			
Log Lactobacillus	5.60±0.58	6.07±0.91	0.000**
Log Bifidobacterium	6.33±0.63	6.51±0.60	0.000**
Log Bacteroidetes	13.26±1.41	13.36±1.47	0.000**
Log firmicutes	9.02±1.08	8.65±1.11	0.020*
Firmicutes/bacteroid ratio	0.69±0.13	0.66±0.15	0.012*

BMI, body mass index; SCFA, short chain fatty acids; WC, waist circumference. *: Significant difference at P less than 0.05. **: Highly significant at P less than 0.01, using paired t-tests.

Table 2 Comparisons between pre and post follow-up among obese females with BMI greater than or equal to 40 Kg/m^2

	Females with BMI equal or more than 40 kg/m^2(*N*=19)		
Variables	Pre Mean±SD	Post Mean±SD	*P* value
Anthropometry:			
Weight (kg)	105.41±10.01	102.45±10.64	0.000**
BMI (kg/m^2)	42.65±1.63	41.43±1.75	0.000**
WC (cm)	128.38±1.56	124.38±5.19	0.054
Lab.:			
SCFA (μmol/L)	29.95±18.43	6.55±1.10	0.000**
Leptin (ng/ml)	19968.42±5389.63	894.74±255.98	0.000**
Leptin methylation %	0.28±0.15	10.44±14.39	0.006**
Microbiota			
Log Lactobasillus	6.02±0.54	6.55±0.79	0.000**
Log Bifidobacterium	6.75±0.78	7.19±0.55	0.001**
Log Bacteroidetes	13.42±0.43	13.49±0.45	0.000**
Log Firmicutes	7.41±0.10	7.60±0.08	0.000**
Firmicutes/bacteroid ratio	0.55±0.03	0.56±0.03	0.000**

BMI, body mass index; SCFA, short chain fatty acids; WC, waist circumference. **: Highly significant at *P* less than 0.01, using paired *t*-tests.

intervention in both groups (↓89.4% among obese <40kg/m^2 and ↓94.7% among obese > 40Kg/m^2). The LEP promoter methylation showed a highly significant increase in both groups (↑1.72% and ↑10.16%, respectively). It was evident that the increase in LEP promoter methylation and the decrease in serum LEP were more obvious among obese females with BMI greater than 40 kg/m^2.

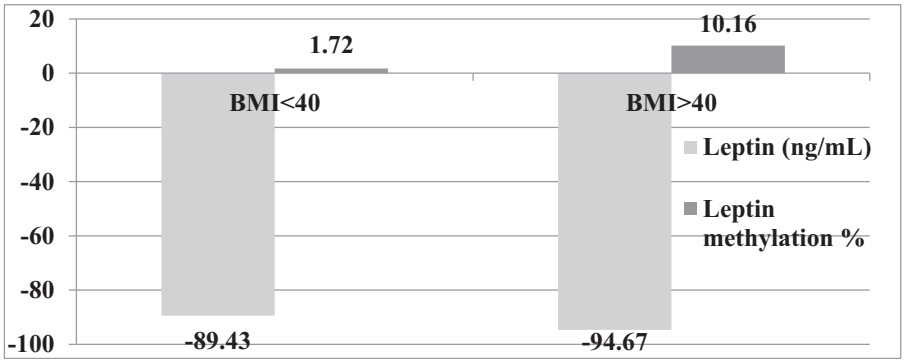
The percentages of changes in the different variables after the intervention among both groups of obesity were presented in Figs. 2 and 3. Among the obese females with BMI less than 40 kg/m^2 (Fig. 2), the most decreased variable was LEP (↓89.4%), followed by Firmicutes/ Bacteroidetes Ratio (↓5.7%), Firmicutes (↓3.6%) and minimal decrease in BMI (↓0.8%), body weight (↓0.8%), and WC (↓0.2%). On the other hand, the most increased variable was SCFA (↓25.0%), followed by Lactobacillus (↑8.0%) and Bifidobacteria (↑3.0%), then LEP promoter methylation (↑1.7%), and Bacteroidetes (↑0.7%).

Among the obese females with BMI equal to or more than 40 kg/m^2 (Fig. 3), the most decreased variable was LEP (↓94.7%), followed by SCFA (↓62.8%), and minimal decrease in WC (↓3.1%), BMI (↓2.8%) and body weight (↓2.8%). While the most increased variable was LEP promoter methylation (↑10.2%), followed by Lactobacillus (↑8.5%) and Bifidobacteria (↑7.1%), then Firmicutes (↑2.6%), Firmicutes/ Bacteroidetes Ratio (↑2.0%), and Bacteroidetes (↑0.5%).

Discussion

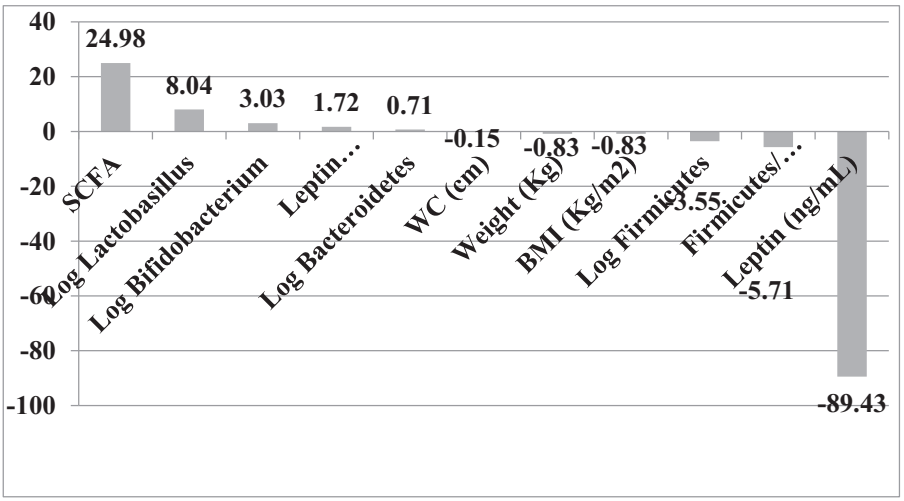
Energy imbalance and excess food intake are not the only causes of obesity; as many studies have implicated epigenetic mechanisms in the development of diet-related obesity via gene-environment interactions [16]. The amazing epigenome role in understanding and then translating the elements of the obesogenic environment –as diet- to distinct functions and phenotypes should open the door to implementing personalized obesity prevention treatments [17,18].

Figure 1



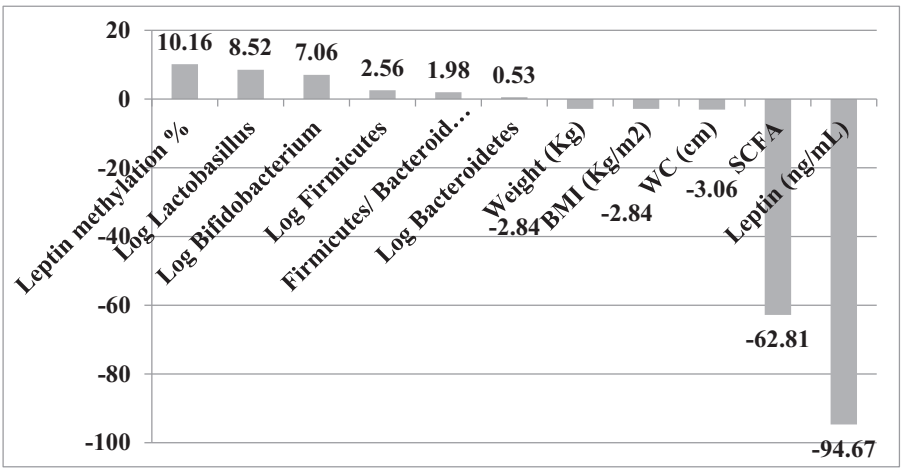
The percentage changes of the Leptin and Leptin methylation % after intervention among the two groups of the Egyptian obese females.

Figure 2



The percentage changes of the different variables after intervention among Egyptian obese females with BMI less than or equal to 40 kg/m^{2}.

Figure 3



The percentage changes of the different variables after intervention among Egyptian obese females with BMI greater than or equal to 40 kg/m^{2}.

SCFAs are the main metabolites produced as result of fermentation of dietary fibers by the anaerobic intestinal microbiota. They exert several beneficial effects on energy metabolism, as they are important fuels for intestinal epithelial cells and represent a major carbon flux from the diet that is decomposed by the gut microbiota. SCFAs affect intestinal motility, barrier function, and host metabolism [19,20]. The current study revealed that serum levels of SCFA were significantly reduced in both obese groups after intervention. This came in agreement with Muscogiuri *et al*. [21] who stated that increased biosynthesis of SCFA, providing an extra source of energy for the host, that is later stored as lipids or glucose and so contributes to the pathogenesis of obesity and its related metabolic disorders. So reduction of SCFA can be used as a marker for weight loss and cessation of obesity complications.

DNA methylation is a crucial epigenetic mechanism, the role of which has been studied extensively in the pathogenesis of many diseases. DNA methylation, specifically in the gene promoter regions, affects gene transcription. This is accomplished by hindering transcription factors from reaching the DNA and engaging transcription-repressive proteins. Aberrant DNA methylation can occur either at the specific or the global gene level; but in the case of obesity, candidate gene methylation, exhibited more consistent findings. LEP is at the top of these candidate genes linked to obesity [8]. Some studies support the concept that DNA methylation patterns,

modified by nutrition, regulate *LEP* gene expression in human cells and consequently be involved in the pathophysiology of obesity [22–24].

It was demonstrated by preclinical studies that high-fat diets and hypercaloric regimens modify the methylation of genes that contribute to appetite regulation and metabolism, such as LEP [8].

High-fat diet given to pregnant rodents demonstrated effects on hypothalamic control of metabolism, energy and body weight of the new generation by causing epigenetic changes to the *LEPR* gene. This change was associated with the prevalence of obesity and inflammation in the offspring, which lasted into adulthood [25].

The study of Houde and his colleagues [23] stated that DNA methylation in most cases applies a repressive effect on gene expression, and in obese subjects was shown to negatively correlate with BMI. In concordance with our study, it was able to show a highly significant decrease in serum LEP levels, accompanied by a highly significant increase in the corresponding LEP promoter DNA methylation percent in both obese groups under study who lost significant weight following 3 months of weight loss intervention program (↓89.4% among obese $<40\,kg/m^2$ and ↓94.7% among obese $>40\,kg/m^2$). A recent cohort study of obese adults by Sadashi and his co-workers [10] and by our study also showed a negative correlation between the percent of the LEP gene promoter methylation and body weight.

Earlier, Melzner *et al.* [26] in an investigation (*in-vitro*) of developing human (pre-) adipocytes, detected an association between reduced promoter region methylation and increased LEP expression. They found that for LEP expression, specific CpG sites demethylation is needed. The relationship between obesity and the LEP methylation status, promoting metabolic diseases and obesity-associated insulin resistance, has been confirmed by several other investigations [27–29].

Current results are in line with Wilhelm *et al.* [30], cross-sectional study as they found marginally but significantly higher DNA methylation of *LEPR* accompanied by a decrease in LEP serum levels in post- versus preoperative patients (within at least 40% excess weight loss following surgery).

In contrast to the current finding, a study by Milagro *et al.* [31] on diet-induced obesity in rats showed at one of the studied CpG sites, a significantly increased demethylation of LEP promoter than in the control rats of normal weight. This state of high methylation was accompanied by lower blood LEP levels

Also, Cordero *et al.* [32] have found that subjects who lost weight after a hypocaloric diet had decreased methylation of LEP gene in adipocytes compared with those who did not.

Another study (longitudinal) of Marchi *et al.* [33] analyzed expression of LEP before and after bariatric surgery in eight patients. Significantly decreased levels of LEP were found postoperatively, but with no alterations in the corresponding pattern of methylation, and the authors suggested that changes in LEP expression induced by weight loss are not methylation-dependent.

The limitation of the current study was: that DNA methylation is influenced by genetic variants, aging, and cell-type heterogeneity. Thus, reviewing the results using a longitudinal analysis as well as by analyzing other gene methylation in future studies is essential.

Conclusion

Weight loss intervention is associated with serum LEP level changes, as well as with LEP promoter methylation. Additionally, severity of obesity (BMI) had a great effect on the degree of association between serum LEP and LEP methylation%. The epigenetic profile of LEP methylation %might be influenced by serum LEP level through a regulating feedback mechanism. Overall, this finding suggests that weight loss is accompanied by altered epigenetic patterns of obesity, acquiring more favorable and improved metabolic, inflammatory, and vascular profiles.

Acknowledgments

Author contribution: N.E.H. is the Principal investigator (P.I.), designed the project and the study as well revised every step of the project, and gave conceptual advice; S.A.E.-M. is the Co-PI of the project from which this data was derived, performed the statistical analysis and shared in tabulation of the data and publication process; W.A.Z., write the primary draft of the manuscript; R.M.M. and M.M. responsible about genetic analysis, N.S..M.responsible about probiotic preparation, M.S.K. responsible about the laboratory investigations, M.A.S.A. and H.T.A. had taken anthropology measurements. M.S., K.A. and A.K. responsible for dietary intervention and

follow-up. All authors contributed to the collection of references and drafting of the article. They have accepted responsibility for the entire content of this submitted manuscript and approved the submission. They have agreed both to be personally accountable for the author's contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Conflicts of interest

There are no conflicts of interest.

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