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Research Article

EFFECT OF DIET BEFORE BARIATRIC SURGERY ON GHRELIN LEVEL THROUGH DNA METHYLATION

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Short Title: Effect of Diet on DNA Methylation of Ghrelin

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Keywords: Diet, Bariatric Surgery, DNA Methylation, Ghrelin

Abstract
Introduction: The ghrelin system, which generates the appetite hormone, is harmed by obesity, a problem of worldwide public health. An efficient way to cure obesity is through bariatric surgery. This randomized controlled study's objective was to assess preoperative diet-related DNA methylation of *Ghrelin (GHRL)* levels in patients undergoing bariatric surgery.
Methods: The 50 patients who volunteered to participate in the trial were randomly divided into two groups. The study group followed the very low-calorie diet (VLCD) for two weeks. The control group did not follow any diet. The physiological parameters, weight, and DNA methylation levels of the patients were assessed.
Results: The percentage of excess weight loss (EWL) in the control and study groups was determined as 47.1% and 51.5%, respectively. The study group's *GHRL* percentage of methylated reference (PMR) was 76.8%, whereas the control group's was 67.3%. It was concluded that the EWL and *GHRL* gene DNA methylation of the diet-treated study group were significantly higher than the control group ($p<0.05$).
Conclusion: According to the findings, the pre-op diet had a favorable effect on the patient's behavior modification. It has also been shown to increase post-operative weight loss and DNA methylation of the *Ghrelin* gene. The ghrelin gene has been muted by methylation, making hunger regulation more manageable.

Introduction

Obesity, defined by the World Health Organization (WHO) as an excessive accumulation of body fat which interferes with bodily functions, is a significant public health issue due to its impact on overall well-being [1]. Body fat ratio, a decisive factor in determining obesity, varies on the basis of gender and age. While newborn infants have 10-15% fat, this percentage increases with age in adulthood for both females and males [2]. There is a wide range of techniques employed in determining body fat levels. Nevertheless, implementing these methods in practice proves to be a challenging task [3]. For this reason, WHO evaluates obesity and being overweight with body mass index (BMI). BMI is calculated by dividing the measured body weight (kg) by the square of the height (m^2). It is a simple and low-cost measure for assessing obesity [4].

WHO has reported that the global incidence of obesity has tripled since 1975. In a decade-long study conducted across various regions of Asia, Africa and Europe, an increase of 10-30% in the incidence of obesity was noted [5]. According to gathered data, it was concluded that over 1.9 billion adults aged 18 and over were overweight in 2016, with over 650 millions of those being categorized as obese. In this case, overweight and obesity were associated with 7–41% of certain malignancies, 23% of ischemic heart disease patients, and 44% of diabetes cases [4]. It has been stated that a 5% decrease in body weight will have a great effect on these complications caused by obesity. Studies have shown that hypertension, one of the most common complications, is strongly associated with obesity. It is known that there is a certain increase in the risk of hypertension with weight gain [6]. Obesity is often defined by a persistent energy imbalance caused by inadequate energy expenditure or excessive energy intake [7]. However, the etiology of obesity is multifaceted to boot. Genetic, physiological, environmental, psychological and economic factors all contribute to the development of obesity [8].

The notion that obesity may arise due to genetic predisposition was first put forward by Von Noorden in 1907. In a study of 540 twins adopted in Denmark, it was observed that although the twins were raised in different households, their body weights correlated more closely to those of their biological parents [9]. Following these twin studies conducted in recent years, it has been concluded that genetics can contribute to the development of obesity by 40-70% [10]. Genome-wide association studies (GWAS) are focused on identifying genetic variants causing obesity. Referring the diversity of biological, psychological, and environmental factors involved in eating behavior, GWAS assume that the genetic risk involved in the development of obesity occurs in the neurobiological regulation pathway by way of controlling appetite [10].

The most important epigenetic mechanisms involved in the regulation of gene activity are DNA methylation, histone modifications and non-coding ribonucleic acid (RNA) [11]. Among the epigenetic modifications, DNA methylation is common in mammals in the process of tissue regeneration. DNA methylation is characterized by the shift of the methyl group in S-adenosyl methionine (SAM) to the 5th carbon of the cytosine (C) base adjacent to the guanine [12].

Epigenetic marks demonstrating a nexus between genes and susceptibility to diseases have been found to be influenced by external factors, such as nutrition. Through epigenetic processes like DNA methylation, these external stimuli can modify the epigenome by changing how genes are expressed [13]. Ghrelin (GHRL), which has a great effect on appetite in epigenetic mechanisms, is a potent growth hormone stimulant. It stimulates nutrition and produces an orexigenic effect. It plays an important role in appetite control, body weight, insulin and glucose metabolism. Ghrelin administration stimulates hunger and food intake in healthy persons as well as in patients with decreased appetite, such as cancer patients [14]. It reduces insulin secretion and increases energy intake by up to 30% [15]. Some approaches assume that ghrelin may play a role in regulating long-term energy balance. Given that obese subjects have been shown to have lower plasma ghrelin levels, recent evidence suggests that obesity is associated with a disruption in the entire ghrelin system [16].

Surgical treatment is an effective treatment for morbid obesity, providing remission of many obesity-related comorbidities, permanent weight loss and improvement in quality of life over time [17]. Surgical procedure aims to restrict the food intake by reducing the stomach volume, inducing an

absorption defect in the intestines and triggering a sensation of early satiety [18]. Regardless of the type of procedure used, it was concluded that there was a high improvement in weight loss levels and obesity-related comorbidities compared with non-surgical treatments [19]. Assessment of nutritional status prior to bariatric surgery is crucial for effective postoperative management [20]. In numerous studies, it has been observed that weight loss in the pre-op period shortens the operation duration, enhances liver parameters and reduces the risk of complications that may occur in the post-op period, as it facilitates surgical maneuvers [21]. There is currently no definite information regarding the optimal diet protocol to be implemented before surgery, nor the duration of which this should be adhered to. Several protocols are advised, including the placement of an intragastric balloon, adherence to a very low-calorie diet (VLCD), implementation of a low calorie diet (LCD), following a very low calorie ketogenic diet (VLCKD), making lifestyle change, and other related options [22].

Further research is necessary before weight loss can be recommended as a preoperative procedure. Therefore, we have opted to examine the post-op effects of a two weeks pre-op diet in our research.

Methods

This study was conducted on morbidly obese patients who applied to Health Sciences University Bakırköy Dr. Sadi Konuk Training and Research Hospital between September 2021 and May 2022. The study was approved by the decision numbered 2021-18-15 of the Clinical Research Ethics Committee of Bakırköy Dr. Sadi Konuk Training and Research Hospital. For estimating sample size, power analysis was performed with the G*power 3.1 program. The effect size for Ghrelin between the control and study groups was found to be 0.72 [23]. In the sample width analysis performed by taking the alpha error probability as 0.05 and the power value as 0.80, the total number of subjects required to be taken was found to be 50 (50/2 = 25 for each group). Fifty patients who were to undergo surgical procedures for the treatment of morbid obesity voluntarily participated in the study. Written voluntary informed consent form was obtained from all participants. The patients involved were randomly divided into two groups, control (n=25) and study (n=25) according to the order of admission to the hospital. VLCD program was implemented on 25 patients who were part of the study group for two weeks before surgery. No nutrition program was provided to 25 patients in the control group during the pre-op period. Patients have undergone sleeve gastrectomy and gastric bypass operations. Biochemical parameters were evaluated in the pre-op period, as well as in the first and third months of the post-op period. Ghrelin levels were recorded using DNA methylation analysis before and three months after surgery.

The Nutrition Program

The VLCD program was applied to the study group for two weeks. The dietitian elaborated on the account of the diet programs face-to-face with the patients. Protein powders (Barifit™) containing 27 g of protein were supplied for use in diet programs. The diet administered to the patients was designed to provide 30% of the energy via a combination of nutrients, with 30% derived from carbohydrates, 30% from fats and 40% from proteins. The list has been arranged to ensure a maximum calorie intake is 700 kcal/day. The content of the diet list is given in Table 1.

Ghrelin Gene and DNA Methylation

The blood collected from the patients before and after surgery, at 3 months post-operation, was drawn into EDTA K2 3 mL tubes. DNA isolation from peripheral blood samples stored in EDTA tubes was performed using a commercial kit in line with the manufacturer's instructions (Zymo Research Quick-DNA Miniprep Plus Kit-D4068). This isolation method is grounded on the spin-column method. After purification of genomic DNAs, bisulfite conversion was performed for GHRL gene methylation analysis. This conversion was carried out in accordance with the EZ-96 DNA Methylation-Gold Kit. The “Sodium Bisulfite Treatment” method is used to measure the methylation status in the nucleotide sequence region or any region of a gene [24]. With this method, all unmethylated cytosines (C) in DNA are converted to uracil (U) by chemical reaction. The methylated C’s in DNA remain unchanged throughout the reaction. After the conversion process is completed, the DNA is amplified by polymerase chain reaction (PCR) and the methylation status of the DNA is determined.

Primer Design

While designing the primer, the reference sequence GHRL (3p25.3, ghrelin and obestatin prepropeptide, NM_016362.5, NP_057446.1) obtained from Ensemble was used. Then, GHRL gene methylation sites from UCSC were determined (Hg19/human ENC DNA Methyl HAIB Methyl450 Track Settings).

It has been ensured that the primers have at least one CpG region and that these regions are situated near the 3' end). Two pairs of primers were used for each amplicon as in Table 2. Forward and reverse methylated primer set containing cytosine in CpG regions and unmethylated primer set containing thymine instead of cytosine in CpG regions were designed as forward and reverse. The GHRL gene was amplified using modified and unmodified DNA.

Bisulfite-converted DNA samples were examined using the Real Time Quantitative Methylation Specific PCR technique to determine the methylation level of the GHRL gene. In Table 3, the forward sequence m-GHLR-F2- has been selected for the GHRL gene primer sequences and m-GHLR-R2- for the reverse sequence. Primer sequences were selected for the control gene β-actin (ACTB) [25].

The GHRL gene region was amplified with methyl-specific primers designed only for methylated CpG sequences, and the resulting fragments were loaded onto agarose gel, and then run in gel electrophoresis to differentiate between methylated and unmethylated regions.

After gel electrophoresis, a screenshot of the gel was taken and interpreted with BioRad, GelDoc-Go imaging system device.

Statistical Analysis

IBM Statistical Program for Social Sciences (SPSS) Statistics 22 program was used for the evaluation of the findings obtained in the study and for statistical analysis. Along with descriptive statistical techniques (mean, standard deviation, frequency), the following were used to analyze the study's data: Student t test, Mann Whitney U test, paired sample t test, Wilcoxon sign test, Fisher's Exact Chi-Square test, Fisher Freeman Halton Exact Chi-Square test, Continuity (Yates) Correction, and Mc Nemar test. Significance was evaluated at the $p<0.05$ level.

Results

Due to poor communication, failure to satisfy eligibility requirements, and other reasons, 8 of the 50 participants in the research were removed from the analysis. The study was conducted with a total of 42 cases, 38 (90.5%) female and 4 (9.5%) male. The mean age is 37.12 ± 14 years. The cases were evaluated under two groups as "Study" (n=18) and "Control" (n=24). The mean age of the control and study groups was 39.5 ± 13.6 and 33.8 ± 14.2, respectively.

Weight Loss and Biochemical Measurements

The pre-op weights of the control and study groups were 120.4 ± 19.9 and 115.2 ± 18.9 kg, respectively. The weight loss of the control group was 14.2 ± 5.9 kg in the first month and 24.8 ± 7.4 kg in the third month. While the post-op weight loss of the study group was 12.4 ± 5.4 kg in the first month, it was 26 ± 7.2 kg in the third month.

During the pre-op period, the study group lost an average of 4.9 ± 1.6 kg due to the diet they followed for two weeks. While the pre-diet BMI values were 46.9 ± 4.4 kg/m^2, the BMI levels decreased to 45 ± 4.1 kg/m^2 shortly before the surgery after the diet.

Considering the percentages of excess weight loss (EWL), the mean of the study group at the 3rd month was found to be significantly higher than the control group ($p:0.028$; $p<0.05$) as given in Table 4.

Biochemical analyzes were performed on the patients in both the control and study groups during pre-op and post-op processes. Based on the assay results, it was concluded that 12 patients in the control group and 15 patients in the study group, whose diabetes was uncontrolled before the operation, was under control with weight loss in the post-op 3rd month. Nine out of the eighteen patients in the study group and six out of the eighteen patients in the control group showed a return to normal LDL-cholesterol levels. Improvement was observed in 4 out of 13 patients and 4 out of 12 patients in the control and study groups with high triglyceride levels, respectively.

Molecular Analyzes

Using normal DNA isolated from peripheral blood and DNA methylated with bi-sulfite modification, the initial amplification trial study involved 3 patients from the third month of the randomized study

group and 3 patients from the third month of the control group, whose biochemical value was unknown. Primers pairs specific to the unmethylated, normal genome were used in this work. The procedure was performed on all patients after receiving adequate results from the trial study. Table 5 provides the primer design for the entire DNA sequence based on the GHRL gene.
In Figure 1, the methylation image of the samples from the study group is shown in the red frame, and the unmethylated (non-methylated) samples from the control group without the proper band are shown in the green frame. The band intensity is less intense in the orange frame compared to the red, indicating incomplete methylation in the study group. Samples that are expected to be generic items are shown as blue-framed bands and are not regarded as qualitatively significant.
The study group's GHRL methylation was discovered to be considerably greater than that of the control group. The evaluation of the GHRL percentage of methylated reference (PMR) is shown in Figure 2.

Discussion

Guidelines concur that all patients must undergo a certain period of medical therapy before bariatric surgery, and that it is also vital to assess the patient's inclination to comply with follow-up programs. However, there are a number of reservations regarding weight loss.
In a study problematizing the necessity of preoperative weight loss, one group of patients underwent a low-calorie diet before surgery, of a piece with our study, whilst the other group did not. The foregoing study contended that the dieting group's operation time was significantly shorter, and their weight reduction was positively affected. At the third month following surgery, the dieting group's EWL (%) value was 44.1 ± 21.8, while the non-diet group's EWL value was 33.1 ± 7.7 [26]. Similar to this, our study found that EWL value of the diet group was significantly higher than that of the control group ($p<0.05$). The systematic analysis of the effects of VLCD on weight loss demonstrated the efficacy of all trials and made a remarkable contribution to people trying to lose weight. However, it was stated that diets with lower calorie values did not cause greater weight loss than diets with higher caloric values [27]. Stefura et al. found that patients with good preoperative weight loss were motivated to attend follow-up examinations and concluded that those who achieved a preoperative ≥5% weight loss were superior to postoperative weight loss [28].
The pathophysiological underpinnings of obesity and other associated illnesses have been theorized to involve DNA methylation patterns that have been altered by environmental influences [29]. However, both lifestyle choices and environmental exposures have the potential to change DNA methylation and cause genome reprogramming in both exposed individuals and subsequent generations. Recent studies have focused on examining the nexus between diet and DNA methylation, and its effects obesity traits and metabolic disorders. The relationship between dietary folate intake and the genome methylation profile was examined by Ramos Lopez et al. The researchers found 51 CpGs linked to folate uptake in the CAMKK2 gene, a milestone in the regulation of metabolic processes such as adiposity, glucose homeostasis. They discovered that the consumption of folate was positively correlated with the level of methylation of this gene [30]. In another study, it was discovered that eating fruit and following a healthy diet index had a negative correlation with TNF- α methylation level. According to the authors' theories, alterations in DNA methylation in TNF- α may be a biological mechanism underlying the advantages of a balanced diet and fruit consumption on glucose tolerance [31].
The DNA methylation analysis methods vary amongst investigations. In tune with our research, most of the studies employed methylation-specific PCR and pyrosequencing of bisulfite-treated DNA (37.2% and 34.9%, respectively), whereas 16.3% used alternative techniques such as spectrometry and enzyme-linked immunosorbent assay, or EIA, Methyllight [32]. Since measuring GHRL level via methylation is the primary goal of our study, there aren't many study methodologies available. One of the few examples of research on this topic is the use of a bisulfite sequencing technique based on chemical alteration of unmethylated cytosines and PCR.
There were some limitations in our study. Firstly, our results have limited generalizability due to the small sample size and differences in gender. Secondly, the intervention period in our study was relatively short. Long-term evaluations can be conducted using a larger number of samples.

In the current study it was concluded that GHRL gene methylation was significantly higher in the study group that applied pre-op diet compared to the control group ($p<0.05$). It was concluded that the study group with higher DNA methylated GHRL gene ratio had a significantly higher percentage of EWL ($p<0.05$). The diet followed before surgery has been shown to promote post-operative weight loss and DNA methylation of the ghrelin gene, which codes for the appetite hormone. Appetite regulation was made simpler by the DNA methylation and silencing of the GHRL gene expression. However, long-term follow-up studies are needed to understand the relationship between diet and the post-operative process.

Statements

Statement of Ethics

Study approval statement: All individual participants in the study provided written informed permission. The study protocol adhered to the Declaration of Helsinki's ethical criteria, and it was approved by the Bakırköy Dr. Sadi Konuk Training and Research Hospital Medical Research Ethics Committee in İstanbul, Türkiye (protocol number 2021/442).
Consent to participate statement: Written informed consent was obtained from all participants included in the study.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

Duygu Güçlü, Nilgün Işıksaçan, and Hakan Seyit have been involved in the design of the study. Duygu Güçlü, Hakan Seyit, Mehmet Karabulut, İrem Erdil, Tamay Seda Taşçı and, Mustafa Yaman were involved in the acquisition of data. Duygu Güçlü, Nilgün Işıksaçan, Hakan Seyit, and Asuman Gedikbaşı reviewed, and edited the manuscript.

Data Availability Statement

All data analyzed during the current study are not publicly available due to privacy reasons but are available from the corresponding author.

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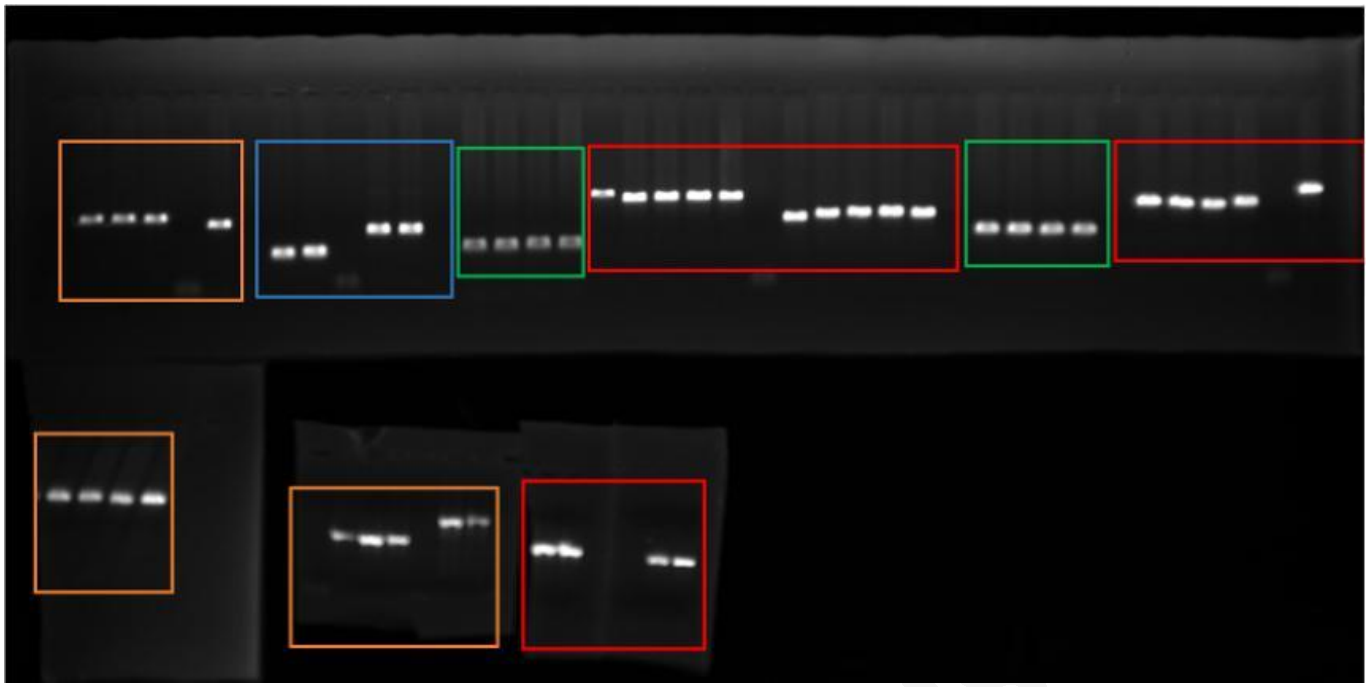
Figure Legends

Fig. 1. Agarose gel image of methylated, partially methylated and unmethylated products with various band Intensities after *GHRL* gene methylation specific PCR of post-op 3rd month patient group samples (orange: incomplete methylation from the study group, red: methylation samples from the study group, green: unmethylated samples from the control group, blue: nonspecific product.)

Fig. 2. GHRL percentage of methylated reference (PMR)



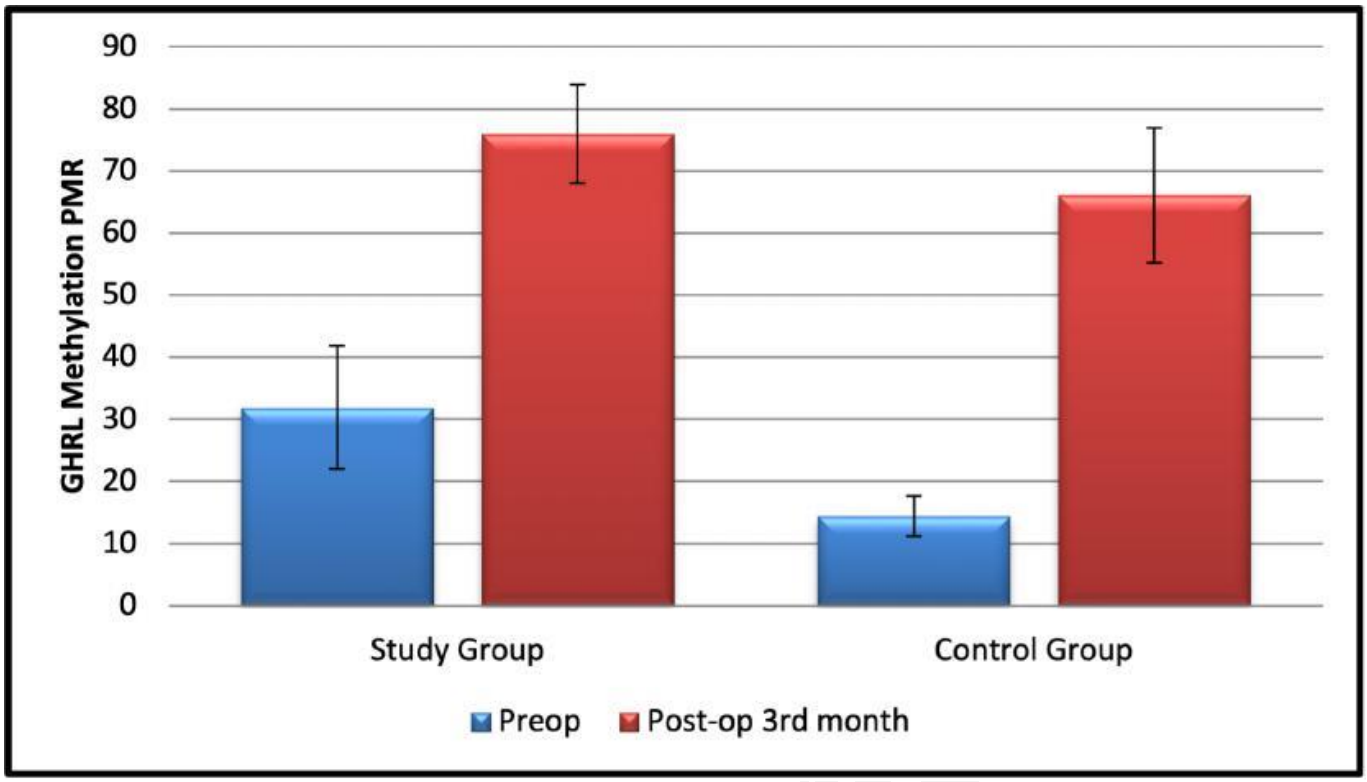


Table 1. The content of the nutrition plan applied to the study group

	Values
Total energy	700 kcal
Carbohydate	57 g (32%)
Protein	70 g (40%)
Fat	21.5 g (28%)

Table 2. Normal primer sequences and post-methylation designed sequences of the *GHRL* gene

	Normal primer	Post-methylation primer
Forward primer	5’- GTG GTC TGG GAC CAA AGC TGT AAT GC -3’	5’-GTG GTT TGG GAT TAA AGT TGT AAT GT-3’
Reverse primer	5’-CCG AAT GAC CAC CTA CCC T-3’	5’-CCA AAT AAC CAC CTA CCC T-3’

Table 3. Primer sequences for the *GHRL* gene and control gene primer sequences

	Primer sequences for *GHRL* gene	**Control gene β-actin primer sequences**
Primer sequences	5’-GTT TTG TAA TTG ATA GGG T-3’	5'-TGG TGA TGG AGG AGG TTT AGT AAG T-3'
Reverse sequences	5’-CCT AAC CAC ATA CCA CA-3’	5'-AAC CAA TAA AAC CTA CTC CTC CCT TAA-3'

Tablo 4. Anthropometric measurement

		Control group	Study group	p
		Ort ± S.S.	Ort ± S.S.	
BMI	Before diet	-	46.9 ± 4.4	
	Pre-op	45.5 ± 5.3	45 ± 4.1	0.747
	Post-op 3rd month	36.2 ± 4.9	34.8 ± 3.8	0.313
EWL (%)		47.1 ± 12.6	51.5 ± 12	0.028*

**p<0.05*

Table 5. GHRL gene primer design (for whole DNA)

Forward primer	5’-TCCAGCCTGCCACTTAGC -‘3
Reverse primer	5’-GGACCCTGTTCACTGCCAC -‘3
Reverse complement primer	3’-GTGGCAGTGAACAGGGTCC -‘5
TCCAGCCTGCCACTTAGCGTGGCAGTGAACAGGGTCC	