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RESEARCH

Relationship between Klotho gene methylation level and diet habit

Klotho gen metilasyon seviyesi ile diyet alışkanlığı arasındaki ilişki

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Abstract

Purpose: This study aimed to determine the relationship between the methylation level of the Klotho gene and nutritional habits.
Materials and Methods: From our healthy sample group consisting of 20 people, two groups were created: 10 people fed with carbohydrates and 10 people had protein. Initially, a food consumption frequency determination form was administered as a survey to individuals. Based on the results of this survey, the amounts of food consumed by the participants (g/cc) were determined. According to the findings of the survey, two groups were formed: those classified as carbohydrate consumers (individuals consuming 33% or more of their diet from carbohydrates) and those classified as protein consumers (individuals consuming 17% or more of their diet from protein). Methylation level of Klotho gene in blood samples of individuals; DNA isolation, RT-PCR and Bisulfite Modification were examined.
Results: In the carbohydrate diet group; there was a very strong and inverse correlation between fat and methylation percentages (r = -0.765, p = 0.05). There was a strong correlation between the percentages of carbohydrate and methylation (r = 0.778, p = 0.004). A strong correlation was also found between BMI and methylation percentage (r = 0.712, p = 0.01). There was a strong inverse correlation between cholesterol and methylation percentages (r =-0.556, p = 0.04). In the protein diet group, there was a strong inverse correlation between BMI and methylation (r =-0.635, p = 0.024).
Conclusion: As a result of the analysis, the Klotho gene methylation percentage (33%) in individuals with a carbohydrate-based diet was found to be higher than that in individuals with a protein-based diet (17%). The data obtained indicate that as carbohydrate consumption increases, the methylation level of the Klotho gene also rises.

Keywords: Diet, methylation, protein, carbohydrate, klotho

Öz

Amaç: Bu çalışma, Klotho geninin metilasyon seviyesi ile beslenme alışkanlıkları arasındaki ilişkiyi belirlemeyi amaçlamıştır.
Gereç ve Yöntem: Sağlıklı bir örnek grubumuzdan oluşan 20 kişiden iki grup oluşturulmuştur: 10 kişi karbonhidratlarla beslenen ve 10 kişi proteinle beslenen. İlk olarak, bireylere bir gıda tüketim sıklığı belirleme formu anket olarak uygulanmıştır. Bu anketin sonuçlarına dayanarak, katılımcıların tükettikleri gıda miktarları (g/cc) belirlenmiştir. Anket bulgularına göre, iki grup oluşturulmuştur: karbonhidrat tüketicileri olarak sınıflandırılanlar (besinlerinin %33'ünü veya daha fazlasını karbonhidratlardan alan bireyler) ve protein tüketicileri olarak sınıflandırılanlar (besinlerinin %17'sini veya daha fazlasını proteinlerden alan bireyler). Bireylerin kan örneklerinde Klotho geninin metilasyon seviyeleri; DNA izolasyonu, RT-PCR ve Bisülfit Modifikasyonu ile incelenmiştir.
Bulgular: Karbonhidrat diyeti grubunda; yağ ve metilasyon yüzdeleri arasında çok güçlü ve ters bir korelasyon vardı (r = -0,765, p = 0,05). Karbonhidrat ve metilasyon yüzdeleri arasında güçlü bir korelasyon vardı (r = 0,778, p = 0,004). BMI ile metilasyon yüzdesi arasında da güçlü bir korelasyon bulundu (r = 0,712, p = 0,01). Kolesterol ile metilasyon yüzdeleri arasında güçlü bir ters korelasyon vardı (r = -0,556, p = 0,04). Protein diyeti grubunda, BMI ile metilasyon arasında güçlü bir ters korelasyon vardı (r = -0,635, p = 0,024).
Sonuç: Analiz sonuçlarına göre, karbonhidrat temelli beslenen bireylerde Klotho geninin metilasyon yüzdesinin (%33) protein temelli beslenen bireylerden (%17) daha yüksek olduğu bulunmuştur. Elde edilen veriler, karbonhidrat tüketimi arttıkça Klotho geninin metilasyon seviyesinin de yükseldiğini göstermektedir.

Anahtar kelimeler: Diyet, metilasyon, protein, karbonhidrat, klotho

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INTRODUCTION

Aging is a process of growth and development with biological, physiological, and psychological dimensions that begins at birth and continues until death. As we get older, the risk of age-related diseases increases[1]. Aging is defined as the accumulation of irreversible structural and functional changes at the molecular, cellular, tissue, organ, and system levels over time[2]. This process progresses at different rates depending on the organism's interaction with its environment and hereditary characteristics. Lifestyle factors such as eating habits, alcohol consumption, and smoking can significantly impact aging. Poor nutrition, imbalances in protein and carbohydrate intake, or caloric extremes can lead to genetic changes, including mutations and epigenetic modifications that alter gene expression. These changes can result in metabolic shifts, potentially leading to metabolic diseases such as diabetes, obesity, and heart disease[3].

The Klotho (KL) gene encodes the KL protein, a transmembrane protein located on chromosome 13 in humans, initially identified as a gene linked to lifespan reduction[4,5]. Although the absence of KL does not immediately cause phenotypic changes in mice, it contributes to senescence-like phenotypes over time[6].

KL protein has roles in adipocyte development and systemic glucose metabolism; it enhances adipocyte differentiation in vitro, and mice with deficient KL gene activity are lean due to reduced white adipose tissue. These mice also show resistance to high-fat diet-induced obesity, suggesting that energy metabolism may be influenced by KL activity[7]. Additionally, consuming a Mediterranean diet has been associated with elevated KL levels[8]. As KL is generally regarded as an anti-aging agent, strategies to prevent its decline may offer new therapeutic approaches for age-related diseases[6].

DNA methylation, an epigenetic mechanism occurring at the C-5 position of cytosine rings in eukaryotic genomes, plays a crucial role in genome stability and gene regulation[9]. In vertebrates, it typically occurs as a result of the addition of methyl groups to CpG sites by the DNA methyltransferase enzyme. S-Adenosyl Methionine (SAM) functions as a methyl donor[10,11]. Non-CpG methylation predominates in embryonic stem cells. Methylation is a system that prevents protein expression by causing DNA inactivation[12].

The present study was designed to assess how dietary habits influence KL methylation. It aimed to examine the relationship between food consumption frequency and the methylation level of the KL gene in carbohydrate-fed and protein-fed individuals. The study hypothesizes that dietary composition, specifically higher carbohydrate versus protein intake, will differentially affect Klotho gene methylation levels. This study aims to contribute to the growing body of literature linking diet to epigenetic modifications, shedding light on how nutritional habits may influence gene expression and health outcomes. Such findings could help inform dietary recommendations that optimize gene expression to support health maintenance and prevent disease.

MATERIALS AND METHODS

Sample

The research was conducted on individuals who applied to the family medicine polyclinic of Necmettin Erbakan University Meram Medical Faculty Hospital. File reliability is ensured by high security protocols in data management; research data are accessible only to authorized persons and analysis processes are carried out in accordance with national and international scientific ethical rules.

Data collection and analysis processes were carried out by trained researchers and experts, and each stage was documented transparently. Informed consent was obtained from all participants. The study included individuals who were considered clinically healthy. Fifteen subjects were excluded from the study after clinical evaluation. These healthy individuals' blood values were sent to the Biochemistry Laboratory (Parameters such as complete blood count, glucose, urea and creatinine, electrolytes, total Cholesterol, AST and ALT, alkaline phosphatase, C-Reactive Protein, TSH, T3, T4, insulin were evaluated). When their hospital procedures were completed and when they were reported as having no disease, the contact was made with each participant and the scope and purpose of the study were explained., The ones who agreed and handed in their consent forms were included in the study.

Procedure

Ethical approval was obtained from the Ethics Committee of Pharmaceutical and Non-Medical Device Research of Necmettin Erbakan University Meram Faculty of Medicine with the decision dated 16.03.2018 and numbered 65-2018/1279.

First, a survey was applied by giving them a food consumption frequency determination form, and in line with this survey, the amount of food consumed by individuals (g/cc) was determined. According to the results of this survey, two groups were formed. Those who consume carbohydrates (those who consume 33% or more carbohydrates) and those who consume protein (those who consume 17% or more protein).

Group 1: It consisted of 10 protein-rich diet volunteers between the ages of 18-60, who had not been diagnosed with any disease and Group 2 consisted of 10 carbohydrate-rich diet volunteers between the ages of 18-60, who had not been diagnosed with any disease.

Height, waist, hip and weight measurements were taken via anthropometric measurements. Fat mass was determined with a caliper and body mass index results were recorded. Body mass index (BMI) was calculated separately for each individual in the study using the formula kg/m2. BMI results were evaluated according to WHO. Participants were instructed to maintain their usual diet during the study and report any deviations.

Chemicals

Chemicals used in experimental studies are as follows; Blood Sv DNA Isolation; Kit (Geneall, Cat No: 108-101). RNA Isolation with Hybrid R; Kit (Geneall, Cat No: 305-101). cDNA isolation from RNA isolated samples; Kit Hyper Script (GeneAll Cat no: 602-105). Real Time PCR; Realamp Sybr Green Mastermix (High Rox Dye) (Cat no: 801-051). Bisulfite Modification; Kit (ZYMO RESEARCH-EZ DNA Methylatio Direct Cat No: D5020). Hot-start PCR was performed with the bisulfite DNAs obtained. Kit (2x AmpMaster Hs-Taq; Cat no: 545-002).

The expressions of the samples were examined by RT-PCR. The study was performed with SYBR Green-based qRT PCR. ACT-B as control primer. KLOTHO-CDNA-F: CACAGAGGTTACAGCATCAG, KLOTHO-CDNA-R: CAGCAAAGTCAACACAGTAGGA, ACTB-GATGGTGGGCATGGGTCAGAAGGA, ACTB-R: CATTGTAGAAGGTGTGGTGCCAGAT

Expression quantification of the KL gene was normalized to the control group using the ACTB gene as a reference. The "2-ΔΔCT Method", which is a relative quantification calculation, was applied.

Bisulfite modification

The bisulfite modification method is a widely used technique for analyzing DNA methylation levels, particularly for genes like the Klotho (KL) gene. Bisulfite treatment converts unmethylated cytosines in DNA to uracil, while methylated cytosines remain unchanged. This chemical conversion enables the differentiation between methylated and unmethylated cytosines upon subsequent analysis.

DNA Extraction: Genomic DNA was extracted from the samples. The quality and quantity of extracted DNA were assessed using a spectrophotometer. Bisulfite Treatment: The extracted DNA was dissolved in Sodium bisulfite solution (pH adjusted to 5.0-6.0) was added to the DNA sample. The reaction mixture was incubated at 55°C for 16 hours to allow for complete conversion of unmethylated cytosines to uracils. Desulfonation: After bisulfite treatment, the DNA was desulfonated by adding sodium hydroxide to neutralize the bisulfite. DNA Purification: Ethanol precipitation was performed to further purify the DNA, and the samples were washed with 70% ethanol. PCR Amplification: Specific primers were designed to amplify the region of interest in the modified KL gene. The PCR was performed using a high-fidelity DNA polymerase. Analysis of PCR Products: The PCR products were analyzed using to determine the methylation status of the KL gene.

Anthropometric measurements

Height, waist circumference, body mass index, weight and caliper measurements were taken. Body Mass Index (BMI): Calculated according to the equation [body weight (kg) / height (m2)]. BMI results were evaluated according to WHO and the ones who were obese were not included in the study.

Caliper Measurement: Measurements were taken from the right arm and measured three times. If there was a difference of more than 5% between the first

and second measurement, it was measured a third time. The average of the measurements was calculated for evaluation.

Statistical analysis

After the data obtained from the samples, they were transferred to Statistical Package for Social Sciences (SPSS) for 16.0 computer program. To compare the methylation levels of the Klotho gene between the two dietary groups (carbohydrate vs. protein consumers), the Mann-Whitney U-test was employed. This non-parametric test was chosen due to the small sample size and the assumption that data may not follow a normal distribution. The Mann-Whitney U-test compares the distribution of methylation levels across the two independent groups. The Pearson correlation analysis was used to explore relationships between various continuous variables, including dietary components (such as percentages of carbohydrate, protein, fat, and cholesterol) and methylation percentages of the Klotho gene. In all analyses, p values that less than 0.05 ($p<0.05$) considered statistically significant.

This study is primarily exploratory, aimed at uncovering relationships between dietary habits and Klotho gene methylation levels. While the sample size of 20 participants provides preliminary insights, it is important to note that a power analysis was not conducted to assess the adequacy of this sample size. Future studies should consider larger cohorts to confirm these findings and enhance the generalizability of the results. To improve the understanding of statistical significance, it is recommended to calculate and report confidence intervals (CIs) for correlation coefficients in addition to p-values. For example, the correlation between carbohydrate percentage and methylation ($r = 0.778$, $p = 0.004$) could be accompanied by a 95% CI to illustrate the robustness of this association. Given the number of correlations performed, adjustments for multiple comparisons were not applied in this study due to its exploratory nature. However, it is advisable for future research to incorporate such adjustments to minimize the risk of Type I errors, ensuring the validity of the findings.

RESULTS

The band images obtained as a result of bisulfite modification are given in Fig. 1.

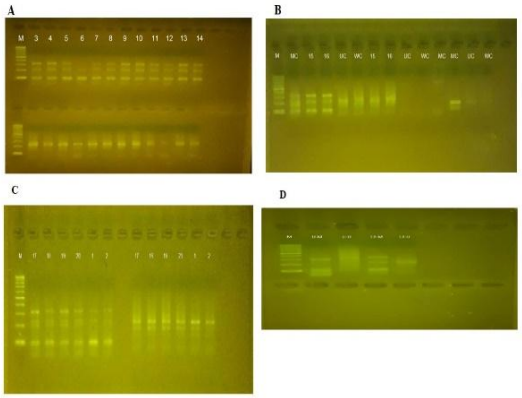

Fig 1. Band images obtained as a result of bisulfite modification are as follows: A. There are samples run with methylated primers in the upper part and unmethylated primers in the lower part. Sample order is valid for the bottom row B. Samples numbered MC-15-16 with methylated primer Samples numbered UC-WC-15-16 with unmethylated primer UC-WC-MC control DNA with control primer sets MC-UC- in the last 3 rows WC bisulfite DNAs were studied with control primer sets. C. The first group was run with methylated and the other group was unmethylated primers. D. Gel image of unmethylated control DNA and KL M and U primers from samples 12.

Dietary component correlation values are given in table 1. In table 2, correlation values between BMI and methylation %.

Table 1. Correlation values between methylation % and the variables of dietary lipid %, dietary carbohydrate %, BMI and cholesterol. (N: Number of samples)

		Lipid %	Carbohydrate %	BMI	Cholesterol mg
Methylation %	Pearson Correlation	-.765	.778	.712	-.556
	P	.005	.004	.01	0.04
	N	10	10	10	10

BMI: Body Mass Index

Table 2. Correlation values between BMI and methylation %. (N: Number of samples)

		BMI
Methylation %	Pearson Correlation	-.635
	P	.024
	N	

BMI: Body Mass Index

In the carbohydrate group: A very strong, significant, and inverse correlation was found between fat percentage and methylation percentage (r = -0.765, p = 0.05). A very strong, significant, and positive correlation was observed between carbohydrate percentage and methylation percentage (r = 0.778, p = 0.004). There was a strong, significant, and positive correlation between BMI and methylation percentage (r = 0.712, p = 0.01). A strong, significant, and inverse correlation was detected between cholesterol percentage and methylation percentage (r = -0.556, p = 0.04).

In the protein group: A strong, significant, and inverse correlation was found between BMI and methylation percentage (r = -0.635, p = 0.024). % methylation, ΔCT distribution and differences between groups are given in Fig 2.

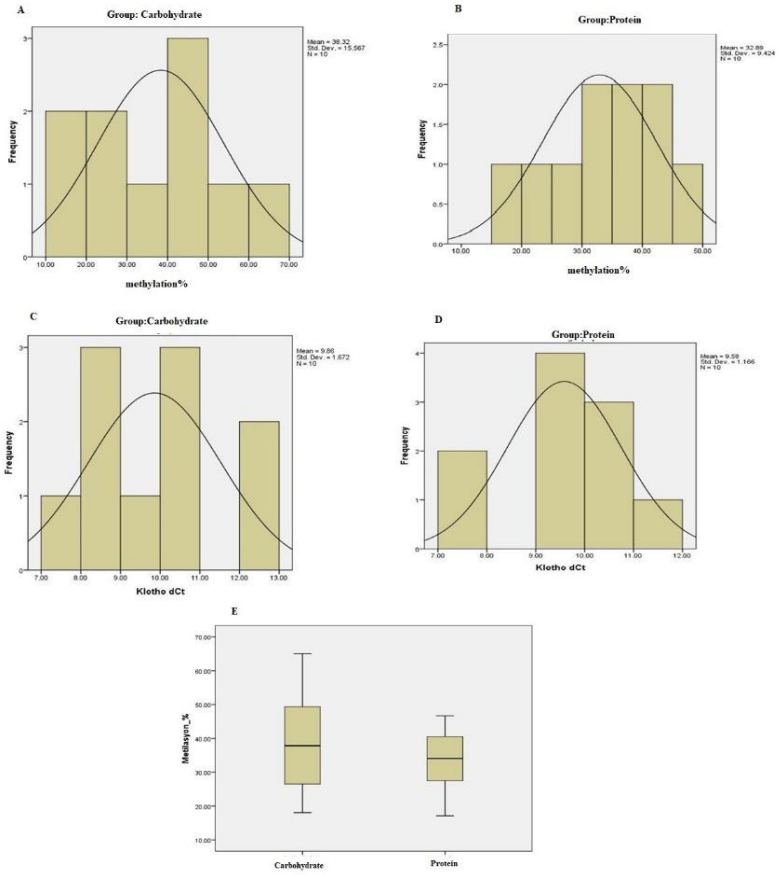

Fig 2. A. % methylation distribution in cases in carbohydrate-fed subjects. B. % methylation distribution in cases in protein fed subjects. C. KL ΔCT distribution in carbohydrate-fed individuals. D. KL ΔCT distribution in protein fed individuals. E. Methylation % values graph of Carbohydrate and Protein group.

The difference between groups was found to be P = 0.004.

The KL ΔCT values of the carbohydrate and protein groups, the difference between the groups was found to be P>0.05.

DISCUSSION

As a result of the analysis of the present study, it was found that the KL gene methylation percentage of protein-fed individuals was lower than the KL gene methylation percentage of carbohydrate-fed individuals, with a statistically significant correlation. This leads us to the conclusion that as the protein consumption rate increases, the KL gene methylation level decreases[13].

DNA methylation is associated with many conditions such as cancer, cardiovascular diseases, and diabetes. The increase in DNA methylation change with age was first noticed in monozygotic twins[14]. These studies also support the idea that DNA methylation exhibits decreasing consistency across the lifespan, resulting in an increase in interindividual variability with an overall decrease in DNA methylation[15].

Regions that gain DNA methylation with age: While more enriched in CpG islands, areas lacking CpG islands are prone to loss of DNA methylation with age[16]. These findings show an interesting structure; while regions that generally show low DNA methylation, such as promoter-associated CpG islands, tend to increase methylation with age; regions with high DNA methylation, such as CpGs that do not contain intergenic islands, are prone to loss of methylation with age. Since most CpGs are located outside CpG islands and are highly methylated, this results in a general loss of DNA methylation in later life, as well as a trend toward a shift in DNA methylation levels with age[17,18]. A notable feature of DNA methylation is that it can be modified by external factors and, in some cases, the resulting symptoms can be transmitted through cell divisions. This balance of heredity and response to stimuli results in the formation of a unique mechanism that bears permanent traces of environmental factors accumulated throughout life[19,20].

Overexpression of the KL gene increases lifespan from twenty to thirty percent[21]. Synthesis of KL protein in large amounts significantly slows down the aging process and prolongs life by providing resistance to oxidative stress[6]. It has been observed that the increase in growth hormone increases the circulating KL level[22]. Low circulating KL levels lead to increased risk of hypertension[23], chronic kidney disease[24], interstitial lung abnormalities[25], progression of diabetic retinopathy[26] and Alzheimer's disease[27]. Additionally, inflammatory conditions have also been observed to significantly alter KL protein expression[28].

A recent study has shown that dietary fiber prevents oxidative stress through its metabolites, which are short-chain fatty acids[29]. It has been determined that there is a relationship between individuals' weight, dietary fiber intake and KL levels[30]. A study in middle-aged adults found that individuals with high carbohydrate intake had higher circulating levels of KL compared to those with lower carbohydrate intake. In the same study, higher serum KL levels were measured only in men at higher intakes of dietary fibre, phosphorus and potassium[31].

In diet practices; incomplete intake of phosphate[32], high sucrose content[33], reduction of calcium[34], diets enriched with keto analogues[35], extra protein restriction[36], low-calorie high-protein diets[37], vitamin D content[38] have been found to be linked to KL gene level.

In this study, we investigated the relationship between Klotho (KL) gene methylation levels and dietary habits among individuals consuming diets with at least 33% carbohydrates or 17% protein. Our findings revealed notable differences between the two groups. In the carbohydrate group, an increase in dietary fat and cholesterol was associated with a decrease in KL gene methylation levels. Conversely, as carbohydrate intake increased, KL gene methylation levels also rose. Additionally, a positive correlation was observed between body mass index (BMI) and KL gene methylation levels; higher BMI was linked to increased methylation. In the protein group, however, an increase in BMI corresponded with a decrease in KL gene methylation levels.

Many studies have been conducted to determine the functions of the KL protein. As a result of, these study it has been seen that the KL protein plays many different roles in cells. Membrane KL proteins form a tetrameric complex with fibroblast growth factor receptors and have a coreceptor function for FGF-23[21,32]. KL proteins take part in body mineral homeostasis together with hormones that function in calcium-phosphate regulation[39]. KL protein deficiency, as mentioned previously, causes diseases

in the human body such as chronic kidney disease, osteoporosis and cardiovascular complications[40,41]. In a study conducted with individuals with stomach cancer, methylation was observed in KL gene promoters in forty-six percent of individuals with cancer, while no methylation was observed in healthy stomach tissues in individuals without it[42]. As a result, it was seen that the KL gene is a new inactivated epigenetic tumor suppressor gene in stomach cancers[6].

In other studies, it was found that while KL gene promoter methylation is normal in healthy cells in the gastric mucosa and colon, the amount of methylation increases in the transformation to malignancy[42]. In research conducted with uremic patients, it is known that the amount of DNA methyltransferase (DNMT) enzymes increases due to the accumulation of uremic toxicity, and KL protein synthesis decreases with the increase in the amount of methylation in the CpG islands of the KL gene[43]. In light of this information, it seems that reducing or inactivating the epigenetic expression levels of tumor suppressor genes by DNA methyltransferase (DNMT) enzymes has an important role in the development of cancers[44]. Regulation of KL protein synthesis, which is thought to be linked to different types of cancer and causes aging-like changes in its deficiency, suggests that it may cure some diseases[23,28,42].

In the present study, when the KL gene expression level of carbohydrate-fed individuals was compared with that of protein-fed individuals, no significant correlation was found in either group. Additionally, the comparison of differences between the groups also revealed no statistically significant difference ($p>0.05$). Although a small increase in KL gene expression was observed in protein-fed individuals compared to carbohydrate-fed individuals, this increase was not statistically significant.

It is thought that this result may be due to different reasons. However, despite the small difference, as a result of the analysis of our study; The KL gene methylation percentage of carbohydrate-fed individuals was found to be higher than the KL gene methylation percentage of protein-fed individuals, and this led us to the conclusion that as carbohydrate consumption increased, the KL gene methylation level also increased. Nutritional habits affected the KL gene methylation level, however, it is thought that comprehensive research should be conducted to elucidate the issue[43,44].

Previous studies have shown the impact of diet on DNA methylation patterns. One study found that high-carbohydrate diets may lead to altered methylation patterns associated with obesity and metabolic disorders [45]. This is consistent with our findings that participants consuming a carbohydrate-rich diet exhibited higher Klotho gene methylation percentages (33%) compared to those on a protein-based diet (17%). The inverse correlation observed between fat intake and Klotho gene methylation in the carbohydrate group ($r = -0.765$, $p = 0.05$) suggests that increased fat consumption may reduce the effects of carbohydrates on methylation, potentially through mechanisms related to metabolic pathways and energy balance.

They also showed that dietary patterns may affect methylation levels in genes associated with aging and longevity, including Klotho. Their findings suggested that a diet rich in refined carbohydrates may increase methylation of certain genes, thereby contributing to age-related decline [46]. Our study supported this view by showing a strong positive correlation between carbohydrate intake and Klotho gene methylation ($r = 0.778$, $p = 0.004$). By providing individuals with adequate and balanced nutrition habits, the incidence of diseases that develop as a result of epigenetic changes, especially cardiovascular diseases, diabetes, cancer and obesity, can be reduced and epigenetic changes that cause aging can be eliminated.

As a result of our analysis, it was found that the percentage of Klotho gene methylation in individuals consuming carbohydrates was higher than in those consuming proteins. This data suggests that as carbohydrate intake increases, the methylation level of the Klotho gene also increases. While dietary habits have been shown to affect the Klotho gene methylation level, further studies are needed to better understand the relationship between dietary habits and the methylation level of the Klotho gene and its impact on aging. The findings may not be generalizable to other populations due to the small and homogeneous sample group, which may not reflect differences in age, gender, ethnicity, or other demographic factors. To derive clearer conclusions about the general population, various external factors such as participants' physical activity levels, stress, sleep patterns, or genetic predispositions should also be evaluated. Methylation changes may occur over longer periods; therefore, the long-term effects on DNA methylation should be assessed. Larger and

more diverse studies are required to validate the results.

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