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Evaluation of the association between obesity and global DNA methylation

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Abstract:

Obesity has become a global epidemic at an increasing rate with the potential to be influenced by epigenetic alterations, including DNA methylation. Considering the lack of local studies investigating the influence of global DNA methylation alterations on obesity, the present study was set out to address this issue.

A total of 90 subjects were involved in the present study. Sixty participants were obese (BMI > 30 kg/m^2) while 30 participants were normal body weight (BMI < 25 kg/m^2). The average age was 31.5 years with a range of (20 – 50 years). Following DNA extraction, global DNA methylation levels were assessed using MethylFlash™ Global DNA Methylation (5mC) ELISA Easy Kit. Other obesity related bio-parameters (including insulin, fasting blood sugar (FBS), insulin resistance, lipid profile, liver enzymes, and total antioxidant capacity(T-AOC) were also compared between obese and non-obese subjects.

The levels of global DNA methylation (5mC%) were significantly ($P<0.0001$) reduced in the investigated obese versus normal weight controls (0.348 ±0.01 vs.0.559 ±0.02). In addition to the significantly elevated levels ($P\leq0.001$) T-AOC in the obese subjects in comparison to normal weight controls (0.191 ±0.01vs. 0.132 ±0.01). Lipid profile parameters analysis showed significantly elevated ($P\leq0.01$) cholesterol and LDL in the obese subjects than their normal weight counterparts (191.65 ±3.95 vs. 109.40 ±7.59, and 129.25 ±5.20 vs. 75.93 ±3.22, respectively). The present study showed that the reduced global DNA methylation level is negatively correlated with body mass index of the investigated subjects ($r = - 0.441$, $p<0.0001$). The present study finding may support the involvement of DNA methylation alteration in the obesity pathogenesis.

Key words: Obesity, global DNA methylation, 5mC%, Insulin, HOMA- IR, T-AOC, Lipid profile

تقييم العلاقة بين السمنة ومثيلة الدنا الكلية

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الخلاصة

لقد أصبحت السمنة وباءً عالمياً بوتيرة ظهورمتزايدة مع إحتمال كبيرلتأثرها بالتغيرات اللاجينية، بما في ذلك مثيلة الحمض النووي. ونظراً لعدم وجود دراسات محلية تبحث في تأثير تغيرات ميثلة الحمض النووي الكلية على السمنة، فقد تم إجراء الدراسة الحالية .

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شارك في هذه الدراسة 90 شخصًا (بما في ذلك 60 شخصًا يعانون من السمنة المفرطة و30 شخصًا من الأصحاء، متوسط أعمارهم 31.5 عامًا ويتراوح من 20 إلى 50 عامًا). بعد استخراج الحمض النووي، تم تقييم مستويات مثيلة الحمض النووي العالمية باستخدام MethylFlash™ Global DNA Methylation (5 mC) ELISA Easy Kit. والمعلمات الحيوية الأخرى المرتبطة بالسمنة (بما في ذلك الأنسولين، سكر الدم الصائم(FBS) ، مقاومة الانسولين، ملف الدهون، انزيمات الكبد، ومضادات الاكسدة الكلية.(T-AOC) انخفضت مستويات مثيلة الحمض النووي الكلية (5mC %) بشكل معنوي (P<0.0001) في الافراد الذين يعانون من السمنة مقارنة بغير البدناء (0.348 ±0.01 مقابل 0.559 ±0.02. بالإضافة إلى المستويات المرتفعة بشكل معنوي لمضادات الاكسدة الكلية T-AOC ((P≤0.001)) في الأشخاص الذين يعانون من السمنة المفرطة مقارنة بالأشخاص الأصحاء (0.191 ± 0.01 مقابل 0.132 ± 0.01). أظهرت النتائج ارتفاعًا معنويا (P≤0.01) في نسبة الكوليسترول والبروتين الدهني منخفض الكثافة (LDL) في الأشخاص الذين يعانون من السمنة المفرطة مقارنةً بغير البدناء (191.65 ±3.95 مقابل 109.40 ±7.59، و129.25 ±5.20 مقابل 75.93 ±3.22 على التوالي) .وارتبط مستوى مثيلة الحمض النووي سلبًا مع مؤشر كتلة الجسم(r = − 0.441 (p<0.0001)للأشخاص الذين تم فحصهم. تدعم نتائج الدراسة الحالية دور تغيير مثيلة الحمض النووي في التسبب في السمنة.

Introduction

Obesity is an excessive fat accumulation that presents a risk to health. Indeed, obesity sheds serous health complications such as cancer[1-4] and cardiovascular diseases that could lead to early death[5]. In addition to many other health issues are linked to obesity that impact individuals physically and emotionally. These including infertility where males with body mass index (BMI) exceeded 30 showed abnormal and low sperm quality in comparison to their lean healthy counterparts[6]. This has been interpreted by the influence of fat deposition on the androgens metabolism, e.g. testosterone, that impacts sperm genomic integrity and causes significant alterations in sperm development[7, 8]. In the same vine, obesity can affect female fertility *via* modulating the reproductive hormones, minimizing the opportunity of natural conception, maximizing the risk of miscarriage, congenital anomalies, and increase pregnancy complications[9, 10].Furthermore, obesity has significant influence on social isolation and loneliness, that affect person's physical and mental well-being[11].
Since obesity has reached global epidemic proportions, with approximately three million dying as a consequence of being overweight or obese every year [12]. Thus understanding the biological characteristics could help to solve the obesity puzzle. In this regard, several lines of evidence suggested the involvement of a number of different genes and their products in obesity pathogenesis[13, 14]. Studies have highlighted the contribution of key cellular pathways in obesity that constitute of genetic susceptibility, biological pathways related to metabolism and body weight regulation, and environmental factors. These pathways include hormones, Intra-enteric neuronal signaling, efferent autonomic signaling through efferent vagus nerve pathways, increased free fatty acids (FFAs) signaling pathway, and increased saturated fatty acids (SFAs) signaling pathway[15].

There are a number of genetic potential biomarkers that have been suggested to overt obesity. Of these, fat mass and obesity-associated protein(FTO) that is believed to has indicated role for in nervous and cardiovascular systems with a strong association with body mass index, obesity risk, and diabetes [16]. However, genetic factors alone cannot be blamed for obesity since obesity is highly affected by epigenetic modifications that govern the genome and regulate its transcription machinery[17, 18]. DNA methylation is an epigenetic modification that involves the addition of methyl group (CH3) to C5 position of the cytosine, to form 5-methylcytosine, that is preceded by guanine in the context of CpG site. Normally, most of the CpG sites that map to genes' promoter (also called CpG island) are methylation

free allowing to active transcription to the associated genes. However, DNA hypermethylation of the promoter CpG island associated with transcription inactivation and gene silencing[19, 20]. Interestingly, 91% of the differentially methylated CpGs were hypomethylated in the obese twin compared with the discordant lean monozygotic twin [21]. Similarly, global hypomethylation in the subcutaneous adipose tissue and leucocytes of obese individuals has been observed[22]. Considering the lack of local studies investigating the influence of global DNA methylation on obesity, the present study was set out to assess the level of global DNA methylation, which refers to the total amount of methyl attached to the genome's CpGs, in obese subjects.

Subjects and Methods

Subjects

Blood samples (5ml) were collected from the participants (60ALLcases and 30 healthy controls) Participants were selected based on strict criteria, as any participant who smoked and suffered from type 1 and 2 diabetes, stroke, angina or MI, kidney diseases, blood pressure, eye diseases use of medications (lipid-lowering - aspirin), use of Nutritional supplements and vitamins. All cases (45 females and 15males) were diagnosed at Al Karkh general hospital, Baghdad, Iraq during the period of 1- November 2023 to 30 march 2024 The age average of the participants was (31,5) years (ranging from 20-50 yrs.) and the age average of healthy controls was (31,7) years. The consent was obtained before any samples were taken. All cases diagnosed by the consultant medical staff.

Ethics approval

This study was approved by the Ethical Committee, Department of Biology, College of Science, University of Bagdad and the Iraqi Ministry of Health, Baghdad, Iraq under the reference number CSEC/1023/0086 in October 29,2023.

Blood Sample Collection

Venous specimens of blood were collected from ALL participants (cases and controls) by using a 5 ml disposable syringe. It was divided into two parts, the first part contain total 2ml of blood was transferred to an EDTA tube for, DNA 5mC measurement and the second part contain total 3ml and pushed slowly into disposable serum tubes containing separating gel which was allowed to clot at room temperature for 10-15 min then centrifuged at 3000 rpm for about 10-15 min, then serum distributed in Eppendorf tubes in equal amounts and stored at -20˚C for later use in serological tests.

Methods

Calculation Body Mass Index (BMI)

The body mass index (BMI) is determined according the equation BMI = weight (kg) / height^2 (m) ^{2} for cases and controls groups and classified according to the World Health Organization (WHO) recommendation[23].

Measuring Biochemical Parameters

Lipids Profile, Liver Enzymes:

•The analyses were performed in serum of cases and controls for Lipid profile [Cholesterol – Triglycerides- Low density lipoprotein (LDL)- Very low-density lipoprotein (VLDL)- High density lipoprotein (HDL)]and Liver enzymes [Alanine aminotransferase (ALT)- Aspartate aminotransferase (AST)- Alkaline phosphatase (ALP)] using (AbbottC4000 device) manufactured by Abbott Diagnostics, (USA), is a clinical chemistry analyser. It typically uses photometric technology to measure various biochemical parameters in clinical samples•. Standard procedures were followed according to the user manual.

Fasting Blood Sugar, F.B.S:

Fasting blood sugar was measured after abstaining from eating and drinking (except water) for 8-12 hours using a contour device (mg/dl) from Ascensia Diabetes Care (UAE).
Fasting insulin serum was measured using Sandwich enzyme immunoassay (ELISA) kit: (ELK Biotechnology, Cat: ELK4963, China). Estimation performed following assay Procedure for all samples (cases and controls).
Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR); where HOMA-IR= [(FBG (mg/dL) x fasting insulin (μU/mL))/405].

Measurement of the Levels of Total Antioxidant Capacity (T-AOC)

The levels of Total Anti-oxidants (TAC) in serum of cases and controls were estimated by using Total Antioxidant Capacity (T-AOC) Assay Kit (Cat No: BC1315) Size:100T/96S manufactured by Solarbio (China). In this assay, within acidic environment, a colorimetric reaction takes place to reduce Fe3+-TPTZ into blue Fe2+-TPTZ. This colour reaction reflects the total antioxidant capacity. Estimation performed following assay Procedure for all samples (cases and controls).

Molecular Study

DNA Extraction

Genomic DNA was extracted from the whole blood samples for both cases and healthy controls by using gSYNC™ DNA Extraction Kit. Extraction performed following manufacturer's instructions. The concentration of extracted DNA was estimated using a Quantus Fluorometer (Fluorescence Method) to determine the quality of samples. 200 µl of diluted Quantifluor Dye was mixed with 1 µl of DNA. Then the mixture was incubated for 5-min at room temperature in a dark environment. After that DNA concentration values were determined.

Global DNA Methylation Assessment

In respect to the estimation of global DNA methylation levels in the extracted DNA samples, MethylFlash™ Global DNA Methylation (5mC) ELISA Easy Kit (Catalog # P-1034, Epigentek, USA) was utilized. This was performed by diluting 100ng of the extracted genomic DNA from each tested sample in the supplied binding solution provided with an eight-well-assay strips kit. In principle, the DNA methylation fraction that binds to the well-assay strips monoclonal antibodies is captured to be detected by the subsequent assay steps. These included the addition of wash solution, detection antibody, enhancer solution, developer and stop reaction solution. Ultimately, global DNA methylation quantification was calculated as proportional to the OD intensity read (at 450nm) using micro-plate reader (Thermo Fisher Scientific Inc.), based on the manufacturer instructions. Global DNA methylation percentage was proportionally measured by subtracting the OD of the positive controls, supplied by the kit, from the OD of each tested sample. To ensure obtaining reliable generated signals, all of the analysed samples were run in duplicate, along with the use of positive and negative controls provided by the kit.

Statistical Analysis:

In this study, a t-Test was used to compare the means between different groups (patients and controls) using the Statistical Analysis System (SAS, 2018) program to assess the effect of group differences on study parameters. Pearson correlation coefficients were calculated using GraphPad Prism version 8 to evaluate the relationship between global DNA methylation levels (5-mC%) and body mass index (BMI). A t-Test was also applied to assess the effect of global DNA methylation (5mC%) on gender among obese and control groups, as

well as to investigate the impact of BMI on global DNA methylation levels in both groups. Additionally, Microsoft Excel 2013 was used to perform t-Tests for comparing the means between 5mC% and several important parameters, including insulin, HOMA-IR, triglycerides, and ALT.

Results

Obesity is Associated with Global DNA Hypomethylation

The levels of global DNA methylation (5mC %) were significantly (P<0.0001) reduced in the investigated obese *versus* none obese healthy controls. This is was evident when the epimethylome of the investigated obese subjects showed to loss DNA methylation by approximately 44.87% than that of non obese (0.348 ±0.01 *vs.*0.559 ±0.02, Figure1). The present study finding support the involvement of DNA methylation alteration in the obesity pathogenesis.

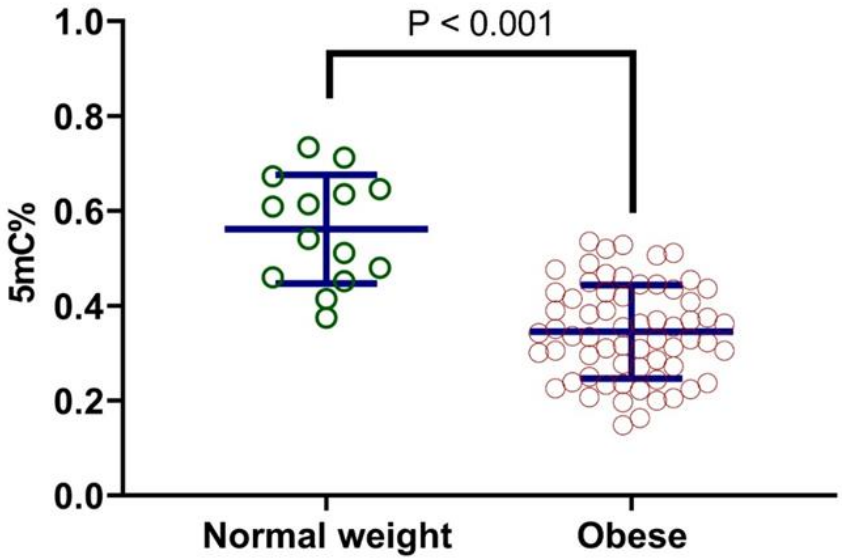

Figure 1: Global DNA methylation levels (5mC%) in high obese subjects and control group(normal weight).

Regarding to the results of the assessed obesity-related metabolic parameters, the present study finding have demonstrated significantly elevated levels ((P≤0.001) of F.B.S and T-AOC in the obese subjects in comparison to the controls (97.73 ±1.46 *vs.* 92.30 ±1.05, and 0.191 ±0.01*vs.* 0.132 ±0.01, respectively, Table 1).Whereas no significant differences were observed in respect to the levels of insulin and HOMA-IR between the two assessed groups (P≥0.05, Table 1).

Table 1: Comparison between obese and the control groups in 5mC, insulin, FBS, HOMA-IR, and TAOC.

Group	Means ±SE				
	5mC (%)	Insulin (µU/mL)	F.B.S. (mg/dl)	HOMA-IR	T-AOC (umol/mL)
Obese	0.348 ±0.01	2.849 ±1.01	97.73 ±1.46	0.734 ±0.29	0.191 ±0.01
Control	0.559 ±0.02	3.158 ±0.46	92.30 ±1.05	0.711 ±0.10	0.132 ±0.01
t-Test	0.0462 **	2.907 NS	4.381 *	0.827 NS	0.020 **
P-value	0.0001	0.833	0.0157	0.955	0.0001

*(P≤0.05) ** (P≤0.01), NS: Non-Significant

Significantly Elevated Cholesterol and LDL Levels in the Assessed Obese Subjects

Lipid profile parameters analysis showed significantly elevated (P≤0.01) cholesterol and LDL in the obese subjects than their healthy counterparts (191.65 ±3.95 *vs.* 109.40 ±7.59, and 129.25 ±5.20 *vs.* 75.93 ±3.22, respectively, Table2. In respect to the triglycerides level, it was higher in obese subjects than in healthy counterparts (135.71 ±9.29 vs. 105.47 ±6.82, respectively); however, the differences were not significant (P≥0.05). Slightly lower levels of VLDL were recorded in the obese subjects than that of the controls (27.27 ±2.24 vs. 29.46 ±1.10, respectively). No differences were identified in respect to the levels of HDL which were almost the same in both obese and the controls groups (Table).

Table 2: Comparison between obese and control groups in lipid profile.

Group	Means ±SE (mg/dl)				
	Cholesterol	**Triglycerides**	**HDL**	**LDL**	**VLDL**
Obese	191.65 ±3.95	135.71 ±9.29	45.86 ±1.83	129.25 ±5.20	27.27 ±2.24
Control	109.40 ±7.59	105.47 ±6.82	45.80 ±1.19	75.93 ±3.22	29.46 ±1.10
t-Test	17.492 **	37.808 NS	7.431 NS	21.1205 **	9.053 NS
P-value	**0.0001**	0.115	0.987	**0.0001**	0.631

** (P≤0.01), NS: Non-Significant.

Interestingly both genders of the investigated obese subjects exhibited lower DNA methylation levels than that of their non-obese counterparts. The levels of total 5mC% were significantly lower in obese males than that of the controls male individuals (0.31± 0.09 *vs.* 0.66± 0.1, respectively, Figure 2). Similarly obese females showed to have drooped DNA methylation level in comparison to their sex matched healthy controls (0.36± 0.09 vs. 0.52± 0.09, respectively, Figure 2). This is suggesting potential general role for the contribution of DNA methylation alterations in obesity.

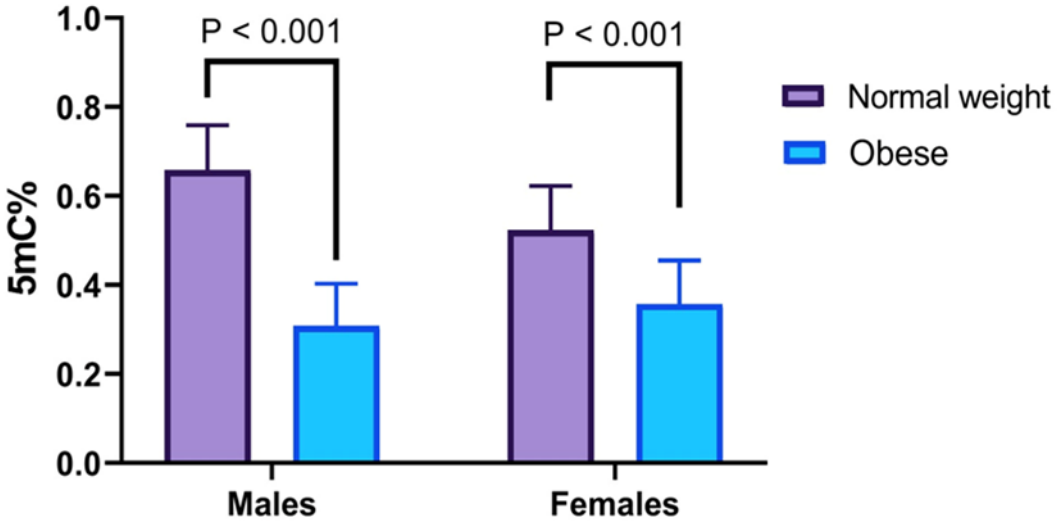

Figure 2: Global DNA methylation levels (5mC %) of the studied obese and their controls by sex.

Individuals with a high BMI have significantly lower global DNA methylation levels compared to those with a normal BMI. The p-value of <0.001 indicates a statistically significant difference, suggesting that the variation in methylation levels between the two BMI categories is not due to chance. This indicates a potential epigenetic impact of BMI on DNA methylation patterns, which could have implications for understanding the metabolic and health outcomes associated with elevated BMI (Figure3).

The graph illustrates a significant decrease in global DNA methylation (5-mC%) as obesity severity increases from normal weight to Class 3 obesity, with the methylation percentage dropping notably between the groups, confirmed by a highly significant p-value ($P < 0.001$). This trend suggests a potential link between higher obesity classes and reduced DNA methylation levels , Figure 3).

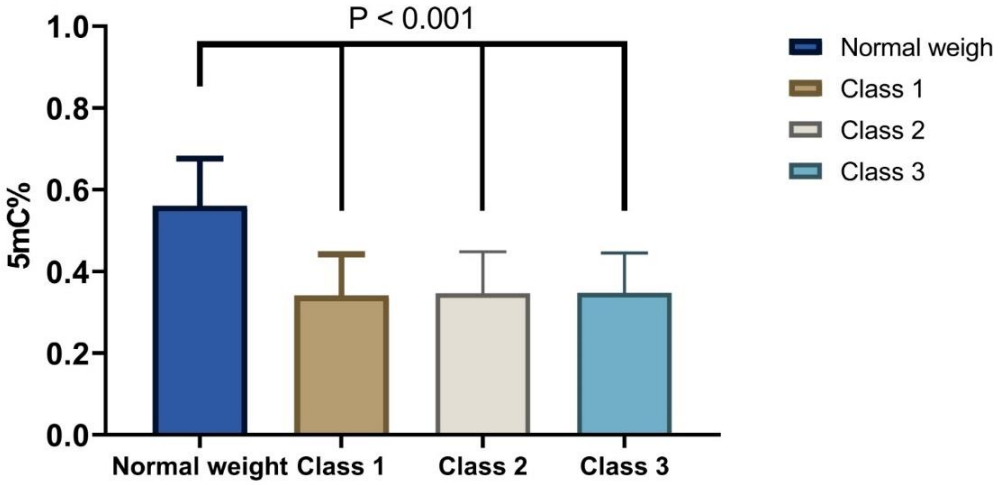

Figure 3: Comparison of global DNA methylation (5mC%) across different obesity Classes (Based on the BMI categorization)

The Reduced Global DNA Methylation Levels are Negatively Correlated with Body Mass Index of the Assessed Subjects

The present study showed that the reduced global DNA methylation level is negatively correlated with body mass index ($r = -0.441$, $p<0.0001$, Figure 4) of the investigated subjects.

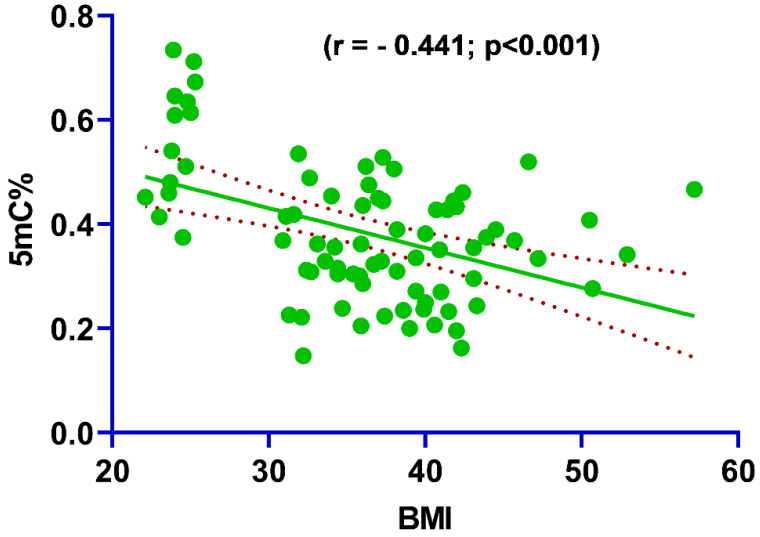

Figure 4: Pearson product-moment correlation coefficient between 5mC% and BMI ($r = -0.441$, $p<0.0001$) of the assessed subjects.

Of interest, the present study findings have proven that the loss of DNA methylation in obese subjects is significantly ($P \leq 0.05$) affects obesity-related physio- pathological parameters (Table1). This is was evident when individuals with very low global DNA methylation (<0.25%)) showed to have higher average levels of insulin, insulin resistance (HOMA-IR),

and ALT (U/l) in comparison to those with relatively higher 5mC% levels (>0.25%-< 0.50%). The insulin average levels exhibited approximately 3.6 folds increase in the assessed obese subjects with lower global DNA methylation than those with comparatively higher ones (7.19 vs. 1.53 µU/mL). Similarly, insulin resistance average levels were increased by more than four folds (1.94 *vs.* 0.37) in the obese individuals having hypo methylated epigenome than those with increased DNA methylation levels. ALT average levels were also increased significantly (P≤ 0.05) in obese subject with very low global DNA methylation levels in comparisons to those with relatively higher levels (38.26 *vs.* 25.59 UL). Similar trend of increasing obesity-related parameters was also observed for the triglycerides levels in obese subjects having lower global DNA methylation levels than those who are not (150.86 vs. 131.10 mg/dl), however, the differences did not reach the significant level (P≥ 0.05, Table 3).

Table3: Obesity-related parameters average levels according to the 5mC% status of the assessed obese subjects

5mC% status	Insulin (µU/mL)	HOMA-IR	Triglycerides (mg/dl)	ALT (U/L)
Low (<0.25%)	7.19	1.94	150.86	38.26
High (>0.25-< 0.50%)	1.53	0.37	131.10	25.59
t-Test (P-value)	0.029	0.020	NS	0.034

Discussion

Globally, the prevalence of obesity has increased markedly especially among children and young adults due to the tendency of consumption of greasy, high calories of fast and processed food that suit the rhythm of modern life[24, 25]. As obesity has a significant adverse impact on different aspects of human health. Thus, understanding the underlying obesity-associated biological alterations could help with the management of this devastating health issue. Epigenetic modifications, including DNA methylation, are considered as the genome guardian and regulator where the transcription activity and gene silencing of key cellular processes/ pathways are tightly controlled by the epigenetic marks[26]. As far as we aware, no pervious local study has addressed the association between global DNA methylation and obesity. Accordingly, the present study was set out to provide an insight about how the epimethylome profile of obese subject is differ in comparison to that of their healthy counterparts. The present study results showed a significant reduction (P= 0.0001) in the levels of global DNA methylation, as it is assessed by the 5mC%, in the obese individuals than the control group. This global DNA hypomethylation demonstrated by the obese subjects in this study seemed consistence with the findings of previous studies that reported hypo methylated signature to the genome of the assessed obese in comparison to those who are not[27-29].

Several lines of evidence support the involvement of epigenetic alterations in the obesity pathogenic. Of these the fat cell epigenetic signature in post-obese women is characterized by global hypo methylation and differential DNA methylation of adipogenesis genes[30]. This was also experimentally proven because high-fat diet induced obesity alters *DNMT1* and *DNMT3A* levels and global DNA methylation in mouse ovary and testis[31].Moreover, studies have identified an inverse association between global DNA methylation and weight loss depending on individual genetic variants for different genes[32].

The current study demonstrated a significantly negative association of global DNA methylation with obesity physio-clinical features including BMI, insulin, HOMA-IR, ALT, cholesterol and LDL. This suggests that the present study finding, if it is validated in larger cohort of obese cases, may contribute to the future development of detection and therapeutic approaches, pending further validation. Furthermore, obese cases with lower levels of global

DNA methylation showed to have significantly higher levels of T-AOC. This could be interrupted by the fact that central obesity causes increase secretion of inflammatory markers such as cytokines which in turn enhance ROS production[33, 34].

Conclusion

Overall, the present study findings showed a link between low methylation and the development of obesity which could suggest relationship between hypomethylation and the pathophysiology of obesity that is potentially impact key cellular components and pathways including insulin resistance, T-AOC, ALT, cholesterol, and LDL that are consider as crucial modifiers of the body of metabolically healthy obese. Large-scale studies are recommended to validate the relationship between global hypomethylation and obesity development.

Conflict of Interest: "The authors declare that they have no conflicts of interest."

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