Investigation of the potential association between the levels of global DNA methylation, vitamin B12 and type 2 diabetes in Iraqi patients

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Abstract---Type 2 diabetes mellitus (T2DM) is an epidemic metabolic disorder that has life threatening health complications if left undetected and untreated. Understanding its molecular landscape alterations, especially DNA methylation that regulates the genome transcription activity, would aid with the prevention and management of this disease. This study was set to investigate the global DNA methylations (assessed by measuring 5mC level), along with other disease-related clinical features, including body mass index (BMI) and serum vitamin B12, in T2DM patients in comparison to healthy controls. One hundred subjects (70 T2DM patients and 30 healthy controls) who attended Baghdad Teaching Hospital/ Department of Consulting Clinic - Endocrinology and Diabetes Consultant and the National Diabetes Center, Al-Mustansiriyah University, Baghdad, Iraq. Global DNA methylation levels were assessed using Methyl flash™ Global DNA methylation (5-mC) ELISA Easy Kits. The results showed significant increase (P=0.0018) in the 5mC level of T2DM patients in comparison to the healthy subjects (0.578±0.208 vs. 0.422±0.110, respectively). The global DNA methylation levels were found to be elevated for all of the assessed BMI categories in T2DM patients in comparison to those of the control group. Significant reduction (t-Test , P= 2.779*10^-15) was also noted in B12 levels in T2DM patients as compared to that of the healthy controls (311.886± 101.494 vs.
628.174 ± 203.279, respectively). Overall, the obtained results suggest an involvement of DNA methylation changes in T2DM pathogenesis, with significant impacts to adiposity and vitamin B12 deficiency.

**Keywords**--- T2DM, DNA methylation, vitamin B12.

**Introduction**

Diabetes mellitus (DM) is a metabolic disorder distinguished by blood hyperglycemia. The International Diabetes Federation (IDF) statistics indicate that the global number of patients with diabetes increased to 382 million in 2013. It is likely that this number will reach to 592 million by 2035 (Shan et al., 2015). It is generally believed that this health problem is resulting from deficiency in the secretion of insulin and/or defect in its function (Qiu et al., 2021). DM is classified as a chronic disease that causes imbalance in the metabolism and can put the macro/microvasculature at high risk of long-term complications, leading to vascular and heart diseases (Osman et al., 2016). Other serious diabetic-associated complications include nephropathy, neuropathy, blindness, ischemic heart disease, stroke, and early death (Reddy, 2017). Common symptoms of diabetes include polydipsia, weight loss, polyuria, and occasional polyphagia (Marathe et al., 2017). Depending on the etiology and clinical characteristics, DM is classified into four major categories (Type 1 diabetes, also known as juvenile diabetes, Type 2 diabetes, Gestational diabetes mellitus and MODY diabetes) (Phillips et al., 2019, Eizirik et al., 2020).

Type 2 diabetes mellitus (T2DM) is a global pandemic disease marked by abnormality or impairment in the regulation of the metabolism of carbohydrates, lipids, and proteins (DeFronzo et al., 2015). The development of T2DM can be related specifically to a combination of reduced insulin secretion from β-cells in the pancreas and insulin resistance of the peripheral target tissues; mostly muscle and liver (Singh, 2011, Eberle and Stichling, 2021). This type of diabetes, formerly referred to as insulin-independent diabetes or type 2 diabetes, affects individuals who have insulin resistance and usually have relative, rather than absolute, insulin insufficiency (Podell et al., 2017, Kobalava et al., 2019). Not all diabetes patients require insulin treatment to survive at the beginning of their diagnosis (Mellitus, 2005). But in advanced clinical cases, T2DM patients rely on insulin treatment, especially those who have significant problems with atherosclerotic cardiovascular disease (ASCVD) and nephropathy, to control their blood glucose (Lingvay et al., 2021). Recent studies have reported that children and adolescents are not safe from this disease due to high levels of obesity and overweight (Abarca-Gómez et al., 2017).

The pathophysiology of type 2 diabetes is known to be influenced by both genetic and epigenetic factors (Davegårdh et al., 2018). Major advances have been made throughout the past few decades in understanding the pathophysiology of T2DM and glucose homeostasis. Recent studies have provided additional evidence with respect to the interactions of many environmental and genetic factors that have remarkable contributions to the development of T2DM by causing particular degrees of dysfunction in pancreatic β-cells as well as the development of insulin
resistance (Zhou et al., 2018, Kim et al., 2021). Epigenetic alterations, especially aberrant DNA methylation marks, have been linked to the pathophysiology of T2DM and other complicated metabolic illnesses via modifying gene expression (Zierath and Barrès, 2011, Ling and Rönn, 2016). Comprehensive studies on the pancreatic islets have identified differential CpG sites that map to genes responsible for regulating pancreatic cell function. Such findings implicate the importance of epigenetic alterations in the pathogenesis of T2D (Volkmar et al., 2012, Dayeh et al., 2014). Also it has been observed that DNA methylation is altered in several tissue types of patients with T2DM. Furthermore, aberrant DNA methylation patterns are thought to contribute to many T2DM-related complications, such as retinopathy, nephropathy, and micro or macrovascular disease (Guo et al., 2019). Accordingly, altered DNA methylation patterns in the adipose tissue, pancreatic islets and leukocytes have been shown to be associated with T2DM (Toperoff et al., 2012, Crujeiras et al., 2017).

Vitamin B12, also recognized as cobalamin, is a necessary micronutrient for human metabolism (Butola et al., 2021) and plays an essential role in DNA synthesis, optimal hemopoiesis, and neurological functions (Yadav et al., 2018, Lata Kanyal and Mujawar, 2019). Its key physiological effects are manifested in mediating two major enzymatic pathways, which are the methylation of homocysteine into methionine and the transformation of methylmalonyl CoA into succinyl CoA. It also contributes to the transformation of dietary folate (methyltetrahydrofolate) into its active metabolic form, the tetrahydrofolate (THF), which is used in the synthesis of purines and pyrimidine's (Badyal and Kumar, 2018, Shailendra et al., 2018). Vitamin B12 is of great metabolic importance, as the deficiency of this micronutrient leads to the accumulation of intracellular homocysteine and the disruption of its methylation (Badyal and Kumar, 2018). B12 deficiency is most commonly related to pernicious anemia, neurological impairment, and hyperhomocysteinemia. Hyperhomocysteinemia is a risk factor for cardiovascular disease and obesity-related complications, including T2DM (Yadav et al., 2018). Recent studies have indicated that vitamin B12 insufficiency may affect more than half of T2DM patients. For that reason, the American Diabetes Association (ADA) recommends that vitamin B12 levels in patients with DM who are using metformin should be checked on an annual basis (Didangelos et al., 2021).

The lifestyle of diabetic patients must be taken into account because it clearly affects both DNA methylation and type 2 diabetes. DNA methylation has been proposed as an interesting molecular mechanism through which diet and exercise immediately affect the transcriptome (Ling and Groop, 2009, Brøns et al., 2010, Milagro et al., 2011). It is generally believed that the development of diabetes is caused by a combination of risk factors, mainly as a result of the interaction between genetics and environmental factors, including lifestyle. In line with the thought that the epigenome is more vulnerable than the genome for such environmental insults, we found it interesting to investigate the potential association between the epigenetic changes, especially DNA methylation, and the clinical/biochemical features of diabetic patients.
Subjects, Materials and Methods

This case-control study was performed at the Postgraduate Laboratories, Department of Biology/College of Science/University of Baghdad, Baghdad Teaching Hospital/Department of Consulting Clinic-Endocrinology and Diabetes Consultant, and the National Diabetes Center, Al-Mustansiriyah University, Baghdad, Iraq. The study cohort included 100 participants and divided into two groups; the first group included 70 T2DM patients while the second consisted of 30 healthy subjects. The average age of the participants was 51.75±22 years (range 22-74 years). The study design was approved by the Scientific Committee designated by the Department of Biology and the Research Ethical Committee (No. CSEC/0921/0050), College of Science, University of Baghdad. Venous peripheral blood (10 ml) was drawn from diabetic patients and healthy control subjects. After that, the blood samples were put into tubes containing EDTA for DNA extraction and HbA1c measurement and other tubes without anticoagulants for biochemical tests. Serum was used for the assessment of FBS and Vitamin B12 levels.

Determination of HbA1c levels was performed using Tosoh G8 Automated Glycohemoglobin Analyzer HLC–723G8, Japan. This is an automated HPLC system that separates and reports stable A1C (sA1C) percentage in whole blood. The determination of FBS was performed by using an automated SELECTRA PRO XL device with glucose kit (GLUCOSE GOD- PAP, provided by BIOLABO, France) based on the Trinder method, also known as the glucose oxidase (GOD) method, which depends on enzymatic colorimetric reactions. An automated ELISA (Immunolight Device) was used to determine serum Vit.B12 concentration by utilizing Human Vitamin B12 (VB12) ELISA kit (MyBiosource, Southern California, San Diego, USA). Following DNA extraction from venous blood samples, the level of global DNA methylation was estimated using the Methyl flash™ Global DNA methylation (5-mC) ELISA Easy Kits (Epigentek Group Inc, USA) according to the manufacturer’s protocol. Statistical analysis was performed by using SPSS version 26 and Microsoft Excel for data entry and analysis.

Results

Assessment of the 5mC levels of total DNA (global DNA methylation) in the blood of the investigated T2DM patients showed a significant increase (t-Test, P=0.0018) in comparison to their healthy counterparts (Figure 1). The average level of 5mC was higher by 26.984% in T2MD subjects than that in the control group (0.578±0.208 vs. 0.422±0.110, respectively).
Figure 1: Blood 5mC levels (global DMAL methylation) in the investigated T2DM patients and healthy controls. Data are presented as mean ± standard deviation (SD). **P<0.01

Further detailed analysis showed a significant increase (t-Test, P=0.0012) in the levels of 5mC of total DNA in males with T2DM in comparison to their healthy control counterparts (Figure 2), with an average increase by 61.7729% (0.584±0.195 vs. 0.361±0.125, respectively). On the other hand, although the average level of 5mC was higher by approximately one fifth (18.7%) in females with T2DM than that of healthy females, these differences were not statistically significant (0.574±0.220 vs. 0.466±0.169, respectively; t-Test, P=0.139).

Figure 2: Blood 5mC levels in the investigated T2DM and the healthy control group according to sex(M=Males, F=Females). Data are presented as mean ± standard deviation (SD). **P<0.01

Of interest, 5mC levels were observed to increase in T2DM patients aged 40 years and older. However, in the healthy control group, the levels exhibited a gradual
rise between the ages of 30 and 50 years, followed by a slight decrease in the older ages (older than 50-74 years) (Figure 3).

With respect to the association between BMI and the levels of 5mC, our study results showed that the level of global DNA methylation was higher in T2DM patients for all of assessed BMI categories than that of the control group (Figure 4). T2DM patients from the healthy BMI category exhibited approximately a three folds increase in the 5mC levels as compared to that in their control counterparts (0.622 ± 0.187 and 0.255 ± 0.035, respectively, t-Test, P=0.0005). Within the overweight BMI category (BMI= 25.0—29.9), the total DNA methylation levels were also higher by 28.56% in T2DM patients in comparison to that of the control group (0.597± 0.195 vs. 0.464 ±0.176, t-Test, P=0.02). While among subjects categorized within the obese BMI (30.0 and above), 5mC raised by about 15% in T2DM patients in comparison to that of the control group (0.538± 0.237 vs. 0.470±0.097).
Regarding the levels of serum vitamin B12 levels, the results showed a significant reduction (t-Test, \(P = 2.779 \times 10^{-15}\)) in T2DM patients compared with that of the healthy controls (311.886± 101.494 vs. 628.174± 203.279, respectively), implying a reduction by about 50% (Figure 5). A Pearson correlation coefficient was computed to assess the linear relationship between Vit.B12 and 5mC. There was a negative correlation between the two variables \((r = -0.141, p = 0.242)\).

![Figure 5: Boxplot of the average serum vitamin B12 levels in T2DM patients in comparison to their healthy controls](image)

**Discussion**

As diabetes has reached epidemic proportions worldwide, efforts need to be concentrated toward understanding such health threat, especially at the molecular level. It is well-acknowledged that the development of diabetes can be attributed to multiple risk factors. These include weight, fat distribution, blood lipid levels and physical inactivity; these factors seem to be largely influenced by how healthy the life style is for the affected subjects. At the molecular level, the transcription activity of the genome is governed and tightly regulated by the epigenome. In this regard, aberrant epigenetic marks are known to be associated with almost all diseases, including diabetes. However, establishing the features and directionality of DNA methylation changes could be of great benefit in disease prevention, early detection, progression, and management. Accordingly, the present study was set to investigate the global DNA methylations (assessed by measuring 5mC level) in T2DM patients in comparison to healthy controls.

Our study results showed significantly higher levels of 5mC in T2DM patients than in their healthy counterparts. Furthermore, the findings of the present study highlight that overweight T2DM patients have significantly increased levels of 5mC compared with their control counterparts. This finding may implicate both causality and directionality effects of global DNA methylation changes in obesity and T2DM pathogenicity. This finding seems to have consistence with that of Wahl and colleagues, who suggested the influence of adiposity on DNA methylation (Wahl et al., 2017). Considering the widespread of overweight and
obesity around the world, it is quite interesting to explore how obesity interrupts the epigenome through altering the pattern of DNA methylation. Several lines of evidence hold the notion that DNA methylation changes may be implicated in the pathogenesis of obesity and T2D (de Mello et al., 2014). Indeed, genome wide DNA methylation studied have revealed differentially methylated genes, including CDKN1A, PDE7B, SEPT9 and EXOC3L2, that were differentially expressed in T2D islets, along with perturbed insulin and glucagon secretion in clonal β- and α-pancreatic cells (Dayeh et al., 2014). Global DNA methylation was shown to be increased in tissues of obese and insulin-resistant (IR) individuals and the methylation levels were correlated positively with IR (Malodobra-Mazur et al., 2019).

Interestingly, the investigated T2DM patients in our study exhibited significant reduction in vitamin B12 levels as compared to the healthy controls. The average level of B12 was found to be lower by about 50% in T2DM patients than that of their healthy counterparts. Recent studies indicate that vitamin B12 levels are linked to global DNA methylation in T2DM patients, suggesting a possible role of vitamin B12 in the mechanism of methylation (Tanwar et al., 2020). The potential relation between the levels of vitamin B12 and DNA methylation was revealed by an elegant experiment conducted by Mahajan et al., who elucidated the effects of the altered dietary ratio of B12 on the expression of transporters and related miRNAs and DNA methylation in C57BL/6 mice. Deficient Vit.B12 mice in both F1 and F2 generations showed an increased the activity of DNA methyltransferase (DNMTs, catalyzing the addition of a methyl group onto cytosine residue and responsible for genomic DNA methylation) (Mahajan et al., 2019). The increases in global DNA methylation level following the modulation of Vit. B12 diet is an evidence of how the epigenetic landscape is altered in response to nutritional influences. Such modulation to epimethylaome was also reported in experiments of endurance training intervention for a relatively short period, 3 months, which resulted in altering the methylation patterns of genes related to the development of the central nervous system, neurogenesis, and neuron differentiation and linked to numerous diseases, such as schizophrenia and Parkinson’s disease (Denham et al., 2015). Further support to the notion that the main factor driving epigenetic remodelling is the induction by external influences came from studies that focused on the influence in lifestyle (Nilsson and Ling, 2017). Collectively, the results presented in our study suggest an involvement of DNA methylation changes in type 2 diabetes pathogenesis, mediated by adiposity and vitamin B12 deficiency. This could be a useful tool to identify individuals at risk for developing the disease and contributes to the development of novel treatments.

References


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