Assessment of global DNA methylation in peripheral blood of type 2 diabetic patients

Amjad Yousuf

ABSTRACT

Type 2 diabetes mellitus (T2DM) is a challenge to global health. About 400 million people are affected globally, and this figure is expected to skyrocket steeply in the coming decades. T2DM patients are at risk of developing serious and life-threatening problems, necessitating increased medical attention, and detecting undiagnosed diabetes and preventing it is particularly important. T2DM's etiology and molecular causes have yet to be fully determined. Its development could be influenced by epigenetic alterations. Hence, in this work, the level of 5-methylcytosine (5mC), has been determined in DNA from the peripheral blood of T2DM patients. Global DNA methylation level was analyzed in peripheral blood leucocytes from 200 patients with T2DM and 120 control participants using a Global DNA Methylation Assay Kit. Levels of global DNA methylation increased in both controlled and uncontrolled T2DM patients compared to healthy controls. However, only the difference in global DNA methylation levels between uncontrolled patients and healthy subjects was statistically significant. In addition, global DNA methylation statistically insignificantly increased in uncontrolled DM patients compared to controlled DM patients. Clinical and biochemical factors had no effect on methylation levels in T2DM patients' DNA. This study has shown that T2DM patients have a higher level of Global DNA methylation on their peripheral blood leucocytes than healthy people, and the degree of methylation is significantly higher in uncontrolled T2DM patients showing that methylation is affected by the level of disease control.

Keywords: T2DM, Epigenetics, Methylation, 5-methylcytosine, Oxidative stress

1. INTRODUCTION

The rapid escalation of type 2 diabetes mellitus (T2DM) prevalence represents a substantial burden on global health and is becoming a major challenge for public health systems. Hence, significant efforts have been made in previous decades to identify the underlying pathological mechanisms of this disease. The International Diabetes Federation reported that 463 million people worldwide were diagnosed with diabetes in 2019, approximately 90% of those cases being T2DM, with the number of diabetes instances expected to reach 700 million by 2045 (Saeedi et al., 2019). T2DM is a chronic illness that affects
various body organs and is characterized by nutrition metabolism dysregulation due to body cells’ resistance to insulin (Saeedi et al., 2019). The disease used to be most common in older persons, but due to increasing obesity levels related to changing lifestyles, such as unhealthy diet and lack of exercise, it is becoming more prevalent in children and young adults (Saeedi et al., 2019). The correlation between growing urbanization, changing lifestyle, and a rapid rise in T2DM prevalence around the world in the recent decade implies that environmental factors are the primary cause of T2DM occurrence.

T2DM is thought to be the outcome of an intricate interaction between environmental and genetics factors (Mambiya et al., 2019). Despite the advances in molecular genetic research methods (Brunetti et al., 2014), elucidating the interactions between environmental and genetic factors in diabetes and its complications is still poor. In contrast to genetic alterations that result in a change to the DNA sequence, epigenetic alterations are susceptible to environmental factors. Epigenetic modification is defined as alterations in genome expression and function that do not result from changes in the DNA sequence. Because epigenetics could explain the interplay between genetics and environmental factors, this is a promising field that may help broaden our understanding of T2DM’s pathophysiology (Karachanak-Yankova et al., 2016).

The most studied epigenetic mechanism is DNA methylation process, primarily at CpG dinucleotides (Suzuki et al., 2008). DNA methylation is implicated in the development of several diseases, with T2DM being one of them, because it directly influences the function of a gene by either activating or silencing its expression (Raciti et al., 2021). DNA methylation changes are reversible but heritable, and known to have a control on metabolic memory. Regarding this memory, patients with a poor history of T2DM control were shown to have epigenetic alterations that persist in subsequent years and predispose them to the development of various complications (Sommese et al., 2017).

Several studies (Akirav et al., 2011; Hidalgo et al., 2014; Toperoff et al., 2012; Volkmar et al., 2012; Wang et al., 2020) have examined the association of gene-specific or global DNA methylation, the total level of 5-methylcytosine (5mC) relative to total cytosine in the genome, with T2D risk in different body tissues. Despite the extensive research on DNA methylation in pancreatic islets cells and insulin-response tissues, it is still difficult to monitor human tissues in vivo, as it is in clinical practice. Peripheral blood could be a target for methylation studies that may discover biomarkers of significant clinical utility due to the ease of obtaining blood, particularly during a routine medical examination. In addition, the changes to DNA methylation in pancreatic islets and insulin-response tissues of T2DM patients were reported in the blood (Wang et al., 2020). This study assessed the association of peripheral blood leucocytes’ global DNA methylation with T2DM in the Saudi population.

2. MATERIALS AND METHODS

Study design
This case control study was performed between April and December 2021 on Saudi subjects (200 T2DM patients and 120 healthy participants). Patients in the T2DM group were being treated at diabetes clinic at Prince Mohammed bin Abdulaziz Hospital (PMAH), National Guard Ministry, Madinah, Saudi Arabia. Healthy participants were visitors who were accompanying patients following up at the same hospital. The following inclusion criteria were applied: (i) T2DM confirmed patients as defined by the American Diabetes Association, (ii) of Saudi origin, and (iii) of ≥ 30-year age. The study excluded anyone with non-Saudi origin, metabolic syndrome, type 1 diabetes, or gestational diabetes. Participants in the control group had to be healthy, without diabetes and renal diseases, and have normal glucose and glycated hemoglobin levels. All participants’ personal information including weight and height was collected using an informed consent form. The guidelines of the Helsinki Declaration (1975) were followed in the study.

Blood samples collection and biochemical measurements
A total of 5 mL of whole blood was collected in plain and EDTA containing tubes from each patient. Blood samples in plain tubes were centrifuged to get serum for fasting blood glucose (FBG) measurements, while blood samples in EDTA containing tubes was used to measure HbA1c level and extract genomic DNA. Levels of HbA1c and FBG were measured at the Clinical Chemistry Laboratory at PMAH.

Nucleic acid extraction and quantification of DNA methylation
The kit Magnetic Beads gDNA Kit for blood (Geneaid, Taipei, Taiwan) was used to extract genomic DNA from 200 μL of blood following the manufacturer’s protocol. The quality and quantity of DNA were determined using the NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA was kept in -20° C freezer until further analysis. Global methylation of 5mC for DNA was analyzed by a Global DNA Methylation Assay Kit (5mC, ab233486, Abcam).

Medical Science, 26, ms160e2249 (2022)
Briefly, DNA-binding solution was mixed with DNA from study participants, six serial dilutions of positive controls, and the negative control. After 60 min incubation at 37 °C for 60 min, three times wash with 150μL of the 1X diluted washing buffer was performed. Then, 50μL of 5mC detection complex (containing anti-5mC antibodies enhancer and signal indicator were pipetted into to each well and incubated at room temperature for one hour. Subsequently, five times wash was performed, and 100μL developer solution was pipetted into each well. After three-minute incubation at room temperature until, 100μL of stop solution were added to each well and the absorbance was read at 405 nm wavelength using a spectrophotometer (ELX800, BioTek, Winooski, VT, USA).

**Statistical analysis**

SPSS statistical software Version 27 (IBM Corp., Armonk, NY, USA) for windows was used to for statistical analysis. Mean age, biochemical parameters (blood glucose and HbA1c), BMI and methylation levels were compared between the groups by applying Student’s t-test. Comparisons of means of the different parameters across more than 2 groups were performed using one-way analysis of variance (ANOVA). For non-parametric variables, Spearman’s rank correlation coefficient (r) was used to determine the power of dependence between variables. A p-value of < 0.05 was considered statistically significant.

**3. RESULTS**

Analysis of global blood DNA methylation was done in the study groups, healthy controls, controlled DM patients, and uncontrolled DM patients. Table 1 show the anthropometric and biochemical characteristics of the study participants. No statistical differences found between the study groups in age and gender. In healthy controls, 76 subjects were males, and 44 subjects were females; the mean age was 45.03 years. In controlled DM patients, 64 subjects were males, and 36 subjects were females, and the mean age was 44.48 years, whereas in uncontrolled DM patients, 61 subjects were males, and 39 subjects were females, with a mean age of 43.56 years. As expected, there was a significant increase in the FBG and HbA1c levels of DM patients compared to controls (P < 0.05). The uncontrolled DM group showed levels of FBG and HbA1c that were significantly higher than the controlled DM and healthy control groups (Table 1). In addition, BMI was significantly higher in DM patients, controlled and uncontrolled groups, than in healthy controls.

**Table 1** Anthropometric and biochemical characteristics of the study groups

<table>
<thead>
<tr>
<th></th>
<th>Healthy Control (n=120)</th>
<th>Controlled DM (n=100)</th>
<th>Uncontrolled DM (n=100)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Overall P-value</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>45.03 (10.79)</td>
<td>44.48 (9.29)</td>
<td>43.56 (8.75)</td>
<td>0.534</td>
</tr>
<tr>
<td>Gender n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>76 (63.33%)</td>
<td>64 (64%)</td>
<td>61 (61%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>44 (36.67%)</td>
<td>36 (36%)</td>
<td>39 (39%)</td>
<td></td>
</tr>
<tr>
<td>FBG (mmol/l)*</td>
<td>5.33 (0.89)</td>
<td>6.97 (1.58)</td>
<td>9.02 (4.14)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.26 (0.63)</td>
<td>6.41 (0.50)</td>
<td>9.29 (1.23)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>28.49 (4.21)</td>
<td>30.41 (5.09)</td>
<td>31.66 (5.0)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Mean (standard deviation)

*P < 0.05

Regarding global blood DNA methylation levels, all patients in the DM group showed statistically insignificant higher levels compared to the healthy control group (P = 0.330) (Fig. 1A). However, classifying DM patients according to the glycemic index, which reflects the metabolic control of the disease (HbA1c < 7 vs. HbA1c > 7), revealed statistically insignificant higher global DNA methylation in controlled DM patients compared to healthy controls (P = 0.131) (Fig. 1B), whereas uncontrolled DM patients showed significantly higher global DNA methylation level than healthy controls (P = 0.014) (Fig. 1C). The global DNA methylation
was statistically insignificantly higher in uncontrolled compared to controlled DM patients (Fig. 1D) \( (P = 0.266) \), a result that was similar to the difference between healthy controls and controlled DM groups. Correlation analysis showed no association between blood DNA methylation levels in the different study groups and different factors (FBG, HbA1c, and BMI) (Table 2).

![Figure 1](image1.png)

**Figure 1** Percentage of global DNA methylation. (A) Global DNA methylation in healthy controls vs. all DM patients. (B) Global DNA methylation in healthy controls vs. controlled DM patients. (C) Global DNA methylation in healthy controls vs. uncontrolled DM patients. (D) Global DNA methylation in controlled DM patients vs. uncontrolled DM patients.

**Table 2** Correlations between levels of 5mC and different factors.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Healthy Control (n=120)</th>
<th>Controlled DM (n=100)</th>
<th>Uncontrolled DM (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG (mmol/l)</td>
<td>( r_s ) = -0.0904</td>
<td>( r_s ) = 0.1970</td>
<td>( r_s ) = 0.0234</td>
</tr>
<tr>
<td></td>
<td>( P )-value = 0.634</td>
<td>( P )-value = 0.345</td>
<td>( P )-value = 0.911</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>( r_s ) = -0.0741</td>
<td>( r_s ) = 0.2281</td>
<td>( r_s ) = -0.2128</td>
</tr>
<tr>
<td></td>
<td>( P )-value = 0.697</td>
<td>( P )-value = 0.271</td>
<td>( P )-value = 0.307</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>( r_s ) = -0.0701</td>
<td>( r_s ) = 0.0062</td>
<td>( r_s ) = 0.1612</td>
</tr>
<tr>
<td></td>
<td>( P )-value = 0.712</td>
<td>( P )-value = 0.979</td>
<td>( P )-value = 0.442</td>
</tr>
</tbody>
</table>
Several previous studies have reported alterations of global DNA methylation levels in the peripheral blood from individuals with T2DM or abnormal metabolic status. However, the majority of these studies were performed by analyzing the level of LINE-1 DNA methylation, which is a useful marker for global DNA methylation (Ohka et al., 2011). In this work, an ELISA-based assay was used to measure global DNA methylation levels in leucocytes extracted from the blood of the Saudi population and to assess the association between methylation levels and T2DM. The results of this study demonstrated that the degrees of global DNA methylation in peripheral cells were different between healthy controls and T2DM patients, and the levels were higher in T2DM patients than in healthy controls.

Previous studies conducted across different body tissues, including blood, and using various methods revealed inconsistent results regarding the associations of global DNA methylation with T2DM and glycemic status. Hence, no solid conclusions could be drawn. Only one study was similar to ours, in which global peripheral blood DNA methylation in T2DM patients was assessed rather than measuring the methylation of LINE-1 (Pinzón-Cortés et al., 2017). By using a fluorometric assay, Pinzón-Cortés and colleagues found that T2DM patients have significantly higher global peripheral blood DNA methylation than healthy controls, and the methylation level in blood DNA of uncontrolled T2DM patients was also higher than that of the uncontrolled ones (Pinzón-Cortés et al., 2017).

It was observed that an increased glucose level resulted in elevated DNA methylation by enhancing the activity of the enzyme DNA methyltransferase (Chiang et al., 2009). Similar to our study, several works have reported an association between increased blood DNA methylation and elevated blood glucose level, a blood biomarker of T2DM, by measuring LINE-1 DNA methylation levels in patients with high blood glucose levels. Pearce and colleagues reported that increased global blood LINE-1 DNA methylation was associated with increasing blood metabolic health biomarkers that are linked with higher risk of T2DM, such as fasting glucose (Pearce et al., 2012). In addition, Carraro et al., (2016) showed that global methylation in LINE-1 was higher in subjects with elevated blood glucose levels as well as high BMI. Furthermore, Ulrich and coworkers demonstrated that LINE-1 methylation was significantly higher in female subjects with high blood glucose levels (Ulrich et al., 2018). Additionally, Zhao et al., (2012) found that global Alu elements in DNA (global DNA methylation marker) increased in the peripheral blood leukocytes of subjects with insulin resistance, suggesting an association of these elements with diabetes.

In addition, it was reported that peripheral blood global DNA methylation represented by Alu elements was associated with insulin resistance, which is the defining feature of T2DM (Zhao et al., 2012). On the other hand, other studies reported either no association or a negative association between increased peripheral blood DNA methylation and T2DM. Using liquid chromatography to measure the DNA methylation methylcytosine/cytosine ratio in blood leucocytes, Luttmer et al., (2013) reported that blood global DNA methylation decreased in patients with T2DM, whereas other studies that used different techniques showed no association with T2DM (Simar et al., 2014; Zhang et al., 2014; Piyathilake et al., 2013).

The analysis of the association of global DNA methylation with T2DM has not only been conducted on peripheral blood leucocytes but also in other tissues. Ribel-Madsen et al., (2012) found an association between skeletal muscle, subcutaneous adipose tissue global DNA methylation, and elevated plasma glucose. An association of blood and tissue global DNA methylation results with hyperglycemia leads to speculation that the relationship between increased global DNA methylation and hyperglycemia could be due to genomic instability (Zhao et al., 2012) because it is believed that alteration in global methylation impacts genomic stability and expression (Jaenisch & Bird, 2003). Because oxidative stress is believed to be involved in metabolic disease development, it is thought that such stress causes alteration of DNA methylation and genomic instability (Rani et al., 2016). Oxidative stress is known to be associated with T2DM and its complications (Hedman et al., 2016).

Due to the fact that age and sex are known confounding factors that affect DNA methylation (Weidner et al., 2014, Zhang et al., 2011), it is important to mention that the strength of this study is that participants were matched for age, distribution of genders across the groups and BMI, to obtain a reliable comparison between the groups. However, these studies have several limitations. A relatively small sample size was used; however. Peripheral blood contains a mixture of leucocyte types, which could confound our methylation results (Adalsteinsson et al., 2012). Furthermore, physical activity, diet, and smoking were demonstrated to alter DNA methylation patterns, were not measured in this work, and this lack of data may have confounded the analysis.

5. CONCLUSION
In conclusion, this study investigated the global DNA methylation levels in the peripheral blood of Saudi subjects with T2DM. It was demonstrated that the global methylation levels in the blood of T2DM patients is higher than healthy people. The level of methylation is significantly higher in uncontrolled T2DM patients which show that the degree of disease control affects the level of
methylation. However, further research at a large scale is required to further investigate whether epigenetic factors may affect the risk of T2D development and understand the intricate pathophysiological link between T2DM and DNA methylation.

Acknowledgements
I gratefully thank all participants for contributing samples, and thank phlebotomy, and laboratory staff for their support in sample collection and processing. In addition, Cambridge Proofreading Worldwide LLC (https://proofreading.org/) is acknowledged for English language editing.

Funding: This study was funded by the Deanship of Scientific Research, Taibah University, Al Madinah Al Munawarah, Kingdom of Saudi Arabia (Grant Number: 5025).

Ethical consideration
The Ethics Committee of the College of Applied Medical Sciences approved this project (approval code: CLS 201725) at Taibah University, Madinah, Saudi Arabia, and written informed consent was collected from participants before their recruitment.

Conflicts of interest: The authors declare that there are no conflicts of interests.

Data and materials availability
All data associated with this study are present in the paper.

REFERENCES AND NOTES
correlated with MGMT promoter methylation and is a better prognostic factor for glioma. PLoS One 2011; 6(8):e23332. https://doi.org/10.1371/journal.pone.0023332


