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THE LACK OF CORRELATION IN TYPE 2 DIABETES MELLITUS BETWEEN NON-CPG METHYLATION AND DIABETES RISK FACTORS, LIPID PROFILE, KIDNEY FUNCTION TEST, HBA1C, AND RANDOM BLOOD SUGAR

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ABSTRACT

Background: Chronic hyperglycemia and insulin resistance are two hallmarks of type 2 diabetes mellitus (T2DM), a complicated metabolic disease. The purpose of the current investigation was to identify Non-CPG methylation in the *ABCC8* and *CAPN10* promoter regions.

Method: Fifty people were divided into two groups: thirty-five were diagnosed with type 2 diabetes mellitus, and fifteen were control group. Utilizing direct bisulfite sequencing to identify Non-CPG methylation in the promoter region and determine the extent of DNA methylation. To ascertain whether a result was statistically significant at the level of significance 0.05, the T-independent test, Spearman's correlation, and Chi square tests were performed.

Results: Hypermethylation of DNA has been found in the diabetes promoter region of the CAPN10 and ABCC8 genes compared to the healthy group. Furthermore, Non-CPG methylation in both genes and the statistically significant CAPN10 gene has not been linked to risk factors or biochemical indicators.

Conclusion: This study concluded that the biochemical biomarkers and risk factors did not influence Non-CPG methylation of selected genes in type 2 diabetes mellitus

KEYWORDS: Non-CPG methylation, type 2 diabetes, *ABCC8*, *CAPN10* and hypermethylation.

1.INTRODUCTION

CpG methylation, specifically DNA methylation, plays a crucial role in the development of Type 2 Diabetes Mellitus (T2DM). Mistakes in DNA methylation can lead to altered gene expression, affecting the response to external stimuli (Ahmed *et al.*, 2020). Epigenetic modifications, such as DNA methylation, have been found to interact with the genome due to environmental influences, contributing to the development of T2DM (Kim 2019). Researchers have explored the potential use of blood-based DNA methylation biomarkers in detecting T2DM (Raciti *et al.*, 2021). The interplay between genetic, non-genetic, and environmental factors leads to changes in the DNA methylome and transcriptome in pancreatic islets, influencing cellular function and contributing to the pathogenesis of T2DM (Bansal & Pinney 2017).

The challenges and concerns surrounding Non-CPG methylation in Type 2 Diabetes Mellitus (T2DM) are significant. Studies have demonstrated that proper nutrition can aid in regulating DNA methylation associated with T2DM and obesity, thereby reducing the risk of developing the disease (Parrillo *et al.*, 2019). However, the factors, both genetic and non-genetic, that influence DNA methylation in humans are intricate and not yet fully comprehended (Raciti et al., 2021). Changes to the mitochondrial epigenome, such as hypermethylation, may lead to reduced gene expression in type 2 diabetes (Davegårdh *et al.*, 2018). The presence of differentially methylated CPGs associated with T2DM highlights the complexity of the epigenetic landscape (Low *et al.*, 2023). Lifestyle modifications that impact DNA methylation have been found to play a role in reducing the occurrence of type 2 diabetes and improving established risk factors (Walaszczyk *et al.*, 2018).

According to Prasad and Groop (2015), the gene Calpain 10 (CAPN10) on chromosome 10 encodes a cysteine protease with mainly unclear roles in glucose metabolism. According to Smail and Muhamad (2023), Non-CPG methylation of CAPN10 has been studied as a possible predictive biomarker for type 2 diabetes, providing information on the epigenetic elements connected to the condition. Non-CPG methylation in the ABCC8 gene was related to type 2 diabetes. It has been determined that ABCC8 and KCNJ11 are essential for the diagnosis of monogenic diabetes in infants (Prasad & Groop 2015).

2. MATERIALS AND METHODS

2.1 Sample collection

Thirty-five people with type 2 diabetes and fifteen healthy people each provided with five milliliters of blood for testing. The study aimed to diagnose type 2 diabetes through biochemical measurements such as HbA1c, while also exploring Non-CpG methylation patterns. Additionally, participants were asked about their age, gender , Body Mass Index (BMI), and family history. The study also examined renal function, lipid profile, and random blood sugar tests In addition to measuring HbA1c blood levels, the study also assessed random blood sugar (RBS) levels, lipid profile (including total cholesterol, triglycerides, HDL and LDL cholesterol) using the ROCHE COBAS-C311 machine, and kidney function tests (measuring levels of urea and creatinine) also using the ROCHE COBAS-C311 machine.

2.2 DNA extraction and Bisulfite conversion

Using Promega Kits, DNA Extraction and Bisulfite Conversion from Blood Samples Promega provides the MethylEdge® Bisulfite Conversion System, which is used to extract DNA and execute bisulfite conversion from blood samples. The kits include high-throughput gDNA purification from large volume blood samples as well as manual approaches for purification from blood (Harrison & Parle-McDermott 2011). The MethylEdge® kits from Promega are ideal for a variety of sample types, including blood, because they are intended for effective DNA purification. It is also a useful technique for bisulfite conversion since it offers a solution-based approach for isolating DNA from white blood cells. Promega's kits are taken into consideration for the assessment of commercial kits for DNA methylation biomarker detection in blood, including bisulfite

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conversion, highlighting the significance of selecting ideal methods for obtaining accurate results (Kresse *et al.*,2023). The Promega Wizard DNA clean-up kit is widely used in bisulfite genomic sequencing to purify bisulfite-treated DNA, underscoring its significance in DNA methylation detection (Li & Tollefsbol 2011).

2.3 Primer design and Bisulfite converted DNA Amplification

MethPrimer, a well-known online tool, is specifically designed for bisulfite-treated DNA. Its purpose is to assist in the creation of primers that selectively amplify DNA strands that have undergone bisulfite conversion. This is crucial for conducting accurate DNA methylation analysis (Kovacova & Janousek, 2012). By employing this primer design technique, the accuracy and efficiency of direct bisulfite sequencing experiments are enhanced (Table 1 and 2).

Bisulfite PCR can now be developed; 12.5 µl of 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM $MgCl_2$ were included in 25 µl of the PCR reaction, along with 10 picomoles of forward (0.9 µl), 10 picomoles of reverse (3 µl bisulfite converted DNA (10-50 ng), and 7.7 1 µl of DNA nuclease-free water. Bisulfite PCR was done with the settings as follows: 95°C for 3 minutes, 40 cycles of (95°C for 30 sec, 54°C for 60 sec and 72°C for 60 sec) and 72°C for 5 minutes. The results of the PCR were examined using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and seen under ultraviolet light. For the *CPAN10* gene, the expected size was 277 bp, while for ABCC8, it was 297 bp (Figure 1)

Table 1: *CAPN10* primer design by MethPrimer based on bisulfite conversion (Bisulfite PCR).

Primer	**Length**	**Start point**	**Product size**	**Tm**	**Sequence (5'->3'**
Left primer	26	26	277	57.58	AAATAGTTGTTATTAGAGGGGTTGAG
Right primer	25	302	277	59.02	TAAAAACTTCAAAAACCAAAAAACC

Table 2: *ABCC8* primer design by MethPrimer based on bisulfite conversion (Bisulfite PCR)

Primer	Length	Start point	Product size	Tm	Sequence (5'->3'
Left primer	24	182	297	57.99	TTTATTTATGTTGGGTGGAGATTT
Right primer	22	487	297	56.70	CCCACCTTCCAAATATAAAAAC

2.4 Statistical analysis

To assess the Non-CPG means of two separate groups, employ Independent-Samples T test. In Graph Pad Prism 8, a significant p-value (< 0.05) indicates a disparity in means. To examine the monotonic relationship between two variables, utilize Spearman's rank correlation coefficient. Additionally, the chi-square to identify correlations between overall Non-CPG Non-CPG methylated and unmethylated groups with a significant p-value (< 0.05). test, HbA1c, random blood sugar, and metformin use.

3. RESULTS

Figure 1 shows the promoter amplification of the CPAN10 and ABCC8 genes using bisulfite PCR stained with ethidium bromide and observed under ultraviolet light. Tables 3 and 2 indicate the levels of Non-CPG methylation in the CPAN10 and ABCC8 genes. On the other hand, Tables 4 and 5 display the overall methylation and unmethylation in both selected genes. Figures 2 and 3 illustrate the correlation between Non-CPG methylation and diabetic risk factors, lipid profile, kidney function.

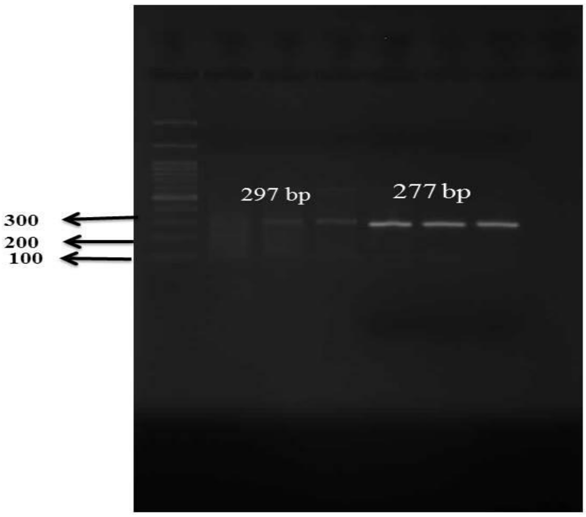


Figure 1: Bisulfite PCR amplified promoter region of *ABCC8* and *CAPN10* gene *Lane 1: 100bp DNA ladder, lane (2,3 and 4):297 bp *ABCC8* promoter region. lane (5,6 and 7):277 bp CPAN10 promoter Agarose gel (1.5%) electrophoresis shows PCR products of the *ABCC8 and CAPN10* gene promoter region after bisulfite PCR amplifies. The expected product size was 297 and 277 bp.

Table 3: Frequency of Non-CPG methylation of CPAN10 gene

			Number of Non-CPG methylation														**Total**	**P value**
			0	1	2	3	4	5	6	8	9	13	14	18	19	20		
Groups	**diabetes**	**Number of samples**	12	4	6	4	2	1	1	2	1	0	1	1	0	0	35	**0.017**
		%	34.3	11.4	17.1	11.4	5.7	2.9	2.9	5.7	2.9	0	2.9	2.9	0	0	100	
	Control	**Number of samples**	3	3	0	0	0	2	3	0	0	1	1	0	1	1	15	
		%	20	20	0	0	0	13.3	20	0	0	6.7	6.7	0	6.7	6.7	100	

Significant = (P≤0.05), using independent sample t-test

Table 4: Frequency of Non-CPG methylation of ABCC8 n gene

			Number of Non-CPG methylation															**Total**	***P* value**
			0	1	2	3	4	5	6	10	12	13	14	17.	22	24	29		
Groups	**Diabetes**	**Number of samples**	12	6	2	3	2	1	1	1	2	1	1	1	1	0	1	35	**0.670**
		%	34.3	17.1	5.7	8.6	5.7	2.9	2.9	2.9	5.7	2.9	2.9	2.9	2.9	0	2.9	100	
	Control	**Number of samples**	9	1	2	1	0	0	0	0	0	0	0	0	0	1	1	15	
		%	60	6.7	13.3	6.7	0	0	0	0	0	0	0	0	0	6.7	6.7	100	

Significant = (P≤0.05), using independent sample t-test

Table 5: Overall Non-CPG DNA methylation status of CPAN10 gene in diabetes groups with control

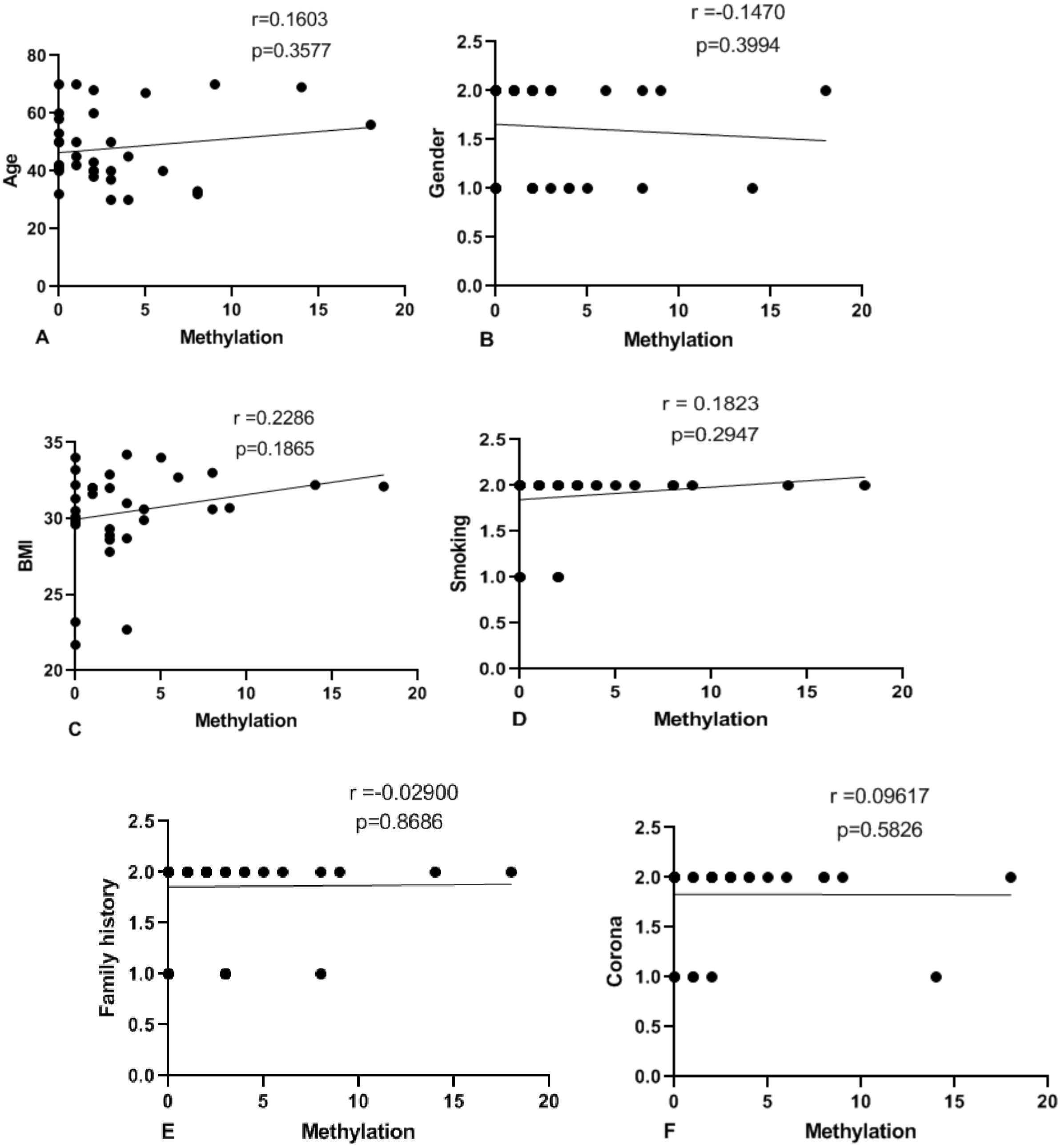
Groups	**Total number of Non-CPG**	**Methylated %**	**Unmethylated %**	Chi square	***P* value**
Diabetes (35)	2310	104(4.5%)	2206(95.5%)	33.9	**0 .00001**
Control (15)	990	97(9.7%)	893(90.3%)		

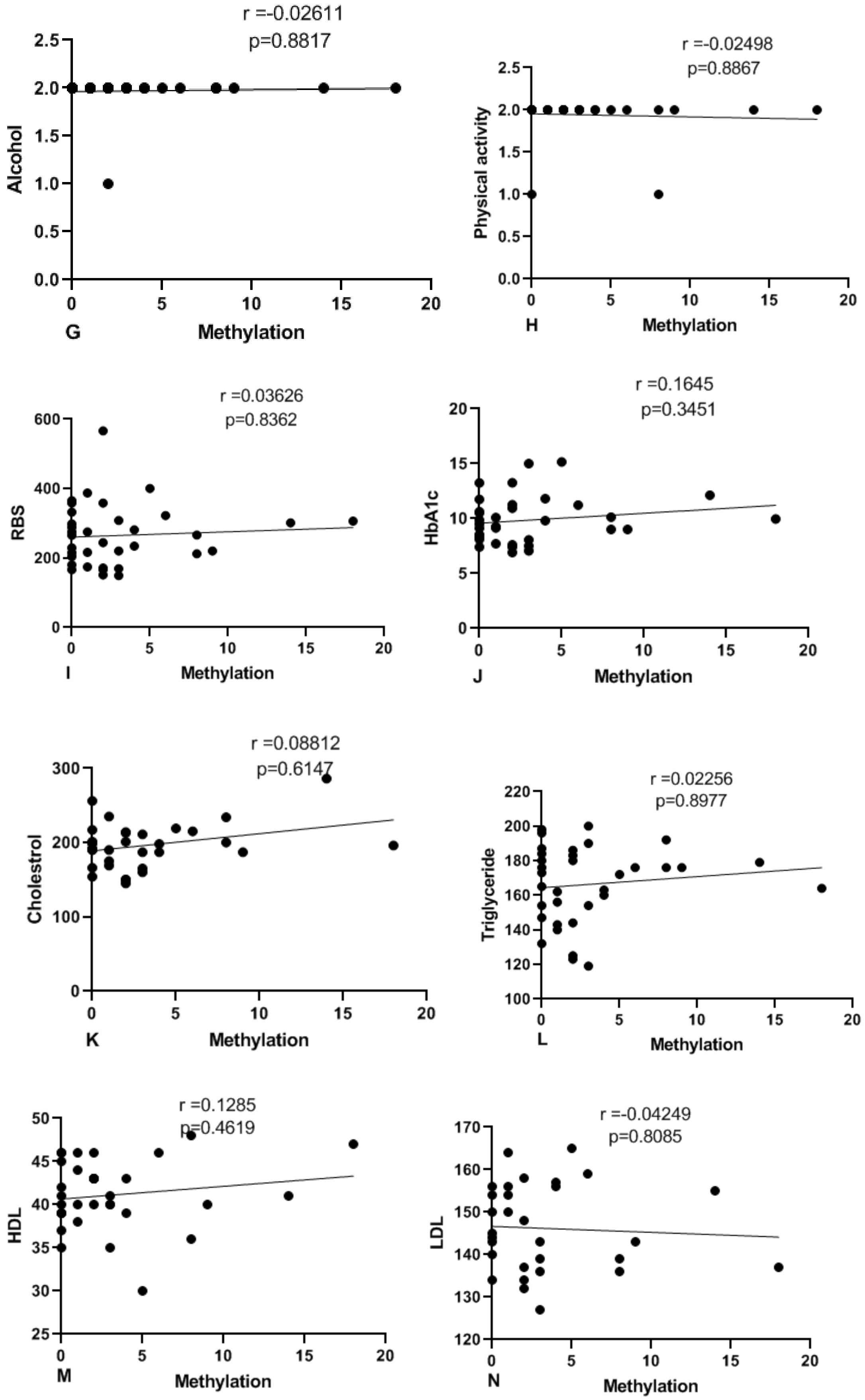
Significant = (P≤0.05), using the Chi square test

Table 6: Overall Non-CPG DNA methylation status of ABBC8 gene in diabetes groups with control

Groups	**Total number of Non-CPG**	**Methylated %**	**UnMethylated %**	Chi square	***P* value**
Diabetes (35)	4095	163(3.9%)	3932(96.1%)	0.84	**0.35**
Control (15)	1755	61(3.4%)	1694(96.6%)		

Significant = (P≤0.05), using the Chi square test




r =-0.02611
p=0.8817
Alcohol
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
G
Methylation
r =-0.02498
p=0.8867
Physical activity
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
H
Methylation
r =0.03626
p=0.8362
RBS
600
400
200
0
0
5
10
15
20
I
Methylation
r =0.1645
p=0.3451
HbA1c
20
15
10
5
0
0
5
10
15
20
J
Methylation
r =0.08812
p=0.6147
Cholestrol
300
200
100
0
0
5
10
15
20
K
Methylation
r =0.02256
p=0.8977
Triglyceride
220
200
180
160
140
120
100
0
5
10
15
20
L
Methylation
r =0.1285
p=0.4619
HDL
50
45
40
35
30
25
0
5
10
15
20
M
Methylation
r =-0.04249
p=0.8085
LDL
170
160
150
140
130
120
0
5
10
15
20
N
Methylation

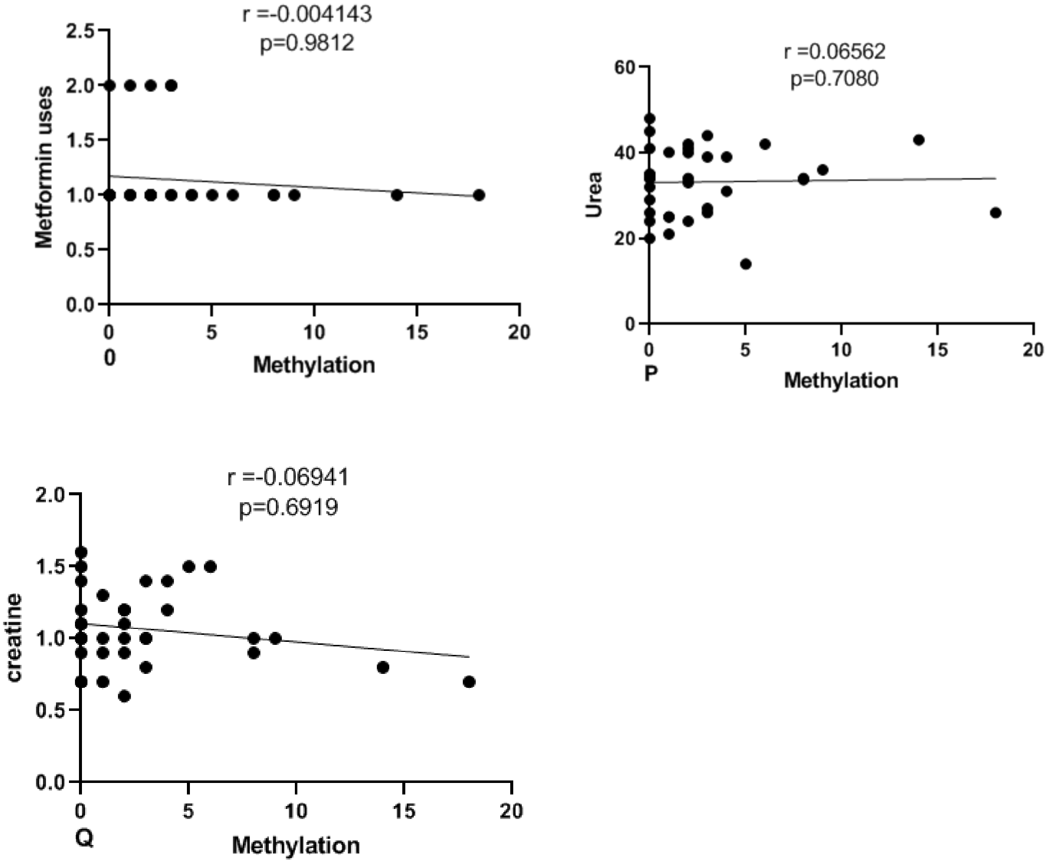
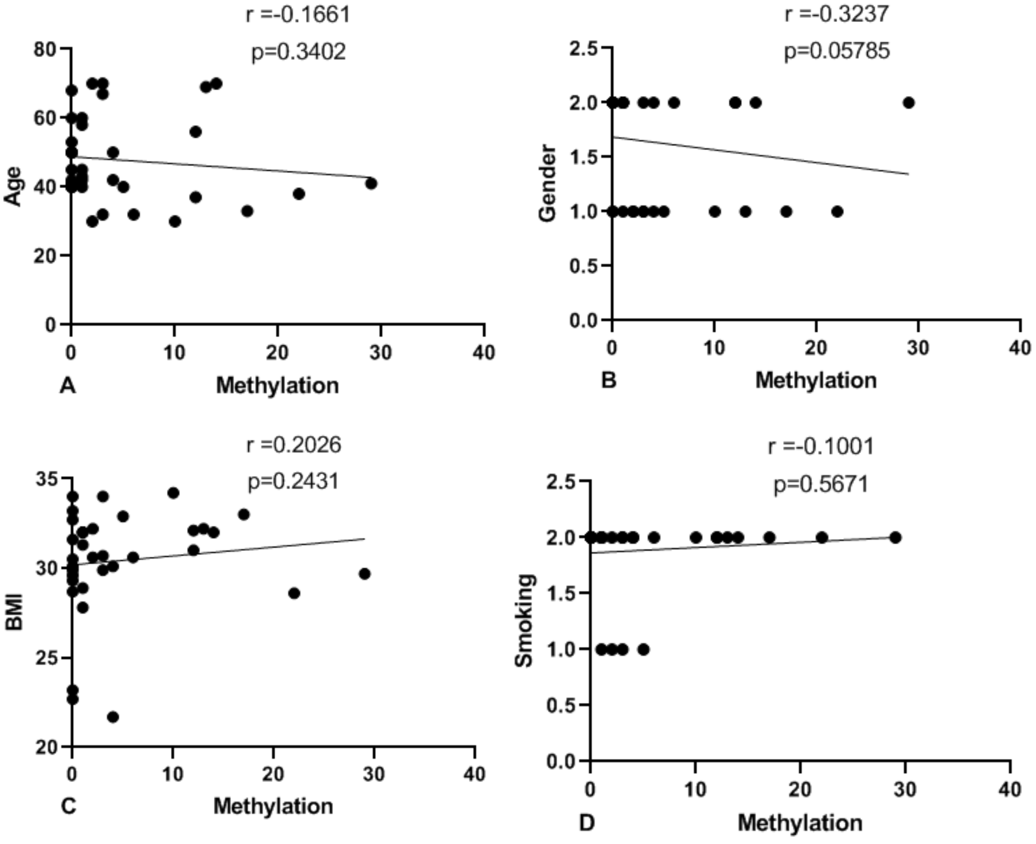
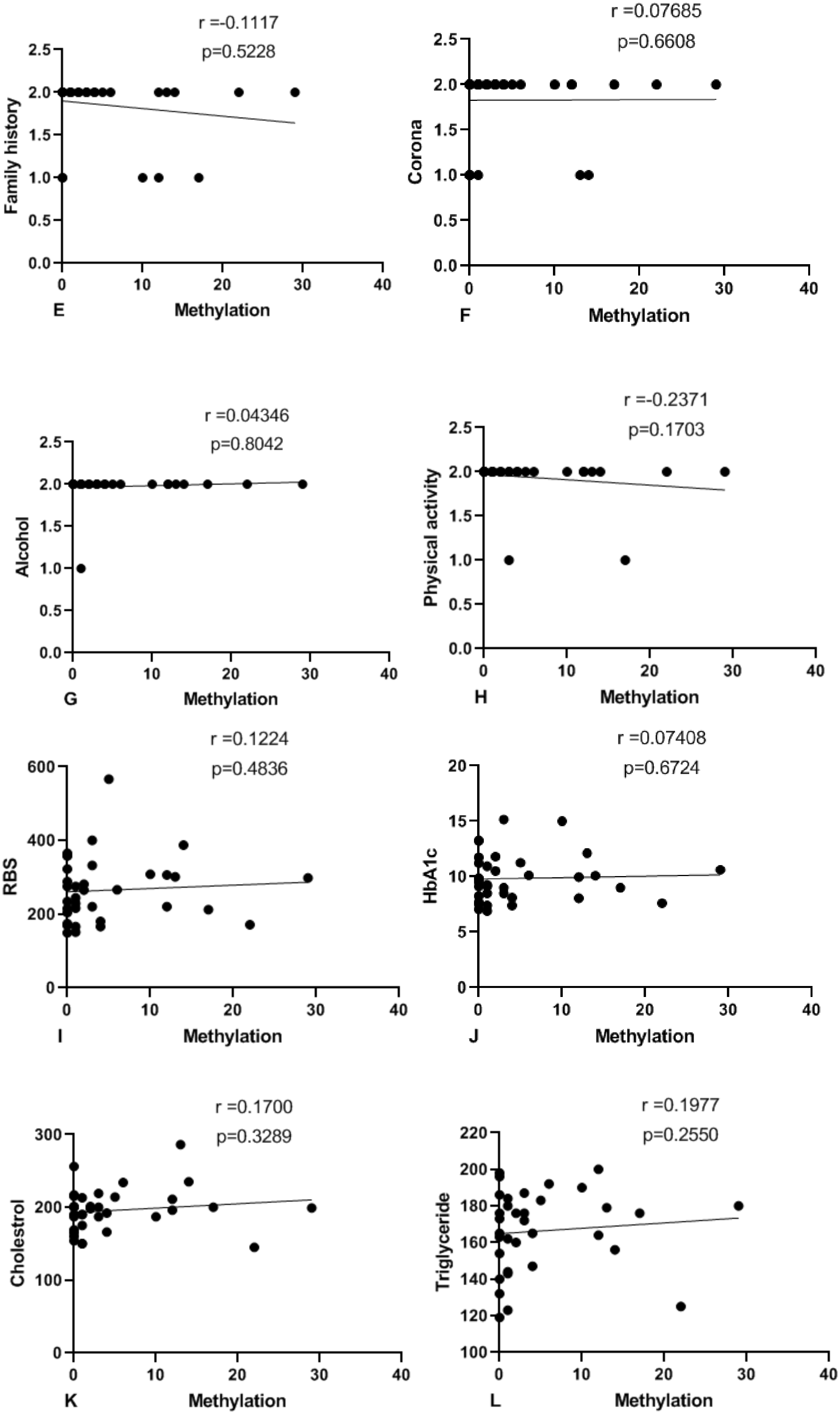


Figure 2: Scatter plot of Spearman's correlation analysis between Non CPG methylation and (A) Age , (B) Gender , (C) BMI , (D) Alcohol , (E) Family history ,(F) Corona , (G) Alcohol , (H) Physical activity , (1) RBS , (J) HbA1c , (K) Cholesterol , (L) Triglyceride , (M) HDL , (N) LDL , (O) Metformin uses , (P) Urea , (Q) creatine in diabetic groups of *CAPN10* gene




r =-0.1117
p=0.5228
Family history
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
E
Methylation
r =0.07685
p=0.6608
Corona
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
F
Methylation
r =0.04346
p=0.8042
Alcohol
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
G
Methylation
r =-0.2371
p=0.1703
Physical activity
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
H
Methylation
r =0.1224
p=0.4836
RBS
600
400
200
0
0
10
20
30
40
I
Methylation
r =0.07408
p=0.6724
HbA1c
20
15
10
5
0
0
10
20
30
40
J
Methylation
r =0.1700
p=0.3289
Cholestrol
300
200
100
0
0
10
20
30
40
K
Methylation
r =0.1977
p=0.2550
Triglyceride
220
200
180
160
140
120
100
0
10
20
30
40
L
Methylation

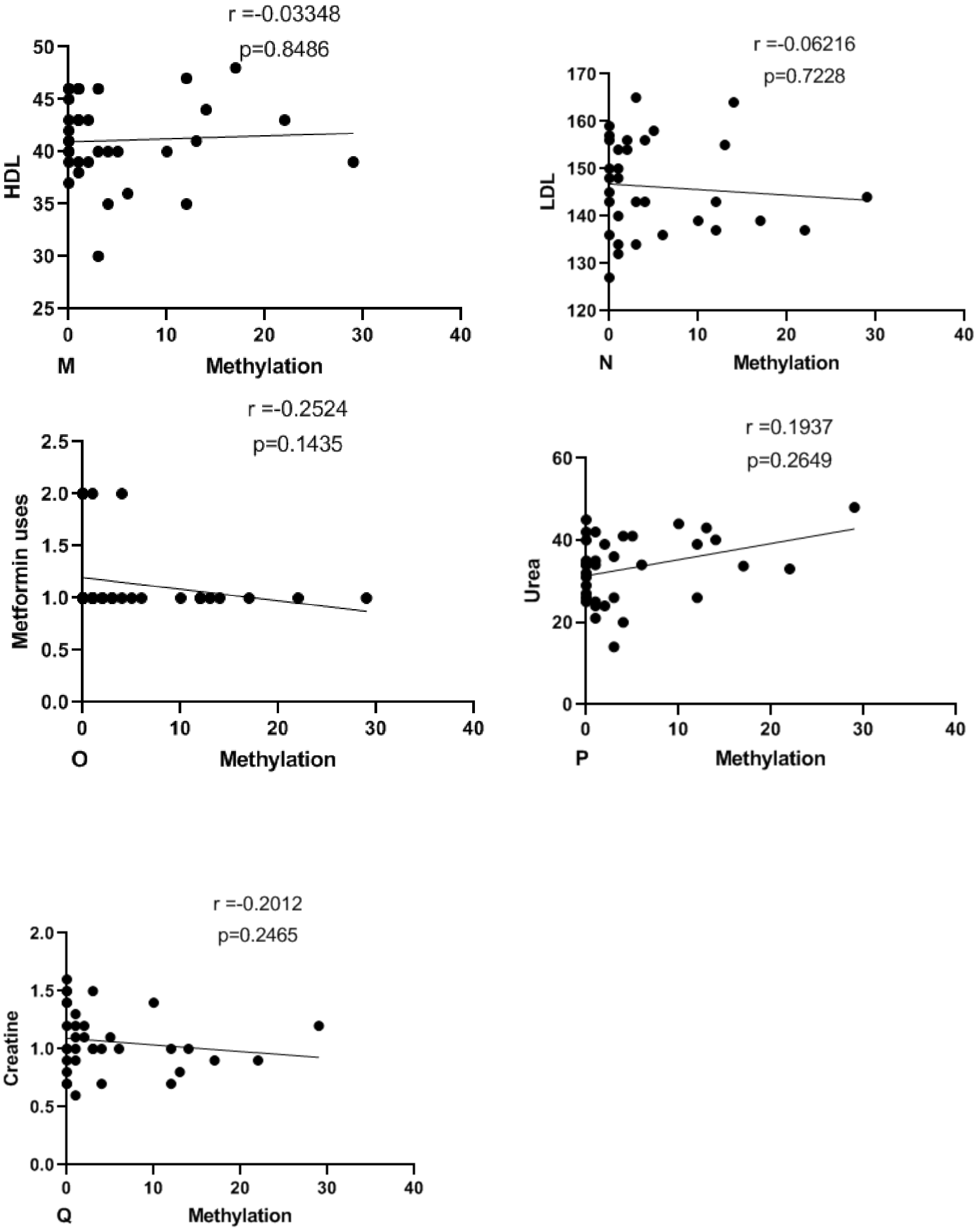


Figure 3: Scatter plot of Spearman’s correlation analysis between Non CPG methylation and (A) Age , (B) Gender , (C) BMI , (D) Alcohol , (E) Family history ,(F) Corona , (G) Alcohol , (H) Physical activity , (1) RBS , (J) HbA1c , (K) Cholesterol , (L) Triglyceride , (M) HDL , (N) LDL , (O) Metformin uses , (P) Urea , (Q) creatine in diabetic groups of ABCC8 gene

DISCUSSION

By using two novel types of primers, the study aimed to ascertain the amount of Non-CPG methylation in type 2 diabetes and compare it with a group of healthy people in both the *CAPN10* and *ABCC8* genes.

Certain genes, such as PGC-1alpha and CAPN10, have been discovered to display Non-CPG methylation in individuals with type 2 diabetes (T2DM). In addition to traditional markers like HbA1c, the study suggested that variations in DNA methylation within the CAPN10 gene could serve as predictive biomarkers for T2DM (Smail &Muhamad 2023). Moreover, in T2DM patients, the hypermethylation of the PGC-1alpha promoter has been associated with reduced mitochondrial content, indicating a potential link between DNMT3B and sudden changes in mitochondrial function (Barrès *et al.,* 2019). By further investigating the genome-wide methylation patterns in the pancreatic islets of diabetic patients, these findings enhance our understanding of the role DNA methylation plays in the pathophysiology of type 2 diabetes (Dayeh *et al.*, 2014 ; Mutize *et al.*, 2018).

The frequency of Non-CPG methylation of the ABCC8 and CAPN10 genes was displayed in Tables 3 and 4. The

Independent-Samples T test indicated that only the CAPN10 gene was statistically significant, with a p value of 0.017. Tables 5 and 6 show that overall Non-CPG unmethylation in both selected genes was higher in healthy participants than in diabetic subjects. The CAPN10 gene showed statistical significance with a p value of 0.00001.

Recent studies have indicated a connection between biochemical markers in type 2 diabetes (T2D) and Non-CPG methylation. Specifically, individuals with T2D have exhibited decreased expression of the PPARG Coactivator 1 Alpha (PPARGC1A) gene, which is associated with heightened Non-CPG methylation within the gene's promoter region. Alterations in DNA methylation have been linked to disturbances in metabolic indicators like HbA1c and fasting glucose levels (Raciti *et al.,* 2021). Additionally, it has been observed that increased PTPN1 promoter methylation is significantly correlated with a higher susceptibility to type 2 diabetes in females, emphasizing gender-specific associations with biochemical markers (Willmer *et al.*, 2018). The current findings, as illustrated in figures 3.2 and 3.3, indicate that there is no relationship between Non-CPG methylation, diabetic risk factor, and biochemical indicators (lipid profile and kidney function test).

The ABCC8 gene's Non-CPG methylation has been proposed as a potential biomarker for type 2 diabetes (T2D). Several studies have investigated the level of DNA methylation in the ABCC8 gene among individuals with T2D, suggesting a potential association with the disease (Patch *et al.,* 2007; Raciti et al., 2021 ; Smail & Muhamd, 2022).

CONCLUSION

The results indicated that there was a correlation between Non-CPG methylation and type 2 diabetes in the *CPAN10* gene, but no correlation was found with the ABCC8 gene. Furthermore, Non methylation did not show any correlation with type 2 risk factors, lipid profile, or kidney function tests. These findings emphasize the need for additional research and the development of more primers using a larger sample size.

List of abbreviations :

ABCC8 : ATP-binding cassette, subfamily C, member 8, CPAN10: calpain 10, T2DM: Type 2 Diabetes Mellitus, CPG: Cytosine-phosphorothioate-guanine, BMI: Body mass Index, RBS: Random Blood sugar, HbA1c: hemoglobin A1C, HDL: High-density lipoprotein, LDL: Low-density lipoprotein.

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