Kurdistan Journal of Applied Research (KJAR)
Print-ISSN: 2411-7684 | Electronic-ISSN: 2411-7706

Original Article

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Effect of the Duration of the Diagnosis on DNA Methylation Changes in Three Different Diabetic Genes in Type 2 Diabetes Mellitus

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Submitted: 7 September 2024
Revised: 18 September 2024
Accepted: 6 November 2024

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Keywords: DNA methylation, type 2 diabetes, Methylation-specific PCR, Bisulfite conversion and diagnosis times.

How to cite this paper: H. O. Samil," Effect of the Duration of the Diagnosis on DNA Methylation Changes in Three Different Diabetic Genes in Type 2 Diabetes Mellitus", KJAR, vol. 9, no. 2, pp. 86-95, December. 2024, doi: 10.24017/science.2024.2.7

Abstract: The primary epigenetic alteration is DNA methylation. This study examined variations in DNA methylation in patients with type 2 diabetes (T2DM) at various time intervals, concentrating on the Calpain-10, ATP binding cassette subfamily C member 8 (ABCC8), and Transcription factor 7-like 2 genes. Since persistent diabetes can lead to aberrant methylation patterns, it is interested in how long these changes may last. The blood samples from 140 individuals with T2DM were collected, and the patients were grouped according to how long they had been diagnosed. Four groups of individuals were created based on their time on the disease: those with T2DM <1 year, those with T2DM 1-3 years, and those with T2DM 3-5 years. The genders and ages of the participants were also noted. Using the Promega technique, bisulfite conversion and DNA extraction were completed. Methylation-specific PCR amplification was used to detect DNA methylation. In the study, receiver operating characteristic curve analysis, Chi-square and Spearman's correlation coefficients, as well as non-parametric tests, were employed to analyze the methylation percentage variation and methylation patterns among groups. A significant threshold of $p < 0.05$ was established. The results of the study indicated that the DNA methylation rates of T2DM patients and the control group differed significantly. Patients with T2DM, particularly those who had just received a diagnosis, showed higher levels of methylation than the control group. The study also found that the length of diagnosis may have an impact on the discriminative strength of the DNA methylation status of the *ABCC8* gene, resulting in varying degrees of T2DM prediction.

1. Introduction

DNA methyltransferases (including DNMT1, DNMT3A, and DNMT3B) add a methyl group to the fifth carbon of cytosine residues that are phosphate-linked to a guanine nucleotide (a CpG dinucleotide), resulting in DNA methylation *Calpain-10 (CAPN10)* is one gene associated with type 2 diabetes mellitus (T2DM) risk [1]. It is one of the few genes that has been shown to have a strong correlation with T2DM, along with *Transcription factor 7-like 2 (TCF7L2)*. Numerous studies have explored the relationship between diabetes risk and single nucleotide polymorphisms in *CAPN10* [2]. In addition, T2DM is linked to variations in the ABCC8 gene that are related to the *ABCC8* gene [3]. Perhaps even more so than hereditary variables, DNA methylation changes are thought to play an even more significant role in T2DM development [4, 5]. DNA methylation plays a crucial role in the pathophysiology of T2DM, as variations in methylation patterns lead to changes in gene expression, which in turn cause the illness [6, 7]. It is evident that DNA methylation alterations occur in response to T2DM disease, even if some

studies do not explicitly state how long these changes take to manifest during the disease's development [8, 9].

Due to the limited number of studies examining the direct DNA methylation alterations in T2DM, it remains possible that factors such as disease course, genetic predisposition, and the environmental influences may also impact how methylation patterns change over time [10]. Diabetic nephropathy is associated with abnormal DNA methylation patterns, suggesting that long-term diabtes may cause methylation alterations [11, 12]. Epigenetic profiling studies show the regions of the genome with different patterns of DNA methylation between diabetic and normal groups. These studies give an overview of the general methylation differences and do not really focus on diagnosis duration [13, 14]. There is currently limited research examining how DNA methylation changes that occur with the length of T2DM diagnosis. As a result, this study tries to look at how the changes in DNA methylation of specific genes of *ABCC8*, *CAPN10* and *TCF7L2* will be in patients diagnosed with T2DM at different time interval of diagnosis.

2. Materials and Methods

2.1. Sample Collection

Blood samples were collected from 140 participants for this study. Participants were divided into groups based on the duration of their T2DM diagnosis. Each group consisted of 35 individuals, along with a control group. Group 1 included T2DM patients within the past year, T2DM patients between one and three years, and control groups. In addition, data regarding the ages and genders of participants were also recorded. Informed consent was obtained orally, as the samples were collected from a private lab recognized by the Ministry of Health in the Kurdistan region of Iraq.

2.2. DNA Extraction and Bisulfite Conversion

The Promega company's protocol was followed while extracting DNA and converting bisulfite from blood samples. The ReliaPrepTM Blood gDNA Miniprep System (Cat. No. A5081, Promega Compa Promega, USA) was used to extract DNA in accordance with the manufacturer's instructions by completing four essential steps, one of which was homogenizing the whole blood before releasing it. DNA, three rounds of wash solution elimination of impurities, DNA binding to the ReliaPrepTM Binding Column, and elution of purified DNA. To ensure accuracy in subsequent analyses, an evaluation of the quality and quantity of DNA was conducted both before and after bisulfite conversion, used a Nanodrop spectrometer to quantify the quantity of DNA, which has an absorbance of 280 with a normal range of 1.7–2.0. One technique for evaluating the purity of DNA was electrophoresis on 1% agarose gel, additionally looked at the quantity and quality of DNA after bisulfite conversion.

2.3. Detection of DNA Methylation

The methylation-specific primers and the Add Star Taq master mix polymerase chain reaction (PCR) kit (addbio, Korea), the methylation-specific PCR amplification reaction was carried out. The procedure was carried out as per the manufacturer's 25 μL. Twelve point five μL of master mix, 3 μL of DNA sample, 7.5 μL of nuclease-free water, and 1 μL of 10 pmol of each primer were used in each PCR reaction. The methylated and unmethylated DNA sequences were targeted by a specific primers utilized in this investigation. The primer sequences were developed using the MethPrimer tool, these primers were created to amplify a 135 bp region for methylated DNA and a 136 bp region for unmethylated DNA. As a guide, the *ABBC8* gene was utilized [15-17].

- Unmethylated forward: TTTTGTGTGAAAGTGTTAGATATGT
- Unmethylated reverse: AATAAAAAAAAATAAAACAAAACATT
- Methylated forward: TTTGTGTGAAAGTGTTAGATACGT
- Methylated reverse: AATAAAAAAAATAAAACAAAACGTT

The PCR protocol was repeated for two other genes, *CAPN10* and *TCF7L2*, with their respective primers and target regions:

CAPN10 gene:

- Unmethylated forward: TTTTTTTAATTATTTGTGGTTATGG
- Unmethylated reverse: CAAACTACAACTCCCAACATACAC
- Methylated forward: GTTTTTTTTAATTATTCGCGGTTAC
- Methylated reverse: CAAACTACAACTCCCAACATACG

Target regions: 114 bp for methylated DNA and 116 bp for unmethylated DNA [17].

TCF7L2 gene:

- Unmethylated forward: TTGGAGTAAGTTTTTGTATTTTTGT
- Unmethylated reverse: CAACTAACAATAATCCTTTCAAACAC
- Methylated forward: TTTCGGAGTAAGTTTTTGTATTTTC
- Methylated reverse: ACTAACAATAATCCTTTCGAACG

Target regions: 171 bp for methylated DNA and 170 bp for unmethylated DNA [17].

Using a thermocycler (Bio-Rad, USA), the PCR reactions were run with the following program: initial denaturation for 5 minutes at 95 °C, followed by 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 52 °C and 54 °C, 30 seconds of extension at 72 °C, and 30 seconds of final extension at 72 °C. Following amplification, a 2% agarose gel in 1× TBE solution was loaded with 4.0 μL of the PCR product for examination.

Three microliters of ethidium bromide (addbio, Korea) were used to stain the gel. Using an electrophoresis machine, electrophoresis was carried out for one hour at 120 volts. A 100 bp DNA ladder was used to determine the PCR amplicons' migration band [18].

2.4. Statistical Analysis

The methylation percentage variance and deviations from the normal pattern between groups were analyzed using the MSP data. Normality of the data were examined using Shapiro-Wilk test. Frequency distributions were used to display categorical variables, and statistical significance was determined using chi-square tests and Spearman's correlation coefficients.

The threshold for significance was fixed at $p < 0.05$. GraphPad Prism version 8 was used to perform receiver operating characteristic (ROC) curve and area under the curve (AUC) analysis to identify the biomarkers for diagnosis periods [19].

3. Results

Significant variations in DNA methylation between different populations were observed in this study. Patients with T2DM, particularly those who were recently diagnosed, showed significantly greater rates of methylation compared to the control group. The highest percentage of methylation cases was observed in patients diagnosed within the first year. Ages three to five came in second place. An exceptionally low p-value from a chi-square analysis indicated a statistically significant relationship between the length of T2DM and methylation status (Table 1).

The percentages of methylation varied across groups with respect to the ABCC8 gene. Patients with T2DM exhibited a peak of 68.6% methylated in those diagnosed three to five years ago. Patients diagnosed less than a year ago scored 31.4%, while those with a follow-up of three to six years received 40%. In contrast, the control group showed a minimum 17.1% methylation rate. The chi-square value is 24.684 and the matching p-value is 0.000391. This implies that there is a statistically significant link between the variables being analyzed (Table 1).

Table 1: Percentage of methylation in patients with T2DM of < 1 year, 1 to 3 years, 3 to 5 years, and controls of the *ABCC8* gene.

Groups	Methylation status			Total	Chi square	P value
	Methylated cases (%)	Unmethylated cases (%)	Partial methylated cases (%)			
T2DM < 1 year (35)	11(31.4%)	20(57.1%)	4(11.4%)	35(100%)		
T2DM 1-3 year (35)	14(40%)	12(34.3%)	9(25.7%)	35(100%)		
T2DM 3-5 year (35)	24(68.6%)	8(22.9%)	3(8.6%)	35(100%)	24.684	0.000391
Control (35)	6(17.1%)	20(57.1%)	9(25.7%)	35(100%)		
Total (140)	55(39.2%)	60(42.9%)	25(17.9%)	140(100%)		

Significant = (P≤0.05), using Chi square test * T2DM: Type 2 Diabetes Mellitus.

The percentage of methylation events for the *CAPN10* gene in each group is presented in table 2. The highest proportion of methylation cases (42.9%) was found in the group of T2DM patients diagnosed during 3–5 years, followed by T2DM patients diagnosed within <1 year (17.1%). The control group has the highest proportion of methylation instances (71.4%), and table 3 displays the percentage of *TCF7L2* gene methylation cases for each group. Notably, T2DM patients within 3–5 years have the highest percentage of methylation instances (40.0%), followed by those during 1–3 years (31.4%) and lowest value was detected in those with < 1 years (8.6%). In comparison to T2DM patients, controls had a lower percentage of methylation events over all time periods. For the *TCF7L2* gene, the chi-square value is 29.616, the corresponding p-value is 0.000046 and the p-value is less than 0.00001. These results indicate a highly significant statistical result with a very low likelihood of observing such an extreme result by chance alone. It is exceedingly improbable that the *TCF7L2* gene would have seen such an extreme outcome by chance alone, as indicated by the p-value, which indicates that the variables under investigation have a statistically significant association (Tables 2 and 3).

Table 2: Percentage of methylation in patients with T2DM of < 1 year, 1 to 3 years, 3 to 5 years, and controls of the *CAPN10* gene.

Groups	Methylation status			Total	Chi square	P value
	Methylated cases (%)	Unmethylated cases (%)	Partial methylated cases (%)			
T2DM < 1 year (35)	6(17.1%)	20(57.1%)	9(25.7%)	35(100%)		
T2DM 1-3 year (35)	5(14.3%)	20(57.1%)	10(28.6%)	35(100%)		
T2DM 3-5 year (35)	15(42.9%)	8(22.9%0	12(34.2%)	35(100%)	37.434	< 0.00001
Control (35)	25(71.4%)	5(14.3%)	5(14.3%)	35(100%)		
Total (140)	51(36.4%)	53(37.9%)	36(25.7%)	140(100%)		

Significant = (P≤0.05), using Chi square test* T2DM: Type 2 Diabetes Mellitus.

Table 3: Percentage of methylation in patients with T2DM of < 1 year, 1 to 3 years, 3 to 5 years, and controls of the *TCF7L2* gene.

Groups	Methylation status			Total	Chi square	P value
	Methylated cases (%)	Unmethylated cases (%)	Partial methylated cases (%)			
T2DM < 1 year (35)	3(8.6%)	19(53.3%)	13(37.1%)	35(100%)		
T2DM 1-3 year (35)	11(31.4%)	16(45.7%)	8(22.9%)	35(100%)		
T2DM 3-5 year (35)	14(40%)	15(42.9%)	6(17.1%)	35(100%)	29.616	0.000046
Control (35)	24(68.6%)	9(25.7%)	2(5.7%)	35(100%)		
Total (140)	52(37.1%)	59(42.1%)	29(20.8)	140(100%)		

Significant = (P≤0.05), using Chi square test* T2DM: Type 2 Diabetes Mellitus.

The relationship (r value) and statistical significance (P value) between age and changes in DNA methylation for the three genes (*ABCC8*, *CAPN10*, and *TCF7L2*) in individuals with T2DM and controls across different time periods after diagnosis was shown in table 4. In individuals with T2DM and controls over different diagnostic intervals, table 5 shows the statistical significance (P value) and correlation (r value) between gender and DNA methylation changes for these three genes (*ABCC8*, *CAPN10*, and *TCF7L2*).

Table 4: Correlating age with DNA methylation changes according to the diagnosis period.

Groups	*ABCC8*		*CAPN10*		*TCF7L2*	
	r value	P value	r value	P value	r value	P value
T2DM < 1 year	-0.313-	-0.067	0.038	0.830	0.167	0.337
T2DM 1-3 year	0.322	0.060	0.004	0.983	0.052	0.767
T2DM 3-5 year	0.189	0.269	-0.066-	0.704	-0.113-	0.511
Control	0.206	0.235	-0.259-	0.133	-0.119-	0.495

The statistical analysis utilized the Pearson correlation. A p-value of 0.05 or lower was considered to be statistically significant.

Table 5: Correlating gender with DNA methylation changes according to the diagnosis period.

Groups	*ABCC8*		*CAPN10*		*TCF7L2*	
	r value	P value	r value	P value	r value	P value
T2DM < 1 year	-0.166-	0.342	0.144	0.490	0.040	0.819
T2DM 1-3 year	0.064	0.717	0.291	0.090	0.332	0.051
T2DM 3-5 year	0.332	0.048*	0.056	0.744	-0.155-	0.367
Control	0.136	0.436	-0.022-	0.898	-0.030-	0.862

The statistical analysis utilized the Pearson correlation. A p-value of 0.05 or lower was considered to be statistically significant.

The p-value and AUC for each chosen gene are displayed in figures 1, 2, and 3 in relation to the various diagnostic times. The *TCF7L2* gene in the group T2DM < 1 year had the greatest AUC of 0.8, while the *ABCC8* gene in the group T2DM 1-3 years had the lowest AUC of 0.5. A greater AUC value denotes a p-value that is extremely significant.

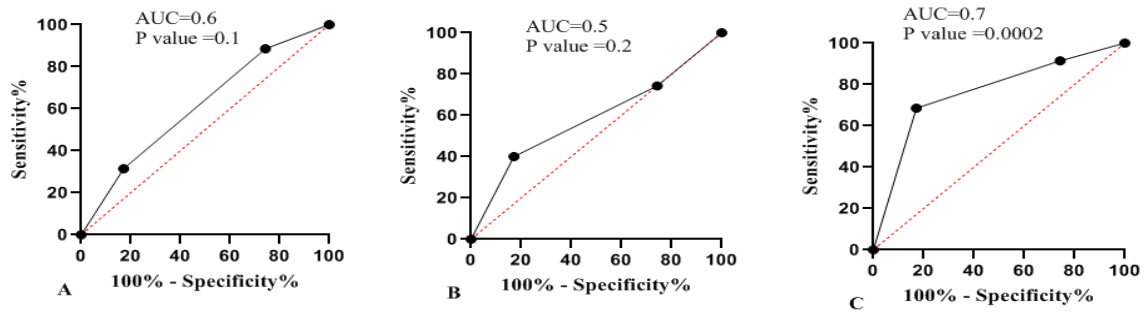


Figure 1: The ROC curve shows the DNA methylation status of the ABCC8 gene for three different groups: (A) T2DM < 1 year, (B) T2DM 1-3 years, and (C) T2DM 3-5 years.

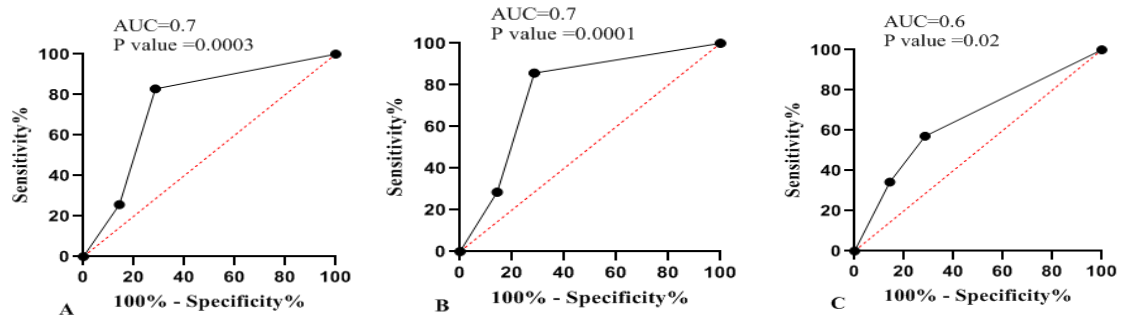


Figure 2: The ROC curve shows the DNA methylation status of the *CAPN10* gene for three different groups: (A) T2DM < 1 year, (B) T2DM 1-3 years, and (C) T2DM 3-5 years.

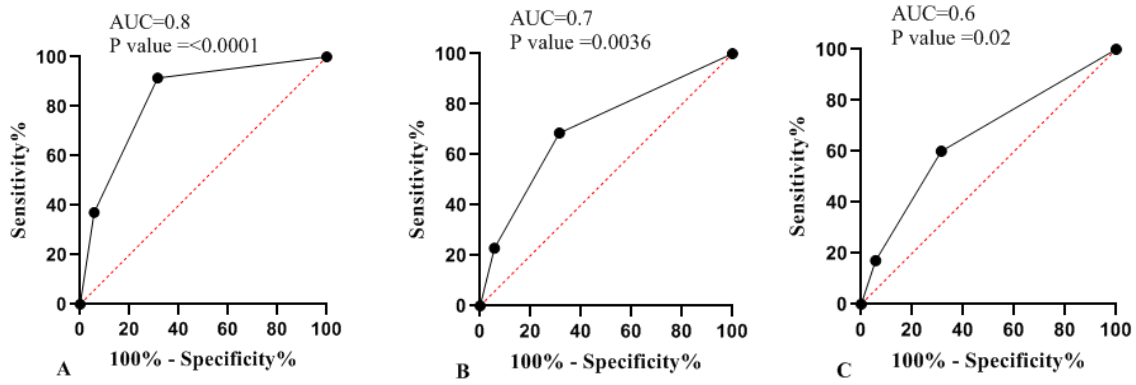
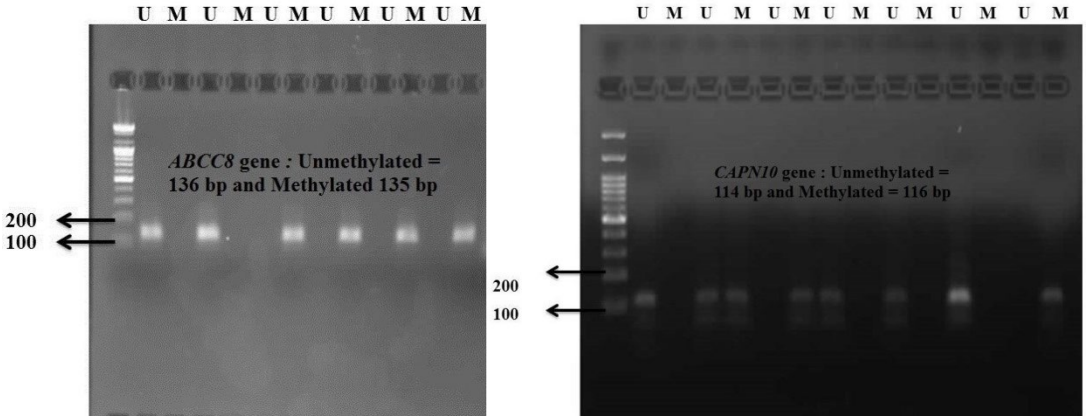


Figure 3: The ROC curve shows the DNA methylation status of the *TCF7L2* gene for three different groups: (A) T2DM < 1 year, (B) T2DM 1-3 years, and (C) T2DM 3-5 years.

After being run on a 2% agarose gel, figure 4 displays the PCR product of the methylation-specific PCR together with an estimated gene size for each of the three chosen genes, both methylated and unmethylated. The 2% agarose gel electrophoresis of MSP products is a representative example. A methylated- specific primer is denoted by M, and an unmethylated-specific primer is denoted by U. For the *ABCC8* gene, the predicted product sizes were 135 bp for the methylation primer and 136 bp for the unmethylated primer. For the *CAPN10* gene, the predicted product sizes were 116 bp for the unmethylated primer and 114 bp for the methylated primer. For the *TCF7L2* gene, the predicted product sizes were 170 bp for the unmethylated primer and 171 bp for the methylated primer.



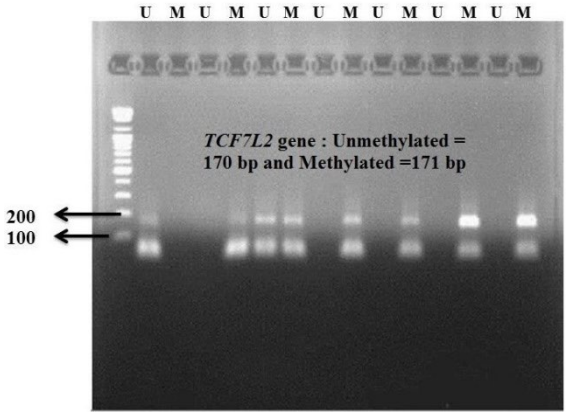


Figure 4: Methylation-specific PCR products of selected genes (*ABCC8* gene, *CAPN10* gene and *TCF7L2* gene) for amplification of both methylated (M) and unmethylated (U) regions. Lane 1 shows the DNA marker of 100 bp.

4. Discussion

The rapid technique of methylation-specific PCR was used to detect DNA methylation in blood samples from diabetic patients, specifically related to the diagnosis period of *ABCC8*, *CAPN10*, and *TCF7L2* genes. DNA methylation of the *ABCC8* gene was shown to be more prevalent in T2DM patients aged 3 to 5 years, whereas partial methylation was more common in T2DM patients aged 1 to 3 years and in the control group (25.7%). However, the control group showed more unmethylated DNA for the *ABCC8* gene, and statistically was significant the p value was 0.000391. Most research currently generating DNA methylation predictors either assumes simply linear additive effects between CpGs or take into account marginal CpG effects [20]. DNA methylation is altered by a number of patients, such as T2DM, which affects DNA regulation and the transmission of infections [21]. DNA methylation may also be influenced by age [22, 23]. DNA methylation is one of the body's regulating mechanisms for healthy cell division and proliferation. This process relies on tightly regulated, cell-specific processes that alter throughout time in response to genetic and environmental changes [24]. Overall, this study found that, for the ABCC8 gene, variations in DNA methylation instances in three selected genes is related to gender in T2DM patients aged 3-5 years, but no discernible effects on age overall.

DNA methylation changes have been observed in people with transient diabetes. These changes have been linked to conditions such as genomic imprinting and transient neonatal hyperglycemia [5]. Additionally, it has been found that diabetic nephropathy is associated with abnormal DNA methylation patterns, suggesting that epigenetic modifications may play a role in the effects of diabetes [7].

Patients with diabetes have long known that changes in DNA methylation are connected to the etiology and consequences of their disease. Hemoglobin A1c levels and DNA methylation levels are associated in the general population, suggesting that dietary and lifestyle factors may influence these alterations and glycemic control [25]. Given that DNA methylation can alter gene expression, these changes might be a main source of variations in diabetes profiles. Furthermore, DNA methylation in T2DM is associated with specific genetic and epigenetic alterations [26, 27].

Figures 1, 2 and 3 present ROC curves, which demonstrate the potential biomarkers of DNA methylation changes of the *ABCC8*, *CAPN10*, and *TCF72L* genes across various groups of T2DM duration diagnosis. The ROC curve evaluates the ability of DNA methylation biomarkers to distinguish between these groups and possibly non-diabetic individuals. Greater AUC values would suggest that *ABCC8* methylation has a better predictive ability to distinguish between the various phases of the course of T2DM. In the *TCF7L2* gene has a higher AUC than the *ABCC8* and *CAPN10* genes, which is 0.8, and a p value of less than 0.0001 indicates that the condition is very significant in people with T2DM who have had the diagnosis for less than a year. Both the *CAPN10* and *TCF7L2* genes exhibited similar AUC values as predictive biomarkers based on the length of diagnosis in people with T2DM for one to three years. People who have had T2DM for three to five years had higher levels of the *ABCC8* gene and *CAPN10* and *TCF7L2* genes.

This study found that *CAPN10* gene methylation in diabetic groups was highly correlated with diagnosis period, particularly for T2DM 3-5 years (42.9%), and statistically significant (p value < 0.00001). DNA methylation in the *TCF7L2* gene increased significantly in tandem with the length of time that T2DM was diagnosed, suggesting a strong relationship between DNA methylation status and the duration of T2DM. A healthy person has a higher percentage of DNA methylation cases than someone with T2DM who has had the disease for a shorter period of time. There appears to be a substantial correlation between the length of T2DM and methylation modifications, as indicated by the chi-square test, which indicates significant differences in DNA methylation across groups (p = 0.000046). This interpretation links the development of T2DM to the TCF7L2 gene's involvement in DNA methylation. Compared to age- and BMI-matched controls, newly diagnosed, drug-naïve T2DM patients have distinct epigenetic changes at the TCF7L2 promoter. The function of epigenetic control of TCF7L2 in T2DM is further illuminated by the correlation between methylation in the TCF7L2 promoter and fasting glucose in peripheral blood DNA [28]. M. Zhu *et al.* [29] reported that compared to healthy people, newly diagnosed T2DM patients had significantly lower levels of methylation at several CpG sites near the *KCNJ11* gene promoter.

DNA methylation variations could serve as diagnostic tools for the early detection and prognosis of diabetes progression. Another way that studies of DNA methylation alterations advance our understanding of the pathophysiology of diabetes is by revealing its molecular mechanisms and underlying consequences [4]. Sometimes specific DNA methylation patterns can act as potential biomarkers, depending on epigenetic profiles [26-30]. Treatment regimens for T2DM may change as a result of these changes [31].

It is not yet possible to routinely employ these indicators in T2DM clinical practice because the majority of the research that are now available cannot be regarded as conclusive. These investigations use a variety of methods to measure DNA methylation. Some have made advantage of the examination of CpG methylation within T2DM-associated genes. Others concentrated on impartial worldwide DNA methylation [32].

The p-value and AUC for each selected gene, in relation to various diagnostic periods. The gene with the highest AUC (0.8) in the T2DM < 1 year group was *TCF7L2*, while the gene with the lowest AUC (0.5) in the T2DM 1-3 years group was *ABCC8*. A very significant p-value is indicated by a larger AUC value. Only in T2DM < 1 year of TCF7L2 gene reached 0.8, but not in other groups; the highest AUC indicated possibility strong biomarkers.

5. Conclusions

It appears that there may be a discrepancy between the diagnostic stage of T2DM patients and controls in their ability to discriminate based on the DNA methylation scores of the *ABCC8* gene. This discrepancy leads to a difference in their suitability for predicting T2DM, with the highest discriminatory skill achieved by group T2DM 3-5 years. The results of the research thus allow us to conclude that it is possible to differentiate the degree of differentiation of the DNA methylation state of the *CAPN10* gene at different diagnostic periods, while the greatest degree of differentiation shows the groups T2DM < 1 year and T2DM 1-3 years, and, based on the data provided by the Group T2DM < 1 year, with a higher discriminatory ability, it can concluded that the expediency and effectiveness of using the DNA methylation state of the TCF7L2 gene as a biomarker for predicting T2DM at different periods.

Data availability: Data will be available upon reasonable request.

Conflicts of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding: The authors did not receive support from any organization for the submitted work.

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