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Identification of DNA methylation of *CAPN10* gene changes in the patients with type 2 diabetes mellitus as a predictive biomarker instead of HbA1c, random blood sugar, lipid profile, kidney function test, and some risk factors

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Objective. Nowadays, type 2 diabetes mellitus (T2DM) is the most common chronic endocrine disorder, affecting an estimated 5–10% of adults worldwide and this disease rapidly increases in the Kurdistan region population. This research aims to identify DNA methylation change in the *CPAN10* gene as a predictive biomarker in T2DM and the association between DNA methylation status with lipid profile and kidney function test.

Methods. The participants (113) were divided into three groups: diabetes group (47), prediabetes group (36), and control group (30). The study was carried out on patients who visited the private clinical sectors between August and December 2021 in the Koya city Kurdistan region of Iraq. To determine DNA methylation status, methylation-specific PCR (MPS) with paired primer for each methylated and unmethylated region was used. The Mann-Whitney U test and Spearman's correlation were performed for statistical analysis of data and a value of $p<0.05$ was considered significant.

Results. The obtained results show that DNA hypermethylation was recorded in the promoter region in the samples of the diabetes and prediabetes groups compared to the healthy group (control). Various factors also affected the level of DNA methylation, such as HbA1c in prediabetes group and body mass index in the control group.

Conclusion. These results indicate that DNA methylation changes in the *CAPN10* gene promoter region may be used as a potential predictive biomarker to diagnose T2DM; however, this study requires further data to support this evidence.

Key words: *CAPN10* gene, type 2 diabetes mellitus, DNA methylation, HbA1c, random blood sugar, lipid profile, kidney function test

In type 2 diabetes mellitus (T2DM), the pancreas still produces insulin (adult-onset diabetes); however, it is not produced enough or does not work properly. Nine out of 10 people with diabetes have T2DM (Saran et al. 2019). The epigenome consists of DNA methylation (DNAm), histone modifications, and RNA-mediated processes. This stability disruption may cause numerous pathologies and contribute to weight problems and T2DM (Ling and Ronn 2019; Smail 2019; Smail and Mohamad 2022). Ageing, obesity, high-energy diets, lack of physical exercise, and an impaired intrauterine environment represent nongenetic risk factors for T2DM. Gene-environment interactions, potentially through epigenetic

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mechanisms, may also affect disease pathogenesis. Indeed, over the last fifteen years, there have been an increasing number of studies supporting the pivotal role of epigenetics in the development of diabetes (Ling 2020).

For the standard form of T2DM in the general population, *CAPN10* (calpain-10) gene is the first T2DM candidate gene identified through genome-wide linkage and positional cloning (Song et al. 2004). *CAPN10* polymorphism is associated with T2DM (Tursinawati et al. 2020). The etiology of disease combines both genetic and lifestyle or environmental factors. Genome-wide association (GWAS) studies have identified *CAPN10* (SNP-19), alpha-ketoglutarate dependent dioxygenase (FTO) (rs-9939609), methylenetetrahydrofolate reductase (MTHFR) (rs1801133) and peroxisome proliferator-activated receptor gamma (PPARG) (rs1801282) genetic polymorphism with susceptibility to T2DM. The exposure will increase by the independent risk of gene polymorphism, obesity, lifestyle factors, and combinations of these factors (Sarkar et al. 2020).

Materials and Methods

Subjects and sample collection. After approval and instructions of the institutional Scientific Committee, 113 blood samples were collected from both men and women, including 47 samples of diabetes and 36 samples of prediabetes groups, and 30 samples from healthy individuals, in Koya city via sterile disposable syringe from an arm vein as these vessels were typically large. All blood samples were placed in the test tubes containing anticoagulant ethylene diamine tetraacetic acid (EDTA) to prevent blood clotting. A concentration of glycated hemoglobin (HbA1c) in blood samples measured by HbA1c analyzer (ichroma™ HbA1c) was used as a parameter to determine a prediabetes and diabetes status of the participants. For people without diabetes (control group), the normal range for the HbA1c level is between 4.0% and 5.6%, a level of 5.7% to 6.4% indicates prediabetes, and a level of 6.5% or more indicates diabetes. Besides HbA1c blood levels, random blood sugar (RBS), lipid profile (levels of total cholesterol, triglycerides, HDL and LDL cholesterol) (ROCHE COBAS-C311) and kidney function test (levels of urea and creatinine) were detected (ROCHE COBAS-C311). In addition, the participants were asked about age, gender, body max index (BMI), smoking, family history, and alcohol consumption.

DNA extraction. DNA extraction was performed using the ReliaPrep™ Blood gDNA Miniprep System (A5081, Promega, Madison, USA) according to the manufacturer's instructions and the isolated DNA samples were kept at –80 °C for further analysis. Briefly, the procedure involves four essential steps, homogenizing whole blood to release the DNA, binding DNA to the ReliaPrep™ binding column, removing impurities with wash solution - repeated three times, and eluting purified DNA.

Bisulfite DNA conversion. Bisulfite modification of DNA was performed by using the MethylEdge™ Bisulfite Conversion System (N1301, Promega, Madison, USA) according to the manufacturer's instructions. In summary, the bisulfite-converted reaction was based on the unmethylated cytosine converted to uracil, while methylated cytosine was unaffected.

Determination of DNA quality and quantity. The concentration of bisulfite-converted DNA in samples was measured at A280 nm using a NanoDrop Spectrophotometer (JANEWAY, U.K.). The sample type was set to RNA-40 because bisulfite-converted DNA contains uracil and is widely single-stranded. The DNA samples (100 ng of each sample) were run on the 1% agarose gel and stained with diamond nucleic™ acid dye to evaluate the level of fragmentation following conversion. The NanoDrop was set to DNA-50 to estimate DNA concentration before bisulfite conversion and stained with ethidium bromide.

Table 1
Primer sequence for methylation-specific PCR

No	Primer	Length	Product size	Sequence (5'->3')
Methylated primer				
1	Forward	25	116	GTTTTTTTTAATTATTCGCGGTTAC
2	Reverse	23	116	CAAACTACAACTCCCAACATACG
Unmethylated primer				
3	Forward	25	114	TTTTTTTAATTATTTGTGGTTATGG
4	Reverse	24	114	CAAACTACAACTCCCAACATACAC

Methylation-specific PCR (MSP) primer. MethPrimer platform was used for designing bisulfite-conversion-based MSP primers for *CAPN10* (Table 1). The PCR total reaction volume of 25 µl contained 12.5 µl of 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM $MgCl_2$, 1 µl of 10 pM of methylated forward or 1 µl of unmethylated forward, 1 µl of 10 pM methylated reverse or 1 µl of 10 pM unmethylated reverse, 3 µl of bisulfite converted DNA (10–50 ng) and 7.5 µl of DNA nuclease-free water. MSP was performed with the conditions as follows: 95 °C for 5 min, 35 cycles of (95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s) and 72 °C for 5 min. The PCR products were analyzed on 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV illumination. The methylation band was present at 116 base pairs and the unmethylation band was 114 base pairs (Figures 1 and 2). Control reactions were prepared using pre-qualified methylated DNA from a source similar to the source of the experimental samples. The Methylated Human Control DNA (N1231, Promega, Madison, USA) was bisulfite-converted in parallel with experimental samples to assess conversion efficiency.

Statistical analysis. The results of MSP were determined by deviation from normal pattern. Next, the difference in methylation percentage expression between all groups and normal ones was analyzed.

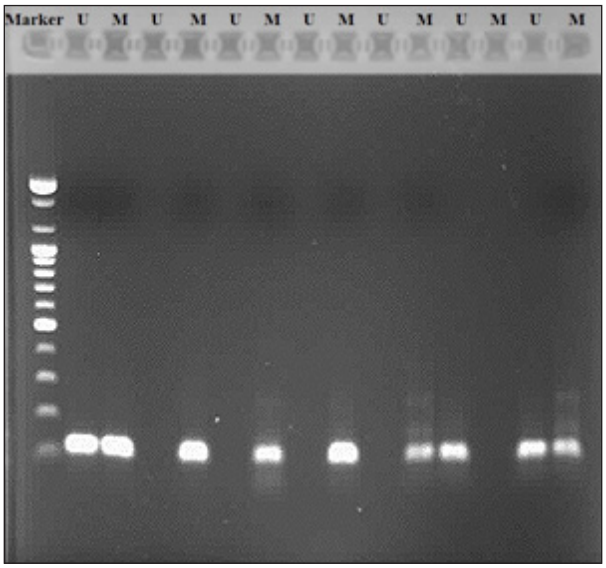


Figure 1. Methylation-specific PCR (MSP) analysis of *CAPN10* gene using 2% agarose gel electrophoresis. Lane 1* represents a DNA marker (3kb DNA ladder). Both products are excellently demonstrated. The expected product size was 114 bp for unmethylated primer (U) and 116 bp for methylated primer (M).

GraphPad Prism 8 was used for statistical analysis of data. According to Shapiro-Wilk or Kolmogorov-Smirnov test, the obtained data had not normal distribution, therefore, differences between treatment groups were determined using Mann-Whitney U test and Spearman's correlation. The categorical variables were displayed as frequency (n) and proportion (%). Descriptive statistics (mean±standard deviation) was used for continuous variables. The receiver operating characteristic (ROC) curve estimated the biomarker test. A p value of <0.05 was considered statistically significant.

Results

General characteristics of the subjects. In the present study, several parameters (age, gender, BMI, smoking, family history, Covid 19, alcohol consumption, physical activity, RBS, HbA1c, lipid profile, and kidney function test) for the participants divided into 3 experimental groups (control, prediabetes, diabetes) were determined for a qualitative identification of DNA methylation. We found highly significant differences ($p=0.000$) in BMI, RBS, and HbA1c along with lipid profile and kidney function test between diabetes group and controls (Table 2). Significant differences ($p<0.05$) were also observed in age, gender, alcohol, and physical activity between diabetes group and controls, while in smoking, family history, and Covid 19 no significant differences were shown between these groups (Table 2). Between prediabetes and control groups, highly significant differences ($p=0.000$) were found in RBS, HbA1c, triglycerides, HDL, LDL, and urea; significant differences ($p<0.05$) in age, gender, BMI, alcohol, and total cholesterol; no significant differences were shown in smoking, family history, Covid 19, physical activity and creatinine (Table 3).

Methylation status of *CAPN10* gene. Data on the methylation status of *CAPN10* gene for all experimental groups are demonstrated in Table 4 and Figure 3. The methylated *CAPN10* gene was significantly more frequent in the diabetes (n=30 cases, 63.8%; $p=0.001$) and prediabetes (n=15 cases, 41.7%; $p=0.004$) groups compared to the control group (n=3 cases, 10%). Similarly, partially methylated state of *CAPN10* gene was found more frequently in diabetes (n=11 case, 23.4%) and prediabetes (n=13 cases, 36.1%) groups in comparison to controls (n=7 case, 23.3%).

Association of *CAPN10* gene methylation status with clinicopathologic characteristics. The relationships between DNA methylation (DNAm) status of

Table 2
General characteristics of the studied population for qualitative identification of DNA methylation in control and diabetes groups

Variable		Diabetes (n=47)	Control (n=30)	p-value
Age (years)		49.4±12.5	41.0±10.6	0.003
Gender	Male (n, %)	18 (38.3%)	21 (70.0%)	0.007
	Female (n, %)	29 (61.7%)	9 (30.0%)	
BMI (kg/m^2)		30.5±2.8	23.8±4.3	0.000
Smoking (n, %)		5 (10.6%)	5 (16.7%)	0.446
Family history (n, %)		7 (14.9%)	5 (16.7%)	0.835
Covid 19 (n, %)		7 (14.9%)	3 (10.0%)	0.536
Alcohol (n, %)		1 (2.1%)	4 (13.3%)	0.053
Physical activity (n, %)		13 (27.7%)	15 (50.0%)	0.048
RBS (mg/dL)		248.7±84.6	113.1±23.7	0.000
HbA1c (%)		9.5±2.0	4.7±0.4	0.000
Lipid profile	TC (mg/dL)	192.10±27.43	153.80±13.33	0.000
	TG (mg/dL)	162.10±21.65	136.70±10.64	0.000
	HDL (mg/dL)	41.47±3.87	53.80±3.90	0.000
	LDL (mg/dL)	146.70±10.47	107.70±12.10	0.000
Kidney function test	Urea (mg/dl)	31.76±9.26	18.19±7.34	0.000
	Creatinine (mg/dl)	1.034±0.259	0.800±0.189	0.000

Abbreviations: BMI – body mass index; HDL – high-density lipoprotein cholesterol; LDL – low-density lipoprotein cholesterol; RBS – random blood sugar; TC – total cholesterol; TG – triglycerides. Data are presented as mean±standard deviation. The Mann-Whitney U test was used for statistical analysis; p value ≤0.05 was considered as statistically significant.

Table 3
General characteristics of the studied population for qualitative identification of DNA methylation change for each control and prediabetes

Variable		Prediabetes (n=47)	Control (n=30)	p-value
Age (years)		46.4±11.4	41.0±10.6	0.039
Gender	Male (n, %)	16 (44.4%)	21 (70.0%)	0.039
	Female (n, %)	20 (50.6%)	9 (30.0%)	
BMI (kg/m^2)		26.0±4.1	23.8±4.3	0.017
Smoking (n, %)		5 (13.9%)	5 (16.7%)	0.756
Family history (n, %)		2 (5.6%)	5 (16.7%)	0.147
Covid 19 (n, %)		9 (25.0%)	3 (10.0%)	0.118
Alcohol (n, %)		0 (0.0%)	4 (13.3%)	0.025
Physical activity (n, %)		13 (36.1%)	15 (50.0%)	0.259
RBS (mg/dL)		145.8±26.8	113.1±23.7	0.000
HbA1c (%)		6.1±0.3	4.7±0.4	0.000
Lipid profile	TC (mg/dL)	161.40±18.49	153.80±13.33	0.041
	TG (mg/dL)	134.20±12.13	136.70±10.64	0.000
	HDL (mg/dL)	42.53±8.43	53.80±3.90	0.000
	LDL (mg/dL)	131.70±13.27	107.70±12.10	0.000
Kidney function test	Urea (mg/dl)	27.12±8.27	18.19±7.34	0.000
	Creatinine (mg/dl)	0.806±0.135	0.800±0.189	0.089

Abbreviations: BMI – body mass index; HDL – high-density lipoprotein cholesterol; LDL – low-density lipoprotein cholesterol; RBS – random blood sugar; TC – total cholesterol; TG – triglycerides. Data are presented as mean±standard deviation. The Mann-Whitney U test was used for statistical analysis; p value ≤0.05 was considered as statistically significant.

CAPN10 gene and clinicopathologic characteristics of the participants within each experimental group are presented in Table 5 and Table 6. In the diabetes group, no statistically significant differences (p>0.05) between DNAm status and any monitored parameter were observed (Table 5). A highly significant differences were recorded between DNAm status and HbA1c levels (p=0.000) and BMI (p=0.013) in prediabetes and control groups, respectively (Table 5). In all experimental groups, no statistically significant differences (p>0.05) between DNAm status and lipid profile or kidney function test were found (Table 6).

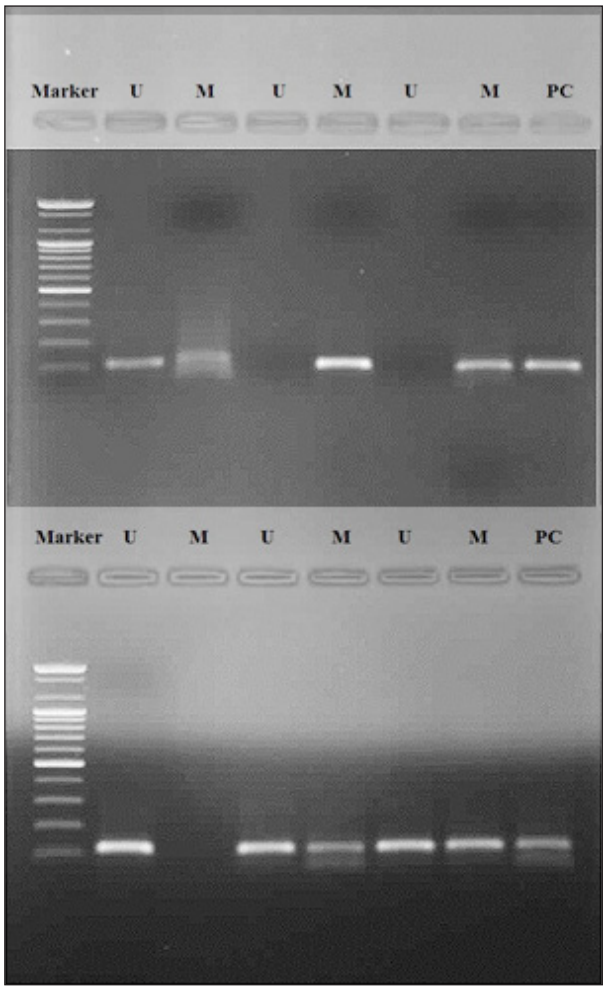


Figure 2. Methylation-specific PCR (MSP) analysis of *CAPN10* gene using 2% agarose gel electrophoresis. Lane 1* represents a DNA marker (3kb DNA ladder). Both products are excellently demonstrated. The expected product size was 114 bp for unmethylated primer (U) and 116 bp for methylated primer (M). P.C. represents a positive control (methylated human control).

The biomarker of DNAm status of *CAPN10* gene by MSP in diabetes and prediabetes groups. The ROC curves of DNAm status of *CAPN10* gene between the diabetes or prediabetes groups and control group are displayed in Figure 4 and Figure 5, respectively. The area under the curve (AUC) was higher in the diabetes group (0.7067) than in the prediabetes group (0.6970) compared to the control group (p=0.0023 and p=0.0077, respectively).

The accuracy of demographic data and laboratory findings for the diagnosis of T2DM. As indicated by the ROC curve analysis, significant correlations between BMI (AUC=0.8660; p<0.0001), age (AUC=0.6989; p=0.0034), and gender (AUC=0.6585;

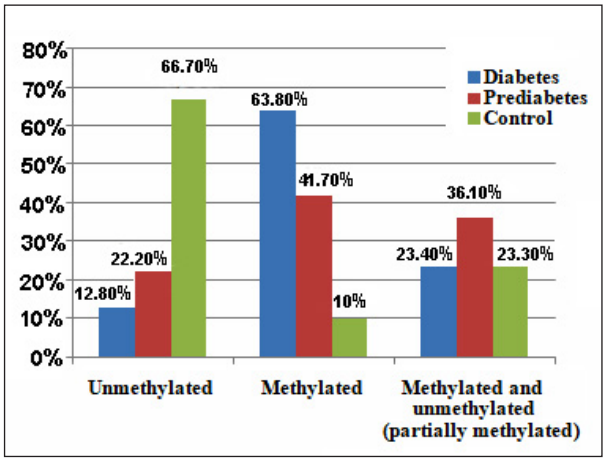


Figure 3. Percentage of methylation status of *CAPN10* gene in diabetes, prediabetes, and control groups.

Table 4
Correlation of methylation status of *CAPN10* gene between the diabetes, prediabetes and the control groups

Group	Methylated state			Total n (%)	p-value
	UM [n (%)]	M [n (%)]	M&U [n (%)] (partially M)		
Diabetes	6 (12.8%)	30 (63.8%)	11 (23.4%)	47 (100%)	0.001
Prediabetes	8 (22.2%)	15 (41.7%)	13 (36.1%)	36 (100%)	0.004
Control	20 (66.7%)	3 (10.0%)	7 (23.3%)	30 (100%)	

Abbreviations: M – methylated; M&U – methylated and unmethylated; UM – unmethylated. The Mann-Whitney U test was used for statistical analysis; p value ≤0.05 was considered as statistically significant compared to control group.

Table 5
Association of promoter region methylation of *CAPN10* gene with clinicopathologic characteristics within the experimental groups

Group		Diabetes				Prediabetes				Control			
Parameter		U	M	U&M	p-value	U	M	U&M	p-value	U	M	U&M	p-value
Age (years)		46.0±13.3	52.1±12.3	43.7±10.9	0.382	47.8±12.5	46.5±12.4	45.5±10.6	0.543	40.1±7.4	55.3±24.4	37.7±7.4	0.887
Gender	Male (n)	3	12	3	0.341	3	8	5	0.881	15	3	3	0.260
	Female (n)	3	18	8		5	7	8		5	0	4	
BMI (kg/m^2)		31.10±1.01	30.54±3.04	30.52±3.37	0.885	25.90±2.77	25.03±4	27.38±4.89	0.166	24.79±4.13	26.17±6.02	20.23±1.86	0.013
Smoking (n)		2	2	1	0.261	2	1	2	0.719	3	0	2	0.601
Family history (n)		1	3	3	0.367	1	1	0	0.277	5	0	0	0.095
Covid 19 (n)		1	5	1	0.604	3	2	4	0.969	2	1	0	0.777
Alcohol (n)		0	0	1	0.120	0	0	0	N	4	0	0	0.145
Physical activity (n)		1	9	3	0.762	1	8	4	0.678	12	1	2	0.130
RBS (mg/dL)		250.0±53.9	247.7±98.6	251.2±57.4	0.883	142.1±24.6	149.4±18.3	144.0±36.5	0.288	117.8±20.8	108.0±13.9	102.0±31.4	0.119
HbA1c (%)		8.96±1.07	9.41±2.10	10.09±2.34	0.354	6.32±0.32	6.24±0.20	5.88±0.24	0.000	4.92±0.33	5.17±0.15	4.69±0.58	0.949

Abbreviations: BMI – body mass index; M – methylated; RBS – random blood sugar; U – methylated; U&M – unmethylated and methylated (partially methylated).
Data are presented as mean±standard deviation. The Spearman's correlation was used for statistical analysis; p value ≤0.05 was considered as statistically significant.

p=0.0196) and the prediction of T2DM were demonstrated. Family history, Covide 19 infection, smoking, and alcohol consumption showed non-significant (p>0.05) results for diabetes group (Figure 6). Based on the ROC curve analysis, HbA1c had the highest AUC (1.000; p<0.0001) from all laboratory parameters monitored; RBS, lipid profile and kidney function test have high AUC (from 0.7543 to 0.9894) and were highly significant (p<0.0001) in the diabetic subject as it is demonstrated in Figure 7.

The accuracy of demographic data and laboratory findings for the diagnosis of prediabetes. Similar to the diabetes group, significant correlations between BMI (AUC=0.6718; p=0.0169), age (AUC=0.6481; p=0.0394) and gender (AUC=0.6278; p=0.0755) and the prediabetes were found. Other population parameters monitored showed non-significant (p>0.05) results in the prediabetes group (Figure 8). Regarding the laboratory parameters, ROC curve analysis showed the highest AUC for HbA1c (1.000; p<0.0001), highly significant results (p<0.0001) for HDL (AUC=0.9370), LDL (AUC=0.909), RBS (AUC=0.8236), and urea (AUC=0.7884) in the prediabetes group (Figure 9).

Discussion

The fasting blood glucose, oral glucose tolerance test, and HbA1c are reliable diagnostic procedures for T2DM. In the present study, the blood samples were collected to determine which participants had diabetes, prediabetes, and which were healthy individuals based on HbA1c and RBS levels. According to our data, HbA1c values less than 5.7% are regarded as normal, values of 5.7% to 6.4% indicate prediabetes, and values higher than 6.5% indicate diabetes. We confirmed significantly increased (p=0.000) HbA1c levels in the diabetes (9.5±2.0%) and the prediabetes (6.1±0.3%) groups compared to the control group (4.7±0.4%). In addition, RBS levels were significantly increased (p=0.000) in the diabetes (248.7±84.6 mg/dL) and the prediabetes (145.8±26.8 mg/dL) groups compared to the controls (113.1±23mg/dL). The normal range is consistent with other recent studies investigating the accuracy of HbA1c in diagnosing diabetes (Thornton-Swan et al. 2022). The optimal value for HbA1c for the diagnosis of prediabetes state remains controversial (Asllanaj et al. 2019).

Moreover, according to Knudsen et al. (2022), the introduction of HbA1c as a diagnostic tool could alter the fundamental epidemiology of T2DM by failing to diagnose patients who would have previously been diagnosed and treated. As there are still limitations to the use of HbA1c and RBS tests for T2DM diagnostic screening, various new T2DM diagnostic biomarkers are still being investigated (Sun et al. 2017).

In addition to HbA1c and RBS, lipid profile and kidney function test were measured for all three groups. Data on age, gender, BMI, smoking, alcohol consumption, family history, and Covid 19 infection of the participants were also included in the laboratory findings. Of these parameters, BMI showed the highest significant differences (p=0.000 and p=0.017, respectively) between the diabetic (30.5±2.8 kg/m^2) or the prediabetes (26.0±4.1 kg/m^2) groups and controls (23.8±4.3 kg/m^2). According to the study of Abbasi et al. (2017), rising obesity has contributed to the increasing incidence of T2D among UK children and young adults, with a fourfold greater risk of developing T2D in obese individuals.

According to our findings, total cholesterol, triglycerides, LDL, and urea levels were significantly increased (p=0.000) and HDL cholesterol was significantly (p=0.000) decreased in the diabetes and the prediabetes groups compared to the controls. A significant difference in creatine levels was found only between the diabetes and the control groups. Another study demonstrated that diabetic individuals had lower levels of serum HDL cholesterol than nondiabetic participants and greater levels of cholesterol, triglycerides, and LDL cholesterol than nondiabetic subjects (Sabahelkhier et al. 2016). A study conducted in Iraq illustrated that both male and female patients with T2DM had higher blood urea levels than those without diabetes, even though these differences were small. Furthermore, a difference in serum creatinine levels was highly significant between diabetics and controls (Azeez et al. 2020).

Calculating HbA1c is crucial for blood glucose control. The American Diabetes Association has recognized HbA1c as a diagnostic standard for diabetes since 2010 (Gao et al. 2022). HbA1c, a protein glycosylated form of hemoglobin, binds hemoglobin (Hb) in red blood cells to blood glucose. But it's still unclear how Hb and HbA1c are related (Bai et al. 2022). HbA1c testing has been proposed as an alternative T2DM screening test. A two- to three-month average of blood glucose levels is represented by the HbA1c level. The presence of hemoglobinopathy or renal failure, laboratory error, and the usage of

Table 6

Association of promoter region methylation of *CAPN10* gene with lipid profile or kidney function test within the experimental groups

Group	Diabetes				Prediabetes				Control			
Parameter	**U**	**M**	**U&M**	**p-value**	**U**	**M**	**U&M**	**p-value**	**U**	**M**	**U&M**	**p-value**
TC (mg/dL)	190.5±16.2	188.4±30.0	203.1±23.2	0.308	149.1±21.2	167.5±16.5	162.1±16.4	0.339	153.0±13.8	152.7±9.5	156.9±14.5	0.629
TG (mg/dL)	168.2±24.0	156.4±21.5	174.1±16.0	0.220	132.9±11.6	136.3±13.7	132.5±11.1	0.478	136.3±11.5	141.3±5.9	136.0±10.3	0.845
HD (mg/dL)	39.50±3.62	41.83±3.59	41.55±4.72	0.415	46.38±3.70	38.93±10.59	44.31±6.24	0.716	53.55±4.36	54.33±3.79	54.29±2.75	0.798
LDL (mg/dL)	149.7±9.2	147.4±11.5	143.3±7.7	0.220	135.9±22.0	129.7±10.5	131.5±9.3	0.528	106.5±12.7	106.0±7.94	111.9±12.3	0.358
Urea (mg/dl)	27.67±6.44	31.77±9.29	33.97±10.37	0.200	22.68±9.84	29.91±9.15	26.64±4.73	0.857	16.47±7.36	20.00±6.08	22.33±6.68	0.067
Creatine (mg/dl)	1.017±0.232	1.017±0.257	1.091±0.291	0.597	0.825±0.167	0.827±0.134	0.769±0.118	0.223	0.840±0.150	0.967±0.057	0.614±0.204	0.107

Abbreviations: HDL – high-density lipoprotein cholesterol; LDL – low-density lipoprotein cholesterol; M – methylated; TC – total cholesterol; TG – triglycerides; U – methylated; U&M – unmethylated and methylated (partially methylated). Data are presented as mean±standard deviation. The Spearman's correlation was used for statistical analysis; p value ≤0.05 was considered as statistically significant.

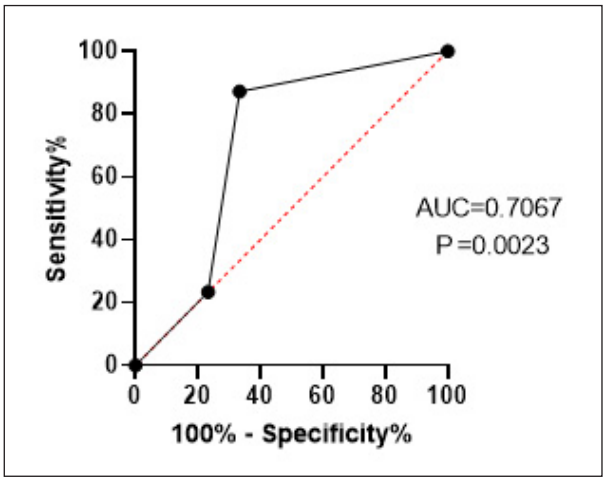


Figure 4. The ROC curve of DNA methylation status of *CAPN10* gene quantified by methylation-specific PCR (MSP) for diabetes group. Abbreviations: AUC – area under the curve.

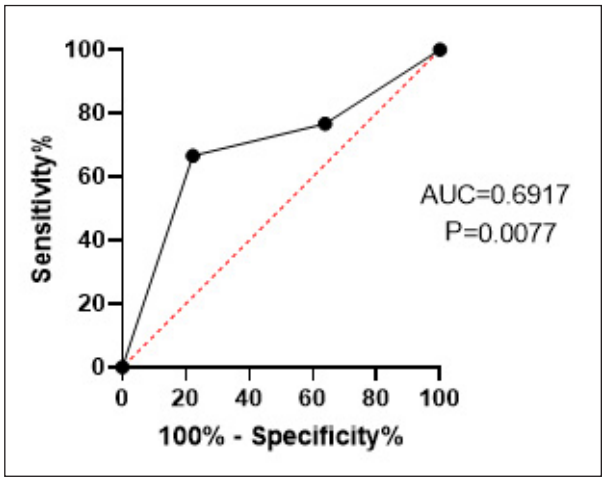


Figure 5. The ROC curve of DNA methylation status of *CAPN10* gene quantified by methylation-specific PCR (MSP) for prediabetes group. Abbreviations: AUC – area under curve.

specific drugs may affect the accuracy of HbA1c analysis (Bennett et al. 2007). Prior research using demographic, clinical, and laboratory information, including age, BMI, HbA1c, high blood pressure, and matching learning methods for predicting diabetes risk, has positive outcomes (Muhammad et al. 2020; Zhang et al. 2020).

The present study qualitatively investigated diabetes-related *CAPN10* gene methylation in blood samples using MSP. Interestingly, *CAPN10* methylation was detected at a high frequency in 30 (63.8%) out of 47 samples in the diabetes group, in 15 (41.7%) out of 36 samples in the prediabetes group compared to 3 (10%) out of 30 samples in the controls. To the best of our knowledge, this is the first study identifying *CAPN10* gene methylation status in residents of Kurdistan region of Iraq. DNAm has been shown to be affected by many factors, including heavy metals, pesticides, tobacco smoke, polycyclic aromatic hydrocarbons, hormones, radioactive substances, viruses, bacteria, basic nutrients, the social environment, maternal care age, gender, and disease state. The conflicting results of various studies may be attributed to the different populations and methods used in these studies (Mutize 2016). In the current study, there were significant differences between DNAm changes in the diabetes and prediabetes with control. Our data demonstrated that the highest DNAm frequencies was detected in the diabetes group (n=47), in 18 females (38.3%) in 12 males (25.5%).

Over the past few decades, the prevalence of diabetes mellitus has risen significantly in nearly all countries and may be considered a growing epidemic. Urbanization and income status are major factors that influence current prevalence rates introducing interesting differences between several population groups (Lovic et al. 2020). Approximately 5% of all diabetes cases are type 1 (T1DM), 90% type 2 (T2DM), and 5% other subtypes. The incidence and prevalence of diabetes have been increasing worldwide over the past 30 years (Mekala and Bertoni 2020). According to the literature, Ditamari people of the Atakora Department of northern Benin are at increased risk of developing T2DM (6.8% versus 2.6% nationally). However, no studies have been conducted to investigate the genetic basis of T2DM in this population. Separate studies on *CAPN10* have demonstrated its role in insulin resistance phenotypes (Chabi et al. 2020).

Aberrant DNAm has been associated with the pathogenesis of T2DM and has been reported to be observable during the subclinical or asymptomatic stage of the disease. Furthermore, the possibility of DNAm acting as a potential biomarker for metabolic diseases such as T2DM and cardiovascular disease (CVD) has been reported (Mutize et al. 2018). On the other hand, the DNAm of eight genes (*IGF1*, *IGF2*, *H19*, *ARHGRF11*, *MEST*, *NR3C1*, *Adiponectin*, and *RETN*) was chosen based on a literature review of candidates doubtlessly involved in gestational diabetes mellitus and obesogenic pathways (Joyce et al. 2019). The biological onset of the disease occurs long before clinical symptoms develop; thus, the search for early diagnostic and prognostic biomarkers, which could facilitate intervention

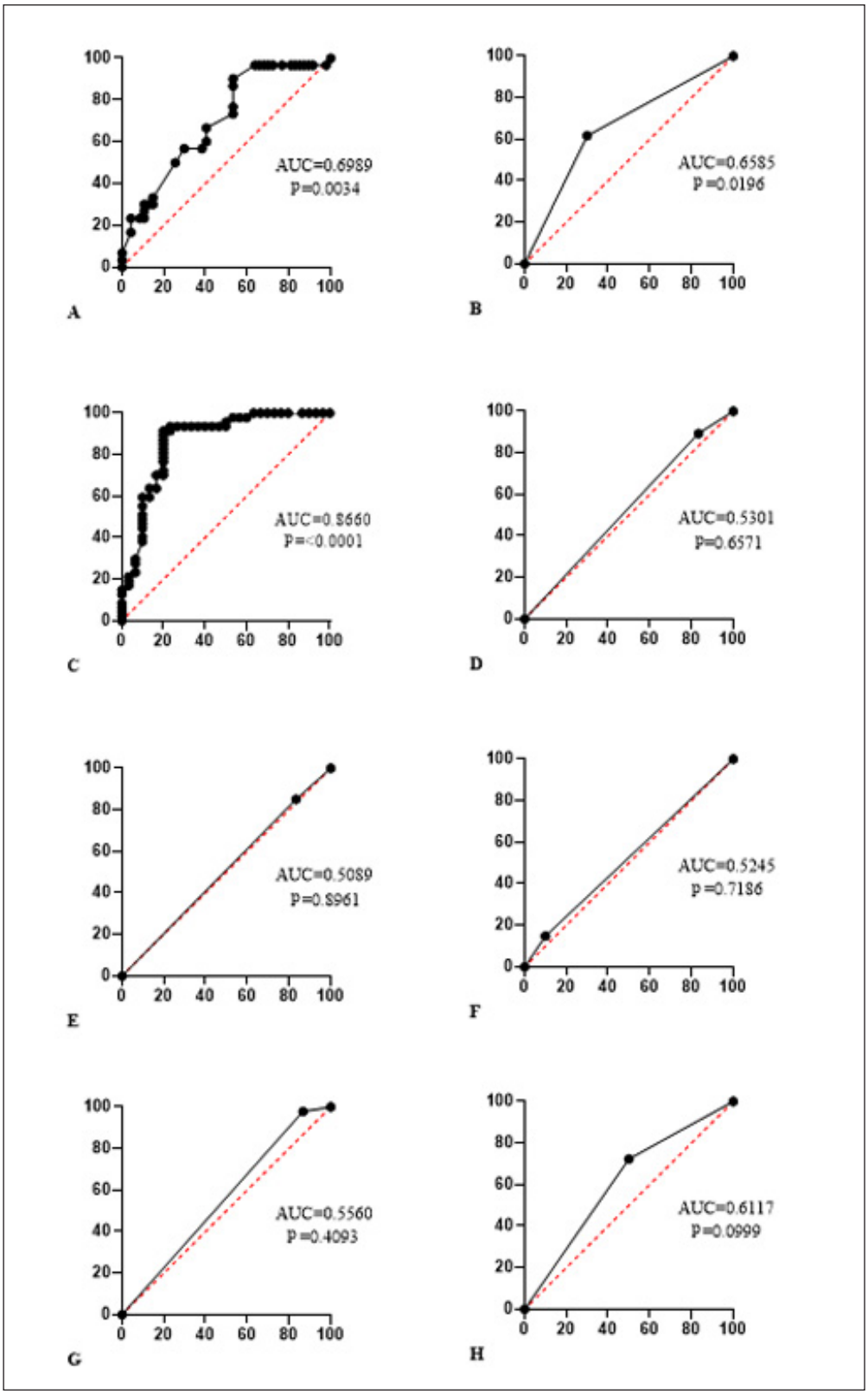


Figure 6. The ROC curves of population characteristics: age (**A**), gender (**B**), body mass index (BMI) (**C**), smoking (**D**), family history (**E**), alcohol consumption (**F**), Covid 19 (**G**), and physical activity (**H**) for the prediction of diabetes. Abbreviations: AUC – area under curve.

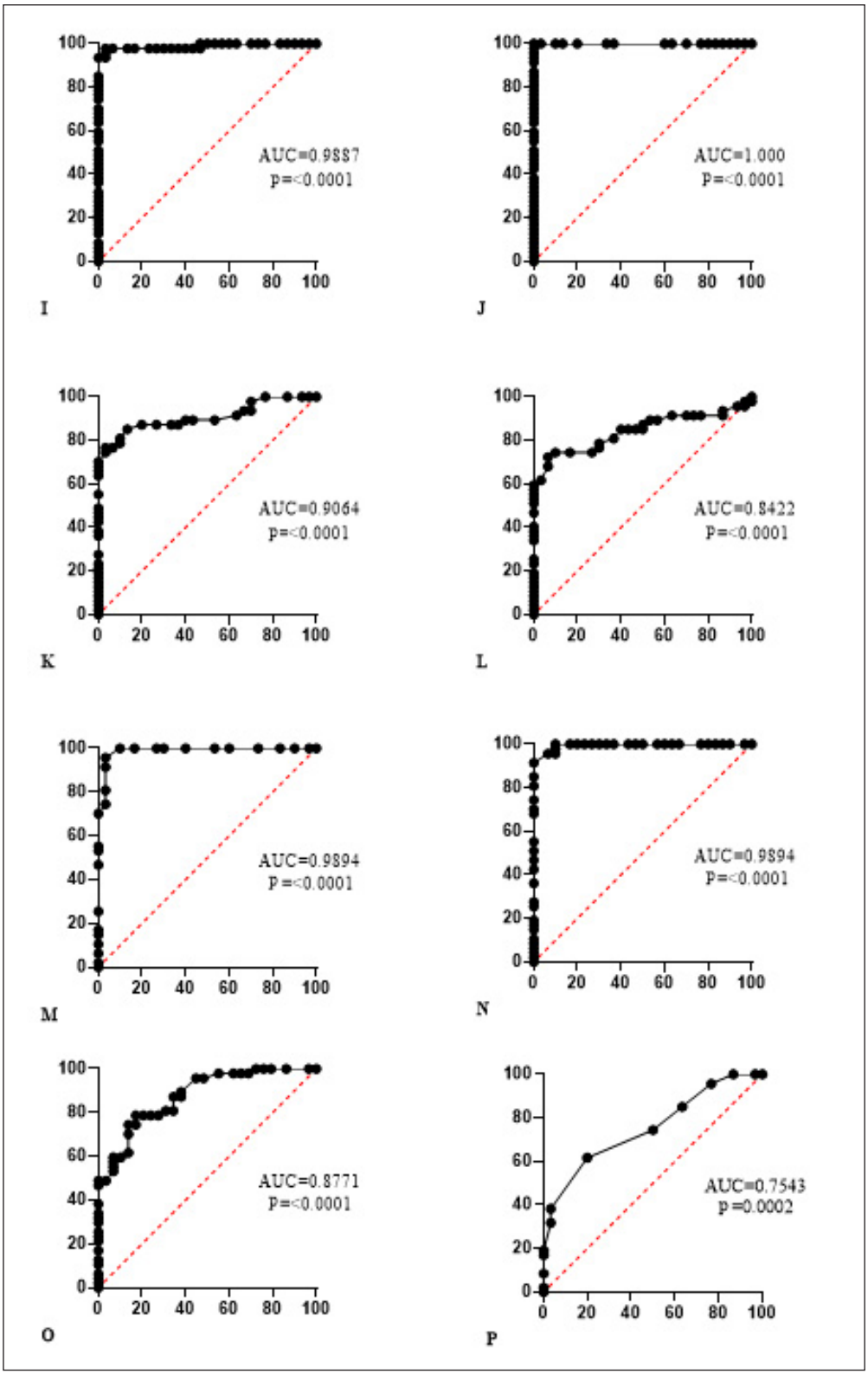


Figure 7. The ROC curves of laboratory parameters: random blood sugar (RBS) (**I**), glycated hemoglobin (HbA1c) (**J**), total cholesterol (**K**), triglycerides (**L**), high-density lipoprotein (HDL) cholesterol (**M**), low-density lipoprotein (LDL) cholesterol (**N**), urea (**O**), and creatinine (**P**) for the prediction of diabetes. Abbreviations: AUC – area under curve.

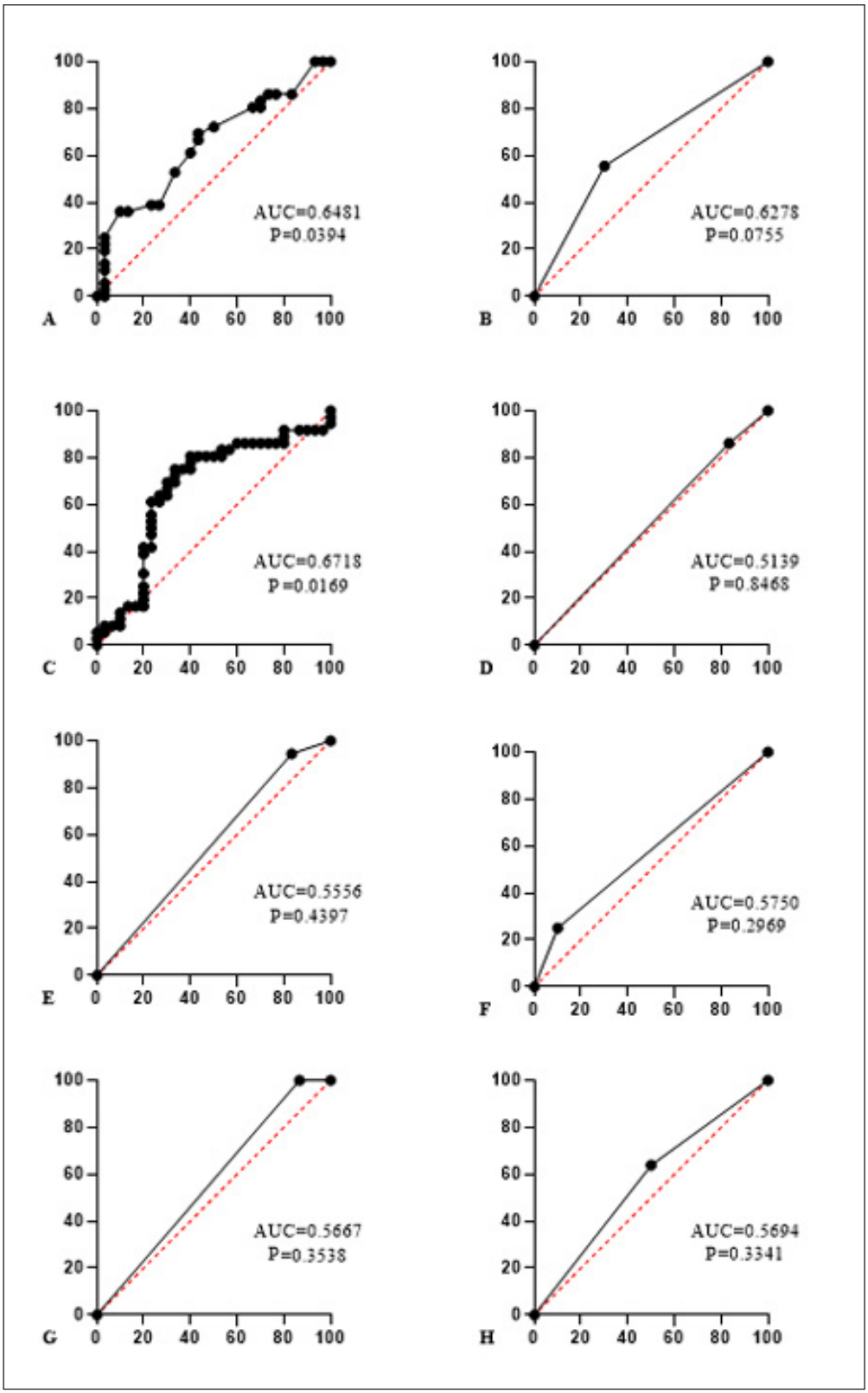


Figure 8. The ROC curves of population characteristics: age (**A**), gender (**B**), body mass index (BMI) (**C**), smoking (**D**), family history (**E**), alcohol consumption (**F**), Covid 19 (**G**), and physical activity (**H**) for the prediction of prediabetes. Abbreviations: AUC – area under curve.

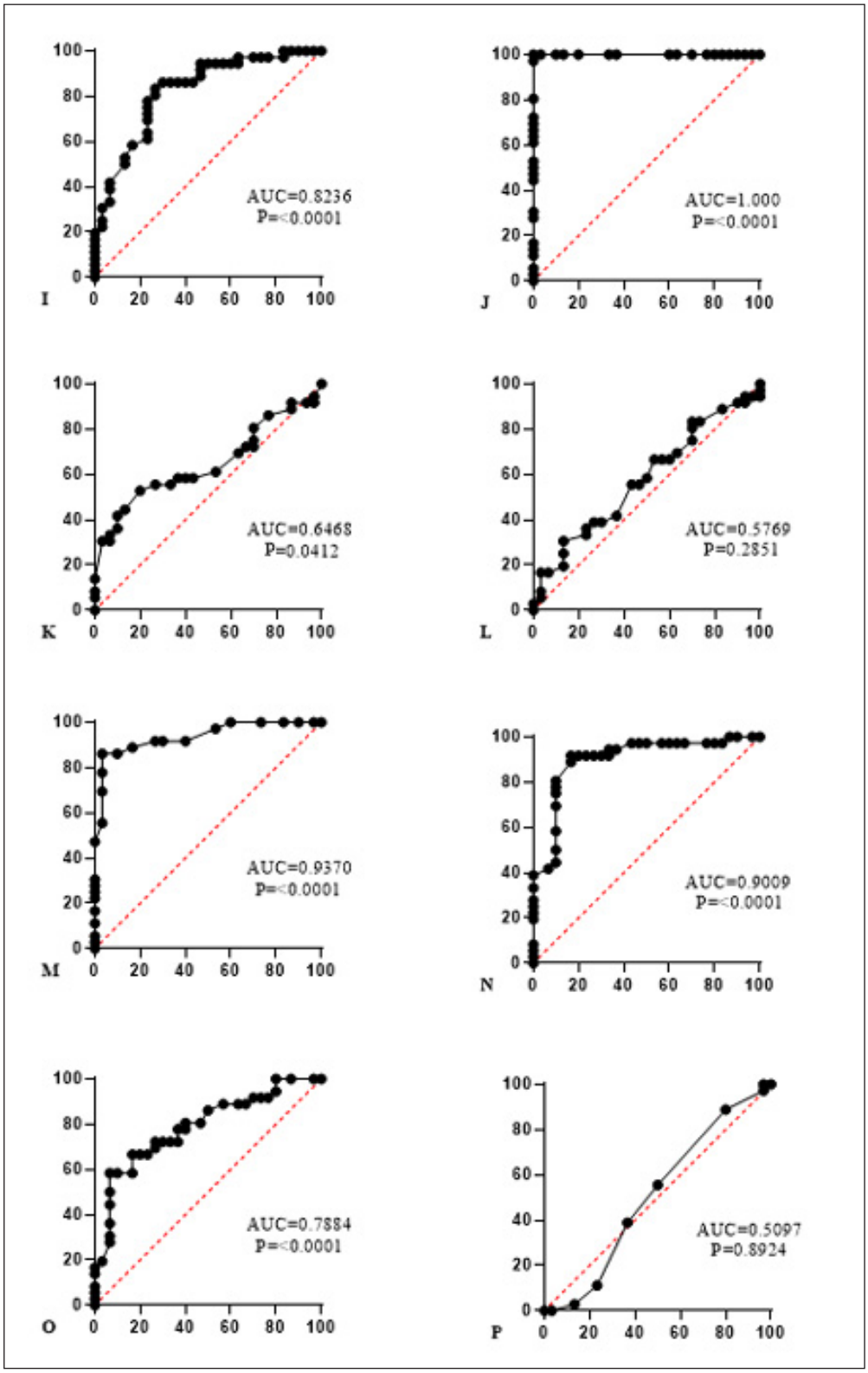


Figure 9. The ROC curves of laboratory parameters: random blood sugar (RBS) (**I**), glycated hemoglobin (HbA1c) (**J**), total cholesterol (**K**), triglycerides (**L**), high-density lipoprotein (HDL) cholesterol (**M**), low-density lipoprotein (LDL) cholesterol (**N**) urea (**O**), and creatinine (**P**) for the prediction of prediabetes. Abbreviations: AUC – area under curve.

strategies to prevent or delay disease progression, has increased considerably in recent years (Willmer et al. 2018). In the present study, the ROC curve analysis of DNAm status of *CAPN10* gene demonstrated significant differences between the diabetes (AUC=0.7067) or the prediabetes (AUC=0.6970) groups and the control group.

Inter-individual DNAm variations were hypothesized to alter individual susceptibility to T2DM and other common human diseases. Methylation profiles are typically established during early developmental stages before major cell differentiation and subsequently maintained through cell divisions. Thus, individual-specific, disease-related methylations may appear not only in the affected tissue(s) but across the human body, including accessible tissues such as blood. Furthermore, given the well-documented effect of DNAm on chromatin activity and gene-expression patterns, such early-onset methylation variation may predispose to disease development in adulthood, like the effect of DNA sequence variations (Toperoff et al. 2012). Several genes involved in (glucose) metabolism have been shown to exhibit differential DNAm in their promoters, e.g., facilitative glucose transporter 4, the major glucose transporter in adipose and muscle tissues, and uncoupling protein 2, a major candidate gene for the development of T2DM (Maier and Olek 2002).

Conclusion. It is assumed that increased DNAm in patients with T2DM may be correlated with the pathogenesis and development of disease symptoms. The results of the present study based on the ROC curve analysis demonstrated that *CPAN10* gene hypermethylation could be used as a potential biomarker instead of lipid profile, kidney function test and some other risk factors that help to diagnose T2DM.

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Conflict of interest: *The authors declare no conflicts of interest.*

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