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Association of Circulating Long Noncoding *RNA GAS5* are Associated with Type 2 Diabetes Mellitus

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Abstract: Hyperglycemia, or abnormally high blood sugar levels, is a hallmark of diabetes mellitus (DM), a serious metabolic illness caused by the body's incapacity to either create or use insulin as intended. This study was designed investigated the relationship between the expression levels of the long noncoding RNA *GAS5* (LNCRNA GAS5) and various biochemical parameters in individuals with type 2 diabetes mellitus (T2DM) compared to a control group of apparently healthy individuals. The study included the collection of blood samples from 50 suspected T2DM patients who attended the AL-Furat General Hospital in Baghdad after medical investigation and 50 apparently healthy individuals as a control group for the period from July 2023 to November 2023. The Serum Biochemical parameters were analyzed after the collection of the sample to observe the changes among T2DM cases and healthy controls. In the present study, the age was classified into three groups 40–50 years, 50-60 years and > 60 years with highly significant differences ($p<0.001$) in biochemical parameters (RBS, HbA1c and C. peptide). the *GAS5* levels were decreased in T2DM calculated ratios for *GAS5* gene fold expression in the patients compared to healthy groups were 0.73, and 1.00, respectively were highly significant differences $p<0.001$. *GAS5* expression profiles did not show a significant correlation with clinical parameters, including random blood sugar (RBS) and HbA1c. The ROC analysis revealed the cutoff GAS5 value of the predictive cut-off value of (0.16) Analysis indicated an area under the curve (AUC) of ROC of 0.81 (95% CI: 72.3%, 92.6%) with 74% sensitivity and 88% specificity with. ($p\leq 0.001$). in distinguishing non-diabetic from diabetic subjects. The positive predictive value is 71.4%. In conclusion, the results of this study indicate that the potential circulating LNCRNA *GAS5* expression level is important in the diagnosis and progression pathogenesis of T2DM. The findings highlight the potential utility of *GAS5* as a biomarker for distinguishing between diabetic and non-diabetic individuals.

Keywords: Long noncoding RNA *GAS5*, Diabetes mellitus (DM) and q PCR.

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Introduction

Type-2 diabetes mellitus (T2DM) presents as a complex condition marked by a diminished mass of pancreatic islets β-cells, leading to impaired insulin release (1). This metabolic disorder is characterized by low-grade inflammation (2) and typically manifests in adults, resulting from either insulin insufficiency or dysfunction. The body's defence mechanisms, primarily orchestrated by Peripheral Blood Lymphocytes (PBLs), play a pivotal role in resisting infective microorganisms (3).The aetiology of (T2DM) is intricate, involving a combination of genetic and environmental factors (4). There is an observed positive correlation between Neck Circumference (NCSA), elevated BMI values, and T2DM in both genders (5). Additionally, T2DM induces heightened lipid peroxidation within the body, subsequently leading to the development of chronic complications attributed to oxidative stress (6). Type 2 Diabetes (T2D) is a progressive condition characterized by

elevated blood sugar levels (hyperglycemia). In individuals with T2D, the body either resists the effects of insulin or fails to produce enough insulin (7). Long non-coding RNAs (lncRNAs), a relatively understudied class of transcripts, are increasingly implicated in the pathogenesis of diabetes. Research suggests that lncRNAs regulate the expression of β-cell-specific transcription factors. The knockdown or overexpression of lncRNAs can impact a network of key genes and pathways involved in diabetes. Gene expression analysis in studies of diabetic models has identified several lncRNAs with roles in β-cell function. Understanding these roles is crucial for comprehending the molecular network of β-cells and advancing novel diabetes treatments (8). The *H19* gene, located in an imprinted region of chromosome 11p15.5 near exon 6 of the insulin-like growth factor 2 (*IGF2*) gene, is one such lncRNA associated with diabetes(9). LncRNAs may either suppress or exacerbate diabetes-associated vascular complications (10). Another lncRNA, growth arrest-specific transcript 5 (GAS5), has been linked to T2DM prevalence, with its serum levels correlated with the condition (11). Notably, the downregulation of *GAS5* has been associated with insulin resistance (12). This underscores the potential significance of lncRNAs in both the development of diabetes and its complications, providing avenues for further research and potential therapeutic interventions.

Materials and methods

Participants (patient and control)

The case-control study conducted at AL-Furat General Hospital in Baghdad involved the enrollment of Type 2 Diabetes Mellitus (T2DM) patients following a medical investigation between July 2023 and November 2023. A total of one hundred blood samples were collected, with 50 T2DM adult patients having a confirmed diagnosis (n = 50) and an additional 50 healthy controls. The gathered patient information included details such as age, sex, name, duration of diabetes, insulin usage, existence of hypertension, hyperlipidemia, genetic factors, and the specific type of drugs being administered.

Sample collection and biochemical tests

Venous blood samples of 5 ml were collected from each participant. Subsequently, each sample underwent a three-part division process. The first part (2 ml) was transferred into an EDTA tube for the assessment of Hba1c and c-peptide. The second part (250 µl) was placed in a 1.5 ml centrifuge tube containing 750 µl of triazole reagent, which was then stored in a deep freeze for RNA extraction scheduled for the following day. The remaining 3 ml of whole blood constituted the third part and was placed in a Gel tube. This portion underwent centrifugation at 3000 rpm for 10 minutes to separate serum for random blood sugar and lipid profile testing. The biochemical analyses were carried out using routine clinical assays colourimetric method. The automated systems employed for these tests were the Accent-200 Analyzer, Cobas c111, and Cobas e411, with procedures conducted according to the instructions provided by the manufacturers.

Primer design for Quantification PCR (qPCR)

Primer has been designed in this study based on the Bioinformatics tools by using the international databases (NCBI) and several tools that are available on the website (online tools and software) the design process for primer was obtained by using primer3 plus for all genes as appear in (Table 1).

Table (1): Primers used in qPCR.

Description	Sequence (5′→3′ direction)	Company and country
	LNCRNAGAS5 gene	Alpha DNA company Canada
Forward	ACACAGGCATTAGACAGAA	
Reverse	CCAGGAGCAGAACCATTA	
	GAPDH (housekeeping gene)	
Forward	TGAGAAGTATGACAACAGCC	
Reverse	TCCTTCCACGATACCAAAG	

The RNA extraction and cDNA synthesis

Total RNA extraction was performed utilizing TransZol UP from TransGen Biotech, China, following the manual extraction procedures outlined in the product manual. The NanoDrop spectrophotometer (NanoDrop Fisher, USA) was employed to assess both the quantity and quality of the isolated RNA. The selection of total RNA extracts for reverse transcription-polymerase chain reaction (RT-PCR) was based on achieving an OD260/OD280 ratio within the specified range.For the RT-PCR process, cDNA was synthesized in accordance with the manufacturer's instructions using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix reagent from TransGen Biotech, China. The generated cDNA was stored until it was utilized as a template for subsequent RT-PCR procedures.

Quantitative real-time (qRT-PCR) for *LNCRNAGAS5* gene and *GAPDH* expression gene

To determine the threshold cycle (Ct), RT-PCR reaction mixtures were prepared using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix and conducted on the Qiagen Rotor gene Real Time PCR System from Germany. Each reaction was carried out individually, and every reaction was performed in duplicate. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as the reference gene. The gene expression reactions for both *GAPDH* and *GAS*5 were performed separately under the following conditions: Enzyme activation at 95 ºC for 1 min, denaturation at 95 ºC for 15 sec, annealing temperature at 56 ºC for 30 sec for 40 cycles, extension at 72 ºC for 15 sec, and a melt curve analysis from 60-95ºC, with fluorescence measurement. The primer sequences were provided by Alpha DNA Company, Canada. Unfortunately, the provided text does not include the actual sequences of the primers. If you have the primer sequences, we may want to include them for a more comprehensive understanding of the experiment.

Statistical analysis

The data were expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS 26.0 (SPSS Inc., Chicago, USA). Statistical significance was determined at a p-value < 0.05. One-way ANOVA was employed to assess the statistical differences between the mean values of control participants and individuals with (T2DM). Correlation analysis was conducted using Pearson correlation tests, and the Chi-square test was utilized for significant comparisons

involving percentages. Additionally, the Least Significant Difference (LSD) test was applied for significant mean comparisons in this study.

Results and discussion

Distribution of T2DM Patients according to age

In this study, a total of fifty Iraqi T2DM patients were included, and their ages ranged from 40 to 80 years. The age distribution was categorized into three groups: 40–50 years, 50–60 years, and >60 years. The statistical analysis revealed a highly significant difference ($p<0.001$), and the corresponding data are presented in (Table 2).

Table (2): The parameters, numbers of samples and age in T2DM patients

Parameters	No. of samples	40-50 Mean ± SD	50-60 Mean ± SD	More than 60 Mean ± SD	P-Value
RBS	12	201.25±69.970	207.60±64.044	249.10±68.122	***
HbA1C	15	6.78± .720	11.79±16.130	7.47±1.053	**
Peptide	23	3.57±1.525	3.58±2.125	4.59±1.777	**
Total	50				
p-value * 0.05 level, p-value **0.01and p-value ***0.001					

The results of the age variable showed that there were significant differences between the age and type 2 DM patients for all the parameter (RBS, HbA1c and c.peptide).

Quantification of *LNCRNA GAS5* expression by Real-time PCR

The Ct value of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), the housekeeping gene used for this work. The Ct values for *GAPDH* among all study groups ranged from 20.44–26.3 with a mean±SD Ct values of (23.367±1.203), and (22.580±1.394) in the Patients and healthy groups. The Ct value of the *GAS5* gene in this study ranged from 23-29, with mean±SD Ct values of (26.543±1,070) and (25.291±1.045) in the Patients and healthy groups. There was a significant difference between these groups in terms of the Ct value means of *GAS5* ($p = 0.001$). The calculated ratios for *GAS5* gene fold expression in the patients and healthy groups were 0.73, and 1.00, respectively. Due to the obvious slight variations in gene fold expression between the research groups, the *GAPDH* gene works as a suitable control gene (Table 3). The *GAS5* gene amplification plots and dissociation curves are depicted in (Figures 1, A and B).

Table (3): Comparison between patients and control groups regarding *GAPDH* and *LNCRNA GAS5* fold expression levels

Group	Mean CT of *GAS5* (Mean±SD)	Mean CT of *GAPDH* (Mean±SD)	ΔCT (mean Ct *GAS5*- mean Ct *GAPDH*)	$2^{-\Delta CT}$	Experimental group/ control group	Fold of gene expression (Mean±SD)
Control	25.291±1.045	22.580±1.394	2.71	0.15	0.15/0.15	1.00
Patients	26.543±1.070	23.367±1.203	3.18	0.11	0.11/0.15	0.73

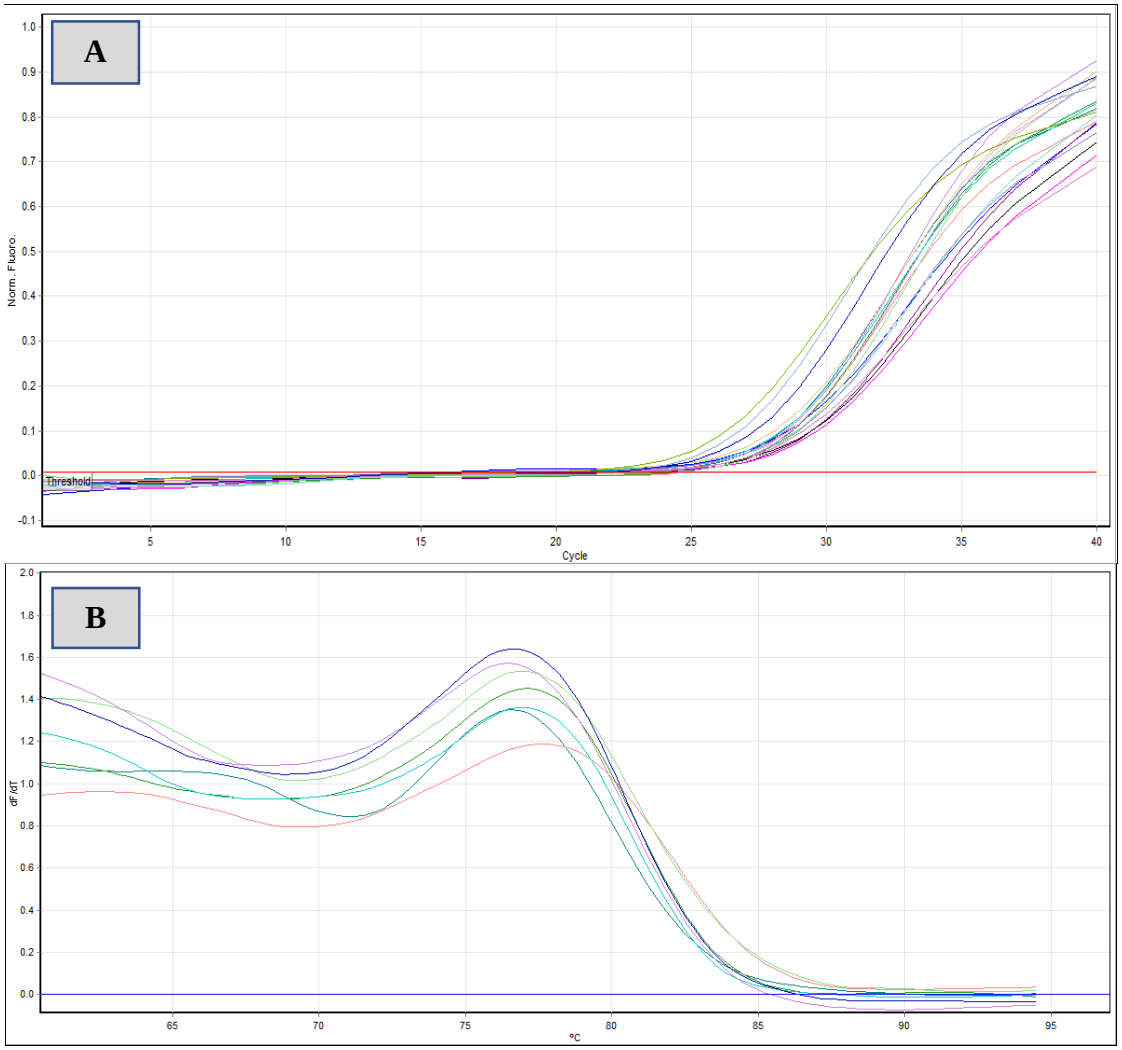

Figure (1): A-*GAS5* gene amplification was plotted using qPCR samples that covered all research groups. The CT values varied between 15 and 17. B-*GAS5* gene dissociation curves using qPCR samples that covered all research groups. Melting temperatures varied from 60°C to 95°C. The images were captured using the Qiagen Rotor Gene Q qPCR apparatus.

Normalization of the *GAS5* gene's Ct (cycle threshold) values

In the ongoing study, quantitative RT-PCR was employed to assess *GAS5* mRNA expression and compare it between the patient and control groups.

in (Table 5), with ΔCt means of (3.176) and (2.711) in the patients' and control groups, respectively. The study groups exhibited a significant difference (p= 0.05). To determine the expression of the *GAS5* gene, $2^{-\Delta\Delta Ct}$ values were utilized for each research group, The relative quantification equation, based on normalizing Ct values of *GAS5* cDNA to *GAPDH* (ΔCt), was used to determine the gene expression fold change (13). The mean ΔCt (normalized Ct values) for each study comparing them to the control group. The outcomes are presented in Table 4. The mean $2^{-\Delta\Delta C}$t value was 0.72 in the patients' group, while the control group's mean $2^{-\Delta\Delta Ct}$ value was 1.00, as shown in (Table 4).

Table (4): Fold of *LNCRNA GAS5* expression Depending on 2-$^{\Delta\Delta Ct}$ Method

Group	Mean CT of *GAS5*	Mean CT of *GAPDH*	ΔCT (mean Ct *GAS5* -mean Ct *GAPDH*)	Mean ΔCT calibrator	ΔΔCT	2^ - ΔΔCt	Experimental group / control group	Fold of gene expression
Patient	26.543	23.367	3.176	5.393	-2.217	4.649	4.649/6.417	0.72
Control	25.291	22.58	2.711	5.393	-3.682	6.417	6.417/6.417	1.00

The present study shows decreased *GAS5* levels of gene expression in Iraqi diabetic two patients, Reference (20) also supports the observed relationship between age and T2DM in Iraqi diabetic patients, particularly when investigating the impact of blood sugar levels on TNFα gene expression and its relation to liver disorders. The current study reveals that long non-coding RNAs (lncRNAs), specifically *H19* and *GAS5*, play cell-specific and diverse roles. Recognizing the crucial regulatory functions of lncRNAs in gene expression, it becomes intriguing to uncover the molecular targets of *H19* and *GAS5* implicated in Type 2 Diabetes Mellitus (T2DM). Another lncRNA, Growth Arrest-Specific Transcript 5 *(GAS5*), is noted for its correlation with T2DM prevalence, as indicated in reference (11). Additionally, its downregulation has been linked to insulin resistance, as reported in reference (21). The identified roles and associations of these lncRNAs, including their correlation with T2DM and involvement in insulin resistance, underscore the potential significance of understanding the molecular mechanisms through which they operate. Further research into the specific molecular targets and pathways influenced by *H19* and *GAS5* can contribute to a deeper understanding of their roles in the context of T2DM. In the present study, it was discovered that the lncRNA *H19* gene was highly expressed (upregulated) in patients with Type 2 Diabetes Mellitus (T2DM), while the lncRNA *GAS5* gene was downregulated (decreased), and there was no significant difference in *H19* and *GAS5* expression levels ($p \leq 0.001$). These findings align with other studies such as (22) and (11), respectively. Reference (22) reported that relative *H19* expression levels were significantly increased in the T2DM group compared to controls. Similarly, *GAS5* levels were decreased in the T2DM group ($p \leq 0.001$). These results are consistent with the present study, highlighting the increased of *H19* and the decrease of *GAS5* in T2DM. Furthermore, a study mentioned in your text found that serum levels of GAS5 were significantly decreased in patients with T2DM compared with healthy control subjects (1). This result aligns with the findings in the present study regarding lncRNA *GAS5* expression levels. These collective results contribute to the growing body of evidence supporting the dysregulation of lncRNAs, particularly *H19* and *GAS5*, in the context of T2DM

A ROC curve was generated to statistically assess the levels of GAS5 in the 100 samples. The ROC curve illustrates the relationship between sensitivity and specificity for *GAS5*. The ROC analysis of *GAS5* resulted in an area under the curve (AUC) of 0.84 (95% Confidence Interval (CI): 0.762, 0.923) with a p-value $\leq$ 0.001. The

optimal cutoff for *GAS5* was determined to be less than or equal to 10 ng/µl, measured as absolute quantification by qPCR. The predictive cut-off value was 0.16, and at this value, the sensitivity was 74%, specificity was 88%, and the positive predictive value (PPV) was 81%, as illustrated in (Table 5) and (Figure 2).

Table (5): Receiver Operating Characteristic curve data of the *GAS5* gene in patients.

Parameters	AUC	Standard Error (Std. Errora)	Sensitivity%	Specificity%	P value	The best Cut off
LNCRNA GAS5	0.81	0.041	85.1%	67.3%	0.001	0.16

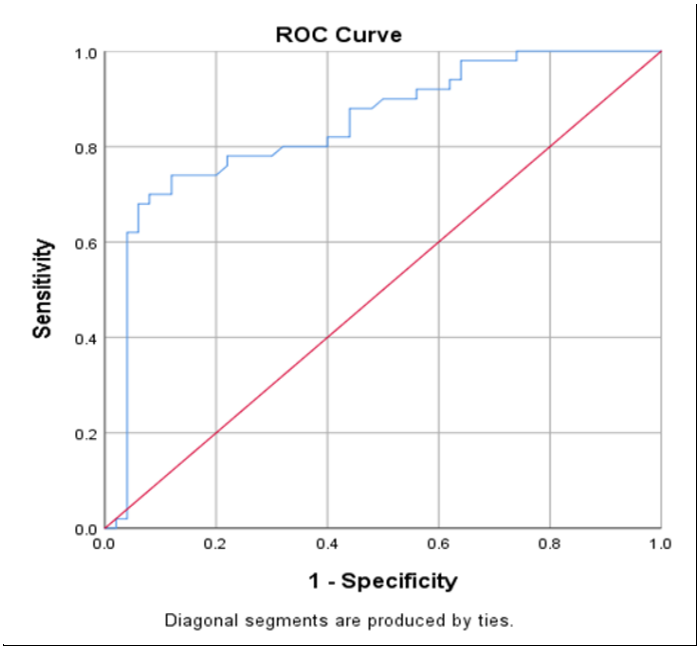

Figure (2): A receiver operating characteristic (ROC) curve was performed on *GAS5* level in plasm from non-diabetic (n=50) and diabetic patients (n=50) to determine the cutoff values. The area under the curve (AUC) of 0.84 (95% CI: 72.3%, 92.6%) sensitivity of 74%, specificity of 88% the positive predictive value (PPV) of 81% .

The present study reported an area under the curve (AUC) or receiver operating characteristic (ROC) of 0.84, with a predictive cut-off value of 0.16, sensitivity of 74%, specificity of 88%, and p-value ≤ 0.001. These findings align with reference (11), where qPCR results indicated that individuals with absolute *GAS5* levels below 10 ng/µl had almost twelve times higher odds of having diabetes (Exact Odds Ratio [OR] = 11.79, 95% CI: 3.97, 37.26, p < 0.001). The analysis in reference (11) indicated an AUC of ROC of 0.81 with 85.1% sensitivity and 67.3% specificity in distinguishing non-diabetic from diabetic subjects and a positive predictive value of 71.4%. The consistent results between the present study and these references support the potential utility of *GAS5* as a biomarker for diabetes, emphasizing its sensitivity and specificity in distinguishing

between diabetic and non-diabetic individuals.

The correlation between *LNCRNA GAS5* gene and some parameters such as RBS and HbA1C

The results showed no significant relationship between *GAS5* gene expression with RBS and HbA1C tests in the Iraqi patients of T2DM with no significant differences (P-0.990) and (p-0.747) respectively. The results regarding the age variable indicated significant differences between age and Type 2 Diabetes Mellitus (T2DM) patients for all the parameters examined, including Random Blood Sugar (RBS), HbA1c, and c-peptide. The prevalence of T2DM tends to increase with age, particularly in developing nations. The highest proportion of individuals with diabetes is typically found in those over 60 years of age. In developed countries, the majority is often discovered in individuals aged 65 years and older. These variations primarily reflect differences in the age distribution of populations between developed and developing countries. The findings of the present study are consistent with the results of references (14) and (15), both of which demonstrated a significant relationship between age and Type 2 DM in Iraqi patients and the control group. However, the current study's results are not in agreement with the findings of reference (16), which reported no significant differences between age and Type 2 DM. Furthermore, the present study contradicts the results of reference (17), which indicated no significant differences between age and HbA1C levels in T2DM patients. These discrepancies in findings may stem from variations in study populations, methodologies, or other factors influencing the association between age and T2DM parameters. which also found no significant difference in the *GAS5* gene concerning clinical parameters, including the HbA1C test.

The obtained results align with reference (18) in a general sense, supporting the notion that elderly individuals with Type 2 Diabetes Mellitus (T2DM) face a higher risk of morbidity and mortality compared to their younger counterparts. This heightened risk is attributed to important age-related immunologic and physiologic changes that complicate the presentation, diagnosis, and management of diabetes in the aged population. The findings of the study highlight those individuals aged fifty years and older are more susceptible to the disease due to the ageing process. It is well-established that, influenced by psychological, physical, and environmental factors. Additionally, obesity serves as a significant risk factor for T2DM, as it contributes to both insulin resistance and β cell dysfunction, as noted in reference (19). The results of the present study are in agreement with reference (22).

Conclusion

In conclusion, this study has identified lncRNA *GAS5* as biomarker of Type 2 Diabetes Mellitus (T2DM), and its levels can be easily measured in the plasma of patients with T2DM. The potential of *GAS5* as a biomarker suggests that it may serve as a valuable tool for predicting the onset of diabetes in adults. Future research could expand on this model, exploring the predictive capabilities of *GAS5* levels in identifying individuals at risk of developing diabetes. Moreover, combining *GAS5* levels with other molecular markers associated with diabetes may enhance the precision of

T2DM diagnosis and prediction. This integrative approach has the potential to provide a more comprehensive understanding of the disease and improve the accuracy of diagnostic procedures. The feasibility of applying this methodology to high-throughput screening of samples further supports its potential utility in a diagnostic context, offering a practical and efficient means of assessing T2DM risk in larger populations.

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