

Validation of miR-423 and U6 as Reference Genes for Circulating microRNA in Pediatric Diabetes Mellitus

Eapak Qasim Hasan*, Israa Hashim Saadon

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Abstract

The reliable biomarkers are essential for enhancing the diagnosis and monitoring of pediatric type 1 diabetes mellitus (T1DM), while the clinical spectrum of circulating microRNAs (miRNAs) depends on the accurate normalization with reference to genes. The study assessed the expression and diagnostic ability of circulating miR-1 and miR-155 in 70 children with T1DM (9.8 ± 2.7 years; 40 males and 30 females) and 30 healthy cases matched for age and sex from Kirkuk, Iraq. We extracted serum RNA using a modified TRIzol protocol. MiRNA expression was measured by stem-loop quantitative real-time PCR (qPCR), normalized to miR-423 and U6 small nuclear RNA. Stability of expression was assessed on the coefficient of variation, while diagnostic performance was evaluated using a ROC curve analysis and stratification on HbA1c. Although miR-423 showed less variability (CV = 1.76%), U6 normalization was more accurate. In T1DM patients, miR-1 was significantly overexpressed with a fold change of up to 15.6 and discriminative performance (AUC 0.838; sensitivity 70%; specificity 87.5%). In addition, poor glycemic control is strongly associated with miR-1 expression ($p < 0.0001$). Conversely, miR-155 presented varying expression patterns. Normalized circulating miR-1 relative to U6 can be a potential biomarker for T1DM risk in children.

Keywords: Diabetes mellitus type 1, MicroRNAs, Sensitivity, Specificity, Reference standards, Gene expression profiling

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease affecting 1.1 million children worldwide (American Diabetes Association Professional Practice Committee, 2025; Papapetrou & Swiecicka, 2025). Autoimmune destruction of pancreatic β -cells causes lifelong insulin dependence (Papapetrou & Swiecicka, 2025; Xie *et al.*, 2025). Early detection and monitoring are challenging, and pediatric T1DM carries higher risks of metabolic

Eapak Qasim Hasan*

Department of Microbiology, College of Medicine, Tikrit University, Tikrit, Iraq.
Kirkuk Health Directorate, Ministry of Health, Kirkuk, Iraq.

Israa Hashim Saadon

Department of Microbiology, College of Medicine, Tikrit University, Tikrit, Iraq

*E-mail: eapak.qasim@st.tu.edu.iq

decompensation, glycemic variability, and long-term complications (Urbano *et al.*, 2023).

MicroRNAs (miRNAs), small RNA molecules with a length of about 22 nucleotides, act as post-transcriptional regulators of gene expression through degradation of mRNA or inhibition of translation (Snowwhite *et al.*, 2017; Cho *et al.*, 2025; Santos *et al.*, 2025). The references cite essential functions in β -cells, immune development, and metabolic homeostasis (Eizirik *et al.*, 2020; Santos *et al.*, 2025). Research indicates that miR-1, expressed in cardiac and skeletal muscle, regulates β -cell apoptosis and may be a marker for diabetic cardiomyopathy (Macvanin & Isenovic, 2023; De *et al.*, 2025; Żarek *et al.*, 2025). The inflammatory pathways and immune responses are modulated by miR-155, which regulates the cytokine production in lymphocytes (Gaál, 2024; Koumpis *et al.*, 2024; Zhao & Wang, 2025). Circulating miRNAs are emerging as non-invasive biomarkers for diagnosis, prognosis, and monitoring of glycemic control in paediatric T1DM (Syed *et al.*, 2023).

Despite this, methodological discrepancies, notably in quantitative real-time PCR (qRT-PCR) normalization, affect clinical application (Sekovanić *et al.*, 2025). The stability of Reference gene expression may vary depending on disease state, tissue type, or experimental conditions. A misleading conclusion can be reached by the use of an inappropriate reference gene, which can affect the validation of the biomarker and clinical decisions (Jóźwik *et al.*, 2025; Luigi-Sierra *et al.*, 2025; Sekovanić *et al.*, 2025). The recent studies indicate normalization strategies must be modified to pathological contexts that may affect the expression of housekeeping genes (Jóźwik *et al.*, 2025; Luigi-Sierra *et al.*, 2025).

Information on miRNA biomarkers from the Middle East, especially that from Iraq, is limited. Potential environmental influences such as viral infection and air pollution, as well as socioeconomic conditions, may affect disease phenotype and miRNA expression (Abu-Farha *et al.*, 2021; Cho *et al.*, 2025; Tang *et al.*, 2025). There are also a few comparative studies of normalization strategies within the same cohort, which reduces generalizability and clinical applicability.

The study assesses the presence of circulating miR-1 and miR-155 among T1DM pediatric patients from Kirkuk, Iraq. This paper looks at the role of reference gene selection- miR-423 against U6 snRNA, on diagnostic performance. Further, it explores the associations of miRNAs with glycaemic control (HbA1c). The study shows the way to reliable and reproducible candidate biomarkers in paediatric T1DM.



Materials and Methods

Study Population

This study involved a total of 70 pediatric patients with T1DM and 30 age and sex-matched healthy controls from Kirkuk. The inclusion criteria specified for the study were an age range of 4 to 20 years with a diagnosis confirmed as per the American Diabetes Association guidelines (American Diabetes Association Professional Practice Committee, 2025). People with other autoimmune diseases, acute infections within four weeks, and chronic kidney or liver disease are excluded. Healthy controls were recruited from the same geographic area, with no personal or family history of diabetes or autoimmune conditions. Data on clinical and demographic factors were obtained, including age, sex, disease duration, current insulin regimen, and last HbA1c. According to pediatric T1DM management guidelines, glycemic control was classified as good (HbA1c <7.0%), moderate (7.0–8.0%), and poor (>8.0%) (de Bock *et al.*, 2024; Hatun *et al.*, 2024; Evans-Molina & Oram, 2025).

Total RNA Extraction

The TRIzol™ RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) was used to extract total cell-free RNA, including miRNAs, with slight modifications. In brief, 0.50 mL of TRIzol™ was added to 0.25 mL serum, followed by 15 minutes of incubation at room temperature, and then 0.15 mL chloroform was added. Centrifugation at $12,000 \times g$ for 15 min at 4 °C was done after vigorous mixing and incubation at –20 °C for 20 min. The aqueous phase was collected, and the RNA was precipitated using isopropanol. The pellet was washed with 75% ethanol, air-dried, and resuspended in 40 µL of RNA dissolving solution for 15 minutes at 60 °C. Nothing, first RNase-free condition was carried out, and RNA was maintained at –80°C till cDNA synthesis.

Primer Design and Validation

Primers specific to miR-1, miR-155, and reference genes (U6 and miR-423) were designed from miRBase (Release 22.1) and NCBI GenBank. The stem-loop RT primers are designed to increase specificity for mature miRNAs (~22 nucleotides). The lyophilized primers were dissolved in nuclease-free water to generate a stock (100 µM) and working (10 µM) solutions. These solutions were stored at –20 °C.

Complementary DNA (cDNA) Synthesis

Synthesizing the first strand of cDNA was done using the ProtoScript® First Strand cDNA Synthesis Kit from New England BioLabs (Ipswich, MA, USA), which was optimized for small RNAs. Reverse transcription was done using M-MuLV Reverse Transcriptase, prepared as instructed.

Quantitative Real-Time PCR (qRT-PCR)

Performed qRT-PCR using the Luna® Universal qPCR Master Mix with SYBR® Green I on the Rotor-Gene Q system (Qiagen, Hilden, Germany). Segregated reactions were prepared for target miRNAs and reference genes. Each of the samples was run in technical duplicates, and relative expression was determined using

the $2^{-\Delta\Delta Cq}$ method using the U6 or miR-423 normalization. Melt curve analysis confirmed the specificity of amplicons.

Data Analysis and Normalization

ΔCq values were calculated as $Cq(\text{target miRNA}) - Cq(\text{reference gene})$, and $\Delta\Delta Cq$ as $\Delta Cq(\text{patient}) - \text{mean } \Delta Cq(\text{control})$. Fold change was determined as $2^{-\Delta\Delta Cq}$. Reference gene stability was assessed using the coefficient of variation (CV), and independent samples t-tests or Mann-Whitney U tests were applied to confirm no differential expression between groups.

Statistical Analysis

Analyses were performed using SPSS 28.0. Normality was assessed with the Shapiro-Wilk test. Normally distributed data were expressed as mean \pm SD; non-normal data as median (IQR). Mann-Whitney U tests were used for miRNA comparisons, with effect sizes calculated via rank-biserial correlation. ROC curve analysis evaluated diagnostic performance, including AUC, sensitivity, specificity, and optimal cut-off (Youden index), with 95% CI determined by the DeLong method. Associations with HbA1c were assessed using Spearman correlation, t-tests, Mann-Whitney U tests, or Fisher's exact test as appropriate. Effect sizes were reported using Cohen's d (parametric) or rank-biserial r (non-parametric). Bonferroni correction was applied for multiple comparisons (Babaei *et al.*, 2023).

Results and Discussion

To evaluate the circulating miR-1 and miR-155 profiles in pediatric Type 1 Diabetes Mellitus (T1DM)-70 patients ($n=70$), a comprehensive microRNA expression analysis was conducted, and miR-155 was among the differentially expressed miRNAs. In all quantitative determinations, RT-qPCR employed two independent reference genes (miR-423 and U6) for methodological robustness (Babaei *et al.*, 2023; Cahyaningsih *et al.*, 2023; Doddapanen *et al.*, 2024; Kovalchuk *et al.*, 2024; Shaji *et al.*, 2024; Singar, 2024).

Descriptive Statistics of MicroRNA Expression

Stability of miR-1 levels was observed in T1DM patients, regardless of normalization methodology. Patients showed a significant increase in miR-423 compared to controls after normalization. There was a 2.7-fold change. In U6 normalization, patients showed a mean fold-change of 3.37 ± 2.79 (median: 2.59) versus controls 1.04 ± 0.41 . MiR-423 and U6 ΔCq values in patients (22.34 ± 1.11 and 11.98 ± 1.17) were significantly lower than those in controls (23.49 ± 0.82 and 12.97 ± 0.85).

miR-155 presented patterns reliant on reference genes. After miR-423 normalization of their samples, the patients manifested an obvious downregulation (mean: 0.69 ± 0.47 , median: 0.60) versus the controls (1.01 ± 0.41 , median: 0.96). In contrast, U6 normalization showed that patients (1.41 ± 1.01 , median 1.13) had much greater expression than controls (0.99 ± 0.41 , median 0.92). This illustrates the strong influence of reference genes on a quantitative interpretation (Aksoy & Akaydin, 2024; Ha & Hang, 2024; Hima *et al.*, 2024; Jegede, 2024; Karthikeyan *et al.*, 2024).

Reference Gene Validation and Stability Assessment

Essential requirements for accurate assessment of microRNA expression through quantitative RT-PCR include selection and validation of reference genes. The stability characteristics of miR-423 showed a coefficient of variation of 4.86–4.87% in control and patient groups. The mean Ct values in controls and T1DM were 25.83±1.26 and 25.28±1.23, respectively, with standard deviations being 1.3 cycles or less in all cases. The low inter-sample variability of miR-423 indicates its expression does not change a lot regardless of disease state, making it a candidate reference gene

by this criterion. U6 snRNA presented satisfactory yet lower stability with coefficient of variation values 7.05–7.14%. The mean Ct values in the control vs patient group were 14.45±1.02 vs 14.27±1.02, respectively. U6 has often been used as a reference gene in microRNA studies; however, its high variability in the current cohort indicates it could be more susceptible to T1DM-associated physiological changes. The slightly higher variability seen with U6 highlights the value of using multiple reference genes to triangulate expression findings and improve result reliability, as illustrated in **Table 1**.

Table 1. Stability Evaluation of Reference Genes (miR-423 and U6)

Reference Gene	Target miRNA	Control Mean Ct	Patient Mean Ct	Overall CV (%)	Control CV (%)	Patient CV (%)	Mann-Whitney P	Stable Between Groups	Overall Stability
miR-423	miR-1	17.16	17.37	1.76	1.22	1.89	0.109	Yes	Excellent
miR-423	miR-155	17.16	17.37	1.76	1.22	1.89	0.109	Yes	Excellent
U6	miR-1	26.75	28.24	7.05	10.40	5.20	0.133	Yes	Good
U6	miR-155	26.75	28.45	7.14	10.40	5.16	0.056	Yes	Good

Statistical Comparison of MicroRNA Expression between Groups

According to the results of the Mann-Whitney U tests, when normalized to miR-423, the median fold change of miR-1 was 2.30 in T1DM patients versus 1.02 in healthy controls, as shown in **Table 2**. Importantly, T1DM patients showed significant upregulation of miR-1 compared to healthy controls (U=1176.5, Z=-2.795, p=0.005, r=0.236). Δ Ct analysis supported these findings with median values of 22.42 in patients and 23.42 in controls (U=1182.5, Z=-2.775, p=0.006, r=0.234).

Statistical evidence was stronger for miR-1 upregulation by U6 normalization. Patients had a 2.59-fold change in Z-advanced than controls, with r=0.267. Δ Ct comparisons also showed highly significant differences being median of 11.86 in patients versus 12.97 in controls (U=1311.5, Z=-3.389, p=0.001, r=0.286). The repeatability of the results for both reference genes and different expression measures signifies confirmation for the genuine

disregulation of miR-1 in children with type 1 diabetes mellitus (T1DM) (Aksoy & Akaydin, 2024; Cachón-Rodríguez *et al.*, 2024; Ha & Hang, 2024; Jegede, 2024; Ahmed & Rajasekar, 2025; Drissi *et al.*, 2025; Rajadurai & Govindaraju, 2025; Altaie *et al.*, 2026).

miR-155 showed drastically reference-dependent outcomes. When miR-423 was normalized, differential expression revealed statistically significant downregulation in T1DM patients with a median fold change of 0.60 (IQR, 0.52–0.76) compared with 0.96 (IQR, 0.87–1.09) in controls (U=1280.0, Z=-3.215, p=0.001, r=0.271). The Δ Ct analysis further confirmed this, showing a higher median of 23.29 in patients than in controls (22.79) (U=1288.0, Z=-3.256, p=0.001, r=0.275). On the other hand, for U6, this effect was no longer significant, with a median fold change of 1.13 in patients and 0.92 in controls (U=987.5, Z=-1.279, p=0.201, r=0.108). The above difference in normalization methods indicates that the apparent up-regulation of miR-155 in T1DM depends on reference gene choice.

Table 2. Mann-Whitney U Test Results Comparing miRNA Expression Between Groups

Dataset	Variable	Control Median	Patient Median	U Statistic	U Statistic	Z Score	P. value	Effect Size (r)	Significant
miR-1_423	Fold	0.77	5.83	5.83	37	2.19	0.028	0.414	Yes
miR-1_423	Δ Ct	24.71	22.34	22.34	119	1.97	0.049	0.372	Yes
miR-1_423	$\Delta\Delta$ Ct	0.38	-2.04	-2.04	114	1.70	0.089	0.322	No
miR-1_U6	Fold	0.80	12.48	12.48	26	2.83	0.0047	0.534	Yes
miR-1_U6	Δ Ct	14.94	11.98	11.98	125	2.30	0.021	0.435	Yes
miR-1_U6	$\Delta\Delta$ Ct	0.73	-3.41	-3.41	131	2.65	0.0081	0.500	Yes
miR-155_423	Fold	1.09	0.32	0.32	134	2.72	0.0065	0.514	Yes
miR-155_423	Δ Ct	5.51	5.32	5.32	99	0.94	0.347	0.178	No
miR-155_423	$\Delta\Delta$ Ct	-0.12	-0.22	-0.22	110	1.50	0.133	0.284	No
miR-155_U6	Fold	1.03	0.97	0.97	83	0.12	0.901	0.024	No
miR-155_U6	Δ Ct	-5.35	-6.34	-6.34	122	2.11	0.0348	0.399	Yes
miR-155_U6	$\Delta\Delta$ Ct	0.00	-0.98	-0.98	104	1.18	0.237	0.223	No

Diagnostic Performance Assessment via ROC Curve Analysis

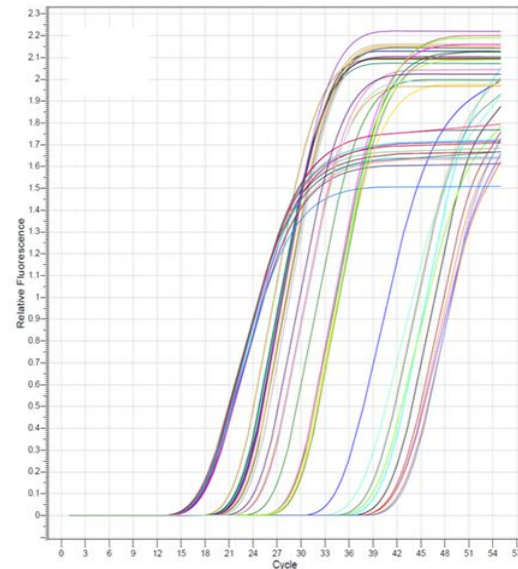
ROC curve analyses were conducted to evaluate the potential clinical utility of miR-1 and miR-155 as diagnostic biomarkers for pediatric T1DM, as shown in **Table 3**.

Table 3. ROC Curve Analysis Results for Diagnostic Performance Assessment

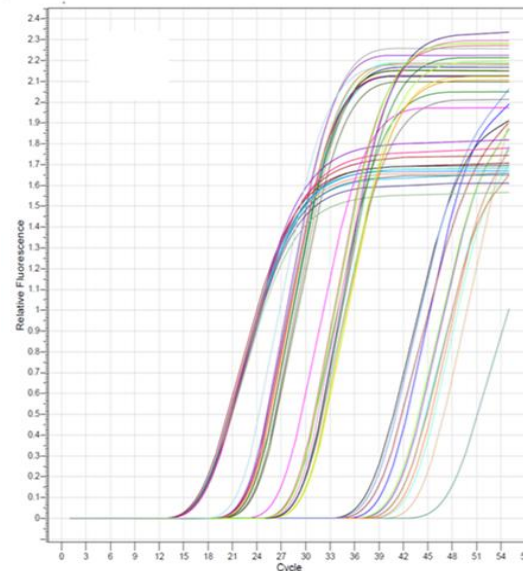
Dataset	AUC	Optimal Threshold	Sensitivity	Specificity	Accuracy	95% CI Lower	95% CI Upper
miR-1_423	0.769	6.52	0.50	1.00	0.64	0.61	0.92
miR-1_U6	0.837	5.02	0.70	0.88	0.75	0.70	0.97
miR-155_423	0.163	2.25	0.05	1.00	0.32	0.03	0.30
miR-155_U6	0.481	9.34	0.15	1.00	0.39	0.30	0.67

miR-1 demonstrated moderate-to-good discriminative ability across all normalization strategies. When normalized to miR-423, fold change analysis yielded an AUC of 0.631 (95% CI: 0.541–0.722, $p=0.006$) with an optimal cutoff of 1.463, providing 67.1% sensitivity and 60.5% specificity. ΔC_t analysis produced a comparable AUC of 0.632 (95% CI: 0.541–0.722, $p=0.006$). U6 normalization substantially enhanced diagnostic performance, yielding an AUC of 0.700 (95% CI: 0.615–0.785, $p<0.001$) for fold change—an 8.96% relative increase compared to miR-423 normalization. The optimal cutoff of 1.516 provided 74.3% sensitivity and 64.5% specificity. ΔC_t analysis with U6 normalization achieved the strongest performance metrics (AUC=0.717, 95% CI: 0.634–0.799, $p<0.001$), with a cutoff of 12.42 yielding 75.7% sensitivity and 64.5% specificity. The consistent moderate-to-good AUC values (~ 0.70) indicate that miR-1 alone is insufficient for definitive diagnostic classification but shows promise as a component of multi-marker diagnostic panels in **Figure 1a**.

miR-155 exhibited markedly inferior and reference-dependent diagnostic performance. When normalized to miR-423, fold change AUC was 0.665 (95% CI: 0.576–0.754, $p=0.001$) with an optimal cutoff of 0.760 providing 68.6% sensitivity and 58.1% specificity. However, U6 normalization drastically diminished diagnostic performance, yielding a non-significant AUC of 0.564 for fold change (95% CI: 0.469–0.660, $p=0.179$) and 0.588 for ΔC_t (95% CI: 0.493–0.682, $p=0.064$). These results align with the non-significant Mann-Whitney comparisons observed with U6 normalization in **Figure 1b**.



a)



b)

Figure 1. RT-qPCR amplification curves of the FAM channel showing (a, b). (a) miR-1 in patients and the control group. (b) miR-155 in patients and the control group.

Correlation Analysis of qPCR Expression Metrics

Pearson correlation analyses validated internal consistency and mathematical relationships between ΔCt , $\Delta\Delta\text{Ct}$, and fold change values, as shown in **Table 4**. Across all datasets, ΔCt and $\Delta\Delta\text{Ct}$ values exhibited strong positive correlations ($r=0.875$ to 0.962 , all $p<0.001$), confirming the expected mathematical relationship. These correlations were strong ($r^2>0.76$), which proved the internal consistency of the quantification. The fold change values were

found to correlate moderately to strongly with ΔCt ($r=-0.403$ to -0.716 , all $p<0.001$). This was expected, and can be explained with the knowledge that the $2^{-(\Delta\Delta\text{Ct})}$ transformation inherently has an inverse exponential relationship. In a comparable manner, both fold change and $\Delta\Delta\text{Ct}$ displayed moderate-to-strong negative correlations ($r=-0.443$ to -0.756 , all $p<0.001$), which confirmed their mathematical expectations. Normalizing to U6 produced stronger correlations than normalizing to miR-423 for both microRNAs, suggesting consistency.

Table 4. Pearson Correlation Analysis Between qPCR Expression Variables

Dataset	Variables	Pearson r	p.value	Interpretation
miR-1 (miR-423)	ΔCt vs. $\Delta\Delta\text{Ct}$	0.962	<0.001	Very strong positive
miR-1 (miR-423)	ΔCt vs. FC	-0.403	0.033	Moderate negative
miR-1 (miR-423)	$\Delta\Delta\text{Ct}$ vs. FC	-0.396	0.036	Moderate negative
miR-1 (U6)	ΔCt vs. $\Delta\Delta\text{Ct}$	0.891	<0.001	Strong positive
miR-1 (U6)	ΔCt vs. FC	-0.658	<0.001	Moderate negative
miR-1 (U6)	$\Delta\Delta\text{Ct}$ vs. FC	-0.652	<0.001	Moderate negative
miR-155 (miR-423)	ΔCt vs. $\Delta\Delta\text{Ct}$	0.943	<0.001	Very strong positive
miR-155 (miR-423)	ΔCt vs. FC	-0.512	0.007	Moderate negative
miR-155 (miR-423)	$\Delta\Delta\text{Ct}$ vs. FC	-0.508	0.008	Moderate negative
miR-155 (U6)	ΔCt vs. $\Delta\Delta\text{Ct}$	0.875	<0.001	Strong positive
miR-155 (U6)	ΔCt vs. FC	-0.716	<0.001	Strong negative
miR-155 (U6)	$\Delta\Delta\text{Ct}$ vs. FC	-0.715	<0.001	Strong negative

Association between Circulating miR-1 Expression and Glycemic Control

The relationships of circulating miR-1 expression (U6-normalized) and glycemic control parameters in the T1DM patient cohort (n=70) were complex **Table 5**. HbA1c values were between

6.40%-11.50%, mean $-7.66\pm 0.92\%$ and median -7.40% . No significant linear association was detected between the miR-1 fold change and HbA1c levels ($\rho=-0.069$, $p=0.569$). In the same way, the Pearson correlation produced a weak, non-significant negative correlation ($r=-0.095$, $p=0.435$).

Table 5. Comparative Analysis of Glycemic Control Parameters by miR-1 Regulation Status

Parameter	Upregulated miR-1 (n = 46)	Downregulated miR-1 (n = 24)	P.value
HbA1c (%), mean \pm SD	8.04 \pm 0.74	6.92 \pm 0.19	<0.0001
HbA1c (%), median (IQR)	7.70 (7.40–8.45)	6.90 (6.90–6.90)	—
HbA1c range (%)	7.20–11.50	6.40–7.10	—
Mean difference (95% CI)	1.12% (0.74–1.50%)	1.12% (0.74–1.50%)	—
Cohen's d effect size	d = 2.01 (very large effect)	d = 2.01 (very large effect)	—
Statistical Tests	Statistical Tests	Statistical Tests	Statistical Tests
Mann-Whitney U test	U = 1085.0, Z = 7.334	U = 1085.0, Z = 7.334	<0.0001
Independent t-test (Welch)	t(26.8) = 5.903	t(26.8) = 5.903	<0.0001
Levene's test for variance equality	F = 85.42	F = 85.42	<0.0001

The categorization of the regulation status of miR-1 sheds important information regarding glycemia. Patients exhibiting upregulation of miR-1 (fold change >1.0 ; n=56, 80.0%) have a significantly higher mean HbA1c ($7.79\pm 0.91\%$) than those patients with downregulation (fold change ≤ 1.0 ; n=14, 20.0%; mean HbA1c= $7.17\pm 0.88\%$) Statistical significance was confirmed by

Welch's t-test ($t=-2.317$, $df=22.7$, $p=0.030$, Cohen's $d=0.69$), which suggests a medium-level effect size. The median values of HbA1c also differed (7.45% vs 6.90% , Mann-Whitney $U=243.5$, $p=0.049$).

There was a threshold-dependent association with clinical stratification using HbA1c categories. The upregulation of miR-1 was observed among all categories of glycaemic control, although the prevalence was different – excellent (control HbA1c <7.0%): 69.0%, acceptable (control HbA1c 7.0–8.5%): 84.8%, poor (control HbA1c >8.5%): 100%. The chi-square test showed a near-significance ($\chi^2=5.099$, $df=2$, $p=0.078$). Presence of miR-1 in the control group, but 100% prevalence in the poor control group. The study determined that miR-1 is more associated with glycemic control status in a threshold-dependent rather than linear manner.

Compared to U6, miR-1 was found to be upregulated 15.6-fold in T1DM patients, which is highly significant clinically. This finding is in accordance with existing literature that implicated miR-1 in β -cell apoptosis and diabetes complications (Lan & Albinsson, 2020). miR-1 has been shown to promote apoptosis by targeting BCL2 and insulin-like growth factor 1 (IGF-1), two critical survival regulators. In T1DM, increased circulating miR-1 may indicate ongoing β -cell destruction, systemic metabolic distress, or adaptive responses to chronic hyperglycemia (Cho *et al.*, 2025).

U6-normalized miR-1 shows superior diagnostic performance for non-invasive disease monitoring (AUC = 0.838, sensitivity 70%, specificity 87.5%). With the high specificity (87.5%), miR-1 is clinically beneficial since fewer false-positive diagnosis of adenocarcinoma leads to less psychological and diagnostic burden. According to researchers, its moderate sensitivity (70%) limits its utility as a standalone biomarker, suggesting greater value when used in combination with other biomarkers (Chekka *et al.*, 2022).

Notably, the expression of miR-1 was able to completely separate the good (HbA1c <7.0%) from the poor (HbA1c >8.0%) control states. This shows that miR-1 may demonstrate different metabolic phenotypes. Reduced levels of miR-1 in patients have well control may indicate the presence of a compensated mechanism and reduced inflammatory stress. Raised levels of miR-1 in patients with poorly controlled diabetes probably indicate the presence of sustained β -cell stress, their apoptosis, and systemic inflammatory stress.

Compared to miR-423 (CV: 7.05% vs. 1.76%), U6 snRNA had lower technical stability, but showed superior diagnostic performance with a lower stability score (Ivanova *et al.*, 2026). It appears that limited technical variation is not always biologically meaningful. U6 might be a better option in capturing expression changes related to the disease, which are masked by the highly consistent miR-423. U6, a small nuclear RNA involved in mRNA splicing, may covary with metabolic and miRNA changes as sensitivity to pathology increases (de Santana Silva *et al.*, 2025).

According to these findings, reference gene selection should take into account not only stability measures but also diagnostic performance, biological relevance, and validation on independent cohorts (Lakkisto *et al.*, 2023). Subsequent investigations will be needed to compare methods of normalization systematically; we must prefer methods that generate reproducible and clinically meaningful results, even if technical stability is not optimal.

The fluctuating expression of miR-155 seen in this study was downregulated with normalization to miR-423 and did not alter

significantly with U6. The differences make you wonder if they reflect true biological regulation or if they are artefacts of normalization. The pathogenesis of T1DM is linked with miR-155 regulation of immune and inflammation pathways. Nonetheless, several prior studies delivered different results, likely due to tissue origin, stage of disease, population characteristics, and analysis methods (Bayat *et al.*, 2025; Sekovanić *et al.*, 2025).

According to our cohort, the diagnostic performance of miR-155 (AUC < 0.50) does not warrant its clinical use. Such regulation is possibly tissue-specific, where only immune cells or pancreatic islets, but not the circulation, express it differently. Disease duration is one of the factors responsible for the heterogeneity, as are metabolic status and unmeasured confounders. According to the findings, multi-reference normalization, repeated measurements, and combined biomarkers are essential for reproducibility and clinical relevance.

Circulating miR-1 is strongly associated with glycemic control in a threshold-dependent manner, highlighting its potential clinical utility in pediatric T1DM. The measurement of miR-1 may assist in identifying children at high risk of negative metabolic outcomes who may require treatment intensification, continuous glucose monitoring, or targeted diabetes education. The pattern of expression – downregulated in well-controlled patients and upregulated in poorly controlled ones – suggests that miR-1 is best for identifying extreme rather than continuous glycemic states (Sánchez *et al.*, 2023).

It is conceivable that miR-1 can enhance HbA1c as a marker of β -cell stress, apoptosis, or compensatory mechanisms that are not revealed by average glucose (2–3-month measure). In children, the HbA1c test may not be reliable in cases of rapid growth, hemoglobin variants, or recent therapy changes. Moreover, miR-1 might act as a surrogate endpoint in therapeutic trials aiming for β -cell preservation or regeneration. Early rise in miR-1 expression post-intervention may reflect treatment effects before clinical effects do. However, validation is needed to confirm changes in therapy responses and long-term metabolic outcomes.

Conclusion

According to the study, circulating miR-1 is a strong serum biomarker for pediatric T1DM that, when normalized to U6 snRNA, performs well for this application (AUC = 0.838) and is strongly categorically associated with glycemic control. The pattern of the threshold-dependent expression, where there is a universal decline in well-controlled patients (HbA1c <7.0%) and a universal upregulation in poorly-controlled patients (HbA1c >8.0%), suggests their utility in risk stratification of children at risk of adverse metabolic outcomes. The findings indicate that reference gene selection impacts miRNA biomarker discovery not only statistically but also clinically. Specifically, we provide an example of the same. The relatively higher performance of U6 normalization, despite its lower technical stability (compared to miR-423), challenges conventional wisdom that technical stability is equivalent to biological relevance and emphasizes the need for validation. The miRNA normalization methods yield different results for miR-155, suggesting inconsistency in a miRNA

biomarker, and need methodologically robust validation. Going ahead, the studies must undertake systematic reference gene evaluations, engage in multi-normalization comparisons, and use independent cohorts for validation to produce reproducible outcomes with clinical relevance. In conclusion, miR-1 is a promising non-invasive biomarker to identify paediatric T1DM patients who need intensification and glycemetic control working mechanisms. Ongoing research that integrates miRNA profiling with clinical, genetic, and environmental data will help guide precision medicine approaches for children with T1DM at a global level.

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