# Downregulation of miR-24-3p and miR-198 in Newly Diagnosed type 2 Diabetes Mellitus

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# Abstract

**Introduction:** Type 2 Diabetes mellitus (T2DM) is a metabolic disorder related to genetic, lifestyle, and environmental factors. It is characterized by hyperglycaemia, primarily due to insulin resistance. Recent studies have shown that microRNAs have been involved in the regulation of post-transcriptional gene expression mainly by repressing protein production. Dysregulated miRNA in type 2 diabetes interrupts the insulin signalling cascade and multiple physiological processes leading to disease progression. miRNAs are released from cells in circulation and are now known as a new class of biomarkers due to their stable nature. miR-24-3p and miR-198 are associated with several diseases but their role in type 2 diabetes remains unclear. This study aimed to compare miR-24-3p and miR-198 expression levels in newly diagnosed Type 2 Diabetes Mellitus patients and Non-T2DM controls.

**Method:** Thirty-five newly diagnosed type 2 diabetic cases and thirty-five Non-T2DM controls were recruited after obtaining due informed consent. Venous blood was obtained under aseptic conditions. Biochemical parameters were analyzed using the autoanalyzer. Expression levels of miR-24-3p and miR-198 were performed using RT-PCR by TaqMan Advanced miRNA assay. miR-16-5p was used as an internal control.

**Results:** The difference between circulating levels of whole blood of miR-24-3p and miR-198 was statistically significant among the study group. miR-24-3p showed a fold change of 0.312 and miR-198 showed a fold change of 0.203. The miRNAs were not correlated with the glycaemic and other clinical parameters.

**Conclusions:** Findings of our study suggests that expression of miR-24-3p and miR-198 are downregulated in newly diagnosed Type 2 Diabetes Mellitus.


# Introduction:

Diabetes has increased significantly in recent years and considered one of the most prevalent global epidemics. Worldwide, more than 400 million individuals are suffering from diabetes mellitus. It was estimated that the incidence of diabetes was 451 million and half of those were undiagnosed. The prevalence is expected to reach 693 million by 2045. In India, the prevalence of DM was 72 million in 2017 [1, 2]. Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic hyperglycaemia due to defects in insulin secretion by beta cells islets of Langerhans or resistance against insulin action. Diabetes is mainly of four types based on its clinical presentation and aetiology i.e. type 1 diabetes, type 2 diabetes mellitus (T2DM), gestational diabetes, and other specific diabetes [3]. T2DM is the most common type with a higher predominance. The pathogenesis of T2DM has been studied extensively for many years. It is a complex disease caused by environmental and genetic factors. Studies have also shown that changes in lifestyle i.e. decrease physical activities, genetic predisposition, unhealthy diet are the most important predisposing factors [4]. Type 2 Diabetes mellitus is a long-term disorder and even after adequate treatment, T2DM patients are at high risk and prone to many

complications like diabetic retinopathy, diabetic nephropathy, stroke, foot ulcer which may lead to lower limb amputations [5, 6]. T2DM should be diagnosed at an early stage to halt disease progression and to prevent its complication. Recently glycated haemoglobin and blood glucose estimation are used as biomarkers for diagnosis of diabetes, but they have some limitations [7, 8]. These biomarkers can detect the disease when it has already established. So, biomarkers are needed for early diagnosis of disease to prevent the severity of the disease. Despite changes in a few years, the novel biomarkers for the early diagnosis of diabetes mellitus remain insufficient. Therefore, new strategies are urgently needed to prevent this metabolic disorder. In recent years, microRNAs (miRNAs) have been shown in the regulation of gene expression post-transcriptionally by binding to the 3'UTR target mRNAs and represses protein production. miRNAs are small (~22 nt) regulatory RNA molecules and they regulate many important cellular processes and can affect major signalling pathways [9]. Dysregulated miRNA in type 2 diabetes mellitus disrupts the cascade of insulin signals and multiple physiological processes leading to disease development and progression [10, 11]. miRNAs are stably present in blood circulation in cell-free form and can be released from cells and so, they represent a novel class of biomarkers. Circulating miRNAs are found to have a significant role in diagnostic, prognostic biomarkers for cancer, cardiovascular disorders, and many other diseases [12]. Some miRNAs like miR-24-3p and miR-198 have been found as mechanistic regulators of insulin secretion and play critical roles in glucose homeostasis and pathogenesis of diabetes, but their status in T2DM patients is unknown [13, 14]. In this study, we aimed to assess the circulating levels of miR-24-3p and miR-198 in newly diagnosed Type 2 diabetes mellitus and assess their role as biomarkers in detecting T2DM.

## Materials And Methods:

# Subjects:

This study had been approved by the Institutional Ethics Committee, All India Institute of Medical Sciences, Jodhpur. This study involved 35 T2DM patients aged 30-60 years, recruited from the Department of Endocrinology and Metabolism, All India Institute of Medical Sciences, Jodhpur, Rajasthan. The American Diabetes Association (ADA) guidelines had been used to diagnose T2DM [15]. Fasting blood glucose (FBG) ≥126 mg/dl, 2h OGTT ≥ 200 mg/dl and HbA1c ≥ 6.5% were diabetic. Also, 35 Non-T2DM controls were taken from healthy individuals aged 30-60 years. The Control group was confirmed to not have suffered from any Diabetes diseases as per the American Association of Diabetes. Those of age <30 years or with serious comorbid conditions including diseases of the lung, kidney, heart, and liver, or those with hematologic or immune disorders, pregnancy, type-1 diabetes, or malignancy were excluded from the study. The blood samples of T2DM patients and Non-T2DM controls were collected in the hospital after due informed consent.

# Blood Collection

From each participant, peripheral whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes (Greiner Bio-one, Germany). Total RNA was isolated within 2 hrs of sample collection. Serum samples were collected and basic investigations like HbA1c (glycated haemoglobin), lipid profile [TC (total cholesterol), TG (triglycerides), HDL-c (high density lipoprotein cholesterol), LDL-c (low density lipoprotein cholesterol)], hs-CRP (high sensitivity C-reactive protein), FBG (fasting blood glucose) was done.

# RNA Extraction and Reverse Transcription

Total RNA, including miRNA, was isolated from 200 µl of whole blood samples using the Trizol LS reagent (Thermo Fischer Scientific, US) [16] following the manufacturer's instruction. The quality and quantity of RNAs were assessed using a NanoDrop One$^{c}$ (Thermo Fischer Scientific, US). All RNA samples were stored at -80$^{o}$C until further processing. Reverse transcription (RT) was carried out using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Thermo Fischer Scientific, US)[17]. cDNA synthesis was the multistep process which includes the poly(A) tailing reaction, in which 2µl of total RNA was mixed with 10X Poly(A) buffer (0.5µl), ATP(0.5µl), poly(A) enzyme(0.3µl), RNase-free water(1.7µl) to a final volume of 5µl. The reaction mixtures were then incubated at 37$^{o}$C for 45 min, at 65$^{o}$C for 10 min, and then held at 4$^{o}$C. The second step was adaptor ligation reaction in which 5µl of poly(A) tailing reaction product mixed with 5X DNA Ligase buffer (3µL), 50% PEG 8000 (4.5µL), 25X Ligation Adaptor (0.6µL), RNA Ligase (1.5µL), RNase-free water (0.4µL) to a total volume of 15µL. Then this mixture was incubated at 16°C for 60 minutes and held at 4°C. Next step was reverse transcription (RT) reaction in which 15µL of adaptor ligation reaction product was mixed with 5X RT buffer (6µL), dNTP Mix (25mM each) (1.2µL), 20X Universal RT Primer (1.5µL), 10X RT enzyme Mix (3µL), RNase-free water (3.3µL) to a total volume of 30µl. The reaction mixture was incubated at 42°C for 15 minutes, stop the reaction at 85°C for 5 minutes, and held at 4°C. The final step for cDNA synthesis was a miR-Amp reaction in which 5µL of the RT reaction product mixed with 2X miR-Amp Master Mix (25µL), 20X miR-Amp Primer Mix (2.5µL), RNase-free water (17.5µL) to a final volume of 50µl. The reaction mixture was incubated at 95°C for 5 minutes for 1 cycle, denature at 95°C for 3 seconds for 14 cycles, anneal/extend at 60°C for 30 seconds, stop the reaction at 99°C for 10 minutes and held at 4°C. undiluted miR-Amp reaction product stored at –20°C until analysis. 1:10 dilution of cDNA template was done before using it in RT PCR (5µL of the miR-Amp reaction product to 45µL 0.1X TE buffer)

# Micro-RNAs profiling by Real-Time PCR

The relative expression of miR-24-3p and miR-198 were assessed using the *TaqMan* Advanced *miRNA Assays kit* (Applied Biosystems; Thermo Fisher Scientific, US)[18]. Expression levels of miRNAs were determined using TaqMan MGB probes and TaqMan Universal PCR Master Mix II (2x) in triplicate. Diluted cDNA (5µl) was used as a template in a 20µl reaction mix containing 10µl of TaqMan® Fast Advanced Master Mix (2X), 1µl of TaqMan® Advanced miRNA Assay (20X) and 4µl RNase-free water (Applied

Biosystems; Thermo Fisher Scientific, US). RT-qPCR reactions were run with Biorad CFX 96 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, US) 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Triplicate measurements were obtained for each sample and mean were taken. The data were analyzed with the automatic threshold cycle (Ct) setting for assigning baseline and threshold for Ct determination. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The expression levels of miRNAs: miR-24-3p and miR-198 were normalized to miR-16-5p, as an internal control.

# Statistical Analysis

Data were presented as the mean ± standard deviation (SD) and median (IQR). Comparisons of anthropometric and biochemical data between cases and controls were analyzed by Mann Whitney U test. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic accuracy of miRNAs and the area under the curve (AUC) was reported for each miRNA. Probability (p) value <0.05 was considered significant. All statistics were performed using GraphPad Prism 8 software.

## Results

In the present study, we included 35 T2DM patients and 35 non-diabetic healthy individuals. The mean ± SD of age of T2DM patients was 45.3 ±7.3. Age-matched Non-T2DM subjects were included. The male/female ratio was 1.2. The comparison of anthropometric parameters between T2DM cases and Non-T2DM controls are presented in Table 1. Anthropometric parameters were estimated for both the groups. Weight and height of the study subjects were taken using a digital weighing balance (in kilograms) and stadiometer (in centimetres which were converted to meters) respectively to determine the body mass index (BMI). The BMI of participants in the T2DM group had a median (IQR) of 27.4 (6.3) whereas the median (IQR) of controls was 24.9 (4.5). Along with BMI, waist to hip circumference ratio (WHR) was also taken using a measuring tape and blood pressure was measured using desk and wall aneroid sphygmomanometer. There were significant differences between the study groups for a series of parameters including BMI, waist to hip ratio, and blood pressure ($p > 0.05$).

# Biochemical parameters:

The findings of biochemical investigations in both T2DM groups and control groups are shown in Table 2. Biochemical parameters were compared between study groups. In terms of glycaemic status, T2DM patients had significantly higher levels of FBG, HbA1c compared with the healthy subjects ($p < 0.0001$). There were no significant differences between the study groups for HDL Whereas TC, TG, and LDL differed significantly between subject groups and were higher in T2DM subjects ($p < 0.0001$). hs-CRP was also significantly higher in the T2DM group compared to control groups.

# Relative Expression of miR-24-3p and miR-198 in Study groups

TaqMan-based RT-qPCR was used to determine the expression of miR-24-3p and miR-198 in peripheral whole blood of T2DM patients and non-diabetic healthy control individuals. miR-24-3p expression (Table 3, Figure 1A and 1B) was significantly downregulated by 0.31-fold in T2DM patients compared with Non-T2DM controls (p =0.0042). Notably, miR-198 expression was also significantly downregulated by 0.20-fold in T2DM patients than the control group. (p = 0.0337).

# Correlation between miR-24-3p and miR-198 with Glycaemic Status and Other Clinical Parameters

To assess the association between expression of miR-24-3p and miR-198 with glycaemic status and other clinical parameters, Spearman's correlation analysis was performed. There was no significant correlation of miR-24-3p and miR-198 with any clinical parameters including glycaemic status, hsCRP, BP, LDL, triglyceride, and total cholesterol. However, there was a significant association between miRNA-24-3p and miRNA-198 (r=0.659; p=0.000009**).

# Evaluation of the Diagnostic Values of circulating miR-24-3p and miR-198

To evaluate the possible roles of miR-24-3p and miR-198 as biomarkers for early diagnosis of T2DM, we evaluated their diagnostic potential using the receiver operating characteristic (ROC) curve analysis (Figure 2). As shown in Figure 2A, the ROC analysis revealed a significant ability for miR-24-3p in discriminating between the subject groups. miR-24-3p discriminated T2DM patients from healthy subjects with an area under the curve was 0.69 (95% CI 0.57–0.81, p = 0.0046). Also, miR-198 revealed a significant difference between T2DM and Non-T2DM controls (Figure 2B). The AUC was 0.65 (95% CI 0.51 -0.78, p =0.0341) in discriminating T2DM patients from healthy subjects.

## Discussion

The rise in T2DM incidence in the world urgently requires efficient biomarkers for early detection of diseases to facilitate future preventive interventions and improve patient care. Micro-RNAs, a group of small non-coding RNA molecules, have been reported to regulate major cell processes and are also involved in many pathological conditions [9, 19]. As miRNAs are released into the bloodstream by cells and can be detected, they have proven to be useful as prognostic, diagnostic, and predictive biomarkers. Studies have shown that peripheral blood miRNAs can be a biomarker of T2DM and other diseases [12, 20, 21]. Previous studies have shown that aberrant expression of several miRNAs affects insulin signalling pathways, insulin resistance and plays a major role in T2DM [10, 22]. In this study, we have

therefore analyzed the expressions of miR-24-3p and miR-198 in peripheral blood for T2DM patients and healthy individuals by TaqMan-based RT-qPCR and assessed their suitability as biomarkers for detection of T2DM. We observed that in T2DM, expression of both miR-24-3p and miR-198 was significantly downregulated as compared to Non-T2DM controls. Decreased in the expression of miR-24-3p in T2DM patients agreed with a previous report by Kokkinopoulou et al. (2019) [23] showing decreased miR-24 in sera from patients with T2DM. These results could suggest that miR-24-3p and miR-198 are involved in the pathogenicity of T2DM. miR-24 is highly expressed in the lungs, heart, pancreases, and kidneys but poorly expressed in the liver and muscle. Consistent with human observations, miR-24 levels were decreased 2-3-fold in STZ-induced mice and db/db diabetic mice lungs, and 5-fold in db/db mice plasma [24]. The study of Zhou et al. (2014) also reported that miR-24 levels were decreased in acute ischemic stroke compared with the controls [25]. There are not many studies of miR-24-3p in diabetes. Thus, the expression or stability of miR-24-3p remains unclear in diabetes, as well as in insulin resistance. Although there are numerous studies of miR-24 in various other diseases like cancer, lung diseases [26, 27]. In-vitro studies with human and murine blood samples had shown that miR-24 influences diabetes complications [28]. Similarly, there are only very few miR-198 studies in diabetes but some miR-198 studies in other illnesses such as cancer exist [29]. Elemeery et al. reported that miR-198 expression is downregulated in T2DM with hepatocellular cancer (HCC) [14]. Further, Cui et al. (2019) showed decreased expression of miR-198 in gastric cancer patients [29]. In the present study, miR-198 expression was significantly downregulated in T2DM patients compared to Non-T2DM controls. As per the findings of the present study, miR-24-3p and miR-198 are found to be downregulated in newly diagnosed T2DM patients. Our study has several limitations for which the findings need to be interpreted with caution. The limitations include low sample size, not measuring the insulin resistance in the study group, not including a pre-diabetic group, and using miR-16-5p for normalisation which is reported to have unstable expression in certain conditions [30]. Finally, it is a cross-sectional observation and needs longitudinal studies with robust design for validation.

# Conclusion

The present study demonstrated that blood miR-24-3p and miR-198 were downregulated in T2DM patients compared with Non-T2DM controls. Although the miRNAs were related to T2DM, but they were independent of hyperglycaemia. This study also provided a preliminary idea regarding the diagnostic ability of miR-24-3p and miR-198, to distinguish between T2DM patients and Non-T2DM individuals. The role of miR-24-3p either alone or in combination with miR-198 should be explored further as biomarkers for early detection of T2DM. For this, validation studies in larger sample sizes with more patient groups (pre-diabetics) are needed before implementing miR-24-3p and miR-198 as diagnostic biomarkers of T2DM. Moreover, the underlying mechanism that may link the downregulation of these miRNAs and the occurrence of T2DM needs to be explored.

# Declarations

# Funding:

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# Conflict of Interest –

The authors declare no conflict of interest.

# Acknowledgement -

None

# Financial disclosure –

None

# Compliance with Ethical Standards:

This study includes human subjects with the approval from Institutional Ethical Committee (IEC), All India Institute of Medical Sciences, Jodhpur, Rajasthan India (AIIMS/IEC/2018/635). The study was performed in accordance with down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

# Informed Consent:

Written informed consent was obtained from all subjects recruited in this study.

# Consent for publication:

All authors consent present for publication of this original article

# Code availability:

Not Applicable

# Authors contribution:

A, PM, IS: conceptualization and methodology; RKGS, A, SS: data curation and preparation; A: writing original draft; A, PM, SS: Visualization and investigation; PM, IS, PS: Supervision; PM: software validation; PM, IS, KKS, PS: writing-reviewing and editing. All authors read and approved the final version of the manuscript.

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# Tables

***Table 1***

*Comparison of Anthropometric data between study groups*

| **Parameters** | | **T2DM (cases) (n=35) Median (IQR)** | **Non-T2DM (controls) (n=35) Median (IQR)** | **P-value** |
|---|---|---|---|---|
| Age | | 45.3 ±7.3 | 45.3 ±7.3 | |
| Male/female ratio | | 1.2 | 1.2 | |
| Weight (kg) | | 73 (22.4) | 72.5 (17) | 0.352 |
| Height (m) | | 1.6 (0.2) | 1.69 (0.1) | 0.032* |
| BMI (kg/m2) | | 27.4 (6.3) | 24.9 (4.5) | 0.0032* |
| Waist circumference(cm) | | 101.6 (17.3) | 89 (10) | <0.0001* |
| Hip circumference(cm) | | 104 (12.3) | 97 (13.5) | 0.047* |
| Waist-to-hip ratio (WHR) | | 0.97 (0.08) | 0.92 (0.07) | <0.0001* |
| BP (mm/Hg) | Systolic | 120 (26) | 110 (10) | 0.0007* |
| | Diastolic | 80 (20) | 70 (10) | 0.023* |

p**< 0.01 highly significant and p*< 0.05 significant; IQR: interquartile range; BP: blood pressure; BMI: body mass index; n: number of patients; Age is expressed as mean ± SD

***Table 2***

*Biochemical parameters among study groups*

| Parameters | T2DM (cases) (n=35) Median (IQR) | Non-T2DM (controls) (n=35) Median (IQR) | P-value |
|---|---|---|---|
| FBG (mg/dl) | 137 (51.5) | 93 (8) | <0.0001** |
| HbA1c (%) | 7.8 (1.3) | 5.6 (0.4) | <0.0001** |
| hs-CRP (mg/l) | 2.83 (7.8) | 1.61 (2.07) | 0.0046* |
| TC (mg/dl) | 206 (72.5) | 153 (21) | <0.0001** |
| TG (mg/dl) | 240 (77.5) | 118 (51.5) | <0.0001** |
| HDL-c (mg/dl) | 38 (12.5) | 39 (12) | 0.872 |
| LDL-c (mg/dl) | 115 (49) | 92 (16.5) | <0.0001* |

p**< 0.01 highly significant and p*< 0.05 significant; IQR: interquartile range; FBG: fasting blood glucose; HbA1c: glycated haemoglobin; hs-CRP: high sensitivity C-reactive protein; TC: total cholesterol; TG: triglycerides; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; n: number of patients

***Table 3***

*Fold changes in the expression of whole blood miRNAs among T2DM (cases) in comparison to Non-T2DM (controls) calculated using relative expression* $2^{-\Delta\Delta Ct}$*. Normalization was done with miR-16-5p.*

| miRNAs | T2DM (cases) (n=35) | Controls (cases) (n=35) | Fold change ($2^{-\Delta\Delta Ct}$) | P-value |
|---|---|---|---|---|
| $2^{-\Delta Ct}$ | Median (IQR) | Median (IQR) | | |
| miR-24-3p | $0.62x10^{-2}$ (0.0101) | $1.51x10^{-2}$ (0.0669) | 0.312 | 0.0042 |
| miR-198 | $0.096x10^{-2}$ (0.0047) | $0.75x10^{-2}$ (0.020) | 0.203 | 0.0337 |

# Figures

*Figure 1A*

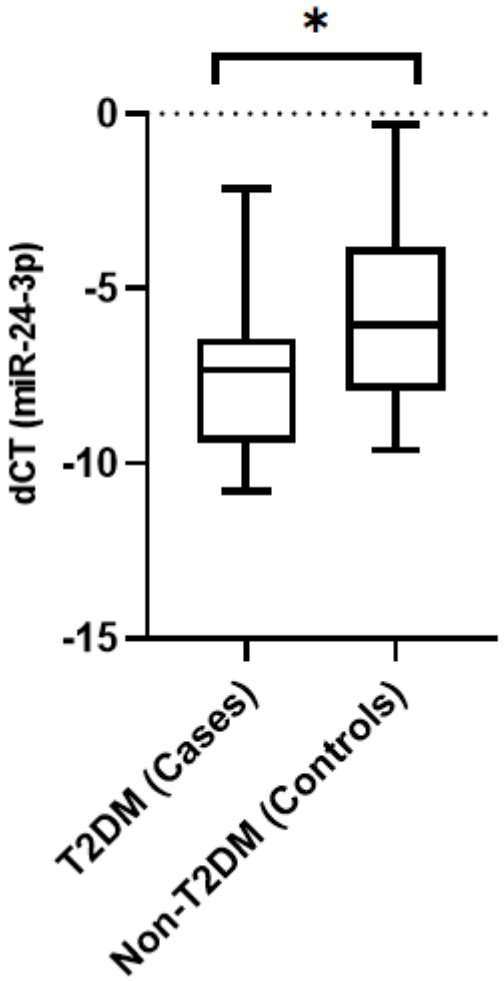
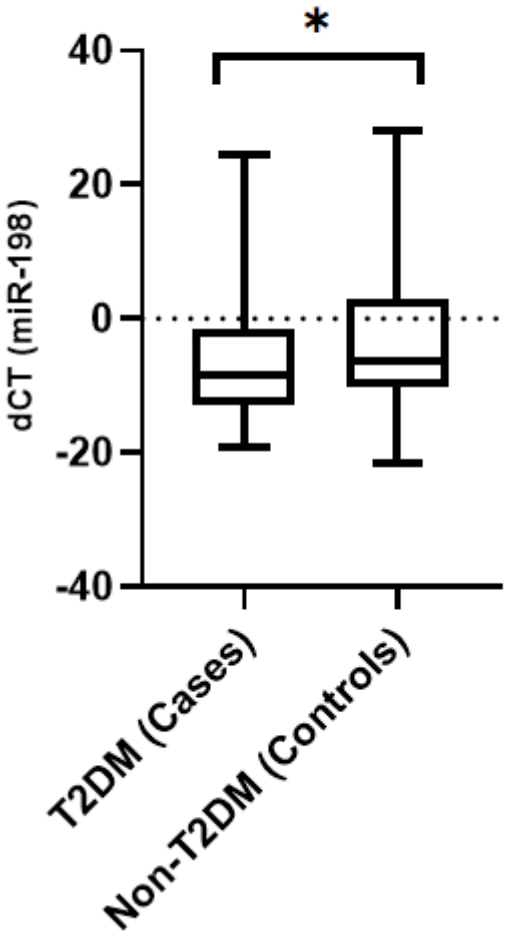


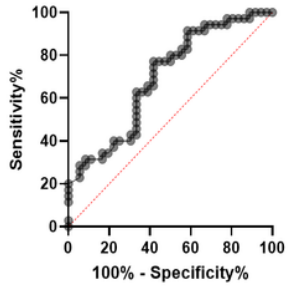

*Figure 1B*




## Figure 1

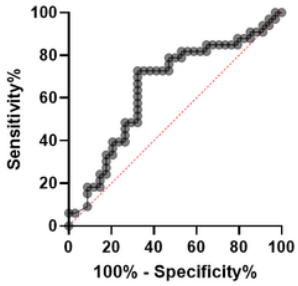
Comparison of miR-24-3p (Fig 1A) and miR-198 (Fig 1B) expression among T2DM (cases) and Non-T2DM (controls). Box and whisker plot show the dCT values of the target miRNA normalised to miR-16-5p for each individual across the study groups. The inner line in the box represents the median and the plot shows the minimum to maximum values,** indicates $p<0.05$

*Figure 2A: ROC curve of miR-24-3p*




| The area under the ROC curve | |
|---|---|
| Area | 0.69 |
| Std. Error | 0.06 |
| 95% confidence interval | 0.57 to 0.81 |
| P - value | 0.0046 |

*Figure 2B: ROC curve of miR-198*




| The area under the ROC curve | |
|---|---|
| Area | 0.65 |
| Std. Error | 0.068 |
| 95% confidence interval | 0.51 to 0.78 |
| P - value | 0.0341 |

## Figure 2

The receiver operating characteristic (ROC) curve analysis. Figure 2A: receiver operating characteristic (ROC) curve analysis of miR-24-3p and Figure 2B: receiver operating characteristic (ROC) curve analysis of miR-198 Figure 2A: ROC curve of miR-24-3p Figure 2B: ROC curve of miR-198