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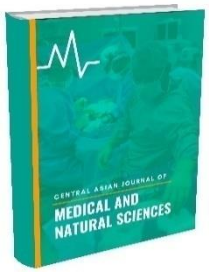

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Evaluation of miR-204 Serum Level in Obese Patients with Type 2 Diabetes

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Abstract: Background The failure of the pancreatic islets to maintain normal blood glucose concentrations is not observed in clinical manifestations until irreversible damage has occurred. There is no effective biomarker yet to predict type 2 diabetes. MicroRNAs (miRNAs) are single non-coding RNA molecules. And miRNAs play a vital role in inhibiting the translation of post-transcriptional mRNAs. Studies in recent years have revealed that these microparticles play an essential role in the pathogenesis of type 2 diabetes. In addition, the presence of miRNAs in serum and plasma also provides a potential target for detecting disease markers. This study aims to investigate miR-204 in obese patients with type 2 diabetes.

Methods: Here we had collected 180 blood samples from 60 normal individuals, 60 patients with T2DM with a BMI of less than 30 and 60 patients with T2DM with a BMI of more than 30. The age of the subjects was 48-72 years . That's 85 males and 95 females. We used real-time Stem-loop PCR to detect miR-204 in the plasma of obese patients with type 2 diabetes.

Results: The results showed that plasma miR-204 levels were increased significantly in patients with T2DM in compared to normal individuals (P>0.001). Then we were also showed that serum miR-204 in Obese- T2DM groups were significantly higher than non-Obese-T2DM group. (2-sided Student's t test P < 0.001). the results showed that there was no significant relationship between age and sex with miR-204 (P=0. 591 and 0.490 for age and sex, respectively). Our results showed that there was a statistical significant difference between BMI of all individuals with miR-204 expression. We showed that 56.1% of none obese individuals were low expression of miR-204 suggesting that increasing BMI leads to increasing level of miR-204 (P=0.001).

Conclusion: The Measuring circulating miR-204 is

provide a relatively simple and straight forward approach, making it attractive for potential wider clinical application. However, identifying miR-204 could provide the necessary sensitivity and specificity to be detected in T2D.

Key words: miR-204, Obesity, Plasma, Type 2 diabetic.

Introduction

Diabetes mellitus is characterized by chronic hyperglycaemia with disturbances of lipid carbohydrate and protein metabolism resulting from defects in insulin secretion, insulin action, or both. (1,2) There are many risk factors that associated with boost risk of type 2 DM : Increasing age, Obesity, Dietary excess, Dietary factors and sedentary lifestyle. (3,4) Type 2 diabetes is results from insulin resistance with a defect in compensatory insulin secretion. (5,6) Several processes are involved in the development of diabetes, including those that damage the pancreatic beta cells and impair insulin secretion and that's lead to insulin resistance. (7) Various degrees of β-cell dysfunction and insulin resistance are commonly associated with overweight and obesity. (8,9) The miRNAs have emerged as powerful regulators of gene expression, including that of pancreatic β-cells.(10,11) Especially miR-204 has been shown to be highly enriched in β-cells. (12) Recently it has been found that miR-204 targets the insulin transcription factor MafA and thereby down regulates insulin production. Moreover, we found that miR-204 also targets PERK and thereby regulates the unfolded protein response and β-cell apoptosis.(13,14) miRNAs can, bind to the 3′ UTR of a multitude of mRNAs, by imperfect base pairing of their seed sequence, that leading to destabilization or translational inhibition of these target genes, we searched for additional putative genes that might be regulated by miR-204. (15)

Methods and materials

We were collected 180 samples in al kut city in Iraq with Three groups : normal individuals (N=60), lean (60) and obese (60) patients with type 2 diabetes.

anthropometric measurements including height and weight were taken from all subjects to calculate m2/ kg BMI. It's done Study of serum miR-204 using Real-time PCR (Stem Loop Real time PCR), here we were used a specific and sensitive quantitative reverse-transcription PCR (RT-qPCR) method for measuring individual microRNAs (miRNAs). each of the RT -qPCR nucleic acid reagents, including the RT-primer, the forward and reverse PCR primers, and the hydrolysis probe, contain design features that, together, optimize miRNA specificity and assay sensitivity. The RT-primer contains a highly stable stem-loop structure that lengthens the target cDNA. (16,17)

RNA extraction

For RNA extraction from serum, we were used TaKaRa commercial kit to extract RNA, which was done according to the protocol proposed by the manufacturer. we are measured the amount of RNA by optical density (OD) and the amount of absorption at 260 nm was measured by spectrophotometer. The final purified solution must have an OD 260 / 280 greater than 1.8 .

An RNA template and primer are used to bind the available A3 hydroxyl group for cDNA synthesis.

Table 1-1 cDNA synthesis

Reagent	Amount
5 × Prime Script™ Buffer (for Real Time)	5µL
Prime Script™ RT Enzyme Mix I	5 µL
Spesific Primer (50 µ M)	4 µL
Total RNA	4µL (700ng)
Rnase free H_2O	2µL
Total Volume	20 µL

Table 1-2 Primer used to this study

Name	Sequence
miR-204 specific cDNA primer	GTCGTATCCAGTGCAGG GTCCGAGGTATTCGCACTGGATACGA CAGGCAT
U6 specific cDNA primer	TCGTATCCAGTGCA*GGGTCCGAGGT*AT TCGCACTGGATACGACAAAATA
Universal Reverse has-miR Primer	-CCA GTG CAG GGT CCG AGG TA
Specific Forward primer of U6	-GCGCGTCGTGAAGCGT TC
Specific Forward primer of miR-204	TTCCCTTTGTCATCCT AGCGT

Master Mix is prepared for this steady according to the table below:

Table 1-3 Master mix ingredient

Material	Amount
Real Qplus 2X master mix SYBR	5 µL
PCR primers (Forward + Reverse)	1 µL
cDNA	2 µL
dH2O	2 µL
Total	10 µL

PCR is performed in three separate steps and these stages were separated due to temperature differences:

1. Denaturing step of two DNA strands.
2. Adherence of primers to the target (Annealing)
3. Making a target complementary string (Extension)

Table 1-4 Real Time PCR program

	Target temperature (°c)	Incubation time
Enzyme activation	95	10 min
Amplification	95	15s
	55	5s
Melting curve analysis	72	30s
	95	15s
	60	60s
Cooling	40	30s

Statistical analysis

All statistical analyses were performed the SPSS version 21 software (SPSS, Inc, Chicago, IL, USA). Data of quantitative RT (qRT)-PCR are analyzed by 2–ΔCt method. All data were presented as mean ± standard deviation (SD) The normal distribution of data was checked by Kolmogorov–Smirnov analysis. Differences in miRNA concentrations between two groups are compared using student t- test. A difference is considered statistically significant at $P < 0.05$.

Results

To ensure the quantity and quality of the extracted RNA that could be suitable for cDNA synthesis, there was determined by Nano-drop spectrophotometry. In qualitative analysis, the presence of two ribosomal 28S and 18S bands indicates non-degradation of RNA.

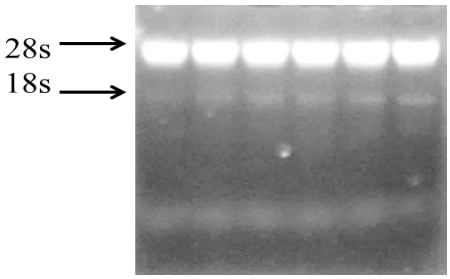

Figure 1-1 RNA electrophoresis extracted on 1% agarose gel

amplification conditions were optimized using RT-PCR reaction, for comparative study of the expression of the miR-204 in plasma of all individuals, the standard curve was drawn for miR-204 and U6, which they are make from one of the cDNA samples and RT-PCR.

Then the Ct curve is plotted against the linear concentration. The analysis curves indicates the efficiency of the desired primers

$$\text{Efficiency} = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1$$

the primers of target genes has effective performance and bind specifically to the microRNA and U6.

Table 1-5 the efficacy of real time PCR

Primer	R	Efficiency
MiR-204	0.947	90.47%
U6	0.982	106.81%

Also, for the desired primers, the standard and calibration diagrams for miR-204 and U6 were drawn as follows.

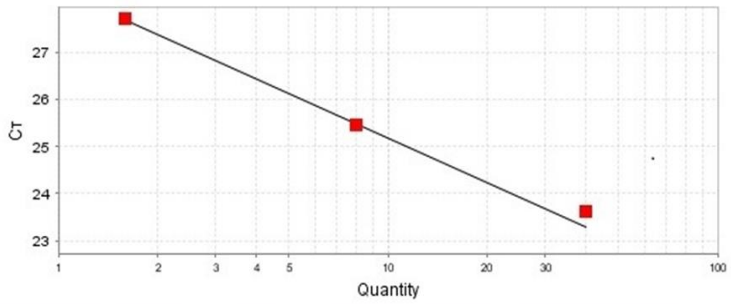

Figure 1-2 Standard curve for calculating Efficiency for revers and forward U6 primers

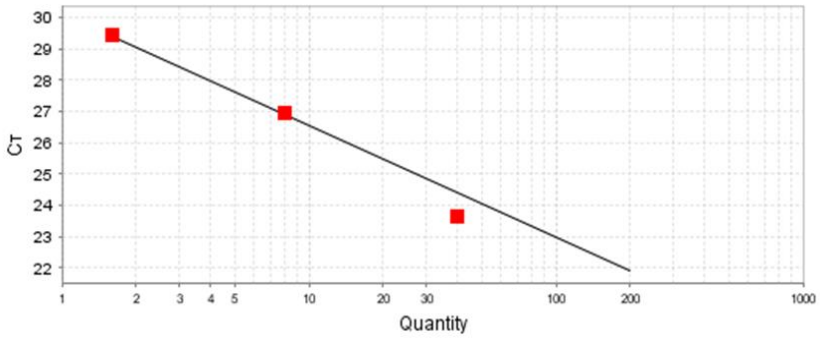

Figure 1-3. Standard curve for calculating Efficiency for miR-204 revers and forward primers

The step of amplification plot and Melting curve analysis of both miR-204 and U6 to investigate the specificity of primers, to ensure the replication of specific components and to investigate the absence of non-specific components and primer dimers in the product, the melting curve for miR-204 and U6 genes was investigated.

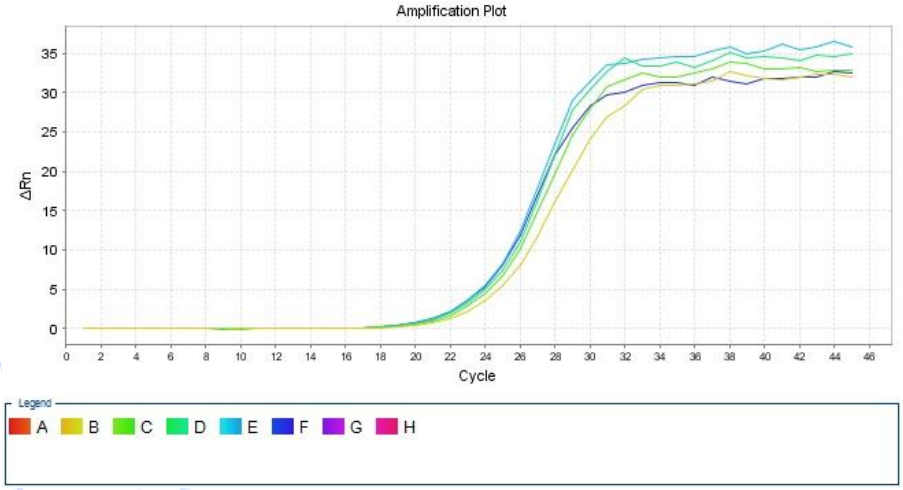

Figure 1-4 Amplification plot for miR-204

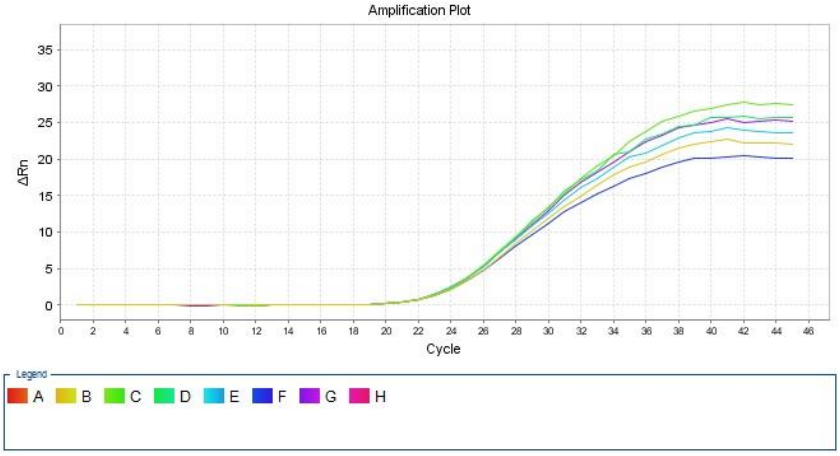

Figure 1-5 Amplification plot for U6

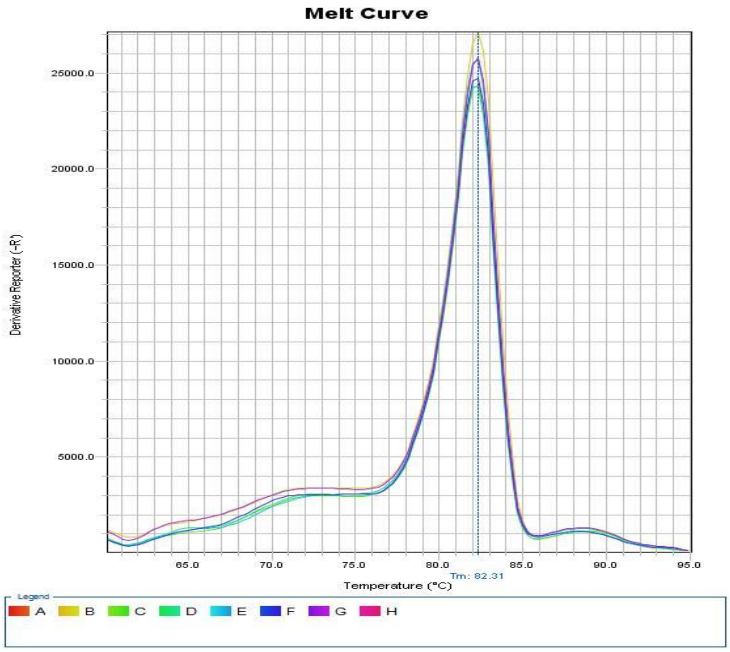

Figure 1-6 Melt curve analysis for miR-204

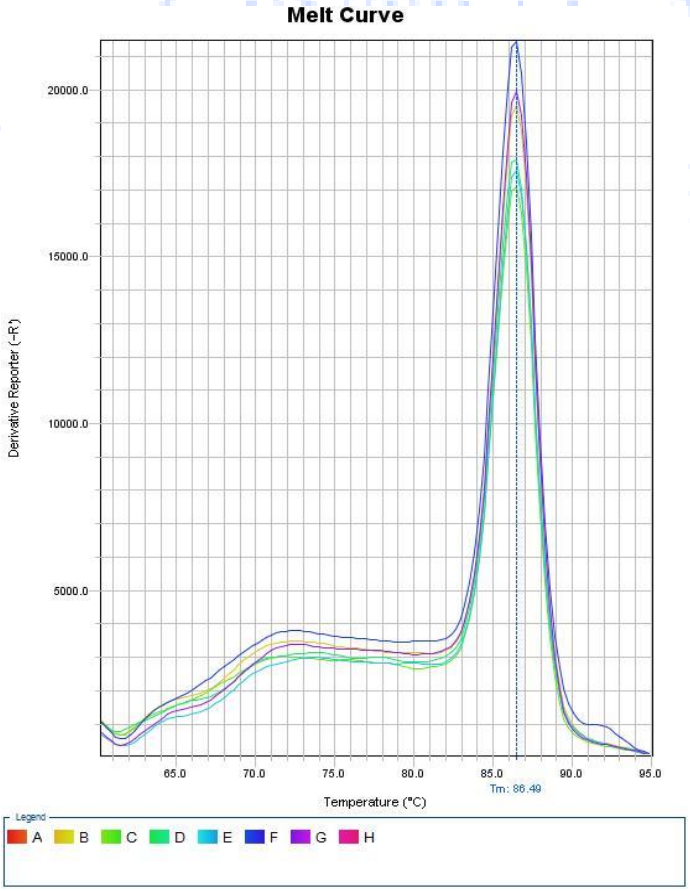

Figure 1-7 Melt curve analysis for U6

We analyzed serum of level of miR-204 in normal individuals and patients with T2DM. The subject demographics are described. Indeed, we found that serum miR-204 was significantly higher in patients with T2D as compared with controls; 2-sided Student's t test $P < 0.001$.

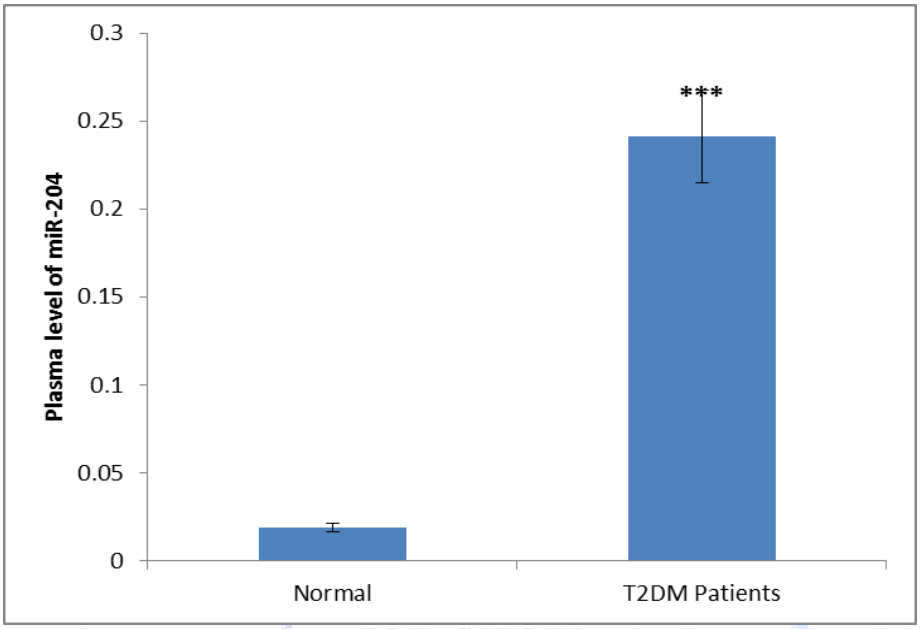

Figure 1-8 plasma level of miR-204 in normal subjects and patients with T2DM. The miR-204 level was adjusted with U6 mRNA as the internal control. *** was stated for statistically difference between 2 groups.

After evaluation of miR-204, the patients were subdivided into two groups. As indicated in Figure 1-9, we found that serum miR-204 in obese- T2DM groups was significantly higher than non-obese-T2DM group. (2-sided Student's t test $P < 0.001$).

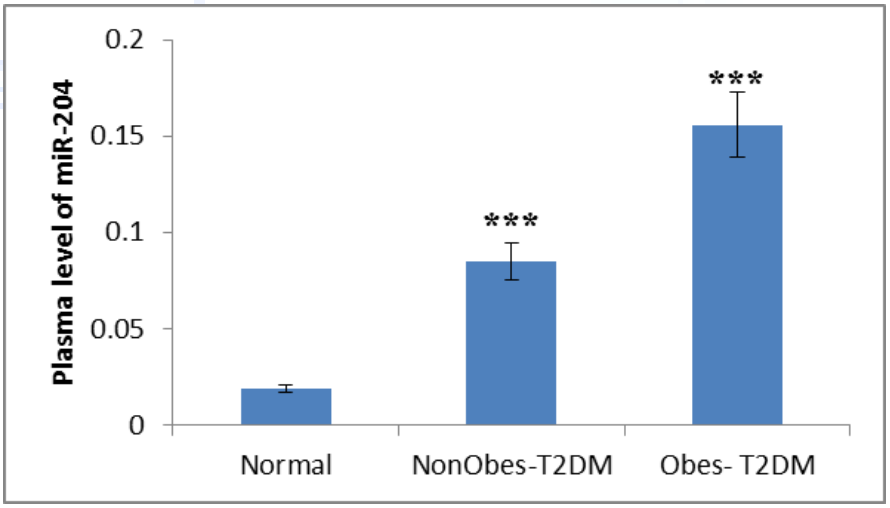

Figure 1-9, plasma level of miR-204 in three groups of normal, Non Obese T2DM and Obese- T2DM groups. The miR-204 level was adjusted with U6 mRNA as the internal control. *** was stated for statistically difference between 2 groups.

The relationship between miR-204 and clinicopathological features

To investigate association between miR-204 with clinicopathological parameters the plasma level of miR-204 were evaluated. As indicated in table 1-6 in this study we have 180 individuals aged between

48 and 72 years old including 85 male and 95 female. As presented in table 1-6 there was no significant relationship between age and sex with miR-204 (P=0.591 and 0.490 for age and sex, respectively). Another parameter is BMI. As presented in table 2-2, our individuals were divided into two groups, the first group was individuals whose BMI were more than 30 and second group was less than 30. Our results showed that there was a statistical significant difference between BMI of all individuals with miR-204 expression. For instance, 56.1% of none obese individuals were low expression of miR-204 suggesting that during increasing BMI, miR-204 were increased significantly (P=0.001).In addition, there was a significant difference between HbA1c of all individuals with miR-204expression. For instance, just 1.7% of none obese individuals were high expression of miR-204 suggesting that during increasing BMI, miR-204 were increased significantly (P=0.0001).

Table 1-6 The relationship between clinicopathological features and miR-204 plasma level

Parameter		Plasma level of miR-204		P value
	No. of cases	≤Average	>Average	
Age (year)				0. 591
≥ 59	96 (53.3%)	68 (37.7%)	29(16.11%)	
<59	84 (46.6%)	49(27.22%)	34(18.88%)	
Sex				0.490
Male	85 (47.22%)	56 (31.11%)	29(16.11%)	
Female	95 (52.77%)	61 (33.88%)	34(18.88%)	
BMI				*0.001*
≥ 30	45(70%)	16 (8.9%)	29(16.11%)	
< 30	135 (30%)	101 (56.1%)	34(18.88%)	
Hb A1C				*0.0001*
≥ 5.7	118(65.6%)	58 (32.2%)	60 (33.3%)	
< 5.7	62 (34.4%)	59 (32.8%)	3 (1.7%)	

Discussion

Our Finding showed that serum miR-204 as an attractive novel biomarker of T2D- in humans. We showed that miR-204 levels were increased in patients with T2DM.

Measuring circulating microRNAs provides a relatively simple and straightforward approach, making it attractive for potential wider clinical application. However, identifying candidate microRNAs that could provide the necessary sensitivity and specificity to detect T2D. Unlike the first identified islet microRNA, miR-375 which is also highly expressed in islet alpha-cells and other more ubiquitous microRNAs identified by typically screening serum samples of children with and without diabetes, miR-204 is primarily expressed in islet beta-cells, providing a strong rationale. (18,19)

On the other hand, not being considered microRNA commonly detected in circulation, miR-204 is often not included in serum profiling efforts, and it is therefore not surprising that it has not been identified by previous screening approaches in the context of T2D. In fact, we only were able to identify and validate serum miR-204 as a potential biomarker by a hypothesis-driven, lengthy, step-by-step process based on previous studies. (13,20)

Consistent with other biomarker studies, including circulating unmethylated insulin DNA and proinsulin/C-peptide ratio, serum miR-204 was elevated in response to massive, acute beta-cell death, such as in the context of recent-onset T1D in children and in autologous human islet transplantation. However, serum miR-204 was also able to detect even mild beta-cell loss as found in adults with

recent-onset T1D and was elevated even before diagnosis in nondiabetic AB+ adults at risk of developing T1D, thereby distinguishing itself as a promising, non-insulin-based biomarker.

It is of note that our results suggest that elevated serum miR-204 is a marker of ongoing beta-cell death, which has the advantage that it is expected to

provide information about a critical aspect in the pathogenesis of T2D, especially during imminent or early onset T1D that is currently not captured by other clinically used diagnostic markers.

Ideally, a good biomarker of T2D-assoicated beta-cell loss would be expected to be enriched in pancreatic beta-cells and, in response to injury, to be released from beta-cells and human islets into the supernatant or into the circulation. Moreover, its circulating levels would be expected to be elevated in the context of recent-onset T2D and its measurement should be sensitive enough to insulin resistance.

Taken together, serum miR-204 represents a novel biomarker that can help assess early T2D and thereby may, in combination with other tests, facilitate more accurate early diagnosis and assessment of T2D activity. As such, it addresses a critical yet unmet need in the personalized management of diabetes.

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