Journal of Pioneering Medical Sciences
Received January 7, 2023 Accept February 11, 2024 Publish February 29, 2024.
Volume 13, Issue 1, Pages 138-143

DOI https://doi.org/10.61091/jpms202413124

Sera Levels of microRNA-124 in Newly Diagnosed Patients with Type 2 Diabetes Mellitus in Iraqi Population

Huda B. Al-Lami[1,*], Ramzi ZEMNI[2] and Ammar Gany Yassin[3]
[1]Department of Chemistry and Biochemistry, Faculty of Medicine, University of Maysan.
[2]Department of Genetics, and Biochemistry Faculty of Medicine University of Soussa.
[3]Department of Biochemistry, Faculty of Medicine, University of Kerbala.

Corresponding author: Huda B. Al-Lami (e-mail: hudailami1993@gmail.com).

Abstract Recently, circulating microRNAs (miRNAs) have emerged as potential new biomarkers for diagnosing and prognosis a wide range of disorders. The study aimed to compare the sera levels of miRNA21 and miRNA124 among newly diagnosed patients e with type 2 diabetes mellitus (T2DM) patients with their levels in apparently healthy subjects using blood samples. The time frame for this case-control research is August 2022 through March 2023, and it took place at Al-Sadr Teaching Hospital/Madina in Masin. One hundred patients with a recent di recently patients with T2DM (50 females) and 100 control participants appeared to be healthy but were really between the ages of 5 and 70. We took 2 ml of blood from each patient and healthy person, placed it in an EDTA tube with 1 ml of Trizole as a preservative, and put the tubes in the freezer at -20 degrees Celsius until we were ready to analyze the samples. Recent studies prove the relationship between miR-124 and inflammatory processes and insulin resistance; our emphasis suggests the role miR-124 in insulin resistance in T2DM. Studies have explored the potential of miR-124 as a diagnostic biomarker for T2DM. Altered expression levels of these miRNAs have been detected in sera of newly T2DM-diagnosed patients, which promises to be a non-invasive technique for the disease. Regarding therapeutic scope, miR-124 may be used as a target for insulin sensitivity. Modulating this miRNA may have a potential therapeutic impact in managing T2DM. It is important to note that significant progress has been made in understanding the involvement of miR-124 in T2DM. New studies may provide additional knowledge on its mechanism to decrease insulin receptor resistance and beta cell function, hence, the pathophysiology of T2DM.

Key Words Circulating microRNAs, Type 2 diabetes mellitus (T2DM), miRNA21, miRNA124, Diagnostic biomarkers, Insulin resistance

1. Introduction

Diabetes mellitus emerges as a global problem around the world, an age-related metabolic condition. As its incidence grows outside of industrialized nations, DM has become a major public health issue worldwide. The International Diabetes Federation (IDF) has released an estimate of the global prevalence of diabetes [1]. Diabetes develops mainly in one of two ways. Ineffective insulin response causes T2DM, while inadequate insulin production causes type 1 diabetes mellitus T1DM [2].

A. Diabetes Mellitus

Diabetes mellitus is a chronic condition that changes how your body handles glucose. The inability of the pancreatic -cells to release sufficient quantities of insulin to control blood glucose levels is a hallmark of both T1DM and T2DM. In addition, the insulin-target tissues, including the liver, adipose tissue, and skeletal muscle, become resistant to insulin in type 2 diabetes mellitus. It is believed that both hereditary and environmental variables have a role in the progression of the illness.

B. MiR-124

Mice were the first to discover miR-124 [3], and subsequent studies have shown that it is very abundant in the brain [4]. There is evidence that it is expressed in a wide range of tissues [5]. There are three members of the miRNA family miR-124; they are miR-124-1, miR-124-2, and miR-124-3. This miRNA's mature form has the same sequence in humans, mice, and rats [5].

C. Function

Additional components of the secretory apparatus, including synaptosomal-associated protein 25 (SNAP25), Rab3A, and

synapsin-1A, are boosted in expression by miR-124a. When glucose is present, miR-124 overexpression increases insulin secretion, but when glucose is absent, it decreases insulin secretion.

The b cells of the pancreas similarly have high levels of miR-124a [6]. Mouse chromosomes 14, 3, and 2 encode the three distinct versions of this gene (miR124a1, a2, and a3). Throughout the stages of pancreatic development, miR-124a2 expression varies. An uptick in miR-124a2 levels has a suppressive effect on FoxA2 expression. By controlling the expression of crucial b- b-cell-specific genes such as Pdx-1, Kir6.2, and Sur1, FoxA2 acts as a master regulator of pancreatic development. Insulin mRNA levels were either reduced by overexpression of miR-124a2 or increased through its inhibition [7].

2. Metrials and Methods

The participants in this case-control research were patients at Al-Sadr Teaching Hospital/Madina in Masin between August 2022 and November 2022. One hundred newly diagnosed T2DM patients (50 females and 50 men) varied in age from 3 to 75 years old. In contrast, one hundred seemingly healthy participants (50 females and 50 males) served as controls in a comparison group ranging in age from 5 to 75.

A. Inclusion Criterion

Newly diagnosed patients with T2DM of both sexes.

B. Exclusion Criterion

Any patient with a known carcinoma or with renal failure.

C. Ethical Approval and Participants' Consent

This study was approved by the Scientific Committee of the Department of Biochemistry, Faculty of Medicine, University of Soussa. Signed written consent has been taken from each patient or their relative and healthy individual participating in the study.

D. Blood Collection and Storage

Two milliliters (ml) of peripheral venous blood samples were collected from every patient. The collected blood was mixed with 1 ml of Trizole preserving material, kept in an EDTA tube, and stored at - 70 $^{\circ}C$ until the time of laboratory investigations of blood miR-21 and miR-124 were performed. The Figure 1 depicts that after thawing of the stored whole blood (EDTA and Trizol) sample, chloroform was added to the sample and centrifuged to collect the total RNA, which was further centrifuged after incubation by using a unique tube which was supplied by the manufactured company kit to collect miRNA, both of the total RNA and miRNA have been stored in a special refrigerator at -70C till the time of investigation of blood miR-21 and miR-124.

E. Instruments

All the instruments and tools which are used in this study are listed in Table 1 with their suppliers:

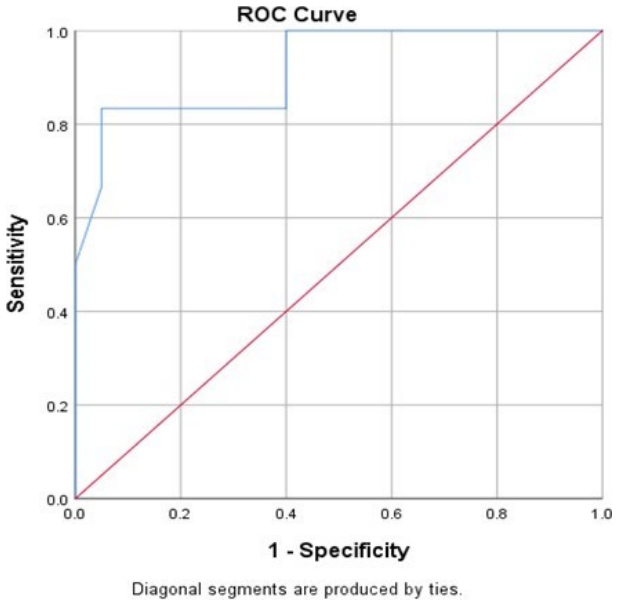

Figure 1: Steps of extraction of small RNA from whole blood (Shahid A et al., 2019)

Equipment	Company	Country
Centrifuge	KOKUSAN (H-19F)	Germany
Cool centrifuge	Shematzu	Japan
Centrifuge tubes (Sterile) - 5ml	Bio basic	Canada
Eppendorf tubes (1.5ml/200µl)	Bioland	USA
PCR Tubes flat cup (Sterile) - 0.2ml	Bio basic	Canada
Spincentrifuge	Qiagene Corbett Rotorgene Q	Germany
Micropipettes	Eppendorff	Germany
Micropipette 1.0-10ul	NEXTY-10	Japan
Micropipette 10 – 100ul	Slamed	Japan
Micropipette 100-1000ul	Slamed	Japan
Micropipette 50 – 200ul	Slamed	Japan
Real time PCR	Qiagene Corbett Rotorgene Q	Germany
Vortex	Scientific industrial	USA
Water bath	Memmert	Germany
Deep freezer	Deep freezer	China
Dirui CS-T180 chemisrty auto- analyzer	Snibe	China

Table 1: Instruments and tools with their suppliers

F. Methods

The separated whole blood preserved in an EDTA tube with Trizole has been used to measure:

1) Mir-U6 by real-time PCR.
2) MiR-21 by real-time PCR.
3) MiR-124 by real-time PCR.

Primers of this study are shown below with their sequences Table 2

Primer	Sequence (5→3 direction)
microRNA	
microRNA21	TAGCTTATCAGACTGATGTTGA
microRNA124	TTAAGGCACGCGGTGAATGCCA
microRNAU6 F.P.	AGAGAAGATTAGCATGGCCCCT
microRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

Table 2: Primers used in the study

G. Total RNA Extraction from the Blood

The total RNA of each blood sample was extracted using the TransZol Up Reagent according to the protocol provided by the manufacturer (TransZol Up), as follows:

1) "Add 250 µl of separated EDTA blood to 1000 µl of TransZol Up. The sample was lifted at -70º C overnight".
2) "Add 200 µl of chloroform to 1 ml Trans Zol reagent with vigors shak- ing of the tube for 30 seconds, then incubation for 3 minutes in room temperature.
3) At 4 ° C, centrifugation performed with 10,000 round per minute RPM for 15 minutes. A pink lower organic phase occcure after separation of mixture, an upper aqueous colorless RNA containing phase and an inter phase. Trans Zol reagent must account 50-60 %.
4) To a fresh RNase-free tube, transfer the upper colorless phase which con- tain RNA. An equal volume of ethanol (96% - 100%) (precipitates might occur in this phase). Gently mixing by inverting tube showed be performed.
5) In to a spin column the resulting solution should be transfer and pre- cipitates together. For 30 seconds in room temperature centrifugation at 12,000 RPM should be performed with discarding the flow-through.
6) To the spin column add 500 µl of clean buffer, in 12,000 RPM centrifu- gation for 30 seconds should be performed with discarding the flow- through.
7) Repeat step 6 once.
1) A 500 µl of Wash buffer added (be sure that ethanol has been added). In room temperature at 12,000 RPM and for 30 seconds centrifugation should be performed with discarding the flow-through.
2) In order to completely remove remaining ethanol centrifugation performed for 2 minutes in room temperature at 12,000 RPM. Then for several min- utes air–dry the column matrix.
3) Into a clean 1.5 ml of RNase-free tube place the spin column then a 100 µl of RNase-free water should be added into the spin column. For 1 minute and at room temperature the matrix must be incubated.
4) To elute RNA, centrifugation for 1 minute at 12,000 RPM should be done.
5) At -70 ºC store the isolated RNA.

H. Micro RNA Extraction From the Blood

1) Add 250 µl of EDTA blood sample to 1000 µl of TransZol Up. Allow the samples to incubate at -70º C overnight.

	Step 2	Step 1	
85 ºC	$42°C$	25 ºC	Temperature
5 Sec.	15 min	10 min	Time

Table 3: Thermal cycler steps of conditions cDNA Reverse Transcription

2) Mix 200 µl of chloroform per ml LB10 by vigorously shaking the tube for 30 seconds. Incubate the mixture at room temperature for 3 minutes.
3) Perform centrifugation at 4 ºC and 10,000 RPM for 15 minutes. This will result in a lower pink organic phase, an upper colorless aqueous phase containing the RNA, and an interphase. Optionally, leave a portion of the aqueous phase in the tube to minimize DNA contamination.
4) Transfer the colorless upper phase containing the RNA to a fresh RNase-free tube. Add an amount equal to 1/3 of the transferred solution in 96% - 100% ethanol. Gently mix by inverting the tubes.
5) Add the entire lysate above to an RNA spin column. Perform centrifugation at 4 ºC and 12,000 RPM for 30 seconds, collecting the flow-through.
6) Measure the volume of the flow-through and transfer it to a clean 1.5 ml RNase-free tube. Add 96% - 100% ethanol to fill the tube's tip (total volume should be 1.5 ml). Gently mix by inverting the tubes.
7) Add the entire lysate to a miRNA spin column. Perform centrifugation at 4 ºC and 12,000 RPM for 30 seconds, discarding the flow-through.
8) Add 500 µl of WB10 into the spin column. Perform centrifugation at room temperature and 12,000 RPM for 30 seconds, discarding the flow-through.

1) In order to remove ethanol residue, In a room temperature centrifugation will be done to the column at 12,000 RPM for 2 minutes then air–dry the miRNA spin column matrix for several minutes.

10- In a clean 1.5 ml of RNase-free tube put the miRNA spin column. Then add 50 µl of RNase-free water into the spin column matrix and incubate 1 minute at room temperature. 11- To elute miRNA, centrifugation for 1 minute with 12,000 RPM. 12- The isolated miRNA should be Store at -70 ºC.

I. Primer Preparation

Using the manufacturer's instructions, we reconstituted the lyophilized primers in nuclease-free water to create a stock solution of 100 M for each primer and stored it at (-21$°C$). After storing the stock primer solution at (-21$°C$), we diluted 10 L of the stock solution into 90 L of nuclease-free water to create a working solution with a concentration of 10 M.

J. Negative Controls

Every reaction was done in duplicate and included a non-template control (NTC), non-amplification control (NAC),

and non-primer control (NPC) as neg- ative controls.

K. Thermal Cycle

10 minutes incubation at $25°C$ for Reaction of random primer, and 15 minutes at $42°C$ for Anchored oligo (dT) primer. Incubate at $85°C$ for 5 seconds to inactivate enzymes, as shown in table 3

L. Quantitative Real-Time PCR (qRT–PCR)

The reverse transcription measured the expression levels of microRNAs using the quantitative polymerase chain reaction (qRT-PCR) method, a sensitive technique for quantifying steady-state miRNA levels. A quantitative real-time qRT-PCR SYBR Green assay was used to confirm the expression of the target gene. The endogenous control gene U6 microRNA levels were amplified and used to normalize the miRNA levels.

M. Statistical Analysis

Blood miR-U6, miRNA21, and miRNA124 have been measured-the difference in levels between miR-U6 and miR-124 in newly diagnosed patients with T2DM.

The correlation coefficient t-test described the association between the variable parameters studied in this study; p 0.05 was considered statistically significant.

We used SPSS for Windows, version 22 (SPSS et al., Illinois, USA) for statistical analysis. Data were presented as mean ± standard deviation (SD). Researchers checked for Gaussianity in the parameters they examined using the Shapiro-Wilk normality test. For this study, we compared the means of the groups using an independent samples t-test. In addition, two tests were used to examine categorical variables. After analyzing variance (ANOVA), we used Scheffe', Tukey's, and Hochberg's GT2 Post Hoc tests for multiple comparisons.

If the data did not follow a normal distribution, the variables were log-transformed for analysis and presented in their original units. We used the Chi-square test to examine the relationship between categorical variables.

Following analysis of variance (ANOVA), multiple comparisons were performed using Tukey's, Dunnett's, and Bonferroni's Post Hoc tests. We employed odds ratios (ORs) with 95% confidence intervals (CIs) to assess any correlations bebetween the study groups and the control group. If the test's P value was less than 0.05, it was declared significant. The relationship degrees between variables were analyzed using Pearson correlation analysis. P-values with two tails less than 0.05 (P 0.05) were judged to be statistically significant.

3. Result

A. Demographic Results

Table 4 shows the sex distribution and mean ± SD value of age of T2DM and control groups. Hundred patients with T2DM (50 females and 50 males) and 100 apparently healthy subjects (50 females and 50 males) with comparable percentages of both sexes. The mean ± SD value of the age of T2DM patients (44±17 years) and healthy controls (42±16 years), without significant differ- ences among them.

Group	Mean age (year)	Females	Males
Patients group (n=100)	44± 17	50 (50 %)	50 (50%)
Healthy subjects (n=100)	42±16	50 (50%)	50 (50%)

Table 4: Mean ± SD value of age and number of studded subjects according to gender

Group	No.	Mean Ct of miR-U6 ± SD	Rang*
Patient T2DM	100	13.706±0.45	12-14
Apparently healthy Subject	100	13.78±0.43	12-14

Table 5: (Mean ± SD) values of Ct of miR-U6 of studded group

B. MicroRNA Results

1) MiR-U6 results
   The nuclear control transcript (housekeeping gene) that was used to search for miR expression in this study was miR-U6; it gives information about the envi- ronment of storage of miR from the day of blood taking to the day of laboratory work and about the normalization of the studying tools and materials. The re- sults showed non-significant differences in mean ± SD value of Ct as well as
   In fold of gene expression of miR-U6 between each of the patient groups with T2DM and healthy controls as shown in Tables 5, and Figure 2.
2) MiR-124 results

The results of the present study revealed a highly significant decline in sera levels of miR-124 in the T2DM group in comparison with its levels in the con- trol group (p-value$>$ 0.001) (Table 3-5). The ΔCt (??) miR-124 in T2DM patients [(Means Ct of miR-124) – (Means Ct of miR-U6)]. Subse- quently. The fold of miR-124 gene expression in T2DM patients decreased (0.007 fold) in comparison with its levels in control group, ROC value of miR- 124 was 92 % (sensitivity =88%, specificity = 95%) cutoff value = 0.0558(P value 0.001, 95%, AUC=0.92) in newly diagnosed patients with T2DM as shown in table 6 and Figure 3.

4. Discussion

Newly diagnosed T2DM patients have considerably lower levels of miR-124 in their blood serum. However, there is a shortage of information on miR-124's function and method of action in diabetes. miR-124a inhibits the release of insulin in response to glucose. T2DM pancreatic islets had higher levels of miR-124a expression, and miR-124a had the highest levels of the three isoforms throughout pancreatic development in mouse embryos. The topic of much research is the involvement of miR-21 and miR-124 in the onset and progression of T2DM in newly diagnosed individuals.

Groups	*Means Ct ofmiRNA12*	*Means Ct of 4U6*	*ΔCt (Means Ct ofmiRNA124)*	*2−ΔCt*	*experimental group/ Control group*	*Fold of gene expres-sion*	*p-value*
Patients	19.235	13.706	5.529	0.021657	0.021657/2.770218	0.007	0.001**
Control	12.31	13.78	-1.47	2.770218	2.770218/2.770218	1.00	

Table 6: Fold of miR-124 expression Depending on $2^{-\Delta Ct}$

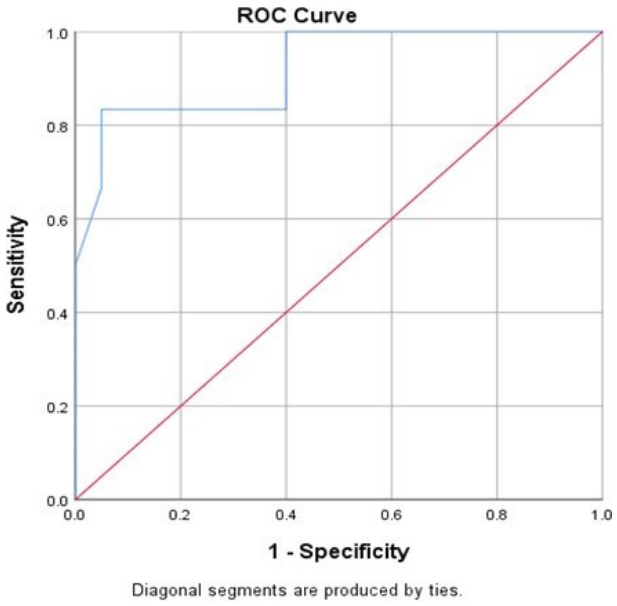

Figure 2: miR-U6 amplification plots by qPCR Samples included studded groups. The photograph was taken directly from the Qiagen Rotor gene qrt- PCR machine

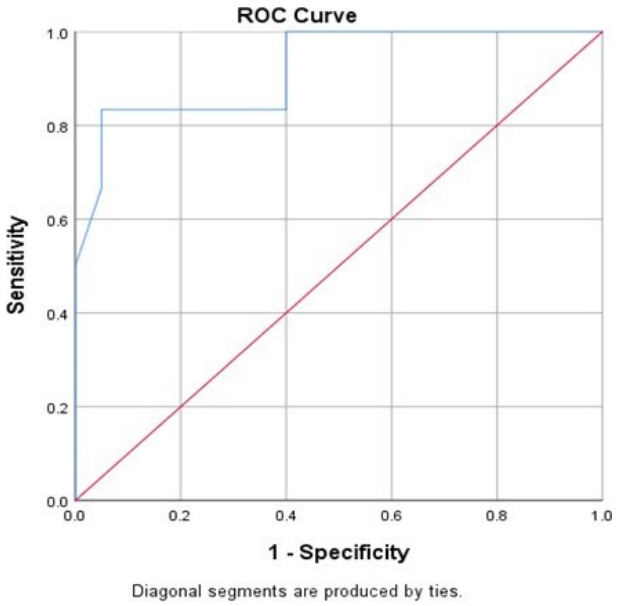

Figure 3: Receiver Operating Characteristic curve of the miRNA-124

Targeting miR-124 may provide a novel concept and potential therapeutic target for diagnosing and treating diabetes because it regulates insulin secretion, proliferation, and apoptosis of islet –cells in response to high glucose [8]. Several research studies have looked at miR-124 and its connection to insulin signaling. There is evidence that miR-124 has a role in insulin resistance and inflammation in type 2 diabetes. MiR-21 and miR-124 have been the subject of research into their possible use as diagnostic biomarkers for type 2 diabetes. These microRNAs' altered expression levels in serum or tissues of newly diagnosed T2DM patients show promise as biomarkers for early disease diagnosis without invasive tissue sampling [8].

The Role of MicroRNA-124 in Growth and Metabolism miRs are crucial regulators of metabolic processes. The presence of miR124 in the liver suggests it plays a critical role in controlling cholesterol and hepatic fatty acid metabolism. Diabetes is characterized by disordered metabolism, and research has shown that miR-124 plays a role in fatty acid and triglyceride hydrolysis by repressing the expression of numerous critical enzymes in the mitochondrial oxidation pathway " [9].In a 2011 study [10], In addition, it has been established that insulin is a potent regulator of lipogenesis and lipolysis and that miR124 is concentrated in pancreatic tissue and is important in pancreas development in humans [11], [12]."

Furthermore, the study analyzed the potential target genes of MiR-124 in beta-cell development and function, focusing on Foxa2. This major beta-cell transcription factor controls beta-cell differentiation and insulin secretion. There was evidence that miR-124 has a role in beta-cell function due to its particular targeting of the Foxa2 gene, which controls insulin production. The observed impact may be attributable to polymorphisms in the miR-124 gene sequence or the sequences of genes immediately next to it. In a recent study [13], researchers found that a particular polymorphism of the miR-124a gene (C/G rs531564) was present in a group of T2D patients. The effects miR-124 on beta-cell activities have only been slightly characterized [14].

suppression of Foxa2 and Mtpn, two miR-124a target genes. While Mtpn controls insulin granule trafficking and docking to the plasma membrane, Foxa2 regulates the potassium channel, ATP-binding cassette, sub-family C genes, and the ATP-dependent K (KATP) channel subunits. We verified the direct binding of miR-124a to the 3'UTR of the Foxa2 and Mtpn genes using luciferase-reporter assays; moreover, the production of miR-124a reduced the amount of luciferase activity regulated by the 3'UTRs of both the Foxa2 and Mtpn genes.

Restoring luciferase activity by altering miR-124 binding regions in the 3'UTR of Foxa2 and Mtpn genes is consistent

with miR-124's unique activity in the 3'UTR of these genes. Additionally, an increase of Foxa2 and Mtpn expression was detected in MIN6-pseudo islets after miR-124a suppression, suggesting that miR-124a negatively affects the expression of these genes. According to these findings, miR-124a may play a role in downregulating Foxa2 and Mtpn expression in T2D pancreatic islets, contributing to decreased insulin production and dysfunctional glucose sensing mechanisms. Although miR-124 is known to adversely regulate Foxa2 and Mtpn, other target genes may be responsible for its effects on pancreatic islets.

5. Conclusions:

Blood miRNA-124 is downregulated in T2DM; the significant decrease in miR-124 is a great diagnostic issue in early diagnosis of T2DM. Regarding the increased number of patients with diabetes mellitus globally, it is essential to develop new methods to prevent the development of disease. MiRNAs might be promising biomarkers to work on.

Conflict of interest

The authors declare no conflict of interest. All authors read and approved the final version of the paper.

Authors Contribution

All authors contributed equally in this paper.

References

[1] Whiting, D. R., Guariguata, L., Weil, C., & Shaw, J. (2011). IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Research and Clinical Practice, 94(3), 311–21.

[2] Chien, H. Y., Lee, T. P., Chen, C. Y., Chiu, Y. H., Lin, Y. C., Lee, L. S., & Li, W. C. (2015). Circulating microRNA as a diagnostic marker in populations with type 2 diabetes mellitus and diabetic complications. Journal of the Chinese Medical Association, 78(4), 204–11.

[3] Sebastiani, G., Ventriglia, G., Stabilini, A., Socci, C., Morsiani, C., Laurenzi, A., ... & Durairaj, S., et al. (2016). Role of microRNA 21 in diabetes and associated/related diseases. Gene, 582.1, 14-18.

[4] Ross, J. S., Carlson, J. A., & Brock, G. (2007). miRNA: the new gene silencer. American Journal of Clinical Pathology, 128(5), 830–.

[5] Zhang, W. H., Jiang, L., Li, M., & Liu, J. (2023). MicroRNA-124: an emerging therapeutic target in central nervous system disorders. Experimental Brain Research, 241(5), 1215-1226.

[6] Martinez-Sanchez, A., Rutter, G. A., & Latreille, M. (2017). MiRNAs in β-cell development, identity, and disease. Frontiers in Genetics, 7, 240782.

[7] Baroukh, N., Ravier, M. A., Loder, M. K., Hill, E. V., Bounacer, A., Scharfmann, R., ... & Van Obberghen, E. (2007). MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic β-cell lines. Journal of Biological Chemistry, 282(27), 19575-19588.

[8] Massart, J., Sjögren, R. J., Lundell, L. S., Mudry, J. M., Franck, N., O'Gorman, D. J., ... & Krook, A. (2017). Altered miR-29 expression in type 2 diabetes influences glucose and lipid metabolism in skeletal muscle. Diabetes, 66(7), 1807-1818.

[9] Andrali, S. S., Sampley, M. L., Vanderford, N. L., & Özcan, S. (2008). Glucose regulation of insulin gene expression in pancreatic β-cells. Biochemical Journal, 415(1), 1-10.

[10] Schultze, S. M., Jensen, J., Hemmings, B. A., Tschopp, O., & Niessen, M. (2011). Promiscuous affairs of PKB/AKT isoforms in metabolism. Archives of physiology and biochemistry, 117(2), 70-77.

[11] Wojcik, M., Mac-Marcjanek, K., & Wozniak, L. A. (2009). Physiological and pathophysiological functions of SIRT1. Mini reviews in medicinal chemistry, 9(3), 386-394.

[12] Andrali, S. S., Sampley, M. L., Vanderford, N. L., & Özcan, S. (2008). Glucose regulation of insulin gene expression in pancreatic β-cells. Biochemical Journal, 415(1), 1-10.

[13] Wang, Z., York, N. W., Nichols, C. G., & Remedi, M. S. (2014). Pancreatic β-cell dedifferentiation in diabetes and redifferentiation following insulin therapy. Cell metabolism, 19(5), 872-882.

[14] Baroukh, N. N., & Van Obberghen, E. (2009). Function of microRNA-375 and microRNA-124a in pancreas and brain. The FEBS journal, 276(22), 6509-6521.