DOI: 10.4274/jcrpe.galenos.2021.2021.0080

Research article

Genetic Indices Relationship to Hyperglycemia-associated Biomarkers: Consistency with miRNA Expression in Egyptian Children with T1D

Barseem NF et al. Circulating miRNAs Markers and T1D

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What is already known on this topic?
MicroRNAs are oligonucleotide sequences that exert a specific biological function.

What does this study add?
New Evidence about insights into miRNA expression patterns. Give an important highlight of relation to be considered a biological marker for T1D. And can be used, for certain studied miRNA molecules, as highly predictive for disease-associated glycemic derangements depending on the reliability of the used methodology.

Abstract
Background: miRNAs are gaining access as novel biomarkers for the spread of autoimmune disorders yet, deviations between individuals at risk or who developed T1D remain to be explored.
Objective: To study the expression pattern of miRNA profiling in plasma obtained from patients with T1D and therefore the matched control subjects
Patients and Methods: Equally divided numbers of T1D patients (90) and healthy-matched control children (90) were analyzed for his or her expression profile of plasma miRNAs; miR-101-5p, 146-5p, 21-5p, miR-375, miR-126, and Let7a-5p using reverse transcriptase (RT-PCR) methodology through quantitative real-time technique.
Results: The 2 studied groups were significantly different in reference to their biochemical parameters, including; FBG, 2hpp, and HbA1c levels ($p < 0.05$). Among the deregulated molecules, miR-101, miR-21 and-375 were highly expressed, whereas, miR-146-5p, miR-126, and miR-Let7a-5p showed significantly low levels of expression in patients compared to control subjects ($p<0.05$). MiR-101, miR-146 were significantly correlated to the age at onset of diagnosis of T1D and disease duration, respectively. Furthermore, multivariate analysis revealed that miR-126 and -Let7a-5p had a significant negative correlation with mean A1C values.
Conclusion: Dysregulation of the analyzed six micro RNAs pointed out their pivotal role as important biomarkers for T1D progression.
Keywords: Type 1 diabetes, miRNAs, plasma, q RT PCR, gene expression

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09.04.2021
17.11.2021
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Published: 17 December 2021

Introduction
Type 1 diabetes mellitus (T1D) is characterized by autoimmune destruction of pancreatic beta-cells by autoimmune mechanisms **[1].** When beta-cell destruction exceeds 80–90% by the infiltrating immune system, the disease can be diagnosed. The development of T1D is slow, with a long latent phase during which it is possible to discover and treat individuals at risk [2, 3].

It's realized that about 80,000 children could develop the disease per year**.** Complications related to T1D vascular drawbacks have a big impact on quality of life, morbidity, and mortality rates, posing an enormous burden on the overall systems of health care. Diabetic nephropathy could also be a number one explanation for the existing end-stage renal disease (ESRD) and surely augments the danger of cardiovascular diseases (CVD). In addition, it should be taken into account the seriously occurring diabetic retinopathy that warrants the possibility of blindness in adult times. Thus, it is urgently required to spot novel targets for qualified treatment options and to obtain innovative noninvasive biomarkers for reinforcing risk prediction, early diagnosis, and prognosis assessment **[4].**

These short (22 nucleotides) non-coding microRNA molecules are shown to be important participant agents that regulate the pattern of organic phenomenon underlying disease pathogenic mechanistic effects through a post-transcriptional way **[5].** Generally, the miRNAs exerted functions passed through binding with the 3' untranslated regions (UTRs) of their specific genes, leading to translational inhibition or direct degradation of the targeted mRNA with a resultant decrease in protein expression **[5, 6].** The observed alteration in miRNA expression has been closely related to the multiplicity of human inflammatory and autoimmune disorders **[7, 8].** Their estimated regulatory control of more than 60% of the protein-coding genes had consequently been linked to many diseases, including cancer, endocrine disorders, and autoimmune diseases, notably T1D [**9**]. miRNA-specific profiles were observed in PBMCs or serum from T1DM patients, and these important molecules seem to modulate mRNA expressions of the major T1DM auto antigens [**10**]. Previously, it was reported that miRNAs, especially those expressed in human pancreatic islets – miR-375 and miR-376 miRNAs may be involved in the regulation of beta cell pancreatic function [11]. Later, a number of miRNAs were discovered to be regulatory factors of beta cell pancreatic function [12]. We have selected these miRNAs, miR-101, miR-21 and-375, miR-146-5p, miR-126, and miR-Let7a-5p as they are considered good indicators of β cell pancreatic function and diabetic state.
On considering these aspects, the present study aimed to investigate the variable pattern of miRNA expression profiling in plasma obtained from patients with T1D and matched control subjects through quantitative real-time PCR.

Subject and methods

This case-control study was prospectively conducted on 90 children with T1D, with a mean age of (10.93 ± 4.51 years) having variable disease duration and variable degrees of glycemic control, who were diagnosed according to ADA criteria (Group I).
A group of apparently healthy age and sex-matched 90children were served as controls (Group II), with a mean age of (10.15 ± 2.56 years). All were enrolled from the Pediatric Department in collaboration with the Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Menoufia University Hospitals, Egypt.
Collection of demographic data, anthropometric measurements, treatment regimens, and other clinically important parameters was done by viewing the medical sheet records.
Cases that were suspected of being diagnosed as MODY, T2D, or secondary diabetes mellitus, or with evidence of chronic systemic/rheumatic diseases, inflammatory disorders, and recent febrile illness, or on long-term steroid therapy, were excluded from the study.
Upon approval of the study protocol from the Ethical Committee of Menoufia University according to the Helsinki II Declaration criteria, written informed consent was obtained from all participants in the study.
Following complete history taking and thorough clinical examination, all studied subjects underwent sampling of 7-10 ml whole blood after 12 h of overnight fasting via sterile techniques and divided into tubes as;
One ml of blood was transferred into a sodium fluoride tube and another sample of blood was obtained after 2 hours for an enzymatic colorimetric determination of blood glucose using the Spinreact kit, SPAIN **[13].**
Another 4ml of blood was transferred into two EDTA tubes: one of them was used for quantitative colorimetric determination of glycated hemoglobin expressed as a percentage of the total hemoglobin by the use of kits supplemented by Teco diagnostics, USA [14], where A1c values of ≥ 6.5% were the limits for diagnosing T1DM **[1].**
For molecular analysis, 2ml of blood was transferred into the other EDTA-containing tube and centrifuged for ten minutes at (4000) r.p.m. The clear supernatant was separated and kept frozen at -80°C until further processing.
Determination of MicroRNA levels was applied throughout a sequence of orders to obtain c DNA via reverse transcription of previously isolated RNA together with the measurement of MicroRNA levels using specific primer sets after being referenced to endogenous control U6B. These steps were applied as:

RNA isolation:

A total RNA, including miRNA molecules was extracted from plasma using Qiagen™ RNA Blood Mini Kit (Qiagen, USA, 2013) according to the manufacturer's instructions.

Reverse transcriptase PCR (RT-PCR):

The Qiagen® miScript II RT Kit (Qiagen, Applied Biosystems, USA, 2012) was used to reverse transcribe RNA. Then, complementary DNA (cDNA) was assayed with the universal SYBR Green Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen).
The preparation of the RT Master Mix is made through: 4 µl 5×miScript HiSpec Buffer, 2µl 10×miScript Nuclease Mix, 2 µl RNase-free water, 2 µl miScript Reverse Transcriptase Mix, then a 10 µl Template RNA to approach a total reaction volume of 20 µl. RT was carried out at 37°C for 60 minutes and 95°C for 5 minutes on an Applied Biosystems 2720 thermal cycler (Bioline, Singapore, USA). Diluted cDNA was the template for 2nd step real-time PCR using Qiagen-produced SYBR Green miScript kit. The addition of universal primers was based on mRNA sequences delivered from the miR-database for (mi RNA 101-5p, mi RNA 146a-5p, mi RNA- 375, mi RNA 21-5p, miR 126, and miR Let 7 a-5p) as shown in Table1(a). Each reaction for real-time PCR was finalized to 25µL volume, as followed: 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µl 10x miScript specific Primer, 2.5 µl 10x miScript primer assay, 4 µl Template c DNA and 3.5 µl RNase-free water. The mixture was incubated at this conditions:95 °C for 15 min (as initial denaturation), then denaturation at 94°C for 15 s duration, annealing for 30 s at a temperature of 55°Cand final extension for 30 s adjusted at 70°C, for designed 60 cycles.
Amplification of small RNA RNU6B was carried out with each sample as an endogenous control. Data analysis was done in the real-time cycler Applied Biosystems®7500 software version 2.0.1 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Validation of the quantified miRNAs:

Then, relative quantification (RQ) of gene expression was performed using the comparative ΔΔCt method, in which the amount of targeted mi RNAs 101-5p, mi RNA146a-5p, mi RNA- 375, mi RNA21-5p, mi RNA 126, and mi RNA Let 7 a-5p were normalized to RNU6B as an endogenous reference among patients and controls. It's to be noted that these miRNAs were chosen based on evidence from available databases and literature that showed the association of these molecules to pathways involved in T1D development in humans **[2, 9, 15].**

Statistical analysis:

We used IBM SPSS statistics version 20 (SPSS Inc., Chicago, USA) for data analysis. The mean and standard deviation were used to express quantitative data. A Chi-square test was used to examine the relationship between qualitative variables. For quantitative data, a comparison between two groups was done using either student t-test or Mann-Whitney test (non-parametric t-test) as appropriate. We tested the correlation between numerical variables using Spearman's correlation method. For the determination of T1D risk, multivariate logistic regression analysis was additionally used aided by the calculation of odds ratio (OR) and 95% Confidence Interval (CI). A p-value ≤ of 0.05 was considered significant.

Results

In this study, a total of 180 children were enrolled, including 90 [46 males and 44 females] diabetic children with T1D (Group I), and 90[55 males and 35 females] apparently healthy control subjects (Group II) who were age and sex matched. All included subjects were investigated for the pattern of expression for the circulating mi-RNAs-101, 146, Let-7a, 21-5p, -375, and 126-5p molecules.

Their ages ranged from 10.93(4.51) and 10.15(2.56) for T1D patients and control groups respectively.

Demographic and clinical data of the studied groups were shown in **(Table2).**

Results of laboratory investigations for the group of T1D children including biochemical indices of; FBG, 2 h-PP, and mean HbA1C**%** were found to be statistically significant in comparison to controls (t-test: 23.985, 23.156 and 14.165, P<0.0001) in order. All of the newly diagnosed cases with T1D disease duration, of no more than6-12months were chosen as being positive for anti-insulin autoantibodies (IAAs), where the levels ranged from 10-130 mIU/L with mean values 10.42±19.59 in those cases and from 0-7.0 mIU/L with mean values 2.08±2.36 in healthy control children(U:7.03, P:0.002).

Comparative results regarding the levels of micro-RNAs studied in both groups revealed that miR-101-5p, miR-21-5p, miR 375 were highly expressed in patients with T1D, with a difference of statistical significance (P<0.05), whereas miR146-5p, miR 126, and miR Let 7a-5p showed down-regulation of their plasma levels (p-value <0.05) in order **(Table 3).** One of the remarkable findings in this study was that our results indicated a significant negative correlation of miR 101-5p with the age of onset (r=-0.264, p=0.015) and with the duration of illness of T1D (r=-0.162, p=0.02) in respect. MiR-146 was correlated with T1D disease duration (r=0.239, p=0.023). On the other hand, miR 126, and miR-Let7a-5p were significantly negatively correlated to mean T1D patients' glycated Hb A1c levels; p value<0.05 **(Table 4).** Results of multivariate logistic regression analysis for T1D risk were shown in **(Table 5),** where miR126-5p and miR-Let7a- markers showed highly significant findings after adjustment of values for age, sex, and mean A1C levels in patients group as evidenced by Odds ratio, CI 95% of 0.016(0.0 – 0.544), p= 0.021for -126-5p and 1.808(1.006 – 3.249) and p value=0.048 for mi-Let7a- in order.

Correlations between some of the studied mi-RNAs and certain parameters of T1D were shown in **figures 1-3**. The Amplification for micro-RNAs expression pattern (normalized fluorescent signal [ΔRn] that was plotted against the number of cycles) was evident in **figures from 4-9** in the corresponding sequence.

Discussion

The role of miRNAs in modulating gene expression has greatly developed and is being implicated in the presentation of various genetic disorders **[16-18].** The level of expression of these circulating molecules favored their ability to be recognized as biomarkers of disease pathogenesis and prognosis **[19, 20].** This was evidenced by the relationship of these molecules to 60% or more of the coding genes that were thought to be in linkage to various endocrinal and autoimmune diseases **[21-23].**

Based on the underlying autoimmune background of T1D, our studied cases with recent disease onset; not more than6-12months duration; showed positivity for insulin autoantibodies, a matter that researchers related to certain miRNAs molecules to be validated as newly developed markers at early phases. Although for some, several miRNAs are tightly associated with glucose homeostasis that determines the progressive pattern in at-risk individuals **[24].**

There is recent evidence of changes in proinflammatory cytokines and autoimmune markers consistent with circulating miRNAs expression profiling in children with type 1 DM [4,25,26].

Also, we found that miR-146a-5p, Let-7a, and miR-126 were down-regulated, whereas miR-101, 21-5p, and miR375a-5p were consistently up-regulated in patients compared to controls.

As for miR-375, its abundance in pancreatic tissue makes it a reflection of B-cell mass and changes in its functions **[27].** In respect to our results, it was found that the level of miR-375 in the plasma of patients with T1D was significantly increased. When analyzed for its correlation to HbA1C, no difference was noticed as evidenced by a coefficient r-value of 0.173 and a p-value of 0.201. The relation of that molecule to mean A1C values was favored by Marchand et al., 2016, who found dysregulated miR-375 levels in the blood of newly diagnosed children with type 1 diabetes when quantified to high levels in human islet tissue and conferred as a hallmark in the etiology of T1D. Furthermore, it may be a marker of the early phases of the disease **[28].**

Of the deregulated molecules in our study, one that was found to be up-regulated in the group of patients with T1D versus control subjects as a difference of statistical significance (p value<0.001, Table 3) was 21-5p, which was examined by Pan et al. for the involvement of enteroviral infection on 21-5p expression and subsequent contribution to T1D **[29].**

Analyzing data from past literature revealed that has-miR-21 seemed to be highly expressed in the plasma of T1D patients in comparison to controls. Osipova et al., 2014 **[30]** found similar results. Ongoing research relates miR21-5p to cytokines of inflammation. In addition to the findings of Backe and coworkers **[31],** it was suggested that miR-21 overexpression was believed to influence the Bax group/ apoptotic signaling pathway, hence inducing pancreatic B-cell death. This process could be served as a new tried target for T1D therapy **[32, 33].**
Another up-regulated miRNA in our study was -101-5p, which targeted reduction of insulin secretion and B-cell mass in a favor of its involvement in cytokine release regulation and altered signaling of STAT3, HGF/C-Met, and Ephrin receptors pathway mechanisms. Adjuvant to our findings of the significant association of miR101-5p to insulin autoantibody-positive cases of recent onset T1D was the largely analyzing study by Santos A et al., who reported that the expression of miR-101 was about 3folds higher in patients with multiple autoantibodies levels **[34].**
Let7 a-5p was one of the studied markers expressed at lower levels in the studied group of patients and demonstrated a statistically significant difference in comparison to that of controls. Similar results were evident in the study done by Tian C et a., where Let -7a was downregulated in both human and mice tissue derivatives **[35].** The latter was known to be involved in the regulation of glucose metabolism. In agreement with our results, it was found to be negatively correlated to A1C by the study done by Erener et al., 2017 **[36].**
Assessing the level of miR-126 expression revealed contradictory findings. Osipova et al., conducted lower urinary levels in patients with T1D, with no significant difference in plasma samples of the studied cases and control groups respectively **[30].** However, the observation of Wang et al. clarified decreased plasma levels of miR-126 in those with chronic ESRD **[37].** Despite the disagreeable findings identified in our study about the significant lower plasma level of the miR-126 in T1D patients to that of Osipova et al., they came in the same line with the proposed mechanism that related decreased level of miR-126 to deranged response to vascular endothelial growth factor (VEGF) and endothelial dysfunction **[30,38,39].** In addition, previous reports considered this marker as a controlling factor for various biological processes **[40-42],** through linkage of decreased circulatory miR-126 levels to micro-vascular change and possibility of later-on long-standing T1D complications **[42, 43].**
The noticed significant negative correlation between miR-126, mi-R Let7a- markers, and high percentages of the mean HbA1C values suggested the significant association of the altered levels of the circulating miRNAs to hyperglycemic state **[36].** These above considerations were nearly similar to the hypothesis of Akerman et al., 2018 who assumed that the expression of miRNAs may be of value regarding their feasibility to be distinguishing complementary markers in risky individuals with abnormal OGTT results **[24].** Satake et al. reported the association of dysregulated miRNAs with Hyperglycemia in children with Type 1 Diabetes that may contribute to further development of diabetic complications [44]**.**
In the issue of T1D, it's still tried to advocate that the miRNAs have a cornerstone step in T1D pathogenesis not merely markers of active B cell dysfunctional outcome **[45].** Previous studies reported the effect of mi- RNAs on pancreatic cellular biology, especially for B-cell differentiation, insulin production, mediation of inflammation, and apoptosis **[46].**
As found in our study, the negative correlation of miR-101 with the age of onset of T1D (r= -0.264, p= 0.015), related studies suggested a greater rate of B-cell turnover and pancreatic injury in young children with T1DM **[47].**
Another important micro-RNA molecule that showed significant downregulation besides being indicated in patients with recent-onset T1D was the miR146 a-5p. This was evident through a lowered expression level in cases; median (IQR) of 0.16(0.02 – 0.43) compared to levels of 0.77(0.0 – 0.88) in control subjects (p<0.001). A possible biological effect explored from its consistent relation to genes linked to apoptotic and innate immune regulatory pathway mechanisms **[47].**

Study limitation

Small sample size was a limitation in our study. Meanwhile, the association between T1D and miRNAs could not be excluded. Other limitations of our study included that we did not proceed for calculation of sample size or power of the study as published data on the link between T1D and miRNAs were not sufficient for an exact calculation of statistical sample size. Lastly, the relationship between miRNAs and the presence and/or levels of autoantibodies had not been investigated. We recommend extended analysis on more patients sampling to clarify the relation of miRNAs and T1D - associated autoantibodies.

Conclusion

miRNA deregulation in our study revealed down-regulation of miRNAs 146-5p, 126-5p, and Let 7a-5p molecules and up-regulation of miR101-5p, 21-5p, and 375- for their significant relationship to T1DM. The fact that miR126-5p and Let7a-5p microRNAs have a significant negative correlation with mean glycated HbA1C values suggests that they could be used as genetic markers of hyperglycemia-associated pathophysiologic changes in T1DM. Given the stability, reliability of these markers, they were preferred for their superiority over other quantification techniques establishment through q-RT-PCR and warranted our choice in that study to be the **1st** estimate for micro-RNA profiling among Egyptian children having type1diabetes, the matter that potentiate pavement of the way to their targeted usage as new intervention therapeutic markers. Of course, further larger-scale functional studies are required for genetic interactions thus improving the quality and life expectancy of children with T1DM.

Acknowledgements

Not applicable.

Authors' contributions

NFB designed the study, analyzed data and drafted the manuscript MMM; IFZ, AEA and EMA participated in the design of the study and coordination of the whole work. NFB and MMM collected and organized patients' data. NFB, IFZ, AEA and EMA performed molecular genetic studies for patients. NFB and MMM analyzed the data. All authors have read and approved the manuscript and ensure that this is the case.

Funding: None.
Availability of data and material: The datasets are available from the corresponding author on reasonable request.
Ethics approval and consent to participate: All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional review committee of Huang et al. BMC Endocrine Disorders (2020) 20:99 Page 7 of 9 the Third Affiliated Hospital of Soochow University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by the Institutional Review Committee of the Faculty of Medicine, Menoufia University.
All subjects gave written, informed consent through their parent or guardian before enrollment in the study.
Consent for publication: Not applicable.
Competing interests: The authors declare that they have no competing interests.
References
1- American Diabetes Association. Classification and diagnosis of diabetes. Diabetes Care 2015. 38 S8–S16. (10.2337/dc15-S005) [PubMed] [CrossRef]
2- Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nature Reviews Endocrinology 2013. 9 513–521. (10.1038/nrendo.2013.86) [PubMed] [CrossRef]
3- Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001. 358 221–229. (10.1016/S0140-6736(01)05415-0) [PubMed] [CrossRef].
4- Barutta F, Bellini S, Mastrocola R , Bruno G, and Gruden G. MicroRNA and Micro-vascular Complications of Diabetes.(2018); International Journal of Endocrinology, https://doi.org/10.1155/2018/6890501.
5-Simranjeet Kaur, Flemming Pociot.(2018); miRNAs regulate development and function of regulatory T-cells in recent onset islet autoimmunity in pre-Type 1 diabetes. doi: 10.21037/ncri.2018.03.06.
6- Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120:15-20.
7- Sebastiani G, Spagnuolo I, Patti A, et al. MicroRNA expression fingerprint in serum of type 1 diabetic patients. Diabetologia. 2012; 55:S48.
8- Nielsen LB, Wang C, Sørensen K, et al. Circulating levels of microRNA from children with newly diagnosed type 1diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. Exp Diabetes Res. 2012:896362.
9- Lin S & Gregory RI. MicroRNA biogenesis pathways in cancer. Nature Reviews Cancer (2015) 321–333. (doi:10.1038/nrc3932).
10-Abuhatzira L, Xu H, Tahhan G, Boulougoura A et al. Multiple microRNAs within the 14q32 cluster target the mRNAs of major type 1 diabetes autoantigens IA-2, IA-2beta, and GAD65. FASEB Journal (2015) 29 4374–4383. (doi:10.1096/fj.15- 273649).
11- Ardekani AM and Naeini M: The role of microRNAs in human diseases. Avicenna J Med Biotechnol 2: 161-179, 2010.
12- Pandey AK, Agarwal P, Kaur K and Datta M: MicroRNAs in diabetes: Tiny players in big disease. Cell Physiol Biochem 23: 221-232, 2009.
13-Trinder P: Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. J Ann Clin Biochem(1969); 6: 24-25.
14-Gonen B and Rubenstien AH: Determination of glycohemoglobin. Diabetologia (1978); 15:1-5.
15-Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas microRNA-target interactions. Nat Methods (2015); 12: 697.
16- Bhatt K, Mi QS and Dong Z. MicroRNAs in kidneys: Biogenesis, regulation, and pathophysiological roles. Am J Physiol Renal Physiol 300: F602-F610, 2011.
17- Butz H, Kinga N, Racz K & Patocs A. Circulating miRNAs as biomarkers for endocrine disorders. Journal of Endocrinological Investigation 2016 39 1–10. (doi:10.1007/s40618-015-0316-5)
18- Carrington JC & Ambros V. Role of microRNAs in plant and animal development. Science 2003 301 336–338. (doi:10.1126/ science.1085242)
19- Lin S & Gregory RI. MicroRNA biogenesis pathways in cancer. Nature Reviews Cancer 2015 15 321–333. (doi:10.1038/nrc3932)
20- Pauley KM, Cha S & Chan EK. MicroRNA in autoimmunity and autoimmune diseases. Journal of Autoimmunity 2009 32 189–194. (doi:10.1016/j.jaut.2009.02.012)
21- Zalts H & Shomron N. The impact of microRNAs on endocrinology. Pediatric Endocrinology Reviews 2011 8 354–362.
22-Akerman L, Casas R, Ludvigsson J, Tavira B, Skoglund C. Serum miRNA levels are related to glucose homeostasis and islet autoantibodies in
children with high risk for type 1 diabetes. PLoS ONE 13(1): e0191067. https://doi. org/10.1371/journal.pone.0191067
23- Esteller M. Non-coding RNAs in human disease. Nature Reviews Genetics 2011 12 861–874. (doi:10.1038/nrg3074).
24-Fan B, Luk A, Chan J and Ching R: Micro RNA and Diabetic Complications: A clinical Perspective. Antioxid Redox Signal.2018 Oct 10; 29 (11): 1041-1063.doi:10.1089/ars.2017.7318.Epub 2017 Dec 1.
25-Pezzolesi MG, Satake E, McDonnell KP, Major M, Smiles AM, Krolewski AS. Circulating TGF-β1-Regulated miRNAs and the Risk of Rapid Progression to ESRD in Type 1 Diabetes. Diabetes. 2015;64(9):3285-93.

26-Diego F. García-Díaz, Carolina Pizarro, Patricia Camacho-Guillén, Ethel Codner, Néstor Soto, Francisco Pérez-Bravo. Expression of miR-155, miR-146a, and miR-326 in T1D patients from Chile: relationship with autoimmunity and inflammatory markers. Arch Endocrinol Metab. 2018; 62/1.
27- M. van de Bunt, K. J. Gaulton , L. Pfarts et al., "The miRNA profile of human pancreatic islets and beta-cells and relationship to type 2 diabetes pathogenesis," PLoS ONE, vol. 8, no. 1, article e55272, 2013.
28- Marchand L, Jalabert A, Meugnier E, Vanden K, Fabien N, Nicolino M, Madec A.M., Thivolet C and Kone S: Mi-RNA-375 a Sensor of Glucotoxicity Is Altered in the Serum of Children With Newly Diagnosed Type1Diabetes. Journal of Diabetes Research. http://doi.org/ 10.1155/2016/1869082.
29- Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, Li J, Zhou H, Tang Y & Shen N. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyl transferase 1. Open Journal of Immunology 2010 184 6773–6781. (doi:10.4049/jimmunol.0904060)
30- Osipova J, Fischer DC, Dangwal S, Volkmann I, Widera C, Schwarz K, Lorenzen JM, Schreiver C, Jacoby U, Heimhalt M, Thum T, Haffner D. Diabetes-associated microRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. J Clin Endocrinol Metab. 2014; 99(9):E1661-5.
31- Backe MB, Novotny GW, Christensen DP, Grunnet LG & Mandrup- Poulsen T. Altering beta-cell number through stable alteration of miR-21 and miR-34a expression. Islets 2014 6 e27754. (doi:10.4161/ isl.27754)
32-Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, Johnson DS, Chen Y & O'Neill LA. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. Nature Immunology 2010 11 141–147. (doi:10.1038/ni.1828).
33-Ventriglia G, Nigi L, Sebastiani G & Dotta F. MicroRNAs: novel players in the dialogue between pancreatic islets and immune system in autoimmune diabetes. BioMed Research International(2015), 749734.
34-Santos A, Cunha N, Fukui R, Ferreira R.P, and Silva M: Increased expression of circulatory micro RNA101-in Type1Diabetes Patients: New Insights Into miRNA-Regulated Pathophysiological Pathways for Type1Diabetes. Frontiers in Immunology, (2019). Doi: 10.3389/ Fimmu. 2019.01637.
35- Tian C, Ouyang X, Lv Q, Zhang Y & Xie W. Cross-talks between microRNAs and mRNAs in pancreatic tissues of streptozotocin-induced type 1 diabetic mice. Biomedical Reports 2015 3 333–342.
36- Erener S, Marwaha A, Tan R, Panagiotopoulos C, Kieffer TJ. Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. JCI Insight. 2017; 2(4):e89656.
37- Wang H, Peng W, Shen X, Huang Y, Ouyang X and Dai Y: Circulating levels of inflammation-associated miR-155 and endothelial-enriched miR-126 in patients with end-stage renal disease. Braz J Med Biol Res 45: 1308-1314, 2012.
38- Harris TA, Yamakuchi M, Ferlito M, Mendell JT and Lowenstein CJ: MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci USA 105: 1516-1521, 2008.
39- Jiang Y, Wang HY, Li Y, Guo SH, Zhang L and Cai JH: Peripheral blood miRNAs as a biomarker for chronic cardiovascular diseases. Sci Rep 4: 5026, 2014.
40-Bartel DP: MicroRNAs: Target recognition and regulatory functions. Cell 136: 215-233, 2009.
41- Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, et al: Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res 107: 810-817, 2010.
42- Bijkerk R, Duijs JM, Khairoun M, Ter Horst CJ, van der Pol P, Mallat MJ, Rotmans JI, de Vries AP, de Koning EJ, de Fijter JW, et al: Circulating microRNAs associate with diabetic nephropathy and systemic microvascular damage and normalize after simultaneous pancreas-kidney transplantation. Am J Transplant 15: 1081-1090, 2015.
43-Barutta F, Bruno G, Matullo G, Chaturvedi N et al: Micro RNA-126 and micro-/macrovascular complications of type 1 diabetes in the EURODIAB prospective complication study. Acta Diabetol.2017 feb; 54 (2): 133-139.doi:10.1007/s00592-016-0915-4.
44-Satake E, Marcus G. Pezzolesi, Zaipul . Md Dom, Adam M. Smiles, Monika A. Niewczas, and Andrzej S. Krolewski. Circulating miRNA Profiles Associated With Hyperglycemia in Patients With Type 1 Diabetes..Diabetes 2018; 67:1013–1023.
45-Kassem S, Ariel I, Thornton P, Scheimberg I, Glaser B: Beta cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes (2000). 49:1325-33.doi: 10.2337/ diabetes.49.8.1325.
46- Tugay K, Guay C, Marques A, Allagnat F, Locke J, Harries L W, et al.: Role of micro RNA in the age-associated decline of pancreatic beta cell function in rat islets. Diabetologia. (2016) 59:161-9. doi:10.1007/s00125-015-3783.
47- Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, Yoshimura A, Baltimore D & Rudensky AY. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. Cell 2010 142 914–929. (doi:10.1016/j.cell.2010.08.012)

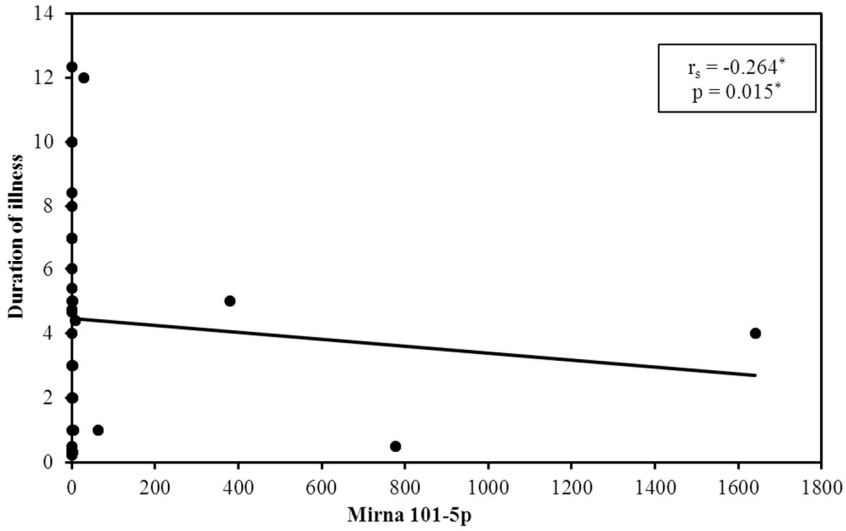

Figure (1): Correlation results between microRNA 101-5p with age of T1D onset.

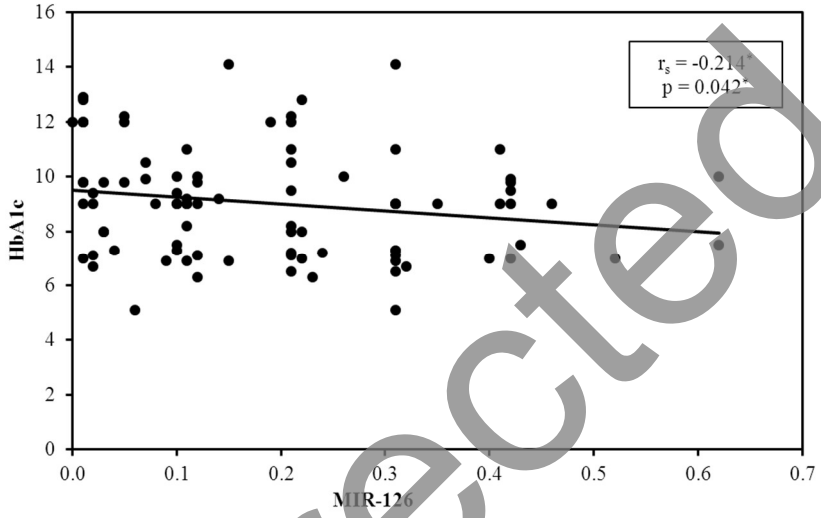

Figure (2): Correlation results between microRNA -126 and HbA1c.

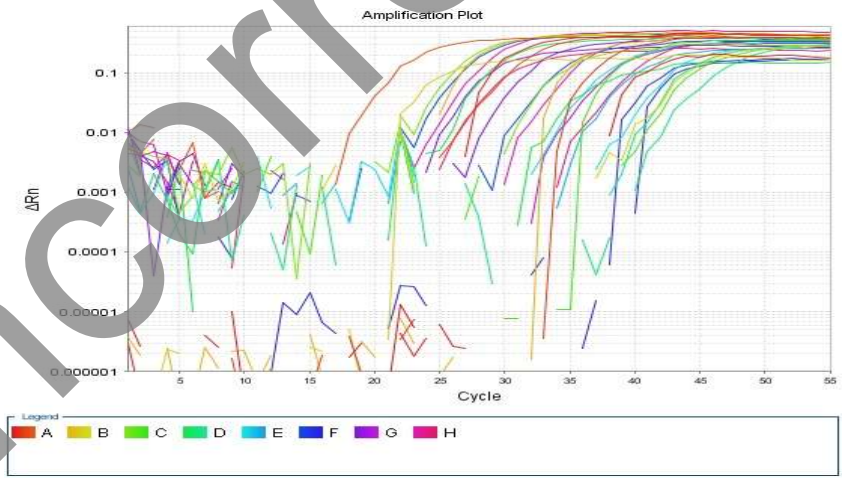

Figure (3): Amplification plot for mi RNA 21-5p expression pattern (normalized fluorescent signal [ΔRn] plotted against the number of the cycle).

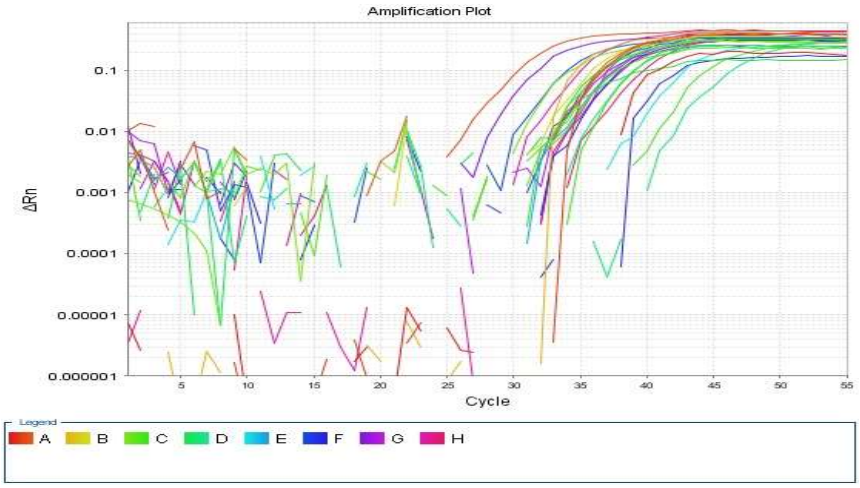

Figure (4): Amplification for mi RNA 101-5p expression (normalized fluorescent signal [ΔRn] plotted against the number of the cycle).

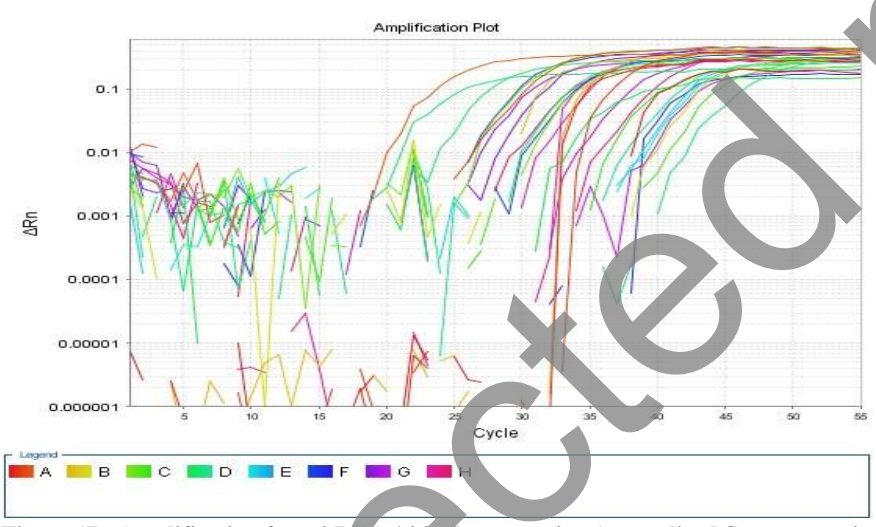

Figure (5): Amplification for mi RNA 146a-5p expression (normalized fluorescent signal [ΔRn] plotted against the number of the cycle).

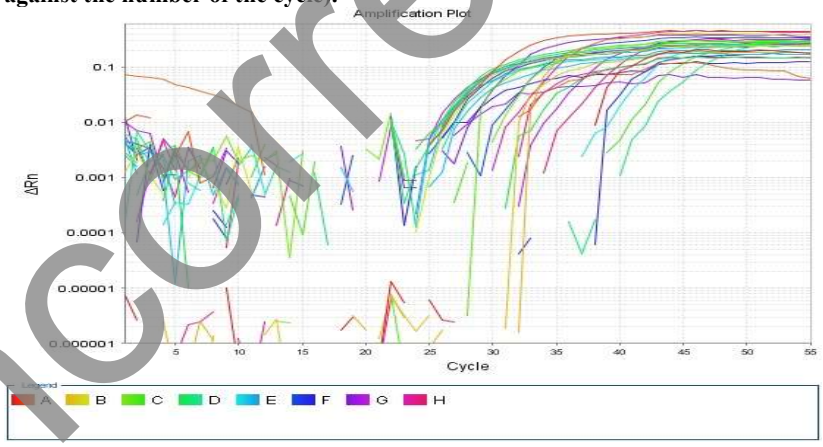

Figure (6): Amplification for mi RNA- 375 expression (normalized fluorescent signal [ΔRn] plotted against the number of the cycle).

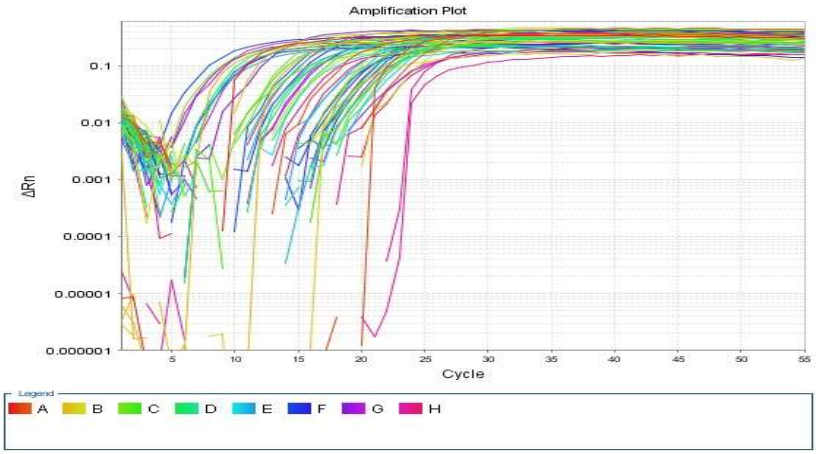

Figure (7): Amplification plot of miR Let 7 a-5p expression (normalized fluorescent signal [ΔRn] against the number of the cycle).

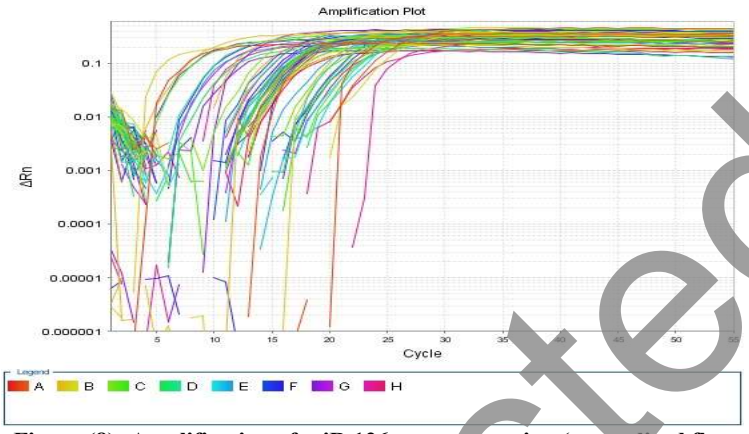

Figure (8): Amplification of miR 126 gene expression (normalized fluorescent signal [ΔRn] against the number of the cycle).

Table (1): Comparison between the two studied group as regarding to demographic, clinical and laboratory data.

	T1DM (n = 90)		Control (n = 90)		Test of sig.	P
	No.	%	No.	%		
Sex						
Male	46	51.1	55	61.1	χ^2= 1.827	0.176
Female	44	48.9	35	38.9		
Consanguinity						
positive	48	48	65	65	2.9	0.006
negative	52	52	35	35		
Family history						
Positive	21	21	0	0	20.1*	<0.001*
Negative	79	79	100	100		
Age (years) Mean ± SD	10.93 ± 4.51		10.15 ± 2.56		t= 1.434	0.154
systolic blood pressure(mm/hg) Mean ± SD.	110.4 ± 6.10		107.0 ± 4.50		t= 3.199*	0.002*
Diastolic blood pressure(mm/hg) Mean ± SD.	67.78 ± 5.95		67.0 ± 6.44		t= 0.841	0.401
BMI (KG/M2)						
Mean(SD)	19.6±3.98		20.22 ±5.51		t=0.91	0.32
Range	13-29		13.3-32.5			

Age of onset						
Mean ± SD.	6.93 ± 3.29					
Median (IQR)	7.0(5.0 – 10.0)					
Duration of illness						
Mean ± SD.	4.41 ± 3.35					
Median (IQR)	4.42(1.0 – 7.0)					
Presentation						
DKA	30	33.3				
Classic symptoms	60	66.7				
HbA1C%						
Mean ± SD.	9.03 ± 2.04		5.94 ± 0.34		t= 14.165*	<0.001*
Median (IQR)	9.0(7.2 – 10.0)		5.95(5.7 – 6.2)			
FBS(mg/dl)						
Min. – Max.	105.0 – 300.0		75.0 – 105.0			
Mean ± SD.	210.52 ± 46.46		91.0 ± 8.76		t= 23.985*	<0.001*
Median (IQR)	200.0(180.0 – 250.0)		91.0(85.0 – 98.0)			
2hr PP(mg/dl)						
Min. – Max.	130.0 – 310.0		140.0 – 172.0			
Mean ± SD.	257.11 ± 40.31		154.80 ± 11.47		t= 23.156*	<0.001*
Median (IQR)	260.0(220.0 – 300.0)		155.5(145.0 – 165.0)			
Micro albuminuria						
No	82	91.1	91	100.0	χ^2= 8.372	FEp= 0.007*
Yes	8	8.9	0	0.0		
Min. – Max.	206.20 – 394.0		-			
Mean ± SD.	265.55 ± 80.19		-		-	-
Median (IQR)	231.0(215.1 – 316.0)		-			

MC: **Monte Carlot** IQR: Inter-quartile range FE: **Fisher Exact** χ^2: **Chi square test**
t: Student t-test U: Mann Whitney test p: p value for comparing between the studied groups *: Statistically significant at $p \leq 0.05$.

Table (2): Comparison between the two studied groups according to different microRNAs expression.

microRNAs expressions	T1DM (n = 90)	Control (n = 90)	U	P
microRNA101-5p				
Min. – Max.	0.0 – 1640.55	0.09 – 1.66		
Mean ± SD.	65.33 ± 270.39	0.39 ± 0.14	3652.0	0.028*
Median (IQR)	0.60(0.08 – 1.95)	0.26(0.10 – 0.35)		
microRNA146a-5b				
Min. – Max.	0.0 – 328.49	0.0 – 1.21		
Mean ± SD.	7.89 ± 48.63	0.63 ± 0.44	2646.0*	<0.001*
Median (IQR)	0.16(0.02 – 0.43)	0.77(0.0 – 0.88)		
microRNA 375a-3p				
Min. – Max.	0.0 – 2127.09	0.07 – 2.93		
Mean ± SD.	70.36 ± 332.53	0.95 ± 1.02	1908.0*	<0.001*
Median (IQR)	2.10(1.08 – 3.11)	0.53(0.09 – 1.05)		
microRNA 21-3p				
Min. – Max.	0.0 – 89.39	0.0 – 0.06		
Mean ± SD.	2.27 ± 13.21	0.03 ± 0.02	2682.0*	<0.001*
Median (IQR)	0.08(0.0 – 0.48)	0.02(0.0 – 0.05)		
microRNA -126				
Min. – Max.	0.00 – 0.62	0.00 – 152.32		
Mean ± SD.	0.19 ± 0.15	4.50 ± 22.39	1569.50*	<0.001*
Median (IQR)	0.15(0.07 – 0.31)	0.69(0.26 – 1.61)		

microRNA -Let 7a-5p				
Min. – Max.	0.0 – 35.80	0.0 – 1.92		
Mean ± SD.	0.94 ± 5.21	0.50 ± 0.51	2356.50*	<0.001*
Median (IQR)	0.09(0.02 – 0.23)	0.12(0.10 – 1.02)		

U: Mann Whitney test p: p value for comparing between the studied groups *: Statistically significant at $p \leq 0.05$

Table (3): Correlation between different parameters and all microRNAs in patients with T1D.

		microRNA 101-5p	microRNA 146a-5b	microRNA 375a-5p	microRNA 21-3p	microRNA -126	microRNA -Let 7a-5p
Duration of illness	r_s	-0.162	0.239	0.095	-0.151	-0.167	-0.034
	P	0.02*	0.023*	0.386	0.167	0.128	0.756
Age of onset	r_s	-0.264	0.149	-0.008	0.211*	0.089	-0.062
	P	0.015*	0.160	0.943	0.046*	0.402	0.563
HbA1c	r_s	0.090	0.183	0.173	0.054	-0.214	-0.216
	P	0.413	0.162	0.201	0.610	0.042*	0.043*
FBS	r_s	-0.194	0.176	-0.147	0.166	-0.032	0.241
	P	0.067	0.097	0.166	0.119	0.764	0.022
2hr pp	r_s	-0.215	0.134	-0.008	0.096	0.006	0.123
	P	0.042	0.209	0.939	0.368	0.954	0.247

r_s: Spearman coefficient *: Statistically significant at $p \leq 0.05$

Table (4): Univariate and multivariate analysis for the parameters affecting T1DM risk (No. = 90)

	Univariate		Adjust Odd`s ratio	
	P	**COR (95%C.I)**	**p**	**AOR# (95%C.I)**
microRNA 101-5p	**0.009***	1.565(1.121 – 2.185)	0.307	1.783(0.587 – 5.414)
microRNA 146a-5p	**0.616**	1.017(0.951 – 1.088)	0.844	1.009(0.924 – 1.101)
microRNA Let 7 a-5p	**<0.001***	1.845(1.370 – 2.483)	0.048*	1.808(1.006 – 3.249)
microRNA 21-5p	**<0.001***	11.62 (3.63.69 – 71.85)	0.026*	7.180(2.554 – 20.187)
microRNA -126	**<0.001***	0.013(0.002 – 0.064)	0.021*	0.016(0.0 – 0.544)
microRNA -375	**0.453**	1.037(0.943 – 1.140)	0.921	1.014(0.776 – 1.324)

OR: Odd`s ratio, C.I: Confidence interval, **AOR#: adjust Odd`s ratio by family history and HbA1C**
*: Statistically significant at $p \leq 0.05$.