Association of exosomal miR-34a with markers of dyslipidemia and endothelial dysfunction in children and adolescents with T1DM

Short title: Exosomal miR-34 and dyslipidemia in T1DM

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What's already known in this topic: Dyslipidemia and endothelial dysfunction are common disorders and major predisposing factors for atherosclerosis and cardiovascular diseases in patients with T1DM. However, their pathophysiology in children and adolescents with T1DM is still under evaluated.

What this study adds: Association of exosomal miR-34a serum expression with markers of dyslipidemia and endothelial dysfunction was identified in children and adolescents with T1DM, suggesting its role in regulation of lipid metabolism and endothelial function in T1DM.

Abstract
Objective: Dyslipidemia and endothelial dysfunction are common disorders and major causative factors for atherosclerosis in patients with T1DM. However, their pathophysiology in young patients with T1DM is still under evaluated. We aimed for the first time to assess the expression of exosomal miR-34a in serum of children and adolescents with T1DM and correlate this expression with markers of dyslipidemia and endothelial dysfunction.
Subjects and Methods: The study included 120 T1DM patients and 100 control subjects. Assessment of exosomal miR-34a was done using qRT PCR. Measurement of Lipid profile was done using automated analyzer and serum endoglin and intracellular adhesion molecule (ICAM) levels were measured using ELISA technique.
Results: Relative expression of miR-34a and serum endoglin and ICAM levels were higher in patients than controls and in in patients with dyslipidemia (42 patients) compared to patients without dyslipidemia (78 patients) (p=0.001, 0.01 respectively). Linear regression analysis revealed strong an independent association between exosomal miR-34a expression with those of total cholesterol, LDL, serum endoglin and serum ICAM after adjustment of other cofactors. The utility of miR-34a as indicator for associated dyslipidemia was tested using ROC curve which revealed AUC: 0.73 with CI:0.63-0.83 and p=0.001.
Conclusion: This is the first study that reported the altered expression of exosomal miR-34a among children and adolescents with T1DM. Moreover, association of miR-34a with markers of dyslipidemia and endothelial dysfunction was identified, suggesting that it could play a role in regulation of lipid metabolism and endothelial function in T1DM.
Keywords: miR-34a, dyslipidemia, endothelial dysfunction, type 1 diabetes mellitus, endoglin, Intracellular adhesion molecule

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1-Introduction
Type 1 diabetes mellitus (T1DM) is a complex multifactorial autoimmune illness. It is one of the most growing diseases among children and adolescents (1). It affects about 35 million persons all over the world with annual increase ranging between 3-5% (2).

Dyslipidemia and endothelial dysfunction are very common metabolic abnormalities in these patients (3,4). Both are major causative factors for atherosclerosis which is a major precursor of cerebrovascular disease (CVD), the leading cause for morbidity and mortality in T1DM (5). However, the pathophysiology underlying the occurrence of dyslipidemia and endothelial dysfunction in young patients with T1DM remains unclear. It is suggested to be caused by an interaction between genetic, environmental and eventually epigenetic factors. Epigenetic factors, including microRNAs, not only represent a key for understanding the complexity of vascular diseases in these patients but also represent new field of investigation to discover new diagnostic and prognostic markers (6).

MicroRNA (miRNA,miR) is a class of small noncoding RNA playing significant role in regulating gene expression. Therefore, miRNAs could contribute in pathogenesis of a number of different physiological and pathological processes (7). Exosomes are nanovesicles originating from all cells whether healthy or diseased and can be found in all body fluids. The lipid bilayer surrounding exosomes enable exosomal enclosed miRNAs to be stably expressed in body fluids much more than free circulating miRNAs. Consequently, recent studies have focused on exosomal enclosed miRNAs as contributing factors in various human diseases (8).

Accumulating data have demonstrated that miR-34a contributes in β-cell apoptotic pathways, suggesting its role in T1DM (9). Among different miRNA candidates, miR-34a is of special interest regarding lipid metabolism and endothelial functions by targeting many genes involved in both pathways (10). Genomic data for exosomal miR-34a were retrieved from the Extracellular Vesicles miRNA database (11,12), while the predicted miRNA target genes were analyzed by using DIANA-miRPath v1.1 webserver (13). Accordingly, its role in adipogenesis, atherosclerosis progression, inflammation and CVD development and progression, has been suggested.

In the current study, we aimed for the first time to assess the expression of exosomal miR-34a in serum of children and adolescents with T1DM and evaluate the association between exosomal miR-34a expression and markers of dyslipidemia such as total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG), as well as the serum levels of markers of endothelial dysfunction including serum endoglin and intracellular adhesion molecule (ICAM).

2-Subjects and Methods

2.1. Subjects

This study is a pilot cross-sectional study. A total of 120 T1DM patients, with disease duration more than 5 years, were randomly selected from Diabetes, Endocrine & Metabolism Pediatric Unit (DEMPU), Pediatric Department, Faculty of Medicine, Cairo University, with respect to inclusion and exclusion criteria. Confirmation of T1DM diagnosis was based on criteria of American Diabetes Association (ADA) (14).

-Exclusion criteria in the current study included, any type of diabetes other than T1DM, T1DM complicated with any microvascular complication, hypertension, heart, liver, or renal insufficiency, acute diabetic complications, systemic inflammatory diseases, endocrine disorders and neoplastic disorders or family history of dyslipidemic diseases.

-One hundred age and sex matched healthy subjects served as control group and were recruited from National Research Centre (NRC) during regular check-up.

-The included patients were subsequently categorized into patients with dyslipidemia (42 patients) and patients without dyslipidemia (78 patients). According to ADA, dyslipidemia was defined by the presence of one or more of the following criteria: TC >200 mg/dL, LDL cholesterol ≥130 mg/dL, HDL cholesterol ≤35 mg/dL, and TG ≥150 mg/dL (15).

-Informed consent was obtained from each participant. This study was approved by ethics committee of National Research Centre (NRC), under approval number 16/285, in accordance with the Declaration of Helsinki 2015.

2.2. Methods

2.2.1-All participants were subjected to full history taking and full clinical examination.

2.2.2-Biochemical analysis:

-Venous blood samples were collected from all subjects after 12 hours of overnight fast. Serum was separated and parameters of dyslipidemia including, TC, TG, HDL-C and LDL levels were quantified using Erba Mannheim XL300 Chemistry Analyzer.

-Markers of endothelial dysfunction including serum endoglin and ICAM concentrations were assessed using enzyme linked immunosorbent assay (ELISA) sandwich technique, supplied by Quantikine, R&D Systems, Minneapolis, USA.

2.2.3.- Assessment of exosomal miR-34a was done by quantitative reverse transcriptase real time PCR technique (qRT PCR):

Exosomes were isolated from eight hundred microliters of serum according to exoRNeasy Serum/Plasma midi kit (Qiagen, Hilden, Germany). The characteristics of isolated exosomes were confirmed by transmission electron microscopy (16). Isolation, purification and elution of exosomal RNA was done according to exoRNeasy

Serum/Plasma midi kit's protocol and 3.5 microns of synthetic spike in control Cel-miR-39 was added to each sample as the internal control. Concentration of isolated RNA was assessed using a NanoDrop 2000c spectrophotometer (ThermoFisherSciectific, USA). Complementary DNA (cDNA) was synthesized using a miScript reverse transcription (RT) kit (Qiagen, Hilden, Germany) and then all specimens were stored at −80 °C. Quantitative PCR was run on Applied technologies, Stratagene Mx3000P using miScript SYBR green PCR kit (Qiagen, Hilden, Germany) for detection of miR-34a (ID:MS00003318). The relative expression of miRNA was described as fold change using the calculated formula; $2^{-\Delta\Delta CT}$. More details were described before (8).

2.3. Statistical analysis

Patients'clinical and laboratory quantitative and qualitative data were presented as mean ± SD and frequencies respectively, while the levels of relative miR-34 expression were presented as median (interquartile range (IQR)). Non parametric tests were used to evaluate expression difference of miR-34a between patients and healthy controls and between patients' groups. To assess the relationships between exosomal miR-34a and different patients' parameters, Pearson's correlation and linear regression were performed. The utility of miR-34a as indicator for associated dyslipidemia among patients was tested using Receiver operating curve (ROC) and area under the curve (AUC) was calculated. All tests are two-sided and a level of $p = <0.05$ was considered statistically significant.

3-Results

-The clinical, demographic and routine laboratory tests for patients and healthy controls were presented in table 1. Both patients and controls were matched for age, gender and BMI. Glycated hemoglobin (HbA1C), TC, TGs and LDL were significantly increased in patients compared to controls while HDL showed no significant difference between the two groups.

-Regarding miRNA expression, miR-34a showed significant higher expression among T1DM patients (median: 22.6, IQR:4.2-111.7 fold change) than healthy controls (median: 6, IQR:0.1-12 fold change) (p=0.001) (Figure 1). Frequency of patients with miR-34a overexpression (defined as expression more than 2 fold change) among T1DM patients was 90%. Association of miR-34a expression and T1DM was confirmed using logistic regression analysis after adjustment of age, gender and BMI (p=0.01).

- On comparison between patients regarding associated dyslipidemia, the relative expression of exosomal miR-34a was higher in patients with dyslipidemia (median:78; IQR:18.1-2388 fold change) compared to patients without dyslipidemia (median:4.8; IQR:3.7-34.2 fold change) (p=0.001) (Figure 1). There was no significant difference between patients with dyslipidemia and those without dyslipidemia regarding age, gender, BMI and hemoglobin A1C. Disease duration, serum TC, TGs and LDL were significantly higher in patients with dyslipidemia compared to patients without dyslipidemia, while HDL showed no significant difference between the two groups (Table 2). The most common disorder of dyslipidemia was hypercholesterolemia (95%). Prevalence of high triglycerides, low HDL and high LDL was 9.5%, 12% and 45% respectively.

- Levels of serum endoglin and serum ICAM were significantly higher in patients with T1DM than healthy subjects (p=0.01 and 0.001) (Table 1) and higher in patients with dyslipidemia compared to patients without dyslipidemia (p=0.01) (Table 2). Serum endoglin and serum ICAM showed no significant correlations with age, BMI, disease duration, glycated hemoglobin and parameters of lipid profile (Table 3).

- Pearson's correlation revealed positive correlation between miR-34a and both serum endoglin and serum ICAM, but failed to demonstrate significant association between miR-34a with parameters of lipid profile (Table 4). Linear regression analysis was used to confirm the association of exosomal expression of miR-34a with parameters of lipid metabolism and endothelial dysfunction in patients with T1DM after adjustment of age, gender, BMI and disease duration. This analysis revealed strong an independent association between exosomal miR-34a with TC, serum endoglin and serum ICAM (Table 4).

- The utility of miR-34a as indicator for associated dyslipidemia was tested using ROC curve which revealed AUC: 0.73 with CI: 0.63-0.83 (p=0.001) (Figure 2).

- To validate and strength our results, the analysis of miRNAs that target different genetic pathways involved in lipid metabolism and endothelial function was done using https://ccb-web.cs.uni-saarland.de/mirtargetlink/index.php. This analysis retrieved miR-34a as one of the three miRNAs that can target both Hepatocyte Nuclear Factor 4 (HNF4) and sirtuin 1 (SIRT1) genes that were identified to play major roles in lipid metabolism (Figure 3) and miR-34a was the only miRNA that targets the three major genes (vascular endothelial growth factor=VEGF,SIRT1 and p53) involved in endothelial function (Figure 4) (17).

4- Discussion

Pathogenesis of associated endothelial dysfunction and dyslipidemia in children and adolescents with T1DM is still under evaluated. This is the first study to evaluate exosomal miR-34a expression in children and adolescents with T1DM and to correlate this expression with markers of dyslipidemia and endothelial dysfunction in studied patients.

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Role of miR-34a in development of diabetes is under the spotlight. Accumulating data indicate that miR-34a plays significant roles in glucose sensing, insulin secretion, and increasing sensitivity of β cells to cytokines-induced apoptosis (18,19,20). In the current study, expression of miR-34a was increased in patients with T1DM compared to healthy subjects, suggesting its role in pathogenesis of T1DM. This is in agreement with other studies that demonstrated overexpression of miR-34a in T1DM (21,22) especially in recent-onset T1DM compared to high-risk and healthy children (23).

Dyslipidemia is a metabolic disorder commonly associated with T1DM, increasing the risk of CVD (5). In our study, the prevalence of dyslipidemia among patients with T1DM was 35%. El Bakry et al reported that 64.0% of Egyptian children and adolescents with T1DM showed association with dyslipidemia (24). The prevalence of dyslipidemia in children and adolescents with T1DM varies from 29% to 66% in cross sectional studies from different countries (25,26,27). The most common frequent type of dyslipidemia among adolescents with T1DM also varies between hypercholesterolemia (24,28), high triglyceridemia (29,30) and high LDL (31,32). Differences in sample size, inclusion and exclusion criteria, degree of glycemic control and ethnicity might be the cause of this wide range of prevalence and difference in frequent type of dyslipidemia in the previous studies.

MiR-34a is known to regulate TC and hepatic lipid metabolism through targeting and inhibition of expression of the NAD+-dependent lysine deacetylase Sirt1, the antiatherogenic mediator that regulates lipid metabolism and endothelial function (33). MiR-34a also down regulates HNF4, that modulates the expression of different genes involved in lipid and glucose metabolism (34). In agreement with the previous data, we have demonstrated for the first time increased miR-34a expression in patients with dyslipidemia compared to those without dyslipidemia. Moreover, miR-34a overexpression was described in different disease associated with dyslipidemia, particularly non-alcoholic fatty liver disease (NAFLD) (33,35S) and coronary artery disease (36) and this expression was correlated with disease severity. Linear regression analysis for our results revealed positive association between miR-34a and TC and LDL, while no correlation or association was detected between the miRNA and TG. Interestingly, Shen et al reported positive correlation of miR-34a expression with LDL and negative correlation with triglycerides in patients with T2DM. However, this can be explained by data mentioned by Xu et al, that miR-34a regulates hepatic TGs via regulating HNF4 expression and its overexpression leads to accumulation of TGs in the liver and subsequently decreasing serum TGs (34).

Endothelial dysfunction precedes and promotes vascular inflammation and therefore atherosclerosis in T1DM and may be considered as independent predictor of associated CVD (3). Endoglin (CD105) is a membrane glycoprotein located on the surface of vascular endothelial cell, acting as a receptor for transforming growth factor beta (TGF-β) which is an important mediator for angiogenesis and responsible for keeping the vascular endothelium healthy (37). ICAM is a surface adhesion molecule expressed on vascular endothelial cells and immune cells, facilitating cell to cell interaction and recruitment of leucocytes to endothelium during inflammation (38). Shedding of endoglin and ICAM1 receptors into the systemic circulation during endothelial injury renders them potential circulatory markers of endothelial dysfunction (37). In this study, levels of serum endoglin and serum ICAM were highly elevated in patients with T1DM compared to healthy controls. In agreement with our results, elevated levels of serum endoglin and serum ICAM in patients with T1DM, was reported in several studies (38,39,40).Another interesting finding was the elevation of serum endoglin and serum ICAM in patients with dyslipidemia compared to patients without dyslipidemia, in agreement to El-Kassas et al, who reported significant positive correlations of endoglin with TC, TG and LDL in children with T1DM (39). In addition, serum endoglin and serum ICAM showed positive correlations with exosomal miR-34a expression in our study. This data was consistent with other reports stating that miR-34a is one of the most important endothelial miRNAs that play significant role in maintaining endothelial cell function by targeting many genes including p53, VEGF and SIRT-1(41). Moreover, TGF-β increases endoglin and can upregulate miR-34a which subsequently promotes vascular inflammation by upregulating vascular cell adhesion molecule-1 (VCAM) and ICAM (10,42). Interestingly, it was reported that miR34a deletion in the endothelium preserve endothelial functions in diabetic mice indicating the responsibility of miR-34a for diabetic vascular dysfunction (10).

Limitations of the study: The partial small sample size and the cross-sectional research design may be considered as limitations for this study.

Conclusions

Based on our knowledge, this is the first study that reported the altered expression of exosomal miR-34a among children and adolescents withT1DM. Moreover, association of miR-34a with markers of dyslipidemia and endothelial dysfunction was identified, suggesting role of miR-34 in the epigenetic regulation of lipid metabolism and endothelial functions in T1DM among children and adolescents. More longitudinal studies with larger sample size and prospective design should be warranted to focus the light on this field. Future studies are recommended to identify the possible use of miR-34a as a therapeutic target in patients with T1DM and cardiovascular diseases.

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6-Authorship Contribution: All authors contributed significantly in study design, methodology and data analysis. All authors revised and approved the final version of manuscript.

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Table (1): Clinical, demographic and biochemical laboratory data of patients and controls

Characteristic	Controls (number=100)	T1DM (number=120)	p value
Age (years)	12.1±2.8	13.5±3.2	0.6
Gender (male/female)	41/59	54/66	0.5
BMI (kg/m^2)	18.1±3.1	18.9±2.3	0.7
Diabetes duration(years)	-	7.6±1.9	-
HbA1c (%)	4±0.6	8±1.2	0.001
Total cholesterol (mmol/l)	127 ±25	181 ±42	0.001
Triglycerides (mg/dL)	75 ±35	88 ±30.7	0.005
HDL (mg/dL)	52 ±4.4	53 ±8.8	0.2
LDL (mg/dL)	80±19.1	106±33	0.001
Serum endoglin(ng/ml)	28.5(16.8-43)	31(19-75)	0.01
ICAM (ng/ml)	231(129-321)	293(240-693)	0.001

T1DM=type 1 diabetes mellitus; BMI=Body mass index; HbA1c=glycated hemoglobin; HDL=High density lipoprotein; LDL=Low density lipoprotein

Table (2): Comparison between patients with dyslipidemia and patients without dyslipidemia

Parameter	Patients without dyslipidemia (78 patients)	Patients with dyslipidemia (42 patients)	p value
Age (years)	13.6±3.2	13.2±3.4	0.5
Gender (male/female)	32/46	22/20	0.2
BMI (kg/m^2)	18.8±3.1	17.9±3.6	0.9
Disease duration (years)	7.2±1.6	8.3±2.2	0.03
HbA1c (%)	7.9±1.1	8.3±1.2	0.1
Total cholesterol (mmol/l)	157± 23.2	227±20.3	0.001
Triglycerides (mg/dL)	80.3±43	103±25.7	0.001
HDL (mg/dL)	52.6± 8.3	54± 9.7	0.2
LDL (mg/dL)	87.8±21.4	139.5±25.3	0.001
Frequency of disorders			
Hypercholesterolemia (>200mmol/l)	-	40/42	
Hypertriglyceridemia (>150 mg/dL)	-	4/42	-
Decreased HDL (<35 mg/dL**)**	-	5/42	

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Increased LDL (>130 mg/dL)	-	19/42	
Serum endoglin (ng/ml)	29(18-57)	48(21-90)	0.02
ICAM (ng/ml)	342(234-509)	435(346-657)	0.005

BMI=Body mass index; HbA1c=glycated hemoglobin; HDL=High density lipoprotein; LDL=Low density lipoprotein; ICAM= Intracellular adhesion molecule

Table (3): Pearson's correlation of serum endoglin and serum ICAM with different parameters in T1DM patients

Parameters	Serum endoglin		Serum ICAM	
	r	p-value	r	p-value
Age (years)	- 0.1	0.1	-0.1	0.2
BMI (kg/m^2)	0.3	0.09	0.09	0.1
Disease duration (years)	0.07	0.4	-0.2	0.6
HbA1c (%)	0.13	0.52	-0.002	0.9
Total cholesterol (mmol/l)	0.09	0.1	-0.03	0.73
Triglycerides (mg/dL)	-0.06	0.6	0.03	0.75
HDL (mg/dL)	0.01	0.8	0.04	0.51
LDL (mg/dL)	0.08	0.1	0.2	0.2

r= Pearson's correlation coefficient; BMI=Body mass index; HbA1c=glycated hemoglobin; HDL=High density lipoprotein; LDL=Low density lipoprotein; ICAM= Intracellular adhesion molecule

Table 4: Pearson's correlation and linear regression analysis of miR-34a with different parameters of dyslipidemia and endothelial dysfunction.

Parameters	Pearson's correlation		Regression analysis	
	r	p-value	β	p-value
Total cholesterol (mmol/l)	0.13	0.03	0.56	0.001

Triglycerides (mg/dL)	0.006	0.3	0.1	0.9
HDL(mg/dL)	0.12	0.07	0.1	0.03
LDL(mg/dL)	0.1	0.03	0.3	0.01
Serum endoglin (ng/ml)	0.6	0.001	0.65	0.001
Serum ICAM (ng/ml)	0.45	0.001	0.51	0.001

r= Pearson's correlation coefficient; β=standardized regression coefficient; HDL=High density lipoprotein; LDL=Low density lipoprotein; ICAM= Intracellular adhesion molecule

Figure legends
Figure (1): Expression of exosomal miR-34a among studied groups. Fig.1A. showed significant higher expression OF miR-34a in patients with T1DM (median:22.6, IQR:4.2-111.7) than healthy controls (median:6, IQR:0.1-12). Fig.1B. showed increased expression of exosomal miR-34a in patients with dyslipidemia (median:78; IQR:18.1-2388) compared to patients without dyslipidemia (median:4.8; IQR:3.7-34.2).
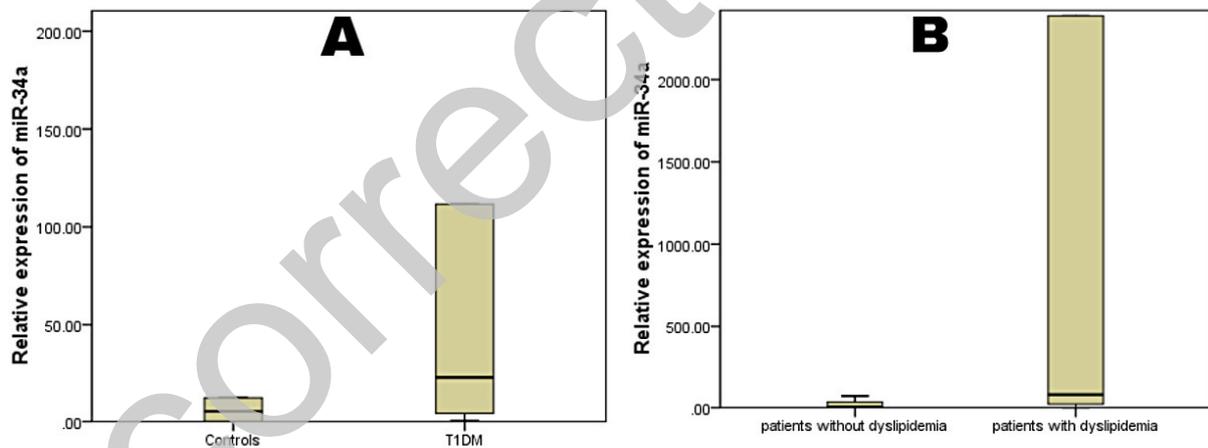
Figure (2): ROC curve of exosomal miR-34a in associated dyslipidemia in T1DM. ROC curve showed the utility of miR-34a as indicator of associated dyslipidemia and revealed AUC: 0.73 with CI:0.63-0.83 (p=0.001).
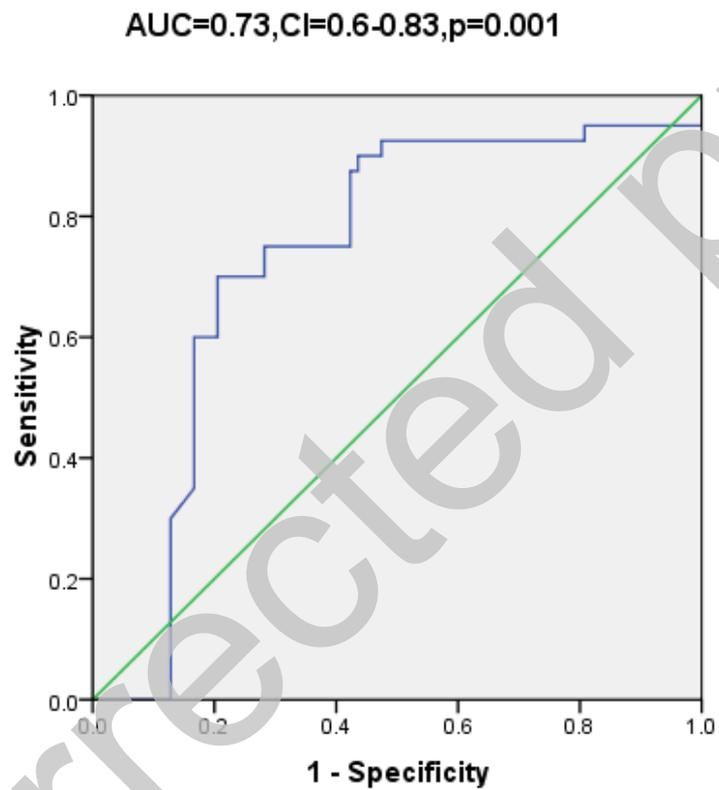

AUC=0.73,CI=0.6-0.83,p=0.001

Figure (3): Interaction network of genes targeted by miR-34a and played significant role in lipid metabolism.
This analysis was done using (miRTargetLinkdatabase)(https://ccb-web.cs.uni-saarland.de/mirtargetlink/index.php)
and retrieved that miR-34a is one of the three miRNAs that can target both Hepatocyte Nuclear Factor 4 (HNF4) and
 sirtuin 1 (SIRT1) genes (green line is indicating strong interaction).
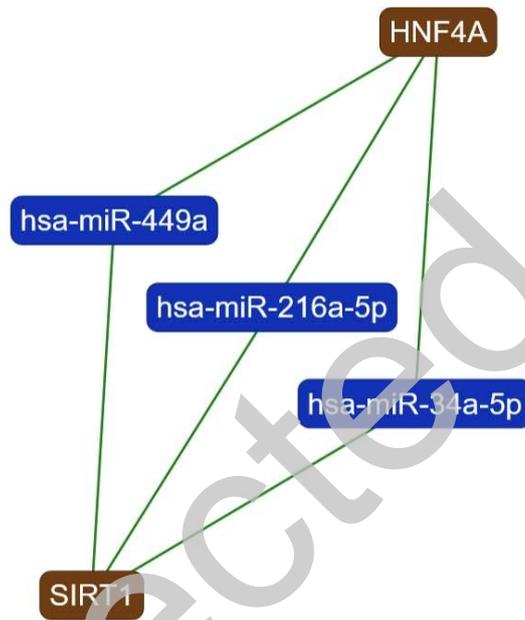
Figure (4): Interaction network of genes targeted by miR-34a and played significant role in endothelial function. This analysis was done using (miRTargetLinkdatabase) (https://ccb-web.cs.uni-saarland.de/mirtargetlink/index.php) and retrieved that miR-34a is the only miRNA that targets the three major genes (vascular endothelial growth factor=VEGF,sirtuin 1=SIRT1 and p53) involved in endothelial function (green line is indicating strong interaction and red line is indicating week interaction).