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ORIGINAL ARTICLE

Decreased Serum Level of miR-155 is Associated with Obesity and its Related Metabolic Traits

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SUMMARY

Background: **Type 2 diabetes is the most common metabolic disease, affecting many of the adult population all around the world. In recent years much attention has been paid to the role of circulating miRNAs as novel biomarkers for various diseases. The aim of this study was to investigate the expression level of miR-155 in serum samples of diabetic and healthy subjects.**
Methods: **42 healthy and 45 type 2 diabetic subjects participated in the study. Serum miR-155 level of the subjects was measured using real-time PCR. The levels of IL-6 and TNF-α were quantified using ELISA.**
Results: **There was no significant difference in the level of miR-155 between the diabetic and non-diabetic groups. The level of miR-155 in non-diabetic obese group was significantly lower than the non-diabetic lean subjects. Correlation analyses in non-diabetic group revealed a significant negative correlation between the amount of miR-155 and body mass index and cholesterol levels after the elimination of the confounding factors. In diabetic group, a negative correlation was found between miR-155 and insulin, HOMA-IR, and waist circumference levels. Furthermore, no significant relationship between miR-155 and inflammatory cytokines (TNF-α and IL-6) was observed in both diabetic and healthy groups.**
Conclusions: **A reduced level of miR-155 might associate with obesity and its related metabolic traits such as hyperinsulinemia and dyslipidemia.**
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KEY WORDS

microRNA-155, circulation, serum, type 2 diabetes, obesity, inflammation

LIST OFABBREVIATIONS

BMI - body mass index
FBS - fasting blood sugar
HDL-C - high density lipoprotein cholesterol
IL-6 - interleukin 6
LDL-C - low density lipoprotein cholesterol
LPS – lipopolysaccharide
miR-155 - microRNA-155

PBMCs - peripheral blood mononuclear cells
TNF-α - tumor necrosis factor alpha
T2D - type 2 diabetes
WC - waist circumference
CRP - C-reactive protein

INTRODUCTION

Type 2 diabetes (T2D) is a multifactorial metabolic disorder that results from the combination of several factors including genetic background, aging, poor lifestyle, and environmental factors [1,2]. The number of people with diabetes is increasing. The reports show that in 2015 415 million adults had diabetes and 5 million people between the ages of 20 and 79 years died from diabetes and its complications [3]. It is estimated that in 2040, there will be more than 642 million people with diabetes worldwide [4].
Obesity has an important role in the pathogenesis of T2D. Activation of the inflammatory signaling has been suggested as the underlying mediator of obesity-induced T2D [5]. Obesity is associated with a state of chronic low-grade inflammation. Low-grade inflammation is characterized by increased levels of circulating inflammatory markers such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), C-reactive protein (CRP), and IL-18 and increased infiltration of macrophages into adipose, liver, pancrease and skeletal muscle tissues [6,7]. These inflammatory factors along with increasing levels of free fatty acids can then interfere with normal insulin function and secretion and thereby induce insulin resistance and β-cell dysfunction in T2D.
MicroRNAs (miRNAs) are a class of non-coding RNAs with unique functions in control of gene expression at the transcriptional and post-transcriptional level. Matured structure of miRNAs includes single-stranded RNA with a length of 19 - 22 nucleotides [8,9]. It has been demonstrated that miRNAs regulate many cellular processes such as proliferation, apoptosis, development and metabolism [8,10,11]. In addition, miRNAs have been reported to regulate the innate and adaptive immune responses as well as inflammatory networks in various cell types [12-14]. In particular, miR-155 has been associated with regulation of different immune-related processes, such as innate immunity and hematopoiesis [15]. Furthermore, miR-155 has been reported to be one of the important regulators of macrophage and monocyte responses to different types of inflammatory stimuli, such as bacterial lipopolysaccharide (LPS), interferon-γ (IFN-γ), and TNF-α [16,17]. Given the important role of miR-155 in regulation of the immune system, alteration of the expression of miR-155 might be associated with several human disorders. In this regard, aberrant expression of miR-155 has been linked to several human diseases such as inflammatory bowel disease, T2D, and cancers [18]. Importantly, a reduced expression of miR-155 has been reported from peripheral blood mononuclear cells (PBMCs) of the diabetic patients [15,19].
Recently, much attention has been paid to the role of circulating miRNAs as novel biomarkers for various diseases. miRNAs have been found in a stable form in tissues, serum, plasma, and other body fluids. The dysregulated expressions of circulating miRNAs have been detected in several disorders such as cancers, infections, and cardiovascular diseases [20,21]. Importantly, aberrant expression of miRNAs in plasma and serum samples of the diabetic patients has also been reported [22-24]. We previously examined the expression of miR-155 in PMBCs of the diabetic patients and our results indicated a reduced expression of this miRNA in PBMCs of the patients with T2D [15]. In the present study, we aimed to investigate the expression level of miR-155 in serum samples of the diabetic and healthy subjects. We also aimed to determine if any observed deregulation was specific to either obesity or diabetes. In addition, we studied the correlation of miR-155 with clinical, biochemical, and inflammatory parameters in both the diabetic and non-diabetic subjects.

MATERIALS AND METHODS

Subjects

A total of 87 subjects (45 type 2 diabetic and 42 non-diabetic subjects) were recruited from the Diabetes Clinic of the Diabetes Research Center, Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences during 2014 - 2015. Inclusion criteria were fasting blood glucose (FBS) lower than 100 mg/dL for non-diabetic group and age between 40 - 65 years and no current or history of acute or chronic inflammatory diseases, malignant diseases, and diagnosed or suspected endocrine disorders in both the diabetic and non-diabetic groups. The Ethics Review Board of the Tehran University of Medical Sciences approved the study; an informed consent was obtained from all participants.
The personal data and clinical measurements such as age, gender, history of cardiovascular diseases, history of glucose, lipid and blood pressure lowering drugs, duration of diabetes, family history of obesity, cardiovascular disease, and history of smoking were recorded. Diabetes was defined as fasting glucose more than 126 mg/dL or use of hypoglycemic medication. Body mass index (BMI) was calculated as weight in kilograms divided by the height in meters squared. Obesity was defined as BMI > 30 kg/m^2. The systolic and diastolic blood pressure was measured in a sitting position and after 5 minutes resting. Height, weight, waist circumference (WC) and hip circumference (HP) were measured with accurate metric tools. Insulin resistance was assessed from glucose and insulin concentrations using the homeostasis model assessment of insulin resistance (HOMA-IR) equation. HOMA-IR = fasting insulin (μU/L) x fasting glucose (mmol/L)/22.5.

Biochemical measurements
All biochemical parameters such as FBS, LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triglycerides (TG), and total cholesterol were measured in serum samples using Pars azmon kits through the enzymatic methods by BIOLIS 24i Premium autoanalyser (Tokyo Boeki Machinery Ltd, Japan). HbA1c was measured by the HPLC method using a Tosoh G8 instrument (South San Francisco, CA). Serum insulin level was measured by the Insulin AccuBind ELISA Kit (Monobind Inc., Lake Forest, CA, USA).

miRNA extraction and cDNA synthesis
Serum miRNAs were extracted using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First strand cDNA was synthesized in two steps. First, a polyadenylation using E. Coli Poly (A) Polymerase (New England Bio Lab) was performed at 37°C for 30 minutes and then the reverse transcription step was conducted using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

Quantitative real-time PCR
Quantitative Real Time-PCR was performed using Real Q Plus 2x Master Mix Green - (Ampliqon, Denmark). miR-16 and miR-155 forward primers were 5-TAGCA GCACGTAAATATTGGCG-3 and 5-TTAATGCTAA TCGTGATAGGGGT-3, respectively. Quantitative Real-Time PCR reactions were performed in a Rotogene Q (Qiagene, Hilden, Germany) in 20 µL of PCR master mix. The relative expression of miR-155 was calculated using the comparative CT method. All tests were run in triplicate to minimize the experimental error. It should be noted that the synthetic miR-39 belonging to the species C. elegans (Caenorhabditis-elegans) with 5-UCACCGGGUGUAAAUCAGCUUG-3 sequence was used as an external control. miR-39 was added to the serums as an external calibrator to monitor the extraction efficiency. Endogenous miR-16 was used as an internal control.

Determination of inflammatory cytokines
Inflammatory cytokines including IL-6 and TNF-α in serum samples were measured using the ELISA kit of Abnova (IL-6, KA0123; and TNF-α, KA0191).

Statistical analyses
All statistical analyses were carried out using SPSS version 22 (SPSS, Chicago, IL, USA); data was expressed as mean ± SD, and $p < 0.05$ was considered significant. The continuous variables that failed the normality test were logarithmically transformed before analysis. The variables transformed were FBS, TG, cholesterol, HDL-C, LDL-C, insulin, HOMA-IR, and $2^{-\text{delta CT}}$ of miR-155. Statistical differences are based on analyses of log-transformed data, but the means of untransformed data are presented in the tables. Comparison between groups was performed using unpaired Student's *t*-test. Analysis of covariance was used to determine the associations while adjusting for age, gender, and BMI. Pearson's correlation analysis was carried out to determine the correlation between logarithmically transformed miR-155 and clinical and biochemical parameters. Partial correlation was used to further explore the relationship between logarithmically transformed miR-155 and clinical and biochemical parameters while adjusting for age, gender, and BMI.

RESULTS

Demographic and biochemical characteristics of the subjects
Table 1 represents the clinical and biochemical parameters of the study population. This study was performed on 45 patients with T2D and 42 healthy subjects. The age of subjects was in the range of 42 to 64 years and a significant difference was observed in the age between patients and healthy subjects ($p = 0.032$). No significant differences were found for WC ($p = 0.135$), HC ($p = 0.061$), systolic ($p = 0.083$) and diastolic blood pressures ($p = 0.062$), total cholesterol ($p = 0.065$), TG ($p = 0.262$), LDL-C ($p = 0.710$), and HDL-C ($p = 0.394$) between the study groups. FBS ($p = 0.000$), HbA1c ($p < 0.0001$), HOMA-IR ($p < 0.0001$), and insulin ($p < 0.0001$) levels were significantly higher in patients with T2D compared to non-diabetic subjects.

Serum miRNA-155 level in diabetic and non-diabetic subjects
Initially, the expression level of cel-miR-39 was evaluated. cel-miR-39 was specifically and consistently detected in all sera with an average raw CT of 21.42 ± 3.68. No significant difference in raw CT values of cel-miR-39 was detected between the diabetic and non-diabetic groups ($p = 0.890$). Our results showed that circulating miR-155 levels in diabetic patients were approximately 60% lower than that of the healthy subjects ($p = 0.004$) (Figure 1). This finding was not statistically significant after adjustment for age, BMI, and gender ($p = 0.385$).

Relationship between serum miR-155 and clinical and biochemical parameters
In the non-diabetic group and total population, a significant negative correlation was observed between the level of miR-155 and cholesterol. These associations remained significant after adjustment for age, gender, and BMI. In the non-diabetic group, there was also a significant negative correlation between BMI and the amount of miR-155 after the elimination of the confounding factors. The correlations of miR-155 with the other clinical and biochemical factors were not significant in the non-diabetic group. In the diabetic group and also in total population, a negative correlation was found between miR-155 and insulin and HOMA-IR levels. It is noteworthy that these correlations remained significant

Table 1. Clinical and biochemical characteristics of the study population.

Parameter	Non-diabetic subjects (n = 42)	T2D subjects (n = 45)	p-value
Gender (M/F)	13/29	26/21	0.032
Age (years)	47.6 ± 5.8	56.5 ± 8.1	0.000
SBP (mmHg)	11.3 ± 14.2	122.4 ±15.1	0.083
DBP (mmHg)	71.8 ± 9.6	75.8 ± 8.7	0.062
WC (cm)	99.0 ± 10.1	95.4 ± 12.7	0.135
HC (cm)	105.4 ± 7.1	101.4 ± 11.8	0.061
BMI (Kg/m^2)	27.3 ± 3.9	28.2 ± 4.8	0.038
WHR	0.93 ± 0.04	0.94 ± 0.05	0.630
FBS (mg/dL)	85.4 ± 8.7	130.1 ± 31.3	0.000
Cholesterol (mg/dL)	151.5 ± 18.6	156.8 ± 49.9	0.650
TG (mg/dL)	120.8 ± 60.4	134.1 ± 55.3	0.263
HDL-C (mg/dL)	43.5 ± 7.0	42.1 ± 9.2	0.394
LDL-C (mg/dL)	118 ± 19.8	125.7 ± 20.7	0.710
Insulin (µIU/mL)	5.74 ± 2.2	9.27 ± 5.7	< 0.0001
HbA1c (%)	5.3 ± 0.4	7.20 ± 0.6	< 0.0001
HOMA-IR	1.25 ± 0.3	2.72 ± 0.9	< 0.0001
Metformin (n)	-	45	-
Statins (n)	-	25	-
Antihypertensive (n)	-	13	-

SBP - systolic blood pressure, DBP - diastolic blood pressure, WC - waist circumference, HC - hip circumference, FBS - fasting blood sugar, TG - triglycerides, BMI - body mass index, WHR - weight-to-hip ratio, HDL - high-density lipoprotein, LDL - low-density lipoprotein. Data are shown as "mean ± SD".

after adjustment for confounding factors such as age, gender, and BMI. In the diabetic group, waist circumference had a negative correlation with the amount of miR-155 after adjustment for confounding factors (Table 2).

Evaluation of serum miR-155 level in obese and non-obese subjects

After observing a significant negative correlation between miR-155 and BMI parameters, the levels of miR-155 in obese and lean groups were studied. The results showed that the level of miR-155 in non-diabetic obese group was 44% lower than that of the non-diabetic lean subjects ($p = 0.023$). The comparison of miR-155 level in obese and lean subjects of the diabetic patients revealed no statistically significant difference (Figure 2).

Comparison of inflammatory cytokines between the diabetic and non-diabetic subjects

To investigate the relationship between miR-155 and the inflammatory factors, the serum levels of TNF-α and IL-6 were measured. Serum TNF-α level in the diabetic patient was significantly higher than the non-diabetic subjects. The level of IL-6 was also different between the two groups (Figure 3).

Correlation between serum level of miR-155 and pro-inflammatory cytokines

Correlation analyses showed no significant relationship between miR-155 and inflammatory cytokines TNF-α and IL-6 in both T2D patients and healthy subjects. The same results were also observed after adjustment for age, gender, and BMI (Table 3).

DISCUSSION

In recent years many attempts have been made to establish whether miRNAs could be used as biomarkers for the diagnosis, prognosis, and treatment of various diseases. This study was designed to investigate the role of miR-155 as a biomarker for T2D. The study was performed on 87 subjects aged 42 to 64 years, including 45 patients with T2D and 42 non-diabetic subjects. As the clinical and biochemical parameters, BMI, FBS, insulin, and HbA1c levels in type 2 diabetic patients were significantly greater than that of the non-diabetic subjects. However, the lipid profile showed no significant difference between the diabetic and healthy groups. This can be due to taking lipid-lowering drugs among patients with diabetes.

Table 2. Correlation between miR-155 and clinical and biochemical characteristics.

	Non-diabetic		Diabetic		Total	
Parameter	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
Age (years)	**-0.312**	**-0.325**	**0.041**	**0.053**	**-0.201**	**-0.202**
SBP (mmHg)	**-0.060**	**-0.503**	**0.108**	**0.188**	**0.095**	**0.085**
DBP (mmHg)	**-0.012**	**0.080**	**0.226**	**0.102**	**0.145**	**0.088**
Weight (Kg)	**-0.243**	**-0.277**	**-0.080**	**-0.091**	**-0.128**	**-0.153**
WC (cm)	**-0.244**	**0.103**	**-0.135**	**-0.474** *	**-0.107**	**-0.054**
HC (cm)	**-0.190**	**0.027**	**-0.098**	**-0.306**	**-0.053**	**-0.038**
BMI (Kg/m^2)	**-0.314**	**-0.352** *	**0.067**	**0.079**	**-0.111**	**-0.135**
WHR	**-0.163**	**0.078**	**-0.131**	**-0.329**	**-0.126**	**-0.040**
FBS (mg/dL)	**-0.307**	**-0.160**	**-0.016**	**-0.036**	**-0.201**	**-0.107**
Cholesterol (mg/dL)	**-0.446** *	**-0.411** *	**-0.150**	**-0.136**	**-0.271** *	**-0.314** *
TG (mg/dL)	**-0.135**	**0.030**	**-0.073**	**-0.078**	**-0.116**	**-0.086**
HDL-C (mg/dL)	**0.124**	**-0.244**	**0.186**	**0.264**	**0.153**	**0.056**
LDL-C (mg/dL)	**-0.197**	**-0.069**	**-0.206**	**-0.219**	**-0.078**	**-0.127**
Insulin (µIU/mL)	**-0.265**	**-0.092**	**-0.361** *	**-0.516** *	**-0.350** *	**-0.313** *
HbA1c (%)	**-0.272**	**-0.175**	**-0.009**	**0.016**	**-0.224**	**-0.135**
HOMA-IR	**-0.275**	**-0.112**	**-0.339** *	**-0.471** *	**-0.373** **	**-0.313** *

SBP - systolic blood pressure, DBP - diastolic blood pressure, WC - waist circumference, HC - hip circumference, FBS - fasting blood sugar, TG - triglycerides, BMI - body mass index, WHR - waist-to-hip ratio, HDL - high-density lipoprotein, LDL - low-density lipoprotein, R1 - unadjusted, R2 - adjusted for age, gender, and BMI. Data were analyzed using log of $2^{-\text{deltadelta CT}}$ of miR-155 and variables in log transformed forms (FBS, TG, cholesterol, HDL-C, LDL-C, insulin, HOMA-IR, and HbA1c). * = p-value less than 0.05, ** = p -value less than 0.01.

Table 3. Correlations between the level of miR-155 with the amount of serum TNF-α and IL-6.

	Non-diabetic		Diabetic	
Parameter	**R1**	**R2**	**R1**	**R2**
IL-6	**-0.182**	**-0.121**	**-0.159**	**-0.125**
TNF-α	**-0.234**	**-0.166**	**-0.158**	**-0.140**

R1 - unadjusted, R2 - adjusted for age, gender, and BMI. Data were analyzed using log of $2^{-\text{delta CT}}$ of miR-155 and variables in log transformed forms (TNF-α and IL-6). * = p-value less than 0.05, ** = p-value less than 0.01.

The results of this study showed that miR-155 level in the serum of patients with T2D was about 60% lower than in healthy individuals. After adjustment for confounding factors such as age, gender, and BMI, the difference was not significant. Aberrant expression level of miR-155 in human serum and plasma has been reported in a number of diseases. Similar to our finding, Baldeon et al. showed that the serum level of miR-155 did not significantly differ between patients with T2D as compared to the non-diabetic subjects [25]. Nunez-Lopez et al. demonstrated that miR-155 was significantly reduced in the circulation of subjects with prediabetes [26]. A reduced expression of circulating miR-155 has also been previously reported in patients with coronary heart disease. Zhu et al. found that the level of miR-155 in PBMCs or plasma was lower in patients with unstable angina pectoris and acute myocardial infarction than in patients with chest pain syndrome [27]. In addition, Weber et al. found that miR-155 was significantly downregulated in the blood of patients with coronary artery disease compared to healthy subjects [28].

In the present study, we demonstrated that miR-155 expression was inversely associated with obesity. The expression level of miR-155 was significantly down-regulated in the serum of non-diabetic obese subjects. Importantly, miR-155 expression was negatively correlated with obesity related factors such as BMI and WC in the non-diabetic and diabetic groups, respectively. In

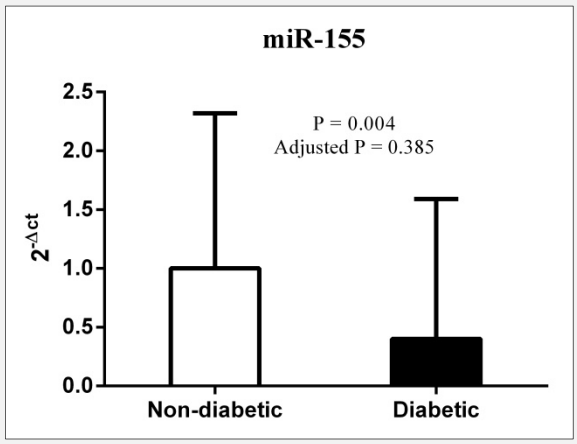

Figure 1. Comparison of circulating miR-155 level in type 2 diabetic and non-diabetic subjects.

The relative expression of miR-155 was measured by real-time PCR. The relative expression levels were normalized to the expression of miR-16. Statistical differences are based on analyses of log-transformed data, but the means of untransformed data are presented in tables. Data are shown as mean ± SD.

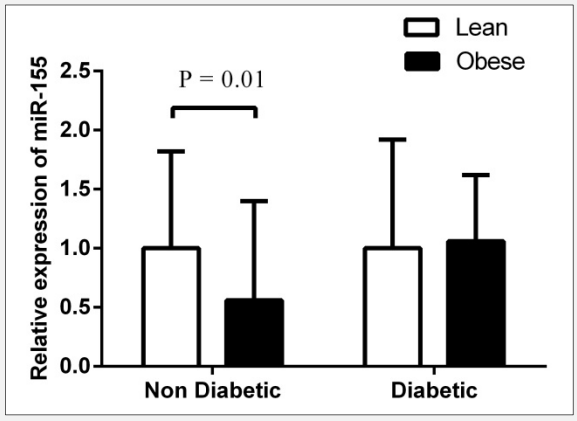

Figure 2. miR-155 level in obese and lean non-diabetic and diabetic subjects.

The relative expression of miR-155 was measured by real-time PCR. The relative expression levels were normalized to the expression of miR-16. Statistical differences are based on analyses of log-transformed data, but the means of untransformed data are presented in tables. Data are shown as mean ± SD.

support of these findings, a reduced expression of circulating miR-155 in obese subjects has been previously reported. In study of Nunez-Lopez et al. a significant negative correlation between miR-155 and measures of body composition such as fat mass was displayed [26]. Furthermore, Murri et al. demonstrated that obesity reduces the serum level of miR-155 in healthy male and female subjects [29]. Overall, these findings suggest that miR-155 is reduced in circulation of obese subjects. Given the close link between obesity and T2D, therefore, a lower circulating level of miR-155 in obese subjects might contribute to future development of T2D. miR-155 has been reported to be a component of the inflammatory responses [30]. To study the relationship

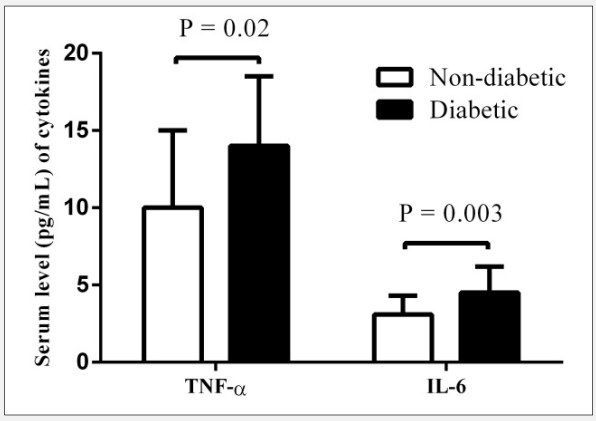

Figure 3. Serum levels of inflammatory cytokines in diabetic and non-diabetic subjects.

The serum levels of TNF-α and IL-6 were measured by ELISA method, as described in Materials and Methods. Data are shown as mean ± SD.

between serum miR-155 and inflammatory cytokines, we measured IL-6 and TNF-α level in the serum of the healthy and diabetic subjects. Our data revealed that miR-155 had no significant association with the inflammatory cytokines in both diabetic and non-diabetic groups. In agreement with these data, one previous study reported no association of miR-155 with inflammatory factors in the context of diabetes or obesity. Nunez-Lopez et al. demonstrated that the serum level of miR-155 was not correlated with IL-6 and IL-8 in diabetic or obese groups [26]. Taken together, these findings suggest that circulating miR-155 might not be associated with inflammatory cytokines in both diabetic and healthy subjects.

The data of this study also revealed a negative correlation between miR-155 and cholesterol, insulin, and HOMA-IR levels in healthy and diabetic groups. To our knowledge, this is the first report on the association between serum miR-155 expression and these biochemical parameters in non-diabetic and diabetic subjects. In support of this finding, it has been reported that miR-155 is implicated in regulation of very-low-density lipoprotein (VLDL) metabolism as shown in a study where the lack of miR-155 in mice fed a high-fat diet increased hepatic steatosis and serum VLDL/LDL cholesterol levels by targeting LXRα [31,32].

CONCLUSION

Our data provide the evidence that the serum level of miR-155 did not statistically differ between T2D and healthy subjects. However, we observed a lower circulating level of miR-155 among obese non-diabetic subjects and this reduced expression appears to be correlated with increased cholesterol levels. In addition, our results suggest that miR-155 has no correlation with the serum inflammatory cytokines in both diabetic and non-diabetic subjects. Altogether, these results suggest that a reduced level of miR-155 might be implicated in obesity related disorders such as hyperinsulinemia and dyslipidemia.

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Declaration of Interest:
Nothing to declare.

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