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Original Article

Investigation of the relationship between miR-33a, miR-122, erythrocyte membrane fatty acids profile, and serum lipids with components of metabolic syndrome in type 2 diabetic patients

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Abstract

Background and purpose: MicroRNAs (miRNAs) are small non-coding RNA molecules acting as critical regulators of post-transcriptional gene expression. MiR-33a and miR-122 have a crucial role in cholesterol and lipid metabolism. Therefore, their dysregulation may contribute to metabolic abnormality and their inhibition may be a useful therapeutic strategy. The objective of the present study was to investigate the relationship between miR-33a, miR-122, erythrocyte membrane fatty acids profile, and serum lipids with components of metabolic syndrome in an Iranian population suffering from type 2 diabetes mellitus (T2DM).
Experimental approach: Expression of miR-33a and miR-122 was measured by real-time polymerase chain reaction and erythrocyte membrane fatty acid profiles were analyzed by gas chromatography-mass spectrometry.
Findings/Results: T2DM patients with and without metabolic syndrome had significantly higher miR-33a and miR-122 levels compared to controls. MiRNAs were significantly correlated with saturated fatty acid (SFAs), total SFAs/total polyunsaturated fatty acids (PUFAs) ratio, fasting plasma glucose, triacylglycerols, insulin and homeostatic model assessment of insulin resistance. In addition, there was a significant negative correlation between miR-33a and miR-122 levels and PUFAs, total PUFAs/total SFAs ratio and omega 6 fatty acids.
Conclusion and implications: Considering the roles of miR-33a and miR-122 in cholesterol and lipids metabolism, it may be concluded that the measurement of their expression may be useful as a potential additional biomarker for cardiometabolic derangement in T2DM patients. In addition, these findings may suggest that the inhibition of these miRNAs by anti-miRNA therapies may be explored as a potential therapeutic strategy.

Keywords: Erythrocyte membrane fatty acid profile; Metabolic syndrome; miR-33a; miR-122; Type 2 diabetes.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by an absolute or relative deficiency in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of lipid metabolism (1). Insulin resistance or deficiency affects key enzymes and pathways in lipid metabolism (2). T2DM is known as a major risk factor for cardiovascular diseases (CVDs) that are in the first place for mortality in developed and developing countries (3-5). Metabolic syndrome (MetS) or cardiometabolic syndrome, is a growing epidemic worldwide and is characterized by CVD risk factors, including obesity, dyslipidemia, elevated blood pressure and impaired glucose tolerance (6).

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Clinically, 97% of T2DM with dyslipidemia is associated with atherosclerosis, the major threat of blood vessels (3,4). It has been suggested that groups of non-coding RNA called microRNAs (miRNAs) act as critical regulators of gene expression at post-transcriptional levels. These non-coding RNAs are small (22 nucleotides) and bind to the 3' untranslated region of mRNA (3' UTR) leading to translation repression and/or mRNA degradation, thus involved in controlling many diseases (7-9). MiRNAs were first discovered in the nematode *Caenorhabditis elegans* and then identified in the genomes of plants, animals, and viruses (7-10). It is anticipated that miRNAs control 30-60% of the activity of total protein-coding genes in humans (10-12). They are stable in plasma and have a potential capacity as biomarkers for fatty liver disease and atherosclerosis (13). Several miRNAs are involved in lipid metabolism including miR-122 and miR-33 (14,15). MiR-122 is recognized as the most abundant miRNA in the liver and accounts for more than 75% of the total miRNA expression in this organ (16). MiR-122 has important roles in a wide range of functions in the liver including cholesterol metabolism, liver cancer, response to stress, viral infections of the liver, and is responsible for the regulation of genes involved in the metabolism of lipids (17-19). Inhibition of miR-122 has resulted in a 25-35% reduction in plasma cholesterol associated with changes of high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). Inhibition of miR-122 causes a significant reduction in genes involved in cholesterol synthesis, including 3-hydroxy-3-methylglutaryl-CoA synthase 1, 3-hydroxy-3-methylglutaryl-CoA reductase, 7-dehydrocholesterol reductase, and squalene epoxidase. In addition, many miR-122 targets are involved in glucose homeostasis and Krebs cycle including aldolase A and citrate synthase (17).

MiR-33 is encoded by an intronic sequence of sterol response element-binding protein (SREBP) genes. In the human body, there are two types of miR-33, miR-33a and miR-33b, that are expressed within intron 16 of the SREBP-2 gene on chromosome 22 and intron 17 of the SREBP-1 gene on chromosome 17, respectively. MiR-33 affects genes involved in cholesterol homeostasis including ABCa1, ABCg1, and Nieman-Pick type C1, and in lipid metabolism including carnitine palmitoyltransferase1A (CPT1A), carnitine O-octanoyl transferase, 5′ adenosine monophosphate-activated protein kinase, and sirtuin 6 (20,21). And also targets the insulin receptor substrate 2 which is an essential component of the insulin signaling pathway in the liver. It was recently shown that miRNAs have been incorporated in lipoproteins in addition to the extracellular matrix. Therefore, it can be hypothesized that miRNAs in the bloodstream can be useful as biomarkers in various diseases including CVD, T2DM, and other metabolic disorders (22). As mentioned, hyperlipidemia with other factors such as obesity and insulin resistance are the major risk factors for atherosclerosis and CVD. Considering the multiple functions of miRNAs, they may be powerful tools to study the pathogenesis of dyslipidemia in the development of metabolic disorders (23). We hypothesized that the expression levels of miR-122 and miR-33a are associated with metabolic abnormalities and may, therefore, potentially serve as the therapeutic targets for metabolic diseases and T2DM. Accordingly, in this study, the relationship between miR-122 and miR-33a gene expression with the profile of erythrocyte membrane fatty acids, serum lipids, and components of MetS in an Iranian population of T2DM patients was investigated.

MATERIALS AND METHODS

Study population

For this cross-sectional study, 150 men between 40-60 years of age referred to the health centers and Amin Hospital in Isfahan city were selected voluntarily after filling out the clinical information questionnaire. Subjects were distributed into three groups: group I included diabetic patients (fasting plasma glucose (FPG) ≥ 126 mg/dL without MetS and apparent signs of complications; group II included diabetic patients with MetS (having at least three criteria of the five criteria, including waist circumference > 102 cm, triglyceride (TG) > 150 mg/dL, FPG ≥ 100 mg/dL,

hypertension (blood pressure >130/85 mmHg), and HDL-C < 40 mg /dL); and the control group included 50 normoglycemic healthy subjects with no history of diabetes and other chronic and acute diseases. Subjects with any recent history of cancer, Alzheimer's disease, dementia, anemia, and hematologic malignancies, tuberculosis and other infectious diseases, insulin therapy, smoking, consuming any special diet, or taking omega-3 supplements were excluded from the study.
The study was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Ethic No. IR.MUI.REC.1394.1.298).

Anthropometric measurements

Participants' weight and height were measured in light clothing and without shoes by a portable calibrated electronic weighing scale and a portable measuring inflexible bar, respectively. Waist (at umbilicus) and hip (at widest point) circumferences were measured on subjects according to standard conditions using a measuring tape, then waist/hip circumference ratio was calculated. Body mass index (BMI) was calculated as the weight of individuals divided by the square of their height (kg/m^2). Blood pressure was measured using a sphygmomanometer after a 15 min rest, the mean of the three measurements of systolic and diastolic blood pressure (SBP and DBP) at intervals of 2-5 min was considered as the blood pressure. All measurements were taken by the same person to avoid subjective error.

Blood collection and processing

Peripheral blood (5 mL) was collected from subjects after an overnight fast (10-12 h) into two tubes containing ethylenediaminetetraacetic acid-K3 (EDTA-K3) as anti-coagulant and were transferred to the laboratory on ice within 2 h. One tube was immediately stored at -80 °C until the analysis of miRNA. Plasma was separated from the other tube by centrifugation at 2000 rpm for 10 min at room temperature. Plasma was used for the measurement of biochemical parameters as follows. FPG was measured by glucose oxidase (GOD) method, total cholesterol (TC), HDL-C, LDL-C, and TG levels were measured using an automatic analyzer (bt-4500, Biotecinca, Italy) and commercial biochemistry kits (Pars Azmoon kit, Iran). For specimens with plasma levels of TG < 400 mg/dL, LDL-C level was estimated indirectly using the Friedwald formula (24), Plasma insulin was measured by an immunoenzymometric assay using Monobind kit (USA). HOMA-IR was calculated using the following equation (25):

$$HOMA\text{-}IR = \text{Insulin}\ (\mu IU/mL) \times \text{glocuse}\ (mmol/L) / 22. \quad (1)$$

After separating the plasma, the remaining contents of the tubes were washed three times with an equal volume of isotonic saline and centrifuged at 2000 rpm for 5 min for removing the residual plasma and buffy coat and preparation of packed cells. The washed packed RBCs (hematocrit = 98%) were then transferred to micro-tubes containing butylated hydroxytoluene (BHT; 0.1 mg/mL of packed RBC) and were immediately stored at -80 °C until the extraction and analysis of erythrocyte membrane fatty acids.

Isolation of miRNAs and reverse transcription miRNA real-time polymerase chain reaction

Total small RNA was isolated from 500 µL of whole blood and 1500 µL RBC lysis buffer using Hybrid-RTM miRNA kit (GeneAll, Korea) according to the manufacturer's instructions, and were quantified using a NanoDrop spectrophotometer. Reverse transcription (RT) miRNA real-time polymerase chain reaction (PCR) was performed to determine the quantity of miR-122 and miR-33a (26). Samples were adjusted to a concentration of 5 ng/µL using nuclease-free water. A 2 µL aliquot of miRNA was used for producing cDNA using the miRCURY LNATM Universal RT microRNA PCR kit (Exiqon, Copenhagen, Denmark). The RT-PCR reactions for the whole blood miRNA assays were performed in a 10 µL final volume containing 4 µL of diluted cDNA using the SYBR Green master mix kit (Exiqon, Copenhagen, Denmark) and specific miR-122 and miR-33a primers (miR-33a primer sequence: GUGCAUUGUAGUUGCAUUGCA and miR-122 primer sequence UGGAGUGUACAAUGGUGUUUG)

UniSp6 RNA spike-in templates were used as the internal controls (artificial reference gene or housekeeping gene) for the assays of miR-122 and miR-33a. The StepOnePlus TM Real-Time PCR Systems (AB Applied Biosystem, USA) instrument was used for RT-PCR assay. The relative expression level of miRNA was quantified using threshold cycle (Ct) values normalized against internal control using the following equation:

$$\Delta CT = CT \text{ (target miR)} - CT \text{ (endogenous control)} \quad (2)$$

Extraction and preparation of erythrocytes fatty acids

RBCs membrane fatty acids were extracted and methylated in a one-step method with saponification according to Raquel and coworkers (27) as described elsewhere (28) with slight modification. Briefly, 150 µL of the packed cell was mixed with 100 µL of BHT (0.5 mg/mL in methanol) as an antioxidant, 25 µg heptadecanoic acid as internal standard and 1 mL methanolic NaOH solution (1 M) in airtight glass tubes. Tubes were flushed with N_2 and saponification of fatty acids was carried out by heating at 85 °C for 8 min. Thereafter, transmethylation was performed by adding 2 mL of H_2SO_4 (1 M) in methanol, flushing with N_2, and heating at 85 °C for 40 min. After cooling at room temperature 0.5 mL of a saturated solution of NaCl was added and the fatty acid methyl esters (FAMEs) were extracted with 2 mL of hexane. The hexane layer was separated and washed with 2 mL of analytical grade water and after removal of water was dried over 1 g of anhydrous sodium sulfate (Na_2SO_4). The hexane layer was removed under a gentle stream of N_2. Finally, the residue was reconstituted in 100 µL of hexane and aliquots of 1 µL were analyzed by gas chromatography-mass spectrometry (GC-MS; HP6890, Agilent Technologies) equipped with an SP-2560 column (100 m × 0.25 mm ID × 0.2 µm film thickness) and MSD detector (HP 5973). FAMEs were identified by comparing their retention times with those of standard FAMEs and also the spectra of known standards in the GC-MS library. FAMEs were quantitated using the area under the curve (ACU) by Chemstation software.

The concentration of each RBC membrane fatty acid was expressed as the average percent of the total area under the peaks (% of total fatty acids) (28). A standard mixture of FAMEs was analyzed and the instrument calibration and tuning were checked daily. In order to assess the repeatability of fatty acid analysis, a blood sample was analyzed on 8 different days spread over the time span of study samples analysis. The coefficient of variation (CV) for all the fatty acids analyzed was less than 7%, except for eicosapentaenoic acid which was 9.3%.

Statistical analysis

All PCR experiments were repeated three times and data were analyzed using the statistical software SPSS (version 23) and appropriate statistical tests. We determined whether each variable was distributed before statistical testing with the Kolmogorov-Smirnov test. General linear model analysis (GLM, Post hoc multiple comparison tests) followed by LSD correction for multiple comparisons was performed to see the differences in clinical indicators and biochemical parameters and also erythrocyte fatty acid composition between groups. The Pearson correlation analysis and analysis of covariance were used to relate variables to each other. The results are expressed as mean ± SD and levels of $P < 0.05$ were considered statistically significant.

RESULTS

Anthropometric and biochemical characteristics

Results of the anthropometric and biochemical variables in the three groups of the study are summarized in Table 1. FBG, TG, insulin, and HOMA-IR were significantly higher in the patients group compared to the control. In addition, there were significant differences in the components of MetS including weight, circumference, BMI, SBP, TG, HDL, insulin, and HOMA-IR between the two groups of patients as expected.

Table 1. Anthropometric and biochemical characteristics of the study subjects. Data are expressed as mean ± SD.

Variables	Control N = 50	Group I N = 50	Group II N = 50	*P*-value Group I *vs* control	*P*-value Group II *vs* control	*P*-value Group I *vs* group II
Age (years)	47.4 ± 9.2	55.9 ± 8.9	57.8 ± 8.5	< 0.001	< 0.001	0.56
Weight (kg)	72.6 ± 12.0	79.1 ± 13.0	86.4 ± 12.2	0.01	< 0.001	0.002
Waist (cm)	92.1 ± 9.7	95.5 ± 8.4	101.1 ± 8.7	0.08	< 0.001	0.002
BMI (kg/m^2)	25.0 ± 3.3	27.0 ± 3.4	30.3 ± 5.7	0.002	< 0.001	< 0.001
SBP (mmHg)	118.0 ± 10.3	119.0 ± 14.7	129.0 ± 17.1	0.73	0.001	0.001
DBP (mmHg)	78.0 ± 5.3	77.0 ± 7.5	80.0 ± 14.2	0.63	0.40	0.15
FPG (mg/dL)	96.4 ± 5.3	165 ± 43.0	156.7 ± 36	< 0.001	0.001	0.2
TG (mg/dL)	99.0 ± 45.5	149 ± 66.5	240.0 ± 126.4	0.007	< 0.001	< 0.001
TC (mg/dL)	174.1 ± 18.4	165.4 ± 34.3	177.0 ± 33.5	0.2	0.65	0.05
HDL-C (mg/dL)	50.7 ± 8.6	49.0 ± 6.6	42.7 ± 7.7	0.30	< 0.001	< 0.001
LDL-C (mg/dL)	102.4 ± 13.9	87.0 ± 34.9	89.5 ± 29.0	0.01	0.03	0.65
Insulin (μIu/mL)	4.15 ± 0.57	13.5 ± 2.2	9.1 ± 1.5	< 0.001	0.03	0.05
HOMA-IR	1.0 ± 0.14	5.8 ± 1.04	3.4 ± 0.43	< 0.001	0.03	0.02
Statins, n (%)	0 (0)	22 (44)	25 (50)	< 0.001*	< 0.001*	0.5

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; * = chi-square test; for patients *vs* controls comparison.

Table 2. Comparison of the miR-33a and miR-122 levels in study subjects.

Variable	Control	Group I	Group II	*P*-value Group I *vs* control	*P*-value Group II *vs* control	*P*-value Group I *vs* group II
ΔCT miR-33a	16.2 ± 3.9	12.3 ± 2.2	12.5 ± 2.8	< 0.001	< 0.001	0.8
ΔCT miR-122	15.2 ± 2.7	14.4 ± 2.1	13.2 ± 2.3	< 0.001	0.01	0.03

ΔCT, logarithmic changes threshold cycle

Characteristics of miR-33a and miR-122

In order to perform statistical analysis on the results of RT-PCR, the logarithmic changes in threshold cycle (ΔCT) data were used (29). A significant difference was observed in miR-33a expression between groups I and II compared with the control group, however, this difference was not significant between groups I and II patients. MiR-122 expression was significantly different between groups I and II of patients and between patient groups with control (Table 2). Lower ΔCT indicates higher expression of miRNAs.

The erythrocyte fatty acid composition

The results of erythrocytes fatty acids composition (% of total fatty acids) are shown in Table 3. No significant differences were observed in erythrocyte levels of stearic acid (C18:0), behenic acid (C22:0), lignoceric acid (C24:0), oleic acid (C18:1n-9), nervonic acid (C24:1n-9), eicosapentaenoic acid (C20:5n-3), docosahexaenoic acid (C22:6n-3), and dihomogamma-linoleic acid (C20:3n-6) between the group I and control and also group II and control except for dihomogamma-linoleic acid (C20:3n-6) which significantly increased in group II when compared to control. Overall, the membrane level of polyunsaturated fatty acids (PUFAs, omega 6 fatty acids (ω6), total unsaturated fatty acids (UFAs, and PUFAs to total saturated fatty acids (SFAs ratio were significantly reduced in groups I and II in comparison with the control while SFAs and SFAs/PUFs ratio were significantly increased.

Correlation between biochemical parameters and erythrocyte fatty acid profile with miRNA levels in all subjects

The results of the correlation between biochemical parameters and erythrocyte fatty acid profile with miRNA levels in all subjects are shown in Table 4. These data showed that miR-33a levels were positively correlated with FPG, TG, insulin, HOMA-IR, SFAs, and

SFAs/PUFAs ratio. Conversely, there were negative correlations with PUFAs, omega 6 fatty acids, and PUFAs/SFAs ratio. Similarly, miR-122 levels were positively correlated with FPG, TG, TC, insulin, HOMA-IR, SFAs, and SFAs/PUFAs ratio. There were negative correlations between miR-122 with levels of HDL-C, PUFA, omega6 fatty acids, and PUFAs/SFAs ratio (Table 4).

Table 3. Comparison of erythrocytes membrane fatty acid composition in the study subjects. Values are given as % of total fatty acids.

Variables	Control N = 50	Group I N = 50	Group II N = 50	*P*-value Group I *vs* control	*P*-value Group II *vs* control	*P*-value Group I *vs* group II
SFA	50.0 ± 1.8	55.3 ± 6.4	56.2 ± 5.7	< 0.001	< 0.001	0.4
Palmitic acid (C16:0)	26.4 ± 1.1	31.1 ± 5.0	31.7 ± 4.4	< 0.001	< 0.001	0.40
Stearic acid (C18:0)	23.4 ± 1.4	24.2 ± 2.2	24.3 ± 2.3	0.06	0.05	0.8
Behenic acid (C22:0)	0.06 ± 0.02	0.06 ± 0.03	0.06 ± 0.03	0.7	0.9	0.7
Lignoceric acid (C24:0)	0.15 ± 0.07	0.17 ± 0.08	0.18 ± 0.08	0.30	0.16	0.65
MUFA	13.1 ± 1.0	13 ± 1.6	12.8 ± 1.5	0.54	0.27	0.57
Oleic acid (C18:1n-9)	13 ± 1.0	12.8 ± 1.5	12.7 ± 1.5	0.50	0.23	0.54
Nervonic acid (C24:1n-9)	0.11 ± 0.06	0.1 ± 0.06	0.1 ± 0.06	0.54	0.62	0.90
PUFA	37.0 ± 1.7	31.6 ± 6	31.0 ± 5.3	< 0.001	< 0.001	0.52
Omega-3 fatty acid	4.6 ± 0.9	4.0 ± 1.7	3.9 ± 1.5	0.07	0.02	0.54
Eicosapentaenoic acid (20:5n-3)	0.16 ± 0.0 4	0.14 ± 0.1	0.13 ± 0.09	0.36	0.20	0.78
Docosapentaenoic acid (C22:5n-3)	1.34 ± 0.2	0.95 ± 0.6	0.94 ± 0.5	0.001	0.001	0.96
Docosahexsaenoic acid (C22:6n-3)	3.0 ± 0.8	2.9 ± 1.2	2.8±1.0	0.56	0.22	0.46
Omega-6 fatty acid	32.4 ± 1.6	27.5 ± 4.7	27.6±6.3	< 0.001	< 0.001	0.91
linoleic acid (C18:2n-6)	11.1 ± 1.7	8.8 ± 2.3	8.6±2.1	< 0.001	< 0.001	0.76
Dihomo-ɣlinoleic acid (C20:3n-6)	1.25 ± 0.3	1.26 ± 0.3	1.45±0.5	0.84	0.01	0.01
Arachidonic acid (C20:4n-6)	17.3 ± 1.5	15.6 ± 2.8	15.2±2.7	0.002	< 0.001	0.36
Docosatetraenoic acid (C22:4n-6)	2.5 ± 0.4	1.9 ± 0.9	1.8±0.8	< 0.001	< 0.001	0.60
Total *UFAs (%PUFAs + MUFAs)*	49.9 ± 1.7	44.5 ± 6.4	43.7±5.7	< 0.001	< 0.001	0.43
PUFAs/SFA ratio	0.74 ± 0. 5	0.58 ± 0.17	0.57±0.15	< 0.001	< 0.001	0.40
SFAs/PUFAs ratio	1 ± 0.08	1.3 ± 0.34	1.3±0.3	< 0.001	< 0.001	0.61

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UFA, unsaturated fatty acid.

Table 4. Correlation between biochemical parameters and RBC membrane fatty acids profile with miRNA levels in all subjects.

Variables	miR-33a		miR-122	
	r	*P*-value	r	*P*-value
FPG (mg/dL)	0.399	< 0.001	0.135	0.05
TG (mg/dL)	0.229	0.006	0.229	0.006
TC (mg/dL)	0.027	0.75	0.188	0.02
HDL-C (mg/dL)	-0.10	0.09	-0.159	0.02
Insulin (µIu/mL)	0.32	0.003	0.24	0.02
HOMA-IR	0.32	0.002	0.20	0.02
SFA	0.258	0.002	0.156	0.03
Palmitic acid (C16:0)	0.284	0.001	0.144	0.04
PUFA	-0.264	0.001	-0.166	0.02
Docosapentaenoic acid (C22:5n-3)	-0.129	0.06	-0.139	0.04
Omega-6 fatty acid	-0.294	< 0.001	-0.189	0.02
Linoleic acid (C18:2n-6)	-0.244	0.003	-0.155	0.03
Arachidonic acid (C20:4n-6)	-0.229	0.005	-0.157	0.03
Docosatetraenoic acid (C22:4n-6)	-0.20	0.01	-0.104	0.1
Total UFAs (%PUFAs+MUFAs)	-0.258	0.002	-0.156	0.03
PUFAs/SFA ratio	-0.263	0.001	-0.171	0.02
SFAs/PUFAs ratio	0.253	0.002	0.14	0.04

FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UFAs: unsaturated fatty acids; r, correlation coefficient.

DISCUSSION

The discovery of circulating miRNAs raised great hope for their use as endocrine signaling molecules and disease markers. In the present study, the relationship between erythrocyte membrane fatty acids and serum lipids profile and their association with miR-33a and miR-122 in T2DM men with and without MetS were investigated. According to the data presented in Table1, all the investigated cardiovascular risk factors e.g. elevated TG, SBP, weight, waist, BMI, and reduced HDL-C showed significant differences in group II in comparison to group I and controls. All the above parameters can, to different extents, adversely affect the normal physiological function of various systems in the body. For example, it is well known that exposure to prolonged hyperglycaemia can cause reversible and then irreversible changes to tissue metabolism and structure (30). Aging is also a major risk factor for the development of T2DM and MetS, and this has led some to believe that glucose intolerance, increased lipoperoxidation, generation of free radicals, and other complications are inevitable outcomes of aging (31).

T2DM is one of the most common causes of dyslipidemia. Vascular complications are believed to be critical for the prognosis of T2DM and its development. Although, it is believed that this depends on several factors such as duration and degree of control (32).

In this study, the mean concentration of LDL-C in both groups of patients was reduced significantly compared to the control. Although this appears to be due to the administration of the lipid-lowering drug statin in patients, adverse qualitative changes in LDL-C may be present. In particular, patients with T2DM and MetS tend to have a higher proportion of smaller and denser LDL particles, which are more susceptible to oxidation and may thereby increase the risk of cardiovascular diseases (33).

In addition, we found that miR-33a and miR-122 expression were increased in patients with T2DM and MetS compared to the control group. Similar findings have been reported in previous studies of metabolic syndrome and T2DM patients (14). There was a significant positive correlation between miR33a and miR-122 with FPG, insulin, HOMA-IR, and TG in all subjects. Moreover, miR-122 showed a significant positive correlation with TC and a negative correlation with HDL-C. MiRNAs have recently been revealed as key regulators of lipid metabolism, playing major roles in regulating cholesterol, fatty acid metabolism, insulin signaling, and glucose homeostasis. MiR-122 is probably the most studied miRNA and the first described for its role in regulating serum cholesterol and hepatic metabolism (14). Changes in miR-33a expression are paralleled to changes in SREBP-2 mRNA (34). Moreover, miR-33a represses the expression of ABCA1 mRNA, which increases the efflux of free cholesterol from cell to apolipoprotein A1. Since miR-33a targets genes involved in the fatty acid metabolism (carnitine palmitoyltransferase 1A, *CPT1A*; peroxisomal carnitine O-octanoyl transferase, *CROT*; and hydroxyacyl-CoA dehydrogenase of trifunctional protein, *HADHB*), its overexpression leads to reduced cellular fatty acid beta-oxidation (14).

In this study, we measured the fatty acids profile in the erythrocyte membrane. Because of the longer lifespan of erythrocytes, it has been suggested that fatty acids in the erythrocyte membrane may offer a better objective measurement than fatty acids measured in plasma, which may be affected by short-term intakes, length of fasting, etc. (35).

Significant differences have been revealed between SFA and PUFA levels and PUFAs/SFAs and SFAs/PUFAs ratios in T2DM and MetS compared to the controls. The highest PUFAs, UFAs, n-3 and n-6 fatty acids levels and PUFAs/SFAs ratios were seen in the controls and the lowest was found in group II patients, suggesting lower membrane fluidity (increased saturation) in group II. Several factors can influence this ratio such as diet, hepatic fatty acid synthesis, desaturase enzymes, hormones, and other chemicals. Erythrocytes in diabetic patients are known to be less flexible due to membrane rigidity, and this is considered to be part of the pathology seen in diabetic microvascular disorders (36). In this study, for the first time, we investigated

the correlation of miR-33a and miR-122 expression with the erythrocyte membrane fatty acids profile and observed significant correlations between the expression of miR-33a and miR-122 with the sum of SFAs and palmitic acid (C16:0) and SFAs/PUFAs ratio. Whereas, there were negative correlations with n6-PUFAs (linoleic acid, C18:2n-6; arachidonic acid, C20:4n-6; and docosatetraenoic acid, C22:4n-6) and PUFAs/SFAs ratio. Some studies have investigated the association between the fatty acid composition of erythrocyte membrane and the risk of diabetes alone and diabetes with MetS. These studies suggest that SFAs are associated with diabetes risk (37) while PUFAs reduce diabetes risk so that PUFAs can have a beneficial effect on the development or control of diabetes as activators of peroxisome proliferator-activated receptor-gamma; which stimulates the differentiation of pre-adipocytes to adipocytes, increases insulin receptors, and thus reducing insulin resistance (38). Omega-6 PUFAs, have anti-inflammatory properties suppressing the production of adhesion molecules, chemokines, and interleukins, all key mediators of the atherosclerotic process (39).

In this study, there were no significant correlations between miR-33a, TC, and LDL-C. In addition, no significant differences in TC were observed between patient groups and control and even LDL-C showed a significant reduction in patient groups compared to the control. This is thought to be due to statin therapy in our study subjects. Nevertheless, a positive significant correlation was found between TC and miR-122, the most abundant miRNA in the liver(40).

In summary, regarding the roles of miR-33a and miR-122 in cholesterol and lipids metabolism and glucose homeostasis, and their importance as risk factors in cardiovascular disorder as reported here and in previous studies, it may be suggested that the measurement of their expression may have value as additional biomarkers of cardiovascular diseases related to T2DM and T2DM with MetS.

In addition, considering that dysregulation of miR-33a and miR-122 may contribute to metabolic abnormalities associated with T2DM, it is suggested that their inhibition, for example by antisense oligonucleotides, may be considered as a therapeutic strategy for ameliorating cardiometabolic disorders, lowering LDL, and raising HDL levels, especially for MetS subjects.

CONCLUSION

Considering the roles of miR-33a and miR-122 in cholesterol and lipids metabolism, as important risk factors in cardiovascular disorders, and their significant correlation with RBC membrane fatty acids and serum lipids, it is concluded that the measurement of miR-33a and miR-122 expression may be used as potential additional biomarkers for cardiovascular disease risk calculations related to T2DM alone and T2DM with MetS. In addition, suppression of miR-33a and miR-122 may be explored as a therapeutic strategy to ameliorate cardiometabolic risk factors *e.g.* high LDL-C and TG or low HDL-C.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

M. Pourfarzam conceived the study. M. Pourfarzam and F. Masoudi designed experiments. F. Masoudi performed the experiments and analyzed the data. M.R. Sharifi contributed to experimental design and data analysis. M. Pourfarzam and F. Masoudi wrote the paper. All authors read and approved the final manuscript.

All authors confirmed that they have contributed to the intellectual content of this

paper and have met the following 3 requirements: significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; drafting or revising the article for intellectual content; and final approval of the published article.

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