

Integrated analysis of long non-coding RNA megacluster, microRNA-132 and microRNA-133a and their implications for cardiovascular risk and kidney failure progression in diabetic patients

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Abstract. Inefficient control of elevated blood sugar levels can lead to certain health complications such as diabetic nephropathy (DN) and cardiovascular disease (CVD). The identification of effective biomarkers for monitoring diabetes was performed in the present study. The present study aimed to investigate the implications of long non-coding RNA megacluster (lnc-MGC), microRNA (miR)-132 and miR-133a, and their correlation with lactate dehydrogenase (LDH) activity and glycated hemoglobin (HbA1c) levels to identify biomarkers for the early diagnosis of diabetes mellitus, induced DN and CVD. The present study included a total of 200 patients with type 2 diabetes, as well as 40 healthy subjects as controls. The diabetic patients were classified into six groups based on their estimated HbA1c level, glomerular filtration rate and LDH activity, while the healthy controls constituted the seventh group. Diabetic patients exhibited significant increases in parameters related to diabetes as fasting blood sugar, HbA1c levels, cardiac injury and kidney failure. Furthermore, the expression levels of TNF- α were significantly increased in the diabetic groups compared with healthy controls. Diabetic patients with cardiovascular dysfunction showed significantly increased expression levels of miR-132, miR-133a and lnc-MGC, compared with the healthy group. The expression of circulating miR-132 in blood was low in the groups of diabetic

patients compared with the healthy controls, and demonstrated a negative correlation with LDH and HbA1c levels. Expression levels of miR-132, miR-133a and lnc-MGC, along with their correlations with LDH and HbA1c levels, could be used to distinguish diabetic patients with reduced CVD from those at early stage diabetes, which indicated their potential as biomarkers for CV complications associated with diabetes mellitus in the future.

Introduction

Diabetes mellitus (DM) is a disease characterized by elevated blood glucose levels and is a prominent challenge for the healthcare system due to its high prevalence (1,2). DM can also result in vascular complications including corneal neuropathy and cardiovascular disease (CVD) (3). Endothelial dysfunction caused by DM is also characterized by increased vasoconstriction and a doubled risk of developing CVD (4,5). CVD pathophysiology encompasses a broad spectrum of disorders affecting the heart and blood vessels, including coronary artery disease (CAD), heart failure, arterial hypertension, atherosclerosis and stroke (6). An important risk factor for CV mortality is chronic kidney disease. A number of studies have previously reported that a lower estimated glomerular filtration rate (eGFR) is strongly associated with CVD and all-cause mortality in patients with DM (7,8). Increased cardiac-specific troponin content and insulin resistance (IR) levels [Homeostasis Model Assessment (HOMA)-IR] are also positively correlated with an increased risk of CVD (9,10). Disease development in patients with kidney neuropathy (DN) is induced by inflammation (11). Activation of nuclear factor NF- κ B, which serves crucial role in the inflammatory process in patients, induces the production of inflammatory chemokines, adhesion molecules and cytokines, such as TNF- α (12). DM is reported to increase the expression levels of TNF- α and nuclear factor erythroid 2-related factor 2 (Nrf2) (13). Induced TNF- α

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contributes to the progression of certain diseases, including psoriasis and rheumatoid arthritis (14). Nrf2 signaling is also involved in attenuating inflammation-associated pathogenesis in certain diseases, such as rheumatoid arthritis, asthma and atherosclerosis (15).

Presently, echocardiography is the most common diagnostic test used for identifying diabetic cardiomyopathy (DCM) (16). This method enables the concurrent identification of both structural and functional alterations in the myocardium (17). However, the use of echocardiography is not a cost-effective approach (18). Therefore, the development of new tools for blood-based diagnosis is needed to allow the identification of patients at risk of developing DCM. Although a number of biomarkers to identify certain inflammatory processes are currently well-known, more precise and novel molecular markers are needed to facilitate the early diagnosis of DM (19). Non-coding RNAs (ncRNAs) can be classified into small size ncRNAs, such as microRNAs (miRNAs) and long ncRNAs (lncRNAs). These ncRNAs are functional RNA molecules transcribed from the genome that are not translated into proteins (20). miRNAs consist of 18-24 nucleotides and regulate gene expression by silencing genes at the post-transcriptional level (21). Altering ncRNA expression in diabetic patients is linked to the pathophysiology of certain diseases and disorders including lung, heart and kidney diseases (22,23). Thus, ncRNAs could potentially act as therapeutic targets based on their regulatory roles. Among miRNAs, miR-132 has been reported to be a negative gene regulator that contributes to the complications of DM (24). miR-132 targets Nrf2, which induces renal injury (25) and mediates the expression of genes involved in TGF- β signaling and cell proliferation, such as Foxo3/p300, which regulates the progression of fibrosis (26).

In response to myocardial infarction, locked nucleic acid (LNA)-based anti-miR-132 treatment improves ejection fraction and ameliorates cardiac dysfunction (27). Additionally, in patients with heart failure, a synthetic LNA-based antisense oligonucleotide against miR-132 was reported to reverse heart failure *in vivo* (27). miR-133a also participates in mediating glucose-induced cardiomyocyte hypertrophy in DM (28). It is the most downregulated miRNA in the failing heart (29), which protects the heart against adverse remodeling (30). It has been previously reported that miR-133a expression is significantly reduced in the hearts of patients with DM (31). Reduced serum miR-133a levels are significantly associated with increased autophagy markers, which results in the exacerbation of DM-induced cardiac hypertrophy (31). Increased miR-133a expression could prevent hypertrophy in the hearts of patients with DM by decreasing the expression of the early cardiac hypertrophy marker β -myosin heavy chain (β -MHC) (32). The lnc-RNA megacuster (lnc-MGC), which is typically >200 nucleotides in length and does not encode proteins, plays a role in controlling DM-induced renal fibrosis, which suggests its involvement in the pathogenesis of kidney diseases (33). Human lnc-MGC shares exons with other lncRNAs, such as maternally expressed gene 8 and maternally expressed gene 9 (34). lnc-MGC is upregulated under conditions associated with DM, such as high glucose and TGF- β levels (34). Inhibition of lnc-MGC decreases the expression of key cluster kidney miRNAs (such as miR-379, miR-494, miR-495 and miR-377) which triggers early DN (35). lnc-MGC upregulation in human

cardiac fibroblasts under mechanical stress conditions was also associated with the downregulation of miR-133a expression (36). ncRNAs are useful as biomarkers as they are easily accessible and can be extracted through liquid biopsies from bodily fluids. They can detect changes that occur as a disease advance and have high specificity for tissue and cell type (37). Compared with the production of novel antibodies to target protein biomarkers, developing new assays for the detection of nucleic acids requires less time and lower costs (38). Overall, this highlights the future potential for the utility of ncRNAs in both clinical and personalized medicine.

To date, the relationship between circulating miR-133a, miR-132 and lnc-MGC expression levels in patients with DM and CVDs, glycemic biomarkers in DN, and the inflammatory biomarkers Nrf2 and TNF- α has not been reported. Thus, the present study aimed to identify the potential of serum miR-133a, miR-132 and lnc-MGC to act as molecular biomarkers for DN and CVDs and examine their interactions with inflammatory biomarkers. This has the potential to contribute to predicting the progression of diabetic cardiomyopathy and nephropathy in the future.

Materials and methods

Experimental design. A total of 200 type 2 diabetic patients who attended the specialized DM and nephrology clinics at the Internal Medicine Department of Beni-Suef University Hospital, (Beni-Suef, Egypt) from November 2021-May 2022 provided written informed consent for participation in the present study and agreed to the use of their samples in scientific research. Eligible patients were classified according to the value of their eGFR into six groups. Furthermore, 40 healthy subjects were included in the study as controls. The study protocol followed the Declaration of Helsinki and good clinical practice guidelines and was approved by the Ethics Committee of Beni-Suef University Hospital (approval no. BSU:7-2021; Beni-Suef, Egypt).

Healthy individuals, diabetic patients without nephropathy [glycated hemoglobin (HbA1c) >6.5%; eGFR ≥ 60 ml/min/1.73 m²], and diabetic patients with nephropathy [HbA1c >6.5%; eGFR <60 ml/min/1.73 m²] were enrolled in the present study. The present study excluded patients with any history of chronic and acute infections, diabetic retinopathy, hepatic diseases, malignancy and other endocrine dysfunctions. According to the clinical data and eGFR values, enrolled participants were classified into seven groups as follows: i) Healthy controls (n=40); ii) G1, eGFR ≥ 90 ml/min/1.73 m² (n=35); iii) G2, eGFR 60-89 ml/min/1.73 m² (n=30); iv) G3a, eGFR 45-59 ml/min/1.73 m² (n=30); v) G3b, eGFR 30-44 ml/min/1.73 m² (n=35); vi) G4, eGFR 15-29 ml/min/1.73 m² (n=30); and vii) G5, eGFR <15 ml/min/1.73 m² (n=40). The G5 group of patients was classed as being in kidney failure. The sample size for the present study was determined based on several factors, including the desired level of confidence, expected effect sizes of the biomarkers, variability within the population and statistical power considerations. Specifically, the aim was to achieve a power of 80% to detect statistically significant differences in biomarker expression levels between diabetic patients with and without complications, as well as among different severity

groups of diabetic nephropathies. With each group comprising 30-40 participants and considering an effect size of 0.5, the sample size was deemed sufficient for detecting medium to large effect sizes.

Blood sample collection. A total of two blood samples (4 ml/sample) were collected from healthy controls and diabetic participants after overnight fasting. EDTA was used in the collection of one of the blood samples and the other was collected using a plain collection tube. Samples were incubated for 30 min at room temperature and blood in the plain tubes was centrifuged at $4,000 \times g$ at 4°C for 20 min to isolate serum. Blood samples in EDTA were used for complete blood count, DNA extraction and HbA1c level measurements. Samples were stored at -80°C until used.

Biochemical analyses. HbA1c was measured using a Stanbio™ Glycohemoglobin (HBA1 and HBA1C) Pre-Fil™ Test (cat. no. SB-P350-50; Stanbio), while fasting blood sugar (FBS), Na^{+} , Ca^{2+} and K^{+} levels were determined in serum samples using a commercial SPINREACT diagnostic kit (cat. nos. 1001380, MD1001065 and #1001390 for Na^{+} , Ca^{2+} and K^{+} , respectively; Spinreact, S.A.U.). Blood uric acid was measured using a commercial SPINREACT diagnostic kit (cat. no. MD41001; Spinreact, S.A.U.). Serum activity of glutamate pyruvate transaminase (sGPT) and glutamate oxaloacetate transaminase (sGOT) enzymes were measured using a commercial SPINREACT GOT/AST diagnostic kit (cat. no. MD41264; Spinreact, S.A.U.) according to the manufacturer's instructions. Blood urea (BUN reagent; cat. no. BK-443350D) and creatinine uric acid levels (creatinine reagent kit; cat. no. BK-472525D) were determined in the serum samples (Diamond Diagnostics). Fasting insulin levels were assayed using Diagnostic Products Corporation radioimmunoassay kits (Coat-A-Count; cat. no. TKIN-4; Diagnostic Products Corporation). Insulin resistance was investigated by calculating the HOMA-IR. $\text{HOMA-IR} = [(\text{fasting insulin, } \mu\text{U/ml}) \times (\text{fasting glucose, mmol/l})] / 22.5$, where 22.5 is the normalizing factor.

The glomerular filtration rate (eGFR) was measured based on serum creatinine levels and calculated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Moreover, the CKD-EPI value was calculated taking into account sex and serum creatinine (39). Females: $\text{Creatinine} \leq 0.7 \text{ mg/dl}$: $\text{eGFR} = 144 \times (\text{creatinine}/0.7)^{-0.329} \times (0.993)^{\text{age}} \times 1.159$ (for Black or African American individuals). $\text{Creatinine} > 0.7 \text{ mg/dl}$: $\text{eGFR} = 144 \times (\text{creatinine}/0.7)^{-1.209} \times (0.993)^{\text{age}} \times 1.159$ (for Black or African American individuals). Males: $\text{Creatinine} \leq 0.9 \text{ mg/dl}$: $\text{eGFR} = 141 \times (\text{creatinine}/0.9)^{-0.411} \times (0.993)^{\text{age}} \times 1.159$ (for Black or African American individuals). $\text{Creatinine} > 0.9 \text{ mg/dl}$: $\text{eGFR} = 141 \times (\text{creatinine}/0.9)^{-1.209} \times (0.993)^{\text{age}} \times 1.159$ (for Black or African American individuals). Adjustments for Black or African American individuals were made in eGFR calculations to account for higher average serum creatinine levels in Black individuals for more accurate kidney function estimates (40). The calculation used for the rest of the individuals studied in this study who were not of Black/African American ethnicity was based on the following CKD-EPI creatinine-cystatin equation (41) $\text{eGFR} = 135 \times$

$\min(\text{SCr}/\kappa, 1)^{\alpha} \times \max(\text{SCr}/\kappa, 1)^{-0.544} \times \min(\text{Scys}/0.8, 1)^{-0.323} \times \max(\text{Scys}/0.8, 1)^{-0.778} \times 0.9961^{\text{Age}} \times 0.963$ [if female], where eGFR=estimated GFR in $\text{ml/min}/1.73 \text{ m}^2$, SCr=standardized serum creatinine in mg/dl , Scys=standardized serum cystatin C in mg/l , $\kappa=0.7$ (females) or 0.9 (males), $\alpha=-0.219$ (females) or -0.144 (males), min=indicates the minimum of SCr/ κ or 1, max=indicates the maximum of SCr/ κ or 1, age=years. ELISA kits were used to measure serum TNF- α (cat. no. SEA133Mu; Cloud-Clone Corp.), cardiac troponin I (Human Cardiac Troponin IELISA Kit; cat. no. ab200016; Abcam) and Nrf2 levels (cat. no. #80593-1-PBS; Wuhan Fine Biological Technology Co., Ltd.).

To investigate hyperglycemia-induced cardiac injury, circulating creatine kinase (CK) activity, CK-myocardial band (MB) activity, lactate dehydrogenase (LDH) activity and troponin I levels were measured. Manufacturers' instructions were followed to determine the ELISA CK-MB (Rat CK-MB ELISA kit; cat. no. DEIA-FN285; Creative Diagnostics), troponin I (cat. no. ELH-Troponin1-1; RayBiotech, Inc.) and LDH (Rat LDH kit; cat. no. LS-F5026; LifeSpan Biosciences, Inc.).

miR-133a, miR-132 and lnc-MGC expression assays. miRNA was extracted from serum samples using the Plus kit (cat. no. R2072; Zymo Research Corp.). Isolated RNA samples were reverse-transcribed, then miR-133a, miR-132 and lnc-MGC expression levels were determined via reverse-transcription quantitative PCR (RT-qPCR) using the One-Step RT-PCR kit (cat. no. 12594100; Thermo Fisher Scientific, Inc.). The cycling conditions for RT-qPCR started with initial HotStar Taq DNA Polymerase activation step at 95°C for 15 min, then 40 cycles each of three steps (94°C for 15 sec, 55°C for 30 sec, and 70°C for 30 sec), and then the dissociation curve stage was added to verify specificity of the PCR products. The primer sequences for GAPDH were obtained from OriGene Technologies, Inc. (cat. no. HP205798) and were used as internal reference controls. The primer sequences for miR-132, miR-133a and lnc-MGC were designed using the National Center for Biotechnology Information Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primer sequences used were as follows: miR-123 (accession no. NC_000017.11) forward (F), 5'-CGACCATGGCTGTAGACTGT-3' and reverse (R), 5'-GTCTCCAGGGCAACCGTG-3'; miR-133a (accession no. NC_000018.10) F, 5'-TTTGGTCCCCCTTCAACC-3' and R, 5'-GAACATGTCTGCGTATCTCA-3'; lnc-MGC (accession no. MW802745) F, 5'-GCTACAGCTGGTTGAAGG G-3' and R, 5'-TGCTTTGCTAGAGCTGGTAAAATG-3'; small nucleolar RNA C/D box 68 (SNORD68; accession no. NC_000016.10) F, 5'-CGTGATGACATTCTCCGGAAT C-3' and R, 5'-AATCAGATGGAAAAGGGTTCAAATG-3'; and GAPDH (accession no. NM_002046) F, 5'-GTCTCC TCTGACTTCAACAGCGC-3' and R, 5'-ACCACCCTG TTGCTGTAGCCAA-3'. SNORD68 was used as an endogenous housekeeping gene for miR-132 and miR-133a as its expression remains stable and does not vary under different experimental conditions or in different states of the same sample (for example, 'disease' vs. 'normal' samples) (42-44). The relative quantities of each target gene were measured and standardized against the specified internal control according to the $2^{-\Delta\Delta\text{C}_q}$ method (45).

Statistical analysis. Statistical analysis was conducted using SPSS (version 16; SPSS, Inc.). Data were presented as mean $\pm$ SEM (46) and statistical comparisons were carried out using a one-way analysis of variance (ANOVA) with Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. Multiple testing corrections were carried out using the false discovery rate (Table SI). Receiver operating characteristic (ROC) curves were generated by plotting sensitivity against 1-specificity at different cut-off values. The diagnostic accuracy for each cut-off point was assessed using the area under the ROC curve (AUC). A performance level of $\geq 50\%$ was deemed acceptable.

Results

Demographic indices and clinical characteristics of the study patients. Based on the clinical characteristics, the study participants were grouped into a healthy control group and six patient groups (Table I). The age range of the study participants was 41-65 years. The mean age of the healthy group (43 years) was significantly lower compared with that of the patient groups (mean range, 50-63 years; $P = 0.001$). The sex distribution of the individuals in the healthy and patient groups was not significantly different. Patient groups demonstrated significantly higher obesity indices as indicated by BMI, compared with the body weights recorded in the healthy group ($P = 0.001$). Furthermore, diabetes-related parameters such as systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, FBS and the activity levels of sGPT and sGOT enzymes were significantly higher in the patient groups compared with the healthy controls ($P < 0.001$), particularly in patient groups G3-G5. According to the present results, both sGPT and sGOT enzyme level in healthy and all diabetic groups were in the range from 21-27 U/l, which is within the normal range of sGPT (7-56 U/l) sGOT (5-40 U/l) (https://www.medicinenet.com/liver_blood_tests/article.htm).

High blood pressure was observed in the patients of G1-G5 with values of 124-146 mmHg for SBP and 82-90 mmHg for DBP. Moreover, there were significant increases in both FBS and HbA1c levels in the diabetic groups compared with the healthy group.

Hyperglycemia-induced kidney failure. Kidney function-related parameters including urine, creatinine, urea, uric acid and mineral (Na^+ , K^+ and Ca^{2+}) levels in serum, as well as GFR, were measured (Table II). There was a significant increase in the concentrations of urea, uric acid, and creatinine in the patient urine in diabetic groups (G1-G5) compared with the healthy group ($P = 0.001$). These increases were more pronounced in the diabetic groups G3b-5, where the concentration of creatinine increased by 78, 233 and 711% ($P = 0.001$), respectively, compared with the healthy group. In G2 diabetic group, there was also a significant increase in serum Na^+ , also known as hypernatremia, of 5.8% ($P = 0.001$), compared with the healthy group. This indicated the presence of a common type of electrolyte abnormality due to osmotic diuresis-induced hypotonic losses increasing serum Na^+ levels (47). Similar to the observed increase in Na^+ levels, the level of serum K^+ was increased, and these increases were positively correlated with the increase in diabetic indices. There was a steady increase in

Table I. Clinical characteristics of healthy controls and groups of patients with diabetes and diabetic nephropathy.

Patient group	Age, years (mean $\pm$ SEM)	Male, n	Female, n	BMI, kg/m^2 (mean $\pm$ SEM)	Systolic blood pressure, mmHg (mean $\pm$ SEM)	Diastolic blood pressure, mmHg (mean $\pm$ SEM)	Fasting blood, mg/dl (mean $\pm$ SEM)	Glycated hemoglobin, % (mean $\pm$ SEM)	Insulin, $\mu\text{U}/\text{l}$ (mean $\pm$ SEM)	Serum glutamic pyruvic aminotransferase, u/l (mean $\pm$ SEM)	Serum glutamate oxaloacetate transaminase, u/l (mean $\pm$ SEM)
Healthy controls	43.0 $\pm$ 1.20	22	18	28.3 $\pm$ 0.36	124.5 $\pm$ 1.13	82.8 $\pm$ 0.62	82.1 $\pm$ 0.98	4.7 $\pm$ 0.06	11.1 $\pm$ 0.10	21.8 $\pm$ 0.7	24.1 $\pm$ 0.6
G1	50.8 $\pm$ 0.73 ^a	15	17	33.3 $\pm$ 0.61 ^a	126.2 $\pm$ 0.99	83.0 $\pm$ 0.74	170.2 $\pm$ 4.59 ^a	8.8 $\pm$ 0.12 ^a	10.4 $\pm$ 0.10 ^a	22.9 $\pm$ 0.8	24.4 $\pm$ 0.82
G2	55.6 $\pm$ 1.16 ^a	18	17	31.8 $\pm$ 0.79 ^a	131.0 $\pm$ 1.65	87.1 $\pm$ 0.92	196.0 $\pm$ 9.22 ^a	9.6 $\pm$ 0.18 ^a	8.4 $\pm$ 0.06 ^a	23.9 $\pm$ 1.16	26 $\pm$ 0.83
G3a	60.1 $\pm$ 0.98 ^b	14	16	31.1 $\pm$ 0.48 ^a	141.7 $\pm$ 2.01 ^a	87.7 $\pm$ 1.08	184.5 $\pm$ 9.36 ^a	9.0 $\pm$ 0.19 ^a	8.05 $\pm$ 0.05 ^a	22.6 $\pm$ 1.17	26.6 $\pm$ 1.11
G3b	62.7 $\pm$ 0.99 ^b	19	14	33.8 $\pm$ 0.73 ^a	143.2 $\pm$ 1.73 ^a	89.5 $\pm$ 1.10 ^a	187.5 $\pm$ 7.06 ^a	9.7 $\pm$ 0.13 ^a	7.9 $\pm$ 0.04 ^a	22.7 $\pm$ 0.85	27 $\pm$ 0.85
G4	63.7 $\pm$ 1.04 ^b	19	13	30.5 $\pm$ 0.57 ^a	147.9 $\pm$ 2.57 ^b	91.6 $\pm$ 1.73 ^a	168.6 $\pm$ 3.78 ^a	9.4 $\pm$ 0.12 ^a	7.8 $\pm$ 0.06 ^b	25.1 $\pm$ 0.85 ^a	26.7 $\pm$ 0.82
G5	58.3 $\pm$ 1.15 ^a	24	14	32.1 $\pm$ 0.80 ^a	146.4 $\pm$ 4.04 ^b	90.1 $\pm$ 2.27 ^a	178.8 $\pm$ 7.59 ^a	9.1 $\pm$ 0.27 ^a	7.9 $\pm$ 0.06 ^a	22.5 $\pm$ 1.1 ^a	23.8 $\pm$ 1.01

Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test to compare the significance among all groups. ^a $P < 0.05$, ^b $P < 0.01$ vs. healthy controls. G1, diabetes without kidney neuropathy; G2, diabetes with mild renal impairment; G3a-diabetes with severe renal impairment; G3b, diabetes with severe renal impairment and mild cardiovascular disease; G4 diabetes with severe renal impairment and moderate cardiovascular disease; G5 diabetes with severe renal impairment and severe cardiovascular disease.

Table II. Kidney function tests of healthy controls and groups of patients with diabetes and diabetic nephropathy.

Patient group	Creatinine, mg/dl	Urea, mg/dl	Uric acid, mg/dl	Na ⁺ , mEq/l	K ⁺ , mEq/l	Ca ²⁺ , mg/dl	Estimated glomerular filtration rate, ml/min/1.73 m ²
Healthy controls	0.9±0.01	22.9±0.55	4.31±0.08	140.4±0.76	4.4±0.07	9.3±0.07	93.0±0.97
G1	0.94±0.02	24.7±0.70	4.95±0.13 ^a	144.2±0.93	4.4±0.08	9.1±0.09	93.1±0.44
G2	1.0±0.03	28.0±0.76	5.39±0.13 ^a	144.3±0.91	4.4±0.09	8.9±0.11	69.5±0.96 ^a
G3a	1.2±0.03	36.3±1.29 ^a	5.88±0.14 ^b	147.7±1.06 ^a	4.5±0.09	8.6±0.12 ^a	52.1±0.83 ^a
G3b	1.6±0.05 ^a	51.3±2.17 ^b	6.30±0.16 ^b	148.6±1.46 ^a	4.6±0.08	8.9±0.11	39.7±0.72 ^b
G4	3.0±0.09 ^b	90.5±2.53 ^c	6.31±0.17 ^b	148.1±1.83 ^a	6.11±0.06 ^a	8.9±0.12	20.2±0.47 ^b
G5	7.3±0.38 ^c	114.2±4.43 ^c	6.51±0.18 ^b	134.3±0.85 ^b	5.7±0.13 ^a	7.9±0.14 ^a	7.9±0.45 ^c

Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test to compare the significance among all groups. ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. healthy controls. mEq, milliequivalents; G1, diabetes without kidney neuropathy; G2, diabetes with mild renal impairment; G3a-diabetes with severe renal impairment, G3b, diabetes with severe renal impairment and mild cardiovascular disease; G4 diabetes with severe renal impairment and moderate cardiovascular disease; G5 diabetes with severe renal impairment and severe cardiovascular disease.

the concentration across different diabetic groups, recording high increased values for the G5 group (29.5%) for K⁺ levels compared with the healthy group. On the other hand, Ca²⁺ levels and GFR, a sensitive indicator of kidney function (a low GFR value indicates that the kidneys are not functioning properly), were significantly decreased (P=0.001) compared with healthy controls. The G5 group demonstrated a lower level of Ca²⁺ (7.9±0.14) compared with the normal serum Ca²⁺ level of the healthy control group (9.3±0.07) and the other diabetic groups (G1-G4) (8.9-9.1 mg/dl). In contrast to the G1 group, there was a significant decrease in GFR across the other diabetic groups and the lowest value was recorded in the G5 group (7.9 ml/min/1.73 m²).

Hyperglycemia increased the risk of heart failure in the diabetic group. To investigate hyperglycemia-induced cardiac injury, the circulating LDH and troponin levels and CK and CK-MB enzyme activity were measured (Fig. 1). Compared with the healthy control group, the diabetic patients, mainly G5, demonstrated a marked increase in the majority of measured cardiac-related parameters. The mean serum LDH cholesterol (P<0.0001) and troponin (P<0.01) levels of diabetic patients in the G5 group, were higher than those compared with the healthy control group, respectively (Fig. 1A and B). Overall, a positive correlation was demonstrated between LDH and HbA1C levels (r=0.48; Table III). The mean serum CK and CK-MB levels were also significantly higher in all diabetic groups (G5) compared with the healthy control group (P<0.0001 and P<0.001, respectively) (Fig. 1C and D). For all the aforementioned parameters, there was an increase demonstrated across the diabetic groups, with the highest levels of CK and CK-MB in the serum of diabetic patients in the G4 and G5 groups. Overall, patients with increased levels of LDH, troponin, CK and CK-MB were potentially associated with the risk of heart failure in the diabetic groups.

Inflammatory cytokine biomarkers. TNF-α and Nrf2 levels were measured as they are cytokine biomarkers of

inflammation (Fig. 2). These results demonstrated that the levels of TNF-α were significantly increased in G2-G5 groups compared with the healthy controls, with the G3a, G4 and G5 groups demonstrating the highest levels of TNF-α. Nrf2 levels were significantly reduced in all diabetic groups compared with the control group, with the lowest values demonstrated in the G4 and G5 groups (-66.6 and -73.33%, respectively).

Expression patterns of ncRNAs as pre-diagnostic molecular biomarkers for CVD. To investigate the molecular biomarkers for CVD, expression levels of the ncRNAs miR-132, miR-133a and lnc-MGC were measured using quantitative RT-qPCR (Fig. 3A-C). Lower expression levels of miR-132 in blood were demonstrated in diabetic patients compared with healthy controls (Fig. 3A). Serum miR-132 expression was steadily decreased across diabetic groups and the lowest expression levels were demonstrated in the G4 and G5 groups of patients (-83.33 and -91.67%, respectively). Compared with the healthy controls, the expression levels of circulating miR-133a were significantly higher in diabetic patients of G2-G5 who showed impaired cardiovascular function (increased LDH and troponin I levels and CK and CK-MB enzyme activity) (Fig. 3B). The expression levels of serum miR-133a in diabetic patients steadily increased across diabetic groups and reached the highest expression level in patients in the G5 group (Fig. 3B). There was an increase in the expression levels of lnc-MGC in diabetic patients compared with healthy controls, with the maximum lnc-MGC expression level recorded in the G5 group (729.29%) (Fig. 3C). The expression level of miR-133a (r=0.326; P<0.01) was negatively correlated with the levels of LDH and HbA1C (Table III). However, serum miR-132 was positively correlated with HbA1C (r=0.382; P<0.01).

Given the upregulated expression levels of miR-132, miR-133a and lnc-MGC in diabetic patients, particularly those of G3-G5, their role as potential prognostic markers was investigated using ROC curves. Serum miR-132 was differentially expressed in participants with DN compared with healthy controls, with a 93.20% sensitivity and a 100.00% specificity

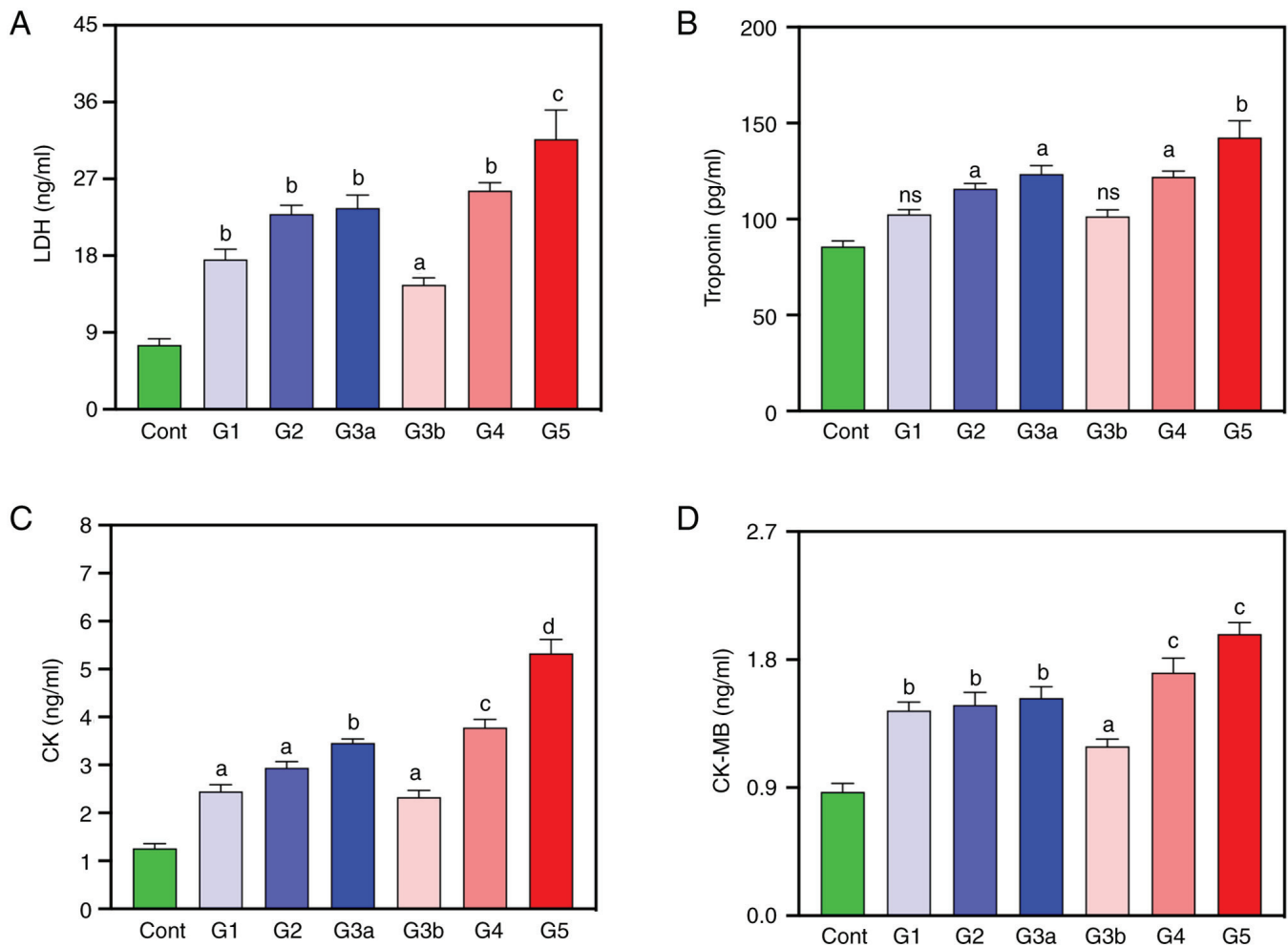


Figure 1. Cardiac profile of healthy controls and diabetic patients. (A) LDH, (B) troponin, (C) CK and (D) CK-MB levels. Data are expressed as mean $\pm$ SEM. (not significant), * $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$ vs. healthy controls. LDH, lactate dehydrogenase; CK, creatine kinase; CK-MB, CK-myocardial band; Cont, healthy controls; G1, diabetes without kidney neuropathy; G2, diabetes with mild renal impairment; G3a-diabetes with severe renal impairment, G3b, diabetes with severe renal impairment and mild cardiovascular disease; G4 diabetes with severe renal impairment and moderate CVD; G5 diabetes with severe renal impairment and severe cardiovascular disease.

(AUC=0.93; 95% CI=0.886-0.95; $P < 0.001$; Fig. 4A). Similarly, serum miR-133a expression levels also differentiated the patients of G2-G5 with DN from healthy controls, with 100.00% sensitivity and 100.00% specificity (AUC=0.998; 95% CI=0.993-1.01; $P < 0.001$; Fig. 4B), thus suggesting its potential utility as a biomarker of CVD. lnc-MGC was differentially expressed in participants with DN compared with healthy controls, with a 97.20% sensitivity and a 100.00% specificity (AUC=0.951; 95% CI=0.891-0.954; $P < 0.001$; Fig. 4C). In addition, serum miR-132 expression levels were shown to differentiate the of G3b, G4 and G5 stages of DN from the G2 and G3a stages of DN with 43.90% sensitivity and a 94.80% specificity (AUC=0.74; $P < 0.001$; Fig. 5A). Similarly, serum miR-133a and lnc-MGC expression level also showed similar results (Fig. 5B-C).

Discussion

Patients with type 2 diabetes are prone to developing heart conditions. Chronic hyperglycemia, as observed in diabetics, can induce DN and potentially lead to chronic kidney and heart failure (48). While several biomarkers are currently

available for monitoring DN and CVD, their efficiency is limited by issues of specificity, sensitivity and validation (49). Thus, the effective diagnosis and early treatment of DN and CVDs requires the identification of more sensitive molecular markers. In the present study, it was demonstrated that serum expression levels of miR-133a, miR-132 and lnc-MGC could potentially be used to detect DN and CVD progression in DM patients with a high sensitivity.

In the present study, HbA1c, creatinine, urea, LDH, troponin I, CK and CK-MB levels increased with GFR used as a measure of CVD severity. In line with the findings of the present study, the aforementioned cardiac biomarker levels were previously reported to be significantly higher in diabetic individuals compared with healthy individuals (50,51), which can lead to impaired cardiac function (52-54). Conversely, fasting insulin, Ca^{2+} and eGFR levels decreased with DN severity. Fasting insulin and eGFR levels were lower in all diabetic groups compared with the healthy group. A decrease in GFR has been linked to increased renal damage and reduced kidney function (55). In diabetic patients, glucose loss through the kidneys induces hyperosmotic urination that results in water and electrolyte loss (56). Similar to the results

Table III. Correlation analysis between biochemical and molecular diabetics and molecular indicators of diabetes and CVD of healthy individuals and groups of patients with diabetes.

A, HbA1C

Correlation analysis	HbA1C	LDH	miR-132	miR-133a	lnc-MGC
r	1.00	480.00 ^b	0.31 ^b	-0.42 ^b	-459.00 ^a
Significant (2-tailed)	N/A	0	0	0	0
N	238	70	179	171	177

B, estimated glomerular filtration rate

Correlation analysis	HbA1C	LDH	miR-132	miR-133a	lnc-MGC
r	-0.51 ^a	-0.61 ^a	-0.76 ^a	0.81 ^b	0.79 ^a
Sig. (2-tailed)	0	0	0	0	0
N	238	70	179	171	177

C, LDH

Correlation analysis	HbA1C	LDH	miR-132	miR-133a	lnc-MGC
r	0.480 [*]	1	0.618	-0.531 ^a	-0.564 ^a
Sig. (2-tailed)	0	N/A	0	0	0
N	70	70	70	69	70

D, miR-132

Correlation analysis	HbA1C	LDH	miR-132	miR-133a	lnc-MGC
r	0.316 ^a	618	1	-0.576 [*]	-0.624 ^a
Sig. (2-tailed)	0	0	N/A	0	0
N	179	70	179	169	175

E, miR-133a

Correlation analysis	HbA1C	LDH	miR-132	miR-133a	lnc-MGC
r	-571 ^b	-0.551 ^a	-620 ^b	1	0.643 ^a
Sig. (2-tailed)	0	0	0	N/A	0
N	171	69	169	171	171

F, lnc-MGC

Correlation analysis	HbA1C	LDH	miR-132	miR-133a	lnc-MGC
r	-0.459	-0.529	-0.631	743 ^b	1
Sig. (2-tailed)	0	0	0	0	N/A
N	177	70	175	171	177

^aP<0.05, ^bP<0.01. HbA1C, glycated hemoglobin; LDH, lactate dehydrogenase; lnc-MGC, long non-coding RNA megacuster; miR, microRNA; N/A, not applicable. N stands for the sample size and r stands for correlation coefficient.

reported in the present study, the clinical presentation of DN has been reported to include albuminuria, hyperkalemia, declining GFR and high creatinine levels (2). When acute

myocardial infarction occurs, the inflammatory reaction leads to the increase of serum inflammatory cytokine levels, such as TNF- α and IL-1 β (57). Increased TNF- α levels can

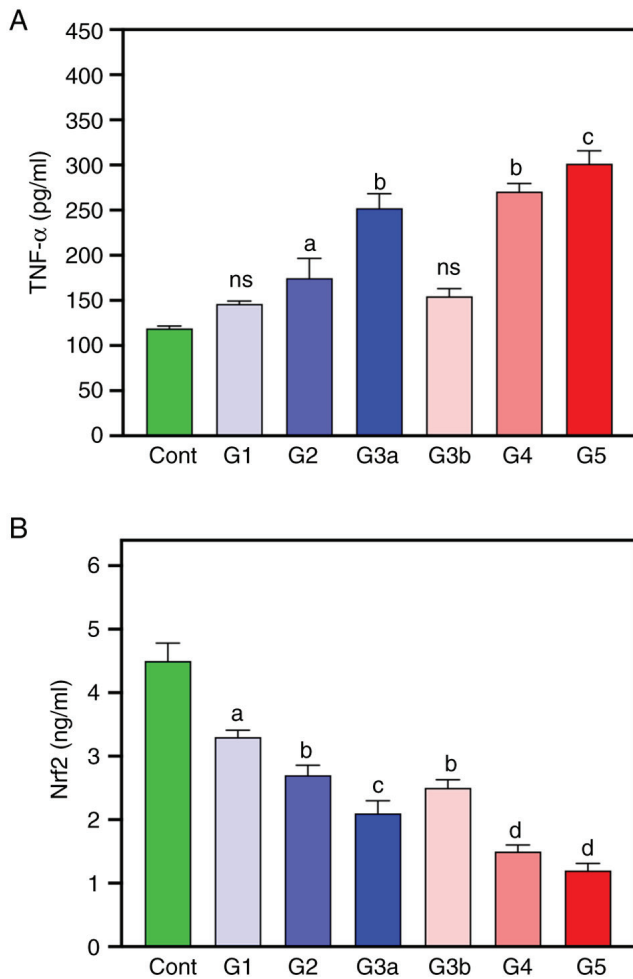


Figure 2. Inflammatory cytokine biomarkers were measured in healthy controls and diabetic patients. (A) 'TNF- α ' and (B) Nrf2 levels were measured in healthy controls and diabetic groups. Data are expressed as mean $\pm$ SEM. ns (not significant), ^aP<0.05, ^bP<0.01, ^cP<0.001, ^dP<0.0001 vs. healthy controls. Nrf2, nuclear factor erythroid 2-related factor 2; Cont, healthy controls; G1, diabetes without kidney neuropathy; G2, diabetes with mild renal impairment; G3a-diabetes with severe renal impairment, G3b, diabetes with severe renal impairment and mild cardiovascular disease; G4 diabetes with severe renal impairment and moderate cardiovascular disease; G5 diabetes with severe renal impairment and severe cardiovascular disease.

activate inflammatory signaling pathways, contributing to complications of DM (58). The present study demonstrated that diabetic patient groups G3-G5 exhibited significantly higher TNF- α levels compared with the healthy group, with a marked variation in TNF- α levels between the late and early stages of DN. A previous study reported that patients with DM-induced chronic kidney disease showed higher serum TNF- α levels (59). However, miR-132 may protect myocardial cells by downregulating expression of the inflammatory factors TNF- α and IL-6, which reduces the inflammatory response (60). miR-132 can also target Nrf2 (25) and mediate genes involved in TGF- β signaling and cell proliferation (26).

miRNAs serve a critical role in cardiovascular diseases such as arrhythmias, hypertrophy, heart failure and atherosclerosis (61). The present study demonstrated a negative correlation between the development of CVD and the expression levels of miR-132 and lnc-MGC, in addition to the decreased expression of miR-133a. Both miR-132 and miR-133 expression levels are

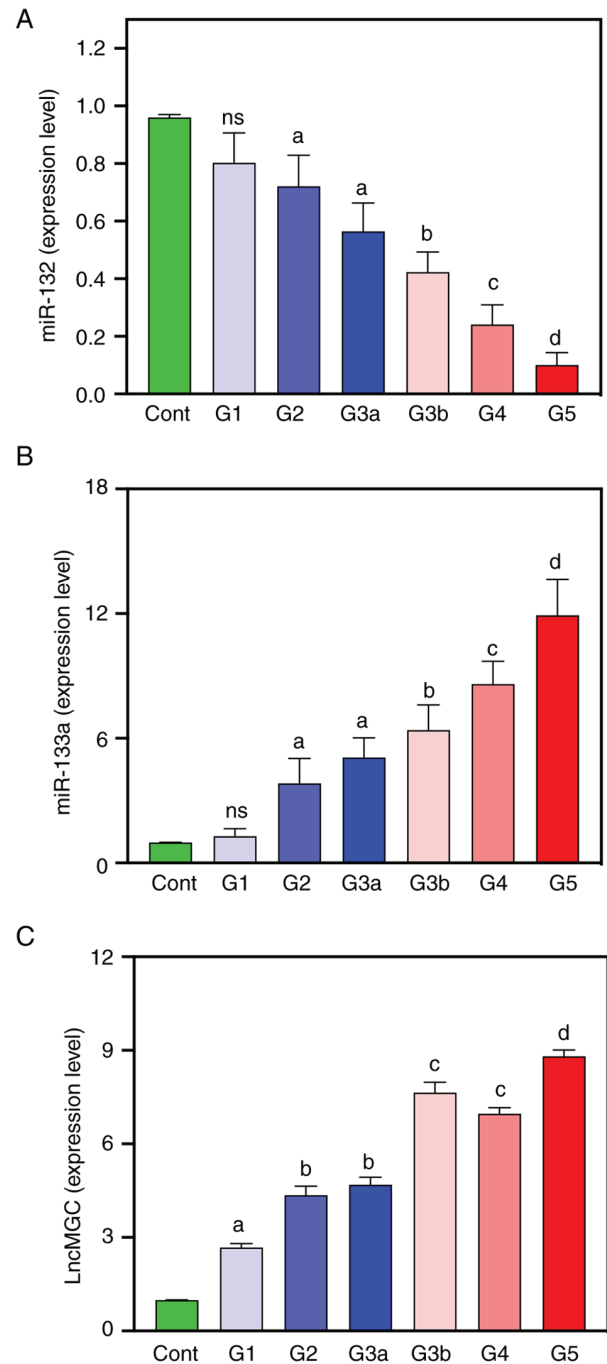


Figure 3. Measurement of non-coding RNA expression levels in healthy controls and diabetic patients as prediagnostic molecular biomarkers for human cardiovascular disease. Expression levels of (A) miR-132, (B) miR-133a and (C) lnc-MGC. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test to compare the significance among all diabetic groups (G1-G5) vs. healthy controls. Data are expressed as mean $\pm$ SEM. ^aP<0.05, ^bP<0.01, ^cP<0.001, ^dP<0.0001 vs. healthy controls. miR, microRNA; lnc-MGC, long non-coding RNA megacuster; cont, healthy controls; G1, diabetes without kidney neuropathy; G2, diabetes with mild renal impairment; G3a-diabetes with severe renal impairment, G3b, diabetes with severe renal impairment and mild cardiovascular disease; G4 diabetes with severe renal impairment and moderate cardiovascular disease; G5 diabetes with severe renal impairment and severe cardiovascular disease.

increased in type 2 DM and CAD (62). In addition, the potential role of miR-132 and miR-133a in vascular pathologies has been previously reported, as they were shown to modulate endothelial

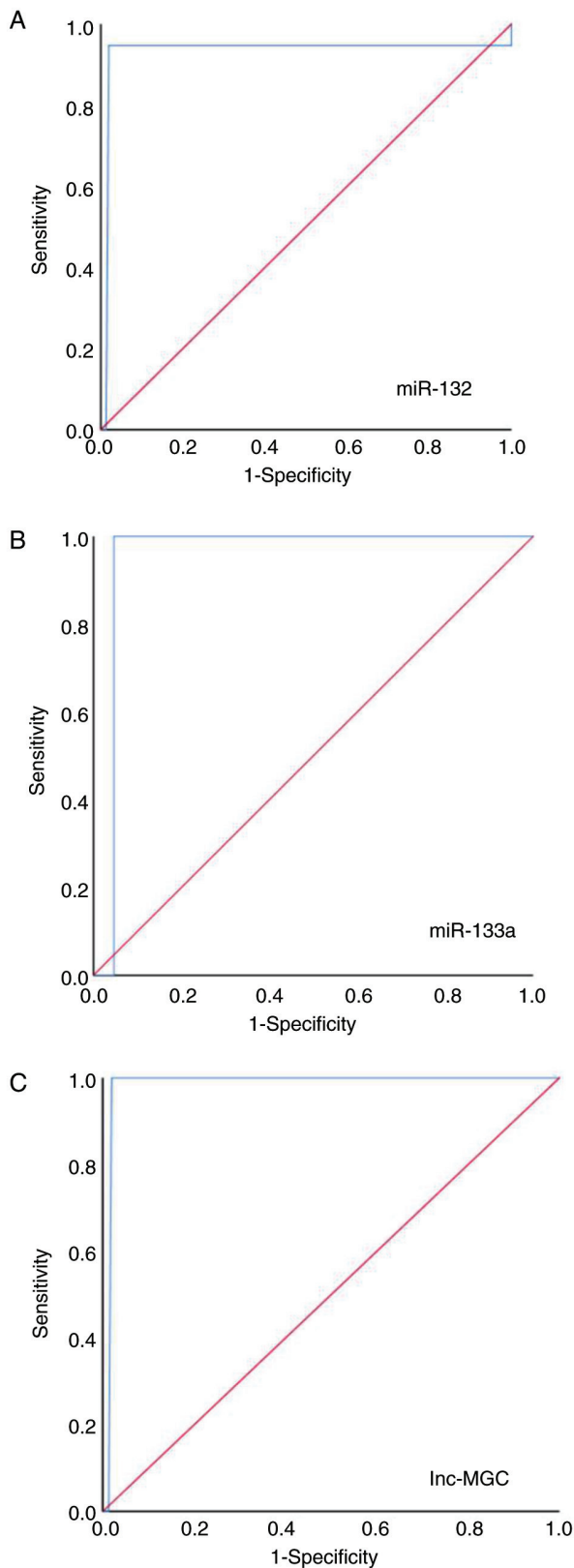


Figure 4. Receiver operating characteristic curves of the expression levels of non-coding RNAs in diabetic patients compared with healthy controls. (A) miR-132, (B) miR-133a and (C) lnc-MGC. miR, microRNA; lnc-MGC, long non-coding RNA megacluster.

cell function and angiogenesis (63,64). Anti-miR-132 treatment improves cardiac function post-myocardial infarction and reverses heart failure (27). miR-132 induces inhibition of PTEN

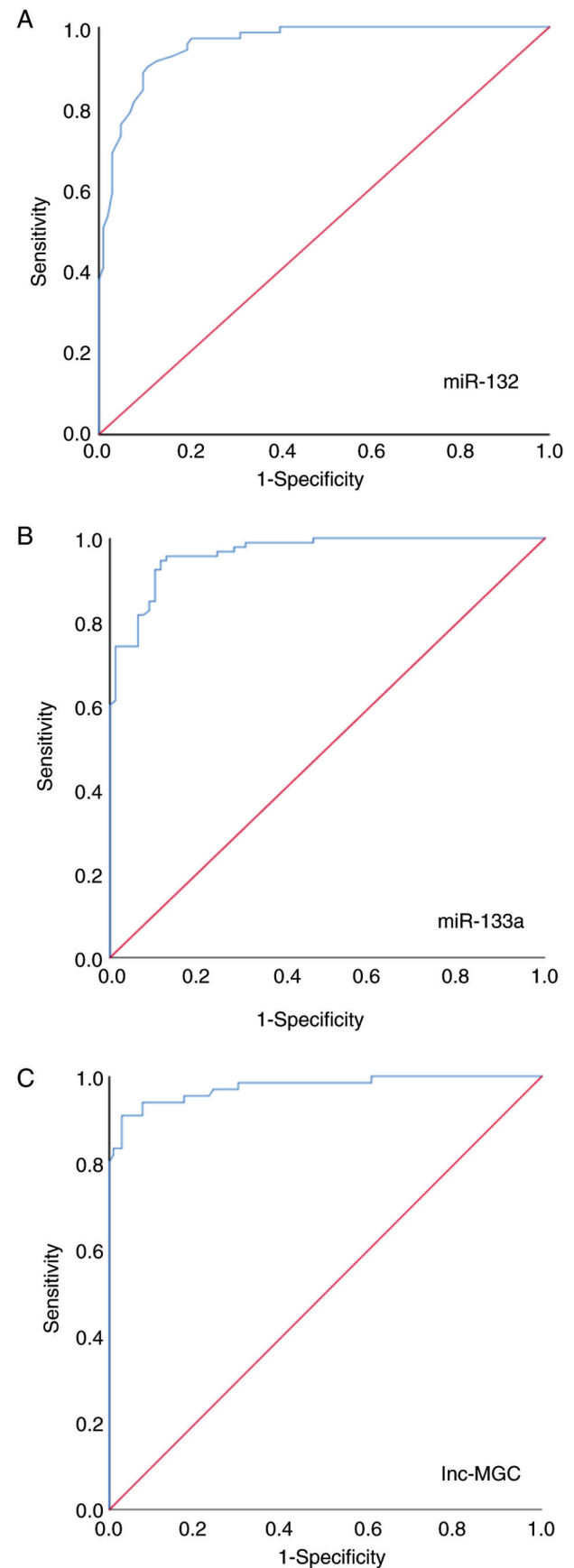


Figure 5. Receiver operating characteristic curves of the expression levels of non-coding RNAs to discriminate between patients with early- and late-stage diabetic neuropathy. (A) miR-132, (B) miR-133a and (C) lnc-MGC. Late stages of diabetic neuropathy were classed as G3b, G4 and G5 patients, whereas early stages were classed as G2 and G3a patients. miR, microRNA; lnc-MGC, long non-coding RNA megacluster.

expression, activating the PI3K/Akt signal transduction pathway. This mechanism facilitates cardiocyte proliferation and reduces apoptosis and cardiac fibrosis (65). Treatment with miR-132 agomiR suppresses the increase in collagen levels, TGF- β and α -smooth muscle actin expression, which are elevated in the hearts of rats with myocardial infarction-induced heart failure (66). Conversely, miR-133a exhibits cardioprotective properties and can maintain lower LDH levels (67) compared with those in patients with diabetes (G2-G5). Moreover, miR-133a is positively correlated with CK, CK-MB and cardiac troponin T (68,69). miR-133a reduces cardiac hypertrophy in streptozotocin-induced diabetic mice by inhibiting glucose-induced upregulation of insulin-like growth factor-1 receptor (IGF1R) and serum- and glucocorticoid-regulated kinase 1 (SGK1) (70). A previous study by Kambis *et al* (71) reported that overexpression of miR-133a in genetically modified mice prevented DM-induced cardiac fibrosis and hypertrophy, which conferred cardioprotective effects. miR-133a also mediates signaling of myocyte enhancer factor 2C in diabetic cardiomyopathy, which is an essential transcription factor underlying myocardial hypertrophy and cardiac fibrosis (72). lnc-MGC upregulation in human cardiac fibroblasts under mechanical stress is associated with miR-133a downregulation (36). lnc-MGC is upregulated in mouse models of DN or mesangial cells treated with TGF- β 1 or high glucose (34).

In conclusion, ROC analysis results demonstrated that serum miR-133a, miR-132 and lnc-MGC levels could potentially be used to differentiate between DN and CVD cases from healthy controls. Serum miR-133a exhibited high sensitivity and specificity values of 99.32 and 100%, respectively, which could suggest that miR-133a serum expression in DN patients may serve as a promising circulating biomarker for detecting and monitoring the progression of diabetes at the early stages of disease.

Overall, the aforementioned biomarkers could potentially assist in predicting the progression of associated complications. Although a correlation between the development of CVD and the expression levels of miR-132, miR-133a and lnc-MGC was demonstrated, the present study has limitations, such as the lack of functional experiments. To address this limitation, *in vivo* studies using genetically modified mouse models, such as miR-132 or miR-133a knockouts or overexpression systems, could be used to elucidate the roles of these miRNAs in cardiovascular health. *In vitro* experiments with cardiomyocyte and endothelial cell cultures could be used to assess the impact of miR-132, miR-133a and lnc-MGC on proliferation, apoptosis and fibrosis. Knockdown or overexpression experiments should be used to investigate how lnc-MGC affects miR-133a levels and contributes to cardiac fibrosis and hypertrophy. Luciferase assays and CRISPR/Cas9-mediated gene editing should be used to identify target genes, such as PTEN (miR-132), IGF1R and SGK1 (miR-133a), and determine their molecular mechanisms of action. The development of miRNA-based therapies, such as miR-132 inhibitors or miR-133a mimics, and the evaluation of these therapies in preclinical and clinical trials could potentially be used for the development of treatments for cardiac fibrosis and other cardiovascular complications in diabetes and related health conditions. Finally, further large-scale studies and clinical trials are required, and the functional impacts of the aforementioned markers on putative target genes and pathways should be thoroughly evaluated.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GA, AA-M and NAH designed the study. GA, NAH, AA-M, MYZ and AM performed the experiments. AM and NAH provided reagents and analytic tools. GA, AM, AH and MYZ analyzed the data. GA and NAH wrote the manuscript. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. GA and NAH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was conducted in compliance with the Declaration of Helsinki and ethical approval was provided by the Ethics Committee of Beni-Suef University Hospital (approval no. BSU:7-2021). The patients were provided with information about the nature and goals of the study, signed an informed consent form and agreed to the use of their samples in scientific research. Staff members of Beni-Suef University are permitted to obtain ethical approval through the University, which encompasses the University Hospital as part of its broader institution.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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