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# Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case-Controlled Study and Bioinformatics Analysis

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**Background:** Diabetic kidney disease (DKD) can result in end-stage kidney disease and renal failure. This study aimed to examine the expression of serum microRNAs (miRNAs), miR-20a, miR-99b, miR-122-5p, and miR-486-5p, and to use bioinformatics data to investigate the pathways involved in DKD.

**Material/Methods:** Serum miRNAs were obtained from 25 healthy volunteers, 50 patients with non-complicated type 2 diabetes mellitus (T2DM), and 42 patients with T2DM and DKD. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of serum miRNAs. Specificity and sensitivity of the association between serum miRNAs in DKD were evaluated by analysis of the receiver operating characteristic (ROC) area under the curve (AUC). Serum miRNAs and clinical parameters of the patients were compared. Bioinformatics data analysis accessed the miRNA targets involved in the pathways related to the pathogenesis of DKD.

**Results:** Serum levels of miR-99b and miR-122 significantly increased, and miR-20a and miR-486 decreased in the DKD group compared with healthy controls. Serum levels of miR-20a, miR-99b, miR-486-5p, and miR-122-5p were significantly correlated with albuminuria, estimated glomerular filtration rate (eGFR), blood glucose and lipid profiles. ROC curve analysis showed that diagnostic accuracy of serum levels of miR-99b for DKD was superior to miR-486-5p, miR-122-5p, and miR-20a, resulting in AUCs of 0.895, 0.853, 0.80, and 0.697, respectively. These four miRNAs regulate several genes affecting oxidative stress, inflammation, and apoptosis.

**Conclusions:** Serum miR-99b, miR-486-5p, miR-122-5p, and miR-20a were differentially expressed in patients with T2DM and DKD and should be evaluated further as potential biomarkers for DKD.

**MeSH Keywords:** **Biological Markers • Diabetic Nephropathies • MicroRNAs**

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## Background

Worldwide, type 2 diabetes mellitus (T2DM) is one of the most prevalent metabolic disorders. The prevalence of diabetes is increasing throughout the world, and from the 285 million individuals diagnosed with T2DM in 2010, the number is expected to rise to 439 million by the year 2030 [1]. The increasing incidence is associated with improvements in living standards and lifestyle changes.

T2DM is associated with multiple complications, with diabetic kidney disease (DKD) being the most prevalent [2]. DKD is the leading cause of end-stage renal disease (ESRD) in patients with diabetes [3]. The incidence of DKD has increased by almost two-fold annually in the past few decades and currently accounts for almost 50% of all cases of ESRD requiring renal transplantation [4,5]. Despite advances diagnosis and treatment, the 5-year survival rate for patients with ESRD due to DKD is less than 50% [6]. Also, patients with T2DM and DKD are at increased risk of macrovascular complications [7]. Therefore, early diagnosis and treatment are important to prevent the progression of DKD. Albuminuria, which is a urine albumin >30 mg/24 hours or random spot albumin/creatinine ratio >30, which is due to a decreased glomerular filtration rate, is a major diagnostic feature of early DKD [8]. In clinical practice, DKD may not be diagnosed at an early stage, due to the lack of sensitive and specific biomarkers, and the lack of minimally invasive diagnostic procedures for DKD.

MicroRNAs (miRNAs) are an endogenously produced novel group of small (20–22 nucleotides) single-strand non-coding RNAs commonly present in both plants and animals [10]. The miRNAs act in a sequence-specific manner to regulate gene expression at the post-transcriptional level by cleavage or translational inhibition of their target mRNAs. Generally, microRNAs are intracellular, but a large number of miRNAs are also found in plasma, serum, saliva, and urine [11–13]. The circulating miRNAs in serum are stable, even when exposed to high temperatures, long-term storage at room temperature, pH changes, and multiple freeze-thaw cycles [11,14].

Also, in humans, microRNAs have a unique expression profile for a wide spectrum of diseases, including neurodegenerative disease [15], cardiovascular disease [16], diabetes mellitus [17], and cancer [18]. Some diseases have been shown to leave specific miRNA fingerprints in the blood of patients suggesting that circulating miRNAs could serve as non-invasive biomarkers for disease diagnosis [14].

Therefore, because miRNAs might be potential diagnostic biomarkers for DKD, this study aimed to examine the expression of serum miRNAs, including miR-20a, miR-99b, miR-122-5p,

and miR-486-5p, and to use bioinformatics data to investigate the potential pathways involved.

## Material and Methods

### Ethical approval and patient consent

The Ethics Committee of the Wuhan Union Hospital approved the study, and informed consent was obtained from all patients who provided clinical information and samples for the study.

### Patients studied

A nested case-controlled study was performed supported by bioinformatic data. A total of 185 patients with type 2 diabetes mellitus (T2DM), aged between 30–70 years, who were admitted between May 2014 and March 2015 to the Wuhan Union Hospital were included in the study. The serum from these patients was obtained during blood sampling for investigative procedures. The study consisted of three groups of patients, including 42 patients (26 men and 16 women) were diagnosed with diabetic kidney disease (DKD), 50 patients (28 men and 22 women) with T2DM without kidney disease, and 25 normal healthy control (HC) subjects (15 men and 10 women). Patients with a history of T2DM and albuminuria >30mg/24 hours on two separate occasions were diagnosed as having DKD. Clinical and demographic patient details recorded included smoking history, biochemical profile, duration of T2DM, type of treatment received, and the presence of diabetic complications.

Patients with comorbid conditions including liver disease, autoimmune disease, or malignancies were excluded from the study. Other exclusion criteria included treatment with antibiotics, nonsteroidal anti-inflammatory drugs, corticosteroids, or cytotoxic drugs at the time of the study. Current use of tobacco products or their use up to six months prior to enrollment was regarded as a positive smoking history, which excluded patients from the study.

Detailed demographic, anthropometric, clinical characteristics and current medications were recorded for both patients and healthy volunteers. The presence of diabetic neuropathy was evaluated by the nerve conduction studies, and diabetic retinopathy was diagnosed by fundoscopy, performed within six months before enrollment. Macrovascular disease was defined as the presence of coronary artery disease, from a history of myocardial infarction or ischemia, cerebrovascular disease, or peripheral vascular disease.

Body mass index (BMI) was calculated as kg/m<sup>3</sup>. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula:

$eGFR = 175 \times (S_{Cr})^{-1.154} \times (\text{age})^{-0.203} \times 0.742 [\text{if female}] \times 1.212 [\text{if black}]$ .

The eGFR was expressed in mL/min/1.73 m<sup>2</sup>, and the standardized serum creatinine (S<sub>Cr</sub>) was expressed in mg/dL.

### Blood samples for serum analysis

For each study participant, 5 ml of fasting venous blood was drawn on the morning of the second day of hospitalization, and clinical and demographic details were also noted at this time. Serum samples were separated from clotted blood by centrifugation at 3,000 rpm for 10 min, aliquoted, and stored at -80°C until required for RNA isolation. During the sample storage, repeated freeze-thawing was avoided to ensure the quality of the samples.

### miRNA expression arrays and miRNA target prediction

Total RNA was extracted from serum samples and miRNA microarray labeling was performed using the miRCURY LNA™ miRNA Hy3/Hy5 Power labeling kit (Exiqon, Vedbæk, Denmark). The labeled samples were hybridized using the miRCURY LNA array version 8.0 (Exiqon, Vedbæk, Denmark), according to the manufacturer's instructions. Following hybridization, the slides were washed using the buffer kit (Exiqon, Vedbæk, Denmark), dried and scanned on a GenePix 4000B array scanner (Molecular Devices Co., Sunnyvale, CA, USA). The average of replicated miRNAs was used, and miRNAs with intensities ≥30 in all samples were chosen for calculating the normalization factor. Data were adjusted using the median normalization. Hierarchical clustering of data points was performed using the Python program.

To further investigate the functional role of upregulated and downregulated miRNAs in the serum of patients with DKD from the 'screening sample set,' bioinformatics analysis was performed to identify the possible miRNA target genes. Target Scan and miRtarbase version 6.1 were used to predict the target genes and molecular pathways of miRNAs. Only the four miRNAs were chosen for validation in the 'validation sample set' to search for potential biological pathways. Enrichment analysis for overlapping genes was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>). Fisher's exact test (P-value <0.01) was used to identify significantly targeted pathways in KEGG and enriched gene target pathways obtained from these databases.

### RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extraction from serum samples was performed using the mirVana Paris™ RNA isolation kit (ThermoFisher Scientific, Waltham, MA, USA). RNA was reverse transcribed using the PrimeScript® RT reagent kit (Takara Japan, Minato-ku, Tokyo, Japan) according to the manufacturer's instructions. For miRNA expression analysis, cDNA prepared by reverse transcription was amplified in real-time PCR using SYBR® Premix Ex Taq™ (Takara Japan, Minato-ku, Tokyo, Japan), template cDNA, and RNase-free water at room temperature, and the RuiBio Primer Assay (Guangzhou RuiBio Corp., China).

The expression of the U6 reference gene, and the expression of miR-20a, miR-99b, miR-122-5p, and miR-486-5p were determined using the 2<sup>-ΔΔCT</sup> method [19], and normalized to the expression of U6 RNA. Primers used for qRT-PCR included: miR-20a: Hsa-miR-20a sequence: UAAAGUGCUUUAUGU GCAGGUAG; miR-99b: Hsa-miR-99b: CACCCGUAGAACCGACCUUGCG; miR-122: Hsa-miR-122-5p: UGGAGUGUGACAAUGGUGUUUG; miR-486: Hsa-miR-486-5p: UCCUGUACUGAGCUGCCCGAG.

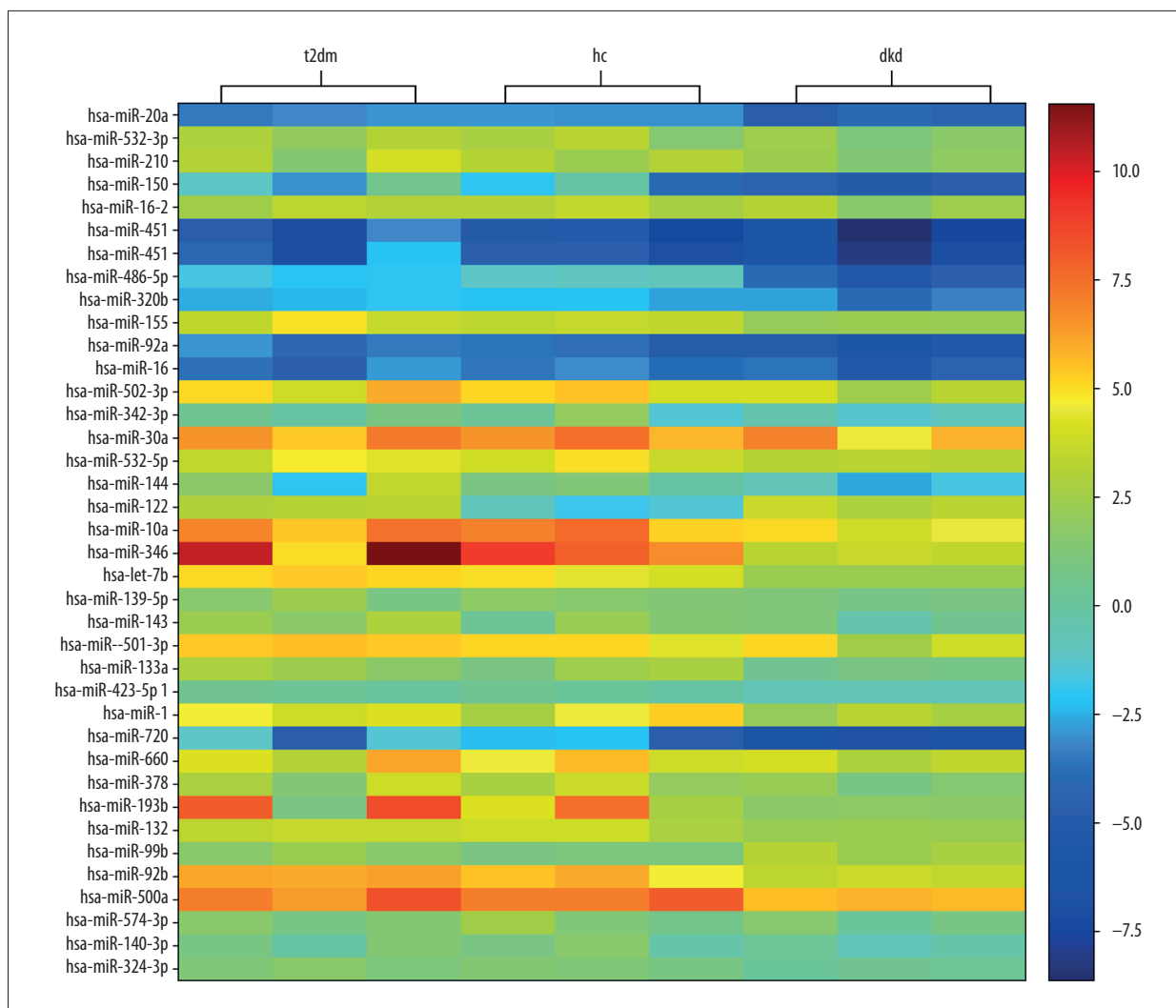
The qRT-PCR was performed in triplicate and the data were presented as the mean ± standard deviation (SD).

### Profiling of miRNA and identification of differentially expressed miRNAs from nine samples

Initially, the expression of 190 miRNAs from serum from the three study groups (HC, T2DM, and DKD), identified the altered miRNA profiles. The microarray results demonstrated that a large number of miRNAs could be detected in human serum samples. Among the miRNAs scanned, 38 miRNAs were observed to be differentially expressed between the patients and the control samples. The levels of serum miRNAs were significantly different between the patient groups and the healthy controls, as shown in Figure 1. Of the differentially expressed miRNAs among the study groups following miRNA profiling, four miRNAs were chosen and validated by qRT-PCR, miR-20a, miR-99b, miR-122-5p, and miR-486-5p.

### Statistical analysis

Data were presented as the mean ± standard deviation (SD), and analyzed using SPSS version 22.0 (IBM, Chicago, IL, USA). Differences between groups were compared by one-way analysis of variance (ANOVA) with a post hoc least significant difference (LSD) test. Spearman's correlation coefficient was performed to test the correlation between serum miRNAs and clinical parameters. Receiver operating characteristic (ROC) curve analysis was used to determine the diagnostic value of



**Figure 1.** Distinct microRNA (miRNA) expression profiles in healthy controls (HC), and patients with type 2 diabetes mellitus (T2DM), and diabetic kidney disease (DKD). Red indicates upregulated expression levels of the microRNAs (miRNAs). Blue indicates downregulated expression levels of the miRNAs.

serum miRNAs in patients with DKD. A P-value <0.05 was considered to be statistically significant.

## Results

### Relative expression of serum microRNAs (miRNAs) in diabetic kidney disease (DKD)

The demographic and clinical characteristics of the patients included in the study are shown in Table 1. There was no significant difference between the healthy control (HC) group, the patients with type 2 diabetes mellitus (T2DM) group, and the group with T2DM and diabetic kidney disease (DKD), including gender, age, and smoking history. The relative expression of serum microRNAs (miRNAs), including miR-20a, miR-99b, miR-122, and

miR-486 were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Serum levels of miR-99b and miR-122-5p were significantly increased, and serum levels of miR-20a and miR-486 were significantly reduced in patients with DKD when compared with the HC and T2DM groups (Figure 2A–2D).

### Correlation between serum miRNAs and clinicopathological features

Spearman's correlation analysis was performed for the four miRNAs and different clinical parameters in the HC, DM and DKD groups. The four miRNAs were compared with each other and with clinical parameters (Table 2). Serum levels of miR-20a and miR-486-5p were negatively correlated with systolic blood pressure (SBP), diastolic blood pressure (DBP), TG, triglyceride (TG), total cholesterol (TC), fasting blood glucose (FBG), glycated

**Table 1.** Clinical and demographic patient data.

	HC group	T2DM group	DKD group
Age in years	47.6±7.9	50.3±10.3	51.5±9.3
Sex (M/F)	15/10	28/22	26/16
History of T2DM	–	4.0±2.3	8.5±4.2#
Smoking History (positive/negative)	8/17	24/26	17/25
Alcohol History (positive/negative)	10/15	10/40	10/32
BMI (kg/m <sup>2</sup> )	22.8±3.4	25.5±4.4*	24.4±3.4
WHR	0.88±0.04	0.93±0.05*	0.94±0.05*
SBP (mmHg)	116.19±10.11	128.77±11.28*	144.37±18.44*#
DBP (mmHg)	78.76±9.20	78.62±14.31	90.88±13.53*#
TG (mmol/L)	1.20±0.21	1.78±0.66*	1.92±0.94*
TC (mmol/L)	4.04±0.13	4.48±0.52	4.62±1.80
FBG (mmol/L)	4.93±0.19	7.53±0.76*	9.11±5.78*
PPBG (mmol/L)	–	13.66±2.34	13.61±5.06
HbA1c%	5.45±0.13	8.97±1.72*	10.08±2.25*#
eGFR (ml/min/1.73 m <sup>2</sup> )	102.53±11.35	107±11.7	68.06±17.9*#
Urine albumin (mg/day)	–	1.81±3.31	225±132.36#

HC – healthy control group; T2DM – T2DM with no complications; DKD – diabetic kidney disease; BMI – body mass index; WHR – waist-hip ratio; SBP – systolic blood pressure; DBP – diastolic blood pressure; TG – triglycerides; TC – total cholesterol; T2DM – type 2 diabetes mellitus; FBG – fasting blood glucose; P2hBG – 2 hours post-prandial blood glucose; HbA1c – glycated hemoglobin A1c; eGFR – estimated glomerular filtration rate. \* P<0.05 when compared with the healthy control. # P<0.05 when compared with the DM group.

hemoglobin (HbA1c), and urine albumin levels. Serum levels of miR-20a and miR-486-5p were positively correlated with levels of estimated glomerular filtration rate (eGFR).

Serum levels of miR-99b were positively associated with SBP, DBP, TG, TC, FBG, HbA1c, and urine albumin levels and negatively associated with eGFR and postprandial blood glucose (PPBG). Serum levels of miR-122-5p were positively associated with FBG, HbA1c, urine albumin, and negatively associated with eGFR. Also, serum miR-20a and miR-99b, miR-99b, miR-20a and miR-486-5p showed a negative correlation, while a positive correlation was found between serum miR-20a and miR-486-5p.

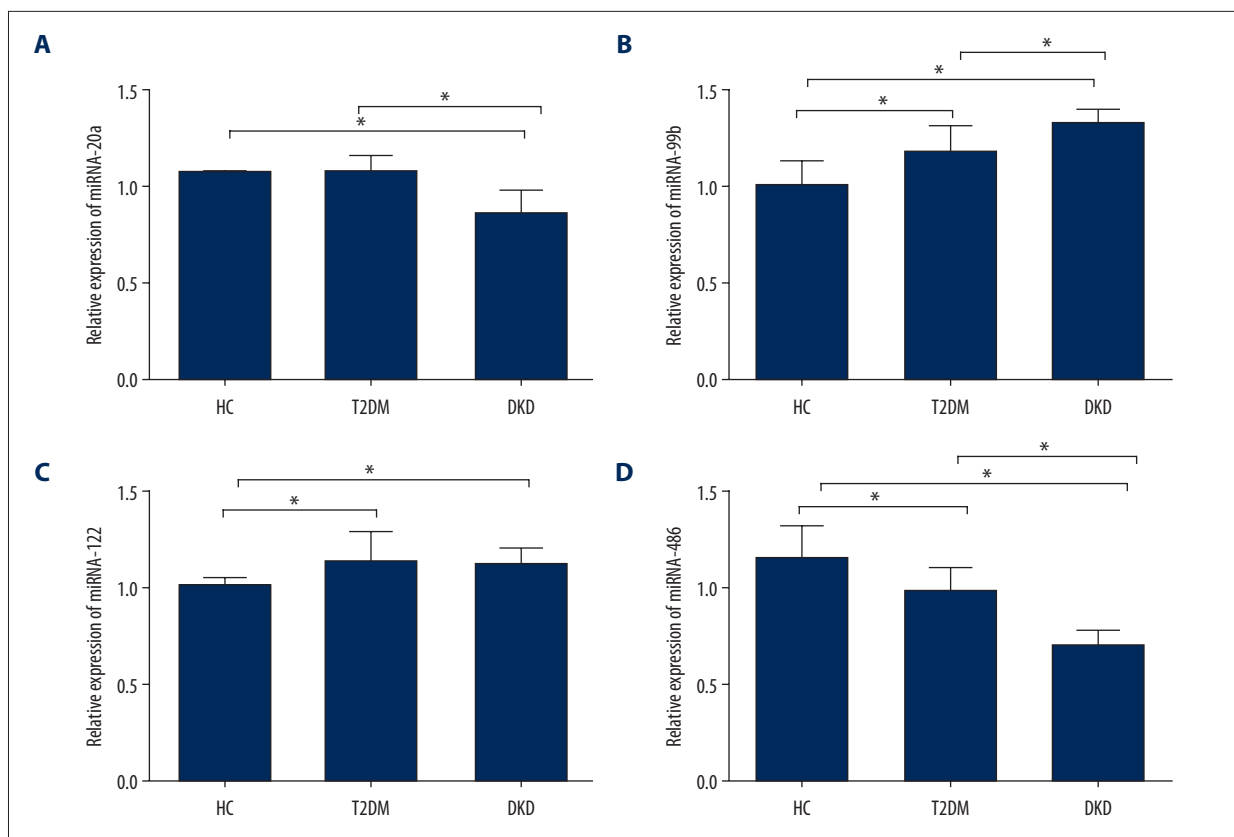
#### Receiver operating characteristic (ROC) curve analysis of the diagnostic value of serum miRNAs in DKD

Receiver operating characteristic (ROC) curve analysis of the diagnostic value of serum miRNAs in DKD, miR-99b, miR-486, miR-122, and miR-20a, are shown in Figure 3A–3D. ROC curve analysis indicated that the diagnostic accuracy of serum levels of miR-99b for DKD was superior to miR-99b, miR-486-5p, miR-122-5p, and miR-20a, with area under the curve (AUC) values of 0.895, 0.853, 0.80, and 0.697, respectively.

#### Target prediction for miRNAs in DKD and their regulatory pathways

The putative targets and pathways modulated by the four validated miRNAs (miR-20a, miR-99b, miR-122, and miR-486) in serum from patients with DKD were retrieved using bioinformatics methods. TargetScan (<http://www.targetscan.org>) was initially used, followed by miRTarBase version 6.1 (<http://miRTarBase.mbc.nctu.edu.tw/>) to predict the target of the four validated miRNAs in serum of DKD patients. Individually, 3,237 target genes were found for miR-20a-3p, 111 target genes were found for miR-99b, 2,226 target genes were found for miR-122-5p, and 1,387 target genes were found for miR-486-5p.

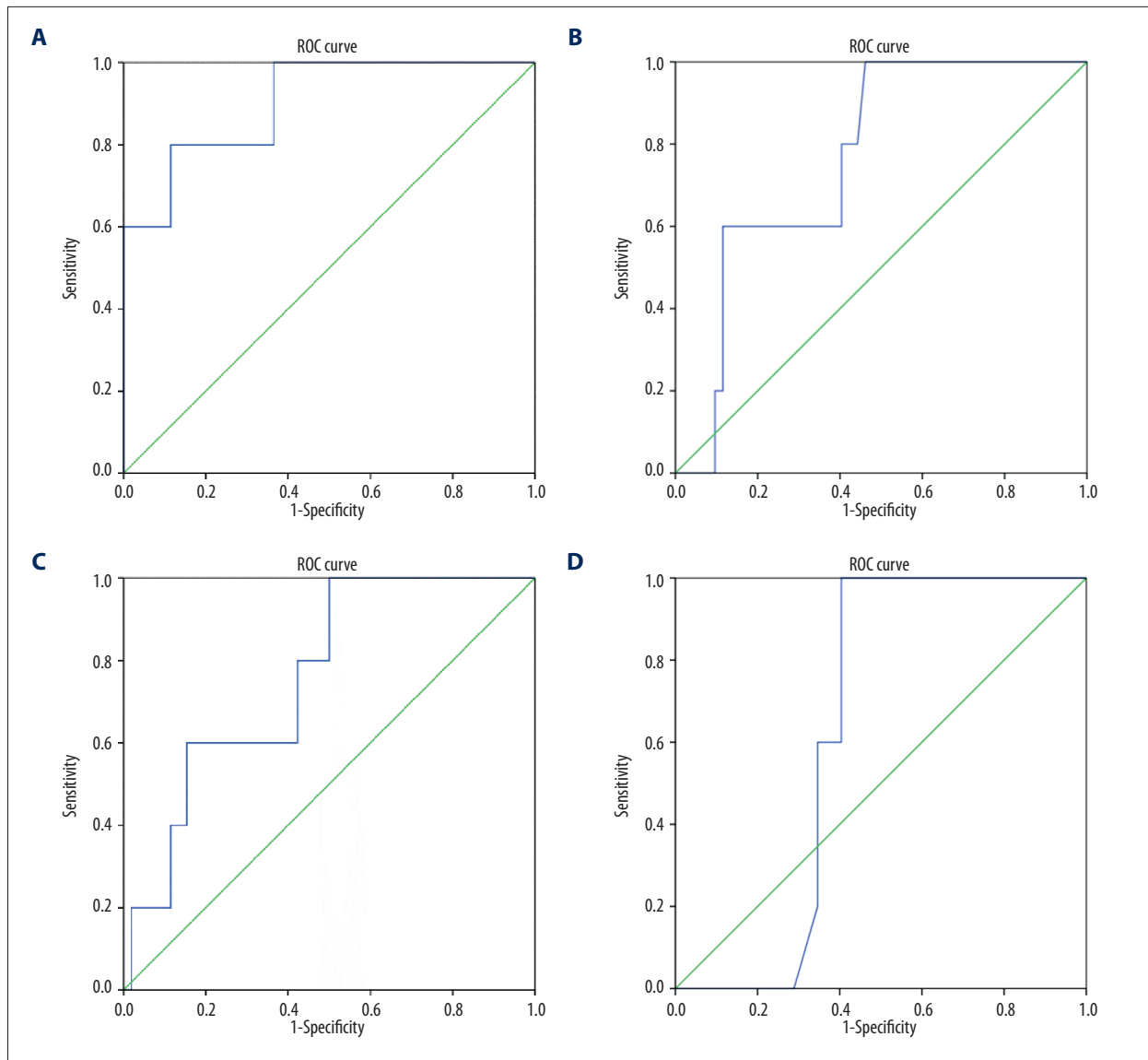
Following target identification, functional enrichment analysis was performed for miRNA targets using pathway maps from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to explore the biological pathways possibly affected by the four validated miRNAs grouped by their expression profile (up-regulation or downregulation). Many of the enriched pathways were known to be related to the pathogenesis of DKD and included PI3K/Akt, p53, 5' AMP-activated protein kinase (AMPK), transforming growth factor-β (TGF-β), forkhead box O (FOXO),



**Figure 2.** The expression of microRNAs (miRNAs) in patients with diabetic kidney disease (DKD). (A) The expression of miR-20a in patients with diabetic kidney disease (DKD) was significantly decreased when compared with other groups. (B) The expression of miR-99b in patients with diabetic kidney disease (DKD) was significantly increased when compared with other groups. (C) Expression of miR-122-5p in patients with diabetic kidney disease (DKD) was significantly increased when compared with other groups. (D) The expression of miR-486-5p in patients with diabetic kidney disease (DKD) was significantly decreased when compared with other groups.

**Table 2.** Correlation between serum microRNAs (miRNAs) and clinicopathological features, using Spearman’s correlation coefficient.

	mir20	mir99	mir122	mir486
SBP	-0.400**	0.488**	0.215	-0.538**
DBP	-0.330**	0.315**	0.024	-0.405**
TG	-0.112	0.331**	0.072	-0.258*
TC	-0.163	0.296**	0.143	-0.337*
FBG	-0.318**	0.576**	0.311**	-0.464**
PPBG	0.081	-0.034	-0.01	0.054
HbA1C	-0.326**	0.627**	0.261*	-0.550**
eGFR	0.410**	-0.382**	-0.287*	0.421**
Urine albumin	-0.623**	0.665**	0.315**	-0.764**
Mir-20a	1	-0.595**	-0.126	0.633**
Mir-99b	-0.595**	1	0.176	-0.686**
Mir-122-5p	-0.126	0.176	1	-0.119
Mir-486-5p	0.633*	-0.686**	-0.119	1



**Figure 3.** Receiver operating characteristic (ROC) curve analysis. Receiver operating characteristic (ROC) curve analysis for (A) miR-99b, (B) miR-486, (C), miR-122, and (D) miR-20a, to evaluate their diagnostic roles.

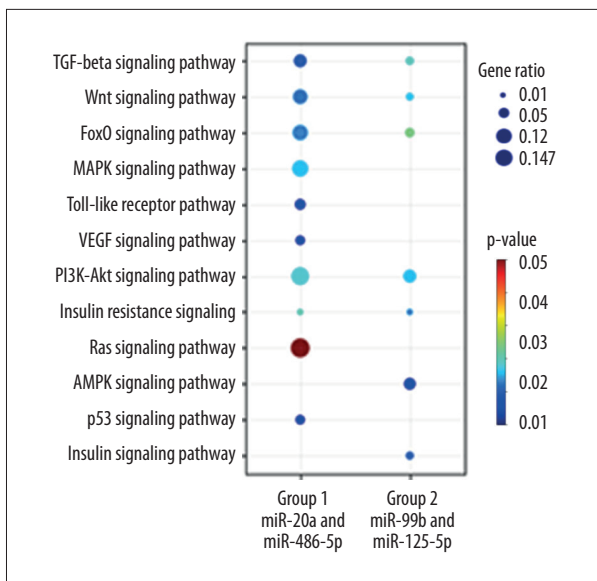
and mitogen-activated protein kinase (MAPK) signaling pathways (Figure 4). Many of these pathways consist of genes known to be related to the pathogenesis of DKD including oxidative stress, inflammation, and apoptosis (Figure 5).

## Discussion

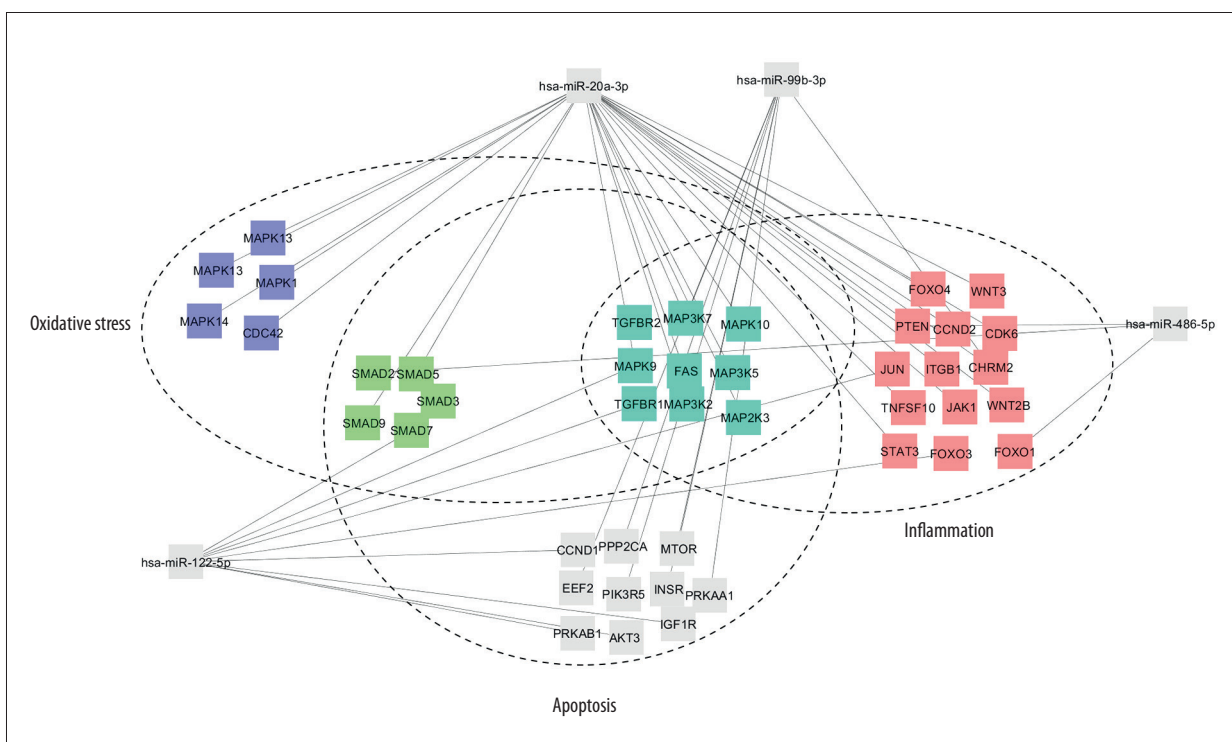
Previously published studies have shown that oxidative stress, inflammation, and apoptosis pathophysiological mechanisms involved in the development and progression of diabetic kidney disease (DKD) [20]. Recently, dysregulation of the expression of microRNAs (miRNAs) has been demonstrated in several diseases, including DKD [21,22], which contribute to the

increased accumulation of extracellular matrix (ECM) proteins that result in renal fibrosis.

The findings of this study showed decreased expression of miRNA-20a in patients with DKD, which is supported by a previous study that showed negative expression of miR-20a associated with the p38 pathway-mediated vascular endothelial growth factor (VEGF)-induced endothelial cell migration and angiogenesis [21], and mitogen-activated protein kinase 1 (MAPK1) expression [22]. Also, previous studies have shown that miR-20a regulated endothelial transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling [23]. TGF- $\beta$  has a key role in the development of renal hypertrophy and the increase in ECM in diabetes [24]. Increased expression of TGF $\beta$ 1 is associated with



**Figure 4.** Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified pathways potentially regulated by miR-20a, miR-99b, miR-122-5p, and miR-486-5p, according to their expression profiles. Upregulated microRNAs (miRNAs) in patients with diabetic kidney disease (DKD): miR-99b, and miR-122-5p. Downregulated miRNAs in patients with DKD: miR-20a and miR-486-5p. The size of the circles denotes the ratio of target genes involved in a given pathway (GeneRatio) and its color represents the q-value of the pathway.



**Figure 5.** Interactions between microRNAs (miRNAs) and their target genes. Raw data used for this analysis were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

loss of renal podocytes that are associated with capillaries in of the glomerulus, which is an early feature of DKD [25]. Gianluca et al. [26] showed that miR-99a and miR-99b were positive modulators of the TGF-β pathway. The findings of the present study showed significantly increased expression of miR-99b in the serum of patients with DKD compared with patients with type 2 diabetes mellitus (T2DM) without DKD (the T2DM group) and the normal healthy controls (the HC group).

A previous study has shown that increased miRNA-122 level inhibited activation of 5'AMP-activated protein kinase (AMPK) [27]. In diabetes, reduced AMPK activity is associated with renal accumulation of triglyceride (TG) and glycogen, which are indicators of diabetic renal hypertrophy [28]. Also, the expression of miRNA-122 is regulated by DNA methylation [29], which is associated with albuminuria and has been implicated in the progression of DKD [30]. The findings for these previous



studies support the findings of the present study and might explain the cause of the increase in miR-122 levels in DKD.

In this study, the expression of miR-486 in serum was decreased in the DM group, which was consistent with the findings from a study by Zampetaki et al., which demonstrated that the expression of miR-486 was reduced in the serum of patients with diabetes [17]. In a study by Xu et al., miR-486 was significantly decreased in the muscles of mice in an animal model of chronic kidney disease (CKD) [31], and similar decreased expression of miRNA-486 was found in the DKD group in this study. The expression of miR-486 has been linked to the PTEN/AKT signaling pathway [32], and the decrease in miR-486 increases the expression of PTEN [33]. Impairment of insulin-induced glucose uptake into podocytes cultured *in vitro* in the presence of high glucose concentrations has previously been shown to be associated with increased PTEN levels in an AMPK-dependent manner [34]. In the present study, correlation analysis showed that miR-486 levels were negatively correlated with albuminuria, and levels of fasting blood glucose (FBG) and glycated hemoglobin (HbA1c). Therefore, the finding of decreased serum levels of miR-486 in this study is in accordance with other studies and decreased expression of miR-486 might be a key marker for DKD.

In the current study, serum levels of miR-99b and miR-122 were significantly increased in the DKD group compared with the healthy control group, while miR-20a and miR-486 levels were significantly decreased. Receiver operating characteristic (ROC) curve analysis showed that the diagnostic accuracy of serum levels of miR-99b for DKD was superior to miR-486-5p, miR-122-5p, and miR-20a, resulting in an area under the curve (AUC) of 0.895, 0.853, 0.80, and 0.697, respectively, indicating that these miRNAs might serve as a potential noninvasive biomarker panel for the diagnosis of DKD.

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Although the circulating levels of miR-20a, miR-99b, miR-122-5p and miR-486-5p appear to have potential as biomarkers for DKD, further controlled clinical studies are needed to validate these findings, and also to determine the correlations with clinical parameters, including systolic blood pressure (SBP), diastolic blood pressure (DBP), triglycerides (TG), total cholesterol (TC), fasting blood glucose (FBG), glycated hemoglobin (HbA1c), estimated glomerular filtration rate (eGFR) and urine albumin levels. The molecular mechanisms of these interactions remain to be elucidated. Confirmation of aberrant expression of these miRNAs in a large prospective study of DKD may provide support for their diagnostic or prognostic role.

## Conclusions

In patients with type 2 diabetes mellitus (T2DM), changes in the expression of circulating microRNAs (miRNAs) detected in serum, miR-20a, miR-99b, miR-486-5p, and miR-122-5p, were shown to be associated with the presence of diabetic kidney disease (DKD). These findings require validation but support the potential role for miRNAs as a minimally invasive blood-based diagnostic biomarker for DKD that may detect early nephropathy in patients with T2DM.

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## Conflict of interest

None.

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