Deregulated profiles of urinary microRNAs may explain podocyte injury and proximal tubule dysfunction in normoalbuminuric patients with type 2 diabetes mellitus

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ABSTRACT

MicroRNAs (miRNAs) are short non-coding RNA species that are important post-transcriptional regulators of gene expression. The aim of the study was to establish a potential explanation of podocyte damage and proximal tubule (PT) dysfunction induced by deregulated miRNAs expression in the course of type 2 diabetes mellitus (DM). A total of 68 patients with type 2 DM and 11 healthy subjects were enrolled in a cross-sectional study and assessed concerning urinary albumin:creatinine ratio (UACR), urinary *N*-acetyl-β-D-glucosamininidase (NAG), urinary kidney injury molecule-1, urinary nephrin, podocalyxin, synaptopodin, estimated glomerular filtration rate (eGFR), urinary miRNA21, miRNA124, and miRNA192. In univariable regression analysis, miRNA21, miRNA124, and miRNA192 correlated with urinary nephrin, synaptopodin, podocalyxin, NAG, KIM-1, UACR, and eGFR. Multivariable regression analysis yielded models in which miRNA192 correlated with synaptopodin, uNAG, and eGFR (R^2=0.902; P<0.0001), miRNA124 correlated with synaptopodin, uNAG, UACR, and eGFR (R^2=0.881; P<0.0001), whereas miRNA21 correlated with podocalyxin, uNAG, UACR, and eGFR (R^2=0.882; P<0.0001). Urinary miRNA192 expression was downregulated, while urinary miRNA21 and miRNA124 expressions were upregulated. In patients with type 2 DM, there is an association between podocyte injury and PT dysfunction, and miRNA excretion, even in the normoalbuminuria stage. This observation documents a potential role of the urinary profiles of miRNA21, miRNA124, and miRNA192 in early DN. Despite their variability across the segments of the nephron, urinary miRNAs may be considered as a reliable tool for the identification of novel biomarkers in order to characterize the genetic pattern of podocyte damage and PT dysfunction in early DN of type 2 DM.

Significance of this study

What is already known about this subject?

- To date, emerging data show that, the mechanisms of albuminuria in diabetes mellitus may be related mainly to an impaired tubular uptake of intact albumin questioning the hypothesis of an increased leakiness of the glomerular filter.
- MiRNAs have a high potential to modulate cellular and biochemical functions, leading to the initiation or progression of various diseases, including diabetic nephropathy.
- There are overexpressed miRNAs in the diabetic kidney, leading to initiation and progression of renal fibrosis, while downregulated miRNAs displayed renal protective effects.

What are the new findings?

- The study demonstrates an association between podocyte injury and PT dysfunction, and miRNA excretion, even in normoalbuminuric patients with type 2 DM.
- This observation documents a potential role of the profiles of miRNA21, miRNA124, miRNA192 in early DN. The expression of miRNA192 was downregulated, while that one of miRNA21 and miRNA124 was upregulated.
- This phenomenon is independent of albuminuria and level of renal function.

INTRODUCTION

Diabetes mellitus (DM) is globally the leading cause of end-stage renal disease, accounting for >40 per cent of patients with both type 1 and type 2 DM undergoing renal replacement therapies.[1–3] Until recently it has been postulated that the underlying mechanism of albuminuria in diabetic nephropathy (DN) is represented by defects in the structure of the glomerular filtration barrier.[4 5] In view of this hypothesis, it is assumed that albuminuria is correlated with the severity of the glomerular lesions, but this phenomenon is not indispensable in preceding

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Significance of this study

How might these results change the focus of research or clinical practice?

- The strength of the study relies on the documentation of a relation between urinary miRNAs and biomarkers of podocyte injury and PT dysfunction in patients with type 2 diabetes.
- This observation points to the clinical importance of urinary miRNAs in the diagnosis of early renal involvement in normoalbuminuric patients with type 2 diabetes, often perceived as a group of patients at low risk of developing DN.
- Urinary miRNAs may be considered as a reliable tool for the the identification of novel biomarkers in order to characterize the genetic pattern of podocyte damage and PT dysfunction in early DN of type 2 DM.

the onset of albuminuria, a fact demonstrated in biopsy studies in normoalbuminuric patients with type 2 DM and impaired renal function.[6 7]

To date, emerging data show that the mechanisms of albuminuria in DM may be related mainly to an impaired tubular uptake of intact albumin questioning the hypothesis of an increased leakiness of the glomerular filter.[8 9] Previously, we demonstrated in normoalbuminuric patients with type 2 diabetes that proximal tubule (PT) dysfunction precedes the occurrence of albuminuria. It is possible that in early DN, sequentially, PT dysfunction may precede glomerular injury.[10 11] Patients with subclinical DN and those with early DN may display more abnormal tubular injury markers that show PT dysfunction, which implies selective albumin processing mainly by the retrieval pathway and less by the degradation pathway of the PT.[12]

The glomerulus normally leaks nephrotic levels of albumin, retrieved and processed by the PT.[8 9] The signaling system from the glomerulus to the PT may turn off the pathway of intact albumin in the course of DM.[13]

Nephrin is a key component of the glomerular filtration barrier. The impairment to the size selectivity of the slit diaphragm, which is located between the foot processes of the podocytes, is due to nephrin alterations.[4] Normoalbuminuric patients with type 1 and type 2 diabetes may display increased levels of nephrinuria, a phenomenon that demonstrates that nephrinuria may precede microalbuminuria.[14–17]

Synaptopodin is an actin-associated podocyte protein that establishes the podocyte specific bundling of the microfilaments in the foot processes.[18]

Podocalyxin is a transmembrane protein that localizes to the apical cell of glomerular podocytes. Podocalyxin maintains the shape of podocytes and of the slit diaphragm. The levels of urinary podocalyxin increase significantly and correlate with DN progression.[19]

MicroRNAs (miRNAs) are endogenously produced short non-coding RNAs of about 21–25 nucleotides that have been shown to play important roles in modulating gene expression, thus affecting almost every key cellular function.[20] miRNAs have a high potential to modulate cellular and biochemical functions, leading to the initiation or progression of various diseases, including DN.[21] miRNA-192 induces upregulation of fibrotic genes, thus promoting increased glomerular sclerosis and fibrosis.[22] Several studies show that upregulation of miRNA-192 expression in tubular cells treated with transforming growth factor (TGF)β1 increased fibrosis.[22] By contrast, miR-192 has been shown to be downregulated in cultured proximal tubular epithelial cells treated with high glucose or TGF β1, a fact that was associated with increased fibrosis. Moreover, there are studies that showed that miR-192 leads to depression of E-cadherin, thus exerting an antifibrotic effect.[23 24]

Apart from miRNA-192, there have been described other profibrotic miRNAs, such as miRNA-21[25] and miRNA-124, which may exert a role in podocyte adhesion damage under mechanical stress.[26] The aim of our study was to establish a potential explanation of podocyte damage and PT dysfunction induced by deregulated miRNAs expression in the course of type 2 DM. The profiles of urinary miRNA21, miRNA124, and miRNA192 were assessed in relation to DN stage.

SUBJECTS, MATERIALS, AND METHODS

Patients' enrollment criteria

A total of 68 consecutive patients with type 2 diabetes (26 patients with normoalbuminuria, 24 patients with microalbuminuria, and 18 patients with macroalbuminuria) recruited from the Outpatient Department of Diabetes and Metabolic Diseases and 11 healthy control subjects were enrolled in a cross-sectional study. The inclusion criteria were duration of diabetes >5 years, categories of albuminuria, such as normoalbuminuria (urinary albumin:creatinine ratio (UACR) <30 mg/g), microalbuminuria (UACR 30–300 mg/g), or macroalbuminuria (UACR >300 mg/g). Patients were classified according to levels of UACR measured in the ambulatory clinic, prior to inclusion in the study. All patients were on ACE inhibitors and/or angiotensin receptor blockers, other antihypertensive agents (including diuretics), and statins. There were no differences in diuretic usage between groups. SGLT-2 inhibitors were not used. Also, all patients were treated with oral antidiabetic agents. There were no significant differences between the therapies across the studied groups.

The biomarkers of PT dysfunction included urinary *N*-acetyl-β-D-glucosaminidase (NAG) and urinary kidney injury molecule-1 (KIM-1), while the biomarkers of podocyte damage assessed were urinary nephrin, urinary synaptopodin, and urinary podocalyxin. UACR and serum cystatin C were also assessed. Serum and urinary biomarkers were determined in specimens frozen at −80°C and thawed before assay. Urinary variables were assessed in triplicate on aliquots from the same first morning urine sample (midstream urine). The ELISA assessments were performed as per protocol indicated by the manufacturer in triplicate assessments for each patient from the same aliquot. The inter-assay or intra-assay coefficients of variance (CVs) were indicated according to the data provided by the manufacturer's brochure.[17 27 28] The estimated glomerular filtration rate (eGFR) was calculated by the combined method that uses serum creatinine and serum cystatin C, according to the KDIGO Guideline for the Evaluation and Management of Chronic Kidney Disease.[29 30]

Biomarkers of podocyte damage

The biological samples were centrifuged at 2000 rpm for 10 min, and the supernatant was aliquoted in 0.5 mL volumes. Samples were stored at −80°C, and aliquots were thawed for ELISA assays. Blanks and standards were assayed according to the manufacturer's instructions for all assays. The test was performed in triplicate. The mean values of absorbance and concentration were plotted and a 4 Parameter Logistic non-linear regression model was used (R^2>0.97 was accepted). The intra-assay variability was <10 per cent. The inter-assay of the test showed a CV <10 per cent.[17 27 28]

Nephrin was assessed by Human Nephrin ELISA Kit; Abbexa, 16 Iceni Way, Cambridge, CB4 2NZ, UK, cat. no.: abx051402; test range: 35–2000 pg/mL. The repeatability of the test showed a CV<10 per cent.[17 27 28]

Synaptopodin was assessed by Human Synaptopodin ELISA Kit; Abbexa, cat. no.: abx055120; test range: 18–1000 pg/mL.

Podocalyxin was assessed by Human Podocalyxin ELISA Kit; Abbexa, cat. no.: abx51856; test range: 26–1500 pg/mL.

Biomarkers of PT dysfunction

NAG was assessed by Human *N*-Acetyl-β-D-Glucosaminidase ELISA Kit; Abbexa, cat. no.: abx53752; test range: 0.06–4 ng/mL.

KIM-1 was assessed by KIM-1 ELISA test kit for the detection of KIM-1 in human urine, cat. no. H-RENA-E-001, Bio Assay Works, Ijamsville, Maryland, USA. A human KIM-1 antibody was used, and the detection level was set at urinary KIM-1<0.150 ng/mL.[17 27 28]

Albuminuria and cystatin C

Albuminuria was measured through immunonephelometry on the BNProSpec System, with N Antiserum to Human Albumin (Siemens Healthcare Diagnostics, Marburg, Germany). Microalbuminuria was defined by UACR between 30 and 300 mg/g, and normoalbuminuria by UACR<30 mg/g. The N Antiserum to Human Albumin was evaluated for the assay of urine on a BN System and yielded a Within-Run CV of 2.2 per cent and a total CV of 2.6 per cent with a mean of 79 mg/L. The results (10 runs, four determinations per run) were evaluated by analysis of variance, according to the manufacturer's brochure. Urinary tract infections were ruled out by negative urine cultures in all patients.[17 27 28]

Cystatin C was assessed in serum with N latex cystatin C kit (Siemens Healthcare Diagnostics) through particle-enhanced immunonephelometry using the BNProSpec System. The reference interval was calculated non-parametrically and was determined to be 0.53–0.95 mg/L. The intra-assay precision was 2.5 per cent CV, and inter-assay precision was 2.0 per cent CV with a total of 2.8 per cent CV. Analytical sensitivity was calculated as 2 SD above the mean signal of 20 replicates of N diluent and was determined to be 0.005 mg/L. A typical detection for N latex cystatin C is 0.05 mg/L.[17 27 28]

miRNAs assessment

Total RNA was isolated from the urine samples using the Urine microRNA Purification Kit, Norgen Biotek Corporation, 3430 Schmon Parkway, Thorold, ON, Canada, cat. no. 29000. Total RNA was stored at −80°C. The quantity and quality of total RNA was verified, and reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems (ABI), Foster City, California, USA). cDNA were amplified using the TaqMan MicroRNA Assay for *specific miRNAs (miRNA21, miRNA124* and *miRNA192)* and TaqMan Universal PCR Master Mix. The PCR reaction was performed on a 7900HT real-time PCR System. Urine samples were run in triplicate, and relative expression level of specific gene was calculated using the SDS software V.2.4, and U6 small nuclear RNA as the endogenous control. Fold-change of expression was calculated using the comparative Ct method (Δct).

Statistical analysis

Data are presented as medians and IQR as for variables with skewed distribution. According to the distribution of the values, differences between subgroups were analyzed with the Mann-Whitney U test for comparison of two groups and the Kruskal-Wallis test for comparison of four groups. Univariable and multivariable logistic regression analyses were conducted in order to evaluate the significance of the relation between miRNAs and other continuous variables. The P values for all hypothesis tests were two-sided, and statistical significance was set at P<0.05. All analyses were conducted with Stata V.9.2.

RESULTS

All patients and controls were included in the analysis.

The demographic, clinical, and biological data of the patients and control subjects are presented in table 1. As there were no significant differences between the therapies across the studied groups, the medications prescribed could not bias the interpretation of data. Whether renoprotective agents such as RAS inhibitors would have any effect on miRNAs in early DN remains to be determined.

Univariable logistic regression showed correlations of miRNA21, miRNA124, and miRNA192 with urinary nephrin, urinary synaptopodin, urinary podocalyxin, UACR, urinary KIM-1, urinary NAG, cystatin C, and eGFR (table 2).

Multivariable regression analysis resulted in a model in which miRNA192 correlated with synaptopodin, uNAG, and eGFR (R^2=0.902; P<0.0001), miRNA124 correlated with synaptopodin, uNAG, UACR, and eGFR (R^2=0.881; P<0.0001), while miRNA21 correlated with podocalyxin, uNAG, UACR, and eGFR (R^2=0.882; P<0.0001). Adjustment for potential confounders, such as lipid profile, HbA_{1c}, and high-sensitive C reactive protein, was performed in order to avoid bias in the interpretation of data. Urinary miRNA192 expression was downregulated, while urinary miRNA21 and miRNA124 expressions were upregulated (table 3).

DISCUSSION

The vast majority of data with regard to miRNAs derive from animal studies, while human studies are lacking and rely mostly on findings from renal biopsies and less from the urine. There are overexpressed miRNAs in the diabetic

Table 1 Clinical and biological data of the patients studied

Parameter	Group 1 (healthy controls)	Group 2 (normoalbuminuria)	Group 3 (microalbuminuria)	Group 4 (macroalbuminuria)	P*	P**	P***	P
Subjects (n)	11	26	24	18	–	–	–	–
Age (years)	58 (50–65)	58.5 (52–61)	58 (53–62)	59.5 (52–62)	0.711	0.557	0.939	0.68
DM duration (years)	–	8 (7–10)	8 (5–12)	10.5 (8–12)	0.50	0.7	0.75	0.07
BMI	25.82 (23.80– 29.75)	32.02 (29.04–34.61)	32.31 (27.88–37.93)	36.7 (31.83–43.44)	0.641	0.003	0.032	0.0001
SBP (mm Hg)	120 (120–130)	132.5 (125–140)	132.5 (125–135)	160 (150–170)	0.600	<0.0001	<0.0001	0.0001
DBP (mm Hg)	70 (70–80)	75 (70–80)	75 (70–80)	87.5 (85–95)	0.791	<0.0001	<0.0001	0.0001
Hb (g/dL)	13.6 (12.5–14.5)	12.85 (12–14.1)	13.5 (12.6–14.15)	10.67 (10.33–11.67)	0.209	<0.0001	<0.0001	0.0001
Serum creatinine (mg/dL)	0.92 (0.78–1.01)	0.82 (0.76–0.96)	1.08 (0.99–1.22)	1.46 (1.34–1.58)	<0.0001	<0.0001	<0.0001	0.0001
eGFR (mL/min/1.73 m^2)	101.15 (92.50–114.63)	94.31 (84.33–107.07)	66.41 (55.10–76.61)	39.51 (28.75–43.20)	<0.0001	<0.0001	<0.0001	0.0001
Glycemia (fasting) (mg/dL)	102 (95–114)	150.5 (117–205)	153 (115.5–222.5)	171 (138–232)	0.853	0.189	0.208	0.0001
HbA_{1c} (%)	6.2 (5.90–6.40)	6.75 (6.30–7.30)	7.23 (6.8–8.4)	8.43 (7.93–9.05)	0.008	<0.0001	0.002	0.0001
Cholesterol (mg/dL)	160 (125–181)	215 (192–246)	239 (194.5–291.5)	275 (244–343)	0.251	0.0002	0.018	0.0001
Triglycerides (mg/dL)	132 (106–148)	153.5 (111–193)	157.5 (119–206)	168 (144–218)	0.620	0.090	0.258	0.0001
hsCRP (mg/dL)	0.85 (0.80–2.78)	4.31 (2.66–8.02)	10.66 (9.26–20.19)	23.85 (19.31–35.72)	0.0001	<0.0001	0.0007	0.0001
UACR (mg/g)	16.02 (14.32–24.45)	27.23 (21.38–28.28)	76.49 (43.47–116.62)	944.95 (518.08–1277.11)	<0.0001	<0.0001	<0.0001	0.0001
Cystatin C (mg/L)	0.66 (0.57–0.67)	0.75 (0.68–0.87)	1.02 (0.96–1.10)	1.66 (1.47–2.00)	<0.0001	<0.0001	<0.0001	0.0001
Nephrin/creat (mg/g)	0.084 (0.035–0.086)	0.11 (0.10–0.15)	0.81 (0.40–1.12)	5.64 (2.82–7.44)	<0.0001	<0.0001	<0.0001	0.0001
Podocalyxin/creat (mg/g)	35.6 (28.6–48.3)	64.95 (58–70.2)	130.15 (100.7–161.5)	422.85 (350.60–950.20)	<0.0001	<0.0001	<0.0001	0.0001
Synaptopodin/creat (mg/g)	9.33 (6.71–10.08)	17.57 (12.7–19.88)	26.81 (24.17–28.60)	67.78 (58.15–138.09)	<0.0001	<0.0001	<0.0001	0.0001
KIM-1/creat (ng/g)	43.82 (26–47.50)	69.8 (59–87.28)	123.82 (108.93–138.56)	649.64 (376.42–853.51)	<0.0001	<0.0001	<0.0001	0.0001
NAG/creat (UI/g)	1.66 (1.64–2.07)	3.08 (2.03–5.08)	10.45 (9.44–12.25)	16.85 (16.35–18.95)	<0.0001	<0.0001	<0.0001	0.0001
miRNA192	2.84 (2.71–2.92)	2.59 (2.14–2.7)	1.53 (1.36–1.74)	0.87 (0.67–0.97)	<0.0001	<0.0001	<0.0001	0.0001
miRNA21	1.02 (0.91–1.02)	1.14 (0.91–1.2)	1.73 (1.49–1.85)	2.34 (2.16–2.41)	<0.0001	<0.0001	<0.0001	0.0001
miRNA124	0.85 (0.77–1.06)	1.48 (1.24–1.85)	2.78 (2.57–2.97)	3.22 (3.18–3.41)	<0.0001	<0.0001	<0.0001	0.0001

Clinical and biological data are presented as medians and IQR, as for variables with skewed distribution.
P: group 1 vs group 2 vs group 3 vs group 4; *P: group 2 vs group 3; **P: group 2 vs group 4; ***P: group 3 vs group 4.
BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitive C reactive protein; KIM-1/creat, urinary kidney injury molecule-1:creatinine ratio; miRNA, microRNA; NAG, urinary *N* -beta-D-acetyl-glucosaminidase/creat; Nephrin/creat, urinary nephrin:creatinine ratio; SBP, systolic blood pressure; UACR, urinary albumin:creatinine ratio.

Table 2 Univariable analysis for the urinary miRNAs

Parameter	Variable	R^2	Coeff β	P
miRNA192	Nephrin/creat	0.401	−0.165	<0.0001
	Podocalyxin/creat	0.480	−0.001	<0.0001
	Synaptopodin/creat	0.489	−0.013	<0.0001
	KIM-1/creat	0.477	−0.002	<0.0001
	NAG/creat	0.882	−0.104	<0.0001
	UACR	0.537	−0.001	<0.0001
	eGFR	0.580	0.021	<0.0001
miRNA124	Nephrin/creat	0.333	0.176	<0.0001
	Podocalyxin/creat	0.366	0.001	<0.0001
	Synaptopodin/creat	0.383	0.014	<0.0001
	KIM-1/creat	0.389	0.002	<0.0001
	NAG/creat	0.834	0.118	<0.0001
	UACR	0.380	0.001	<0.0001
	eGFR	0.550	−0.024	<0.0001
miRNA21	Nephrin/creat	0.395	0.112	<0.0001
	Podocalyxin/creat	0.539	0.001	<0.0001
	Synaptopodin/creat	0.533	0.009	<0.0001
	KIM-1/creat	0.476	0.001	<0.0001
	NAG/creat	0.781	0.066	<0.0001
	UACR	0.571	0.0008	<0.0001
	eGFR	0.507	−0.013	<0.0001

eGFR, estimated glomerular filtration rate; KIM-1/creat, urinary kidney injury molecule-1:creatinine ratio; miRNA, microRNA; NAG, urinary *N*-beta-D-acetyl-glucosaminidase/creat; Nephrin/creat, urinary nephrin:creatinine ratio; UACR, urinary albumin:creatinine ratio.

kidney, leading to initiation and progression of renal fibrosis, while downregulated miRNAs displayed renal protective effects.

To the best of our knowledge, this is the first study to demonstrate an association between podocyte injury and PT dysfunction, and miRNA excretion, even in the normoalbuminuria stage in patients with type 2 DM. This observation documents a potential role of the profiles of miRNA21, miRNA124, and miRNA192 in early DN. The expression of miRNA192 was downregulated, while that of miRNA21 and miRNA124 was upregulated. This phenomenon is independent of albuminuria and the level of renal function.

miRNA21 and miRNA124 are upregulated, while miRNA192 is downregulated and correlate with podocyte damage biomarkers in early DN

Urinary elimination of nephrin is highly indicative of podocyte lesions in the course of DN. In our study, nephrinuria correlated directly with miRNA21 and miRNA124, and indirectly with miRNA192. The levels of nephrin were increased in all patients, including normoalbuminuric patients, and the correlations were found across all study groups. These data are in keeping with a previous study performed in patients with type 2 diabetes in whom we demonstrated that nephrinuria showed significant correlations with UACR, biomarkers of PT dysfunction, renal function, and urinary advanced glycation end products.[17]

In another study conducted by us in patients with type 2 DM, urinary podocytes correlated with the levels of urinary alpha$_1$-microglobulin and urinary KIM-1, even in patients with high-to-normal levels of albuminuria. This observation points to a concomitant podocyte injury and PT dysfunction, the latter phenomenon showing the crucial role of the PT in albumin processing in early DN.[27] Furthermore, in patients with type 2 DM, there is an association between urinary podocyte-derived mRNA levels, and podocyte injury and PT dysfunction biomarkers.[28]

The levels of urinary synaptopodin and urinary podocalyxin paralleled those of urinary nephrin and followed the same correlation pattern with the miRNAs studied. Urinary synaptopodin levels increase as DN advances, thus proving that urinary synaptopodin is a sensitive biomarker of DN, predicting disease progression.[18]

In a study performed in patients with type 2 DM, urinary podocalyxin was increased in 53.8 per cent normoalbuminuric patients, 64.7 per cent microalbuminuric patients, and 66.7 per cent macroalbuminuric patients, respectively, thus showing early lesions to the podocytes in patients with diabetes.[19] There are few data in the literature that show that miRNA21 is involved at the glomerular level in DN.

Table 3 Multivariable analysis for the urinary miRNAs

Parameter	Variable	Coeff β	P	95% CI	Prob>F	R^2
miRNA192	Constant	2.286	0.0001	1.985 to 2.587	<0.0001	0.902
	Synaptopodin/creat	−0.005	0.03	−0.0180334 to −0.0070125		
	NAG/creat	−0.088	0.0001	−0.099 to −0.077		
	eGFR	0.005	0.0001	0.002 to 0.008		
miRNA124	Constant	1.729	0.0001	1.338 to 2.120	<0.0001	0.882
	Synaptopodin/creat	0.1305	0.01	0.0032 to 0.2930		
	NAG/creat	0.123	0.0001	0.105 to 0.142		
	UACR	−0.0005	0.0001	−0.0007 to −0.0002		
	eGFR	−0.007	0.0001	−0.011 to −0.003		
miRNA21	Constant	1.729	0.0001	1.338 to 2.120	<0.0001	0.881
	Podocalyxin/creat	0.0007	0.04	−0.0008 to −0.0024		
	NAG/creat	0.123	0.0001	0.105 to 0.142		
	UACR	−0.0005	0.0001	−0.0007 to −0.0002		
	eGFR	−0.007	0.0001	−0.011 to −0.003		

eGFR, estimated glomerular filtration rate; KIM-1/creat, urinary kidney injury molecule-1:creatinine ratio; miRNA, microRNA; NAG, urinary *N*-beta-D-acetyl-glucosaminidase/creat; Nephrin/creat, urinary nephrin:creatinine ratio; UACR, urinary albumin:creatinine ratio.

miRNA21 was found strongly related to hyperglycemia, thus contributing to renal cell hypertrophy and matrix expansion.[31] Most likely, miRNA21 displays the same activity on the podocytes, thus explaining the high levels of nephrinuria, urinary synaptopodin, and urinary podocalyxin found in our study. However, controversial reports show that deletion of miRNA21 induces severe cellular damage, especially to the podocytes, due to upregulation of the pro-apoptotic targets of miRNA21, thus leading to podocyte loss, mesangial expansion, and albuminuria.[32]

As for miRNA192, this is for the first time that this miRNA was studied in relation to podocyte damage biomarkers. As far as our study is concerned, urinary miRNA192 levels were downregulated in our patients and correlated indirectly with urinary nephrin, synaptopodin, and podocalyxin, a fact that could allow for the speculation that miRNA192 may exert protective effects within the glomerulus, in early stages of DN.

According to the results of our study, it may be hypothesized that miRNA192 expression varies across the segments of the nephron by exerting a protective effect mainly within the tubulointerstitial compartment, but also within the glomerulus.

Podocytes are highly specialized glomerular cells, a component of major importance of the glomerular filtration barrier. It has been demonstrated that they adhere to the glomerular basement membrane through cell–matrix adhesion receptor INTEGRIN α3β1. This receptor is a target of miRNA124 and explains a potential intervention of miRNA124 in podocyte adhesion damage under mechanical stress.[26] In our study, nephrinuria correlated directly with miRNA124 and this correlation holds true for synaptopodin, as well as for podocalyxin excretion. We assume that this phenomenon may be attributed to miRNA124 involvement in podocyte structure and function.

miRNA profiles present with high variability in association with PT dysfunction in early DN

In early DN, the mechanisms of albuminuria rely on an impairment in the retrieval pathway of albumin in the PT that precedes glomerular damage.[9]

In early DN, urinary NAG and urinary KIM-1 are biomarkers that allow for an accurate detection of PT dysfunction. Several studies performed in normoalbuminuric patients with type 2 DM showed that these patients displayed increased levels of NAG[12 33] and of urinary KIM-1,[17 34] thus demonstrating that tubular functional defects precede the onset of albuminuria. Moreover, other studies reported that in patients with type 2 diabetes, urinary tubular biomarkers were independently associated with albuminuria in the early stage of DN.[34]

Higher levels of urinary KIM-1 were found in type 2 diabetes patients with glomerular hyperfiltration as compared with patients with normal eGFR, showing that this glomerular phenomenon is a trigger for PT dysfunction in early DN.[35] Also, urinary NAG has been proved a reliable biomarker of PT dysfunction in normoalbuminuric patients with type 2 diabetes.[36 37]

In our study, urinary nephrin, synaptopodin, and podocalyxin correlated with the levels of urinary NAG and urinary KIM-1, even in patients with high-to-normal levels of albuminuria. This observation points to a concomitant podocyte injury and PT dysfunction, the latter phenomenon showing the role of major importance of the PT in albumin processing in early DN. The miRNA expression profiles of miRNA21, miRNA124, and miRNA192 correlated with urinary NAG and urinary KIM-1, thus proving a potential molecular signature of these miRNAs at tubulointerstitial level.

Several studies carried out in experimental models and in human studies have provided data that show that inhibition of miRNA192 decreased renal fibrosis.[22 24]

On the contrary, in other studies, it has been postulated that loss of miRNA192 might promote fibrogenesis in advanced DN.[38] miRNA192 was shown to be downregulated in cultured proximal tubular epithelial cells treated with high glucose or TGFβ1, a phenomenon that was associated with increased fibrosis.[38] In another study, however, it was reported that miRNA192 expression in tubular cells treated with TGFβ1 increased tubulointerstitial fibrosis.[39] In our study, the indirect correlation of miRNA192 with the PT dysfunction biomarkers in normoalbuminuric patients leads to the hypothesis that miRNA192 might display a certain protective effect against tubulointerstitial fibrosis, starting from the early stages of DN.

These controversial results with regard to miRNA192 could be explained in part by the high variability of miRNA expression according to cell-type-specific effects of miRNAs,[40] cell-specific transcription factors, and differences in the experimental models studied.[39] We assume that in our patients miRNA192 expression varied with the type of cells involved, presumably most prominently within the tubulointerstitial compartment compared with the glomerulus, namely the podocytes.

The same controversial aspects have been forwarded concerning miRNA21, which has been shown to promote and increase fibrosis, inflammation, and albuminuria in type 2 diabetes,[25 31] while Lai observed that miRNA21 correlated with podocyte injury, but not with tubulointerstitial fibrosis.[32]

Our patients followed this trend, miRNA21 being correlated directly with albuminuria, podocyte damage, and with PT dysfunction biomarkers.

In the KK-AY mouse model of DN, miRNA21 played an important role in renal fibrosis by targeting matrix metalloproteinase-9 and metalloproteinase inhibitor 1.[25]

Fiorentino *et al* showed in a mouse model of type 1 diabetes, as well as in kidney biopsies from patients with DN that miRNA 21 was upregulated, thus initiating and promoting renal fibrosis.[41]

miRNA124 was upregulated in our study and correlated with albuminuria and PT dysfunction biomarkers. Very few data emerge from the literature with regard to miRNA124, mainly related to podocyte damage, as discussed above. We assume that, according to our data, miRNA124 may be involved in tubulointerstitial fibrosis as well, a fact that should be proved by further studies.

It is likely that the PT delays the expression of podocyte injury, although patients display podocyte damage biomarkers and a particular miRNA profile, while remaining normoalbuminuric. Presumably, factors that drive changes to the glomerulus are the same as those that affect PT cells processing of albumin. Therefore, the onset of albuminuria

and its progressive increase may be considered a matter of sequence in the course of DN.

miRNA21, miRNA124, and miRNA192 are independent of the renal function decline

The biomarkers of PT dysfunction are associated with renal function as assessed by eGFR and cystatin C, irrespective of the level of albuminuria.[42 43] Moreover, the same applies for podocyte injury biomarkers as was reported by several studies and by our results with regard to nephrin, synaptopodin, and podocalyxin. Other podocyte injury biomarkers studied in patients with type 2 diabetes and DN, such as urinary podocalyxin positive element, correlated with albuminuria, serum cystatin C, and eGFR.[44]

Previously, we showed that podocyte injury biomarkers, such as nephrin and vascular endothelial growth factor, may be associated to renal function decline independently of albuminuria. We assumed that this association is most likely related to PT dysfunction, the PT being a key player in albumin, as well as in podocyte injury biomarkers uptake and processing.[17]

It has been reported that decreased eGFR may correlate with the degree of albuminuria within normal range.[10 11 17] The results of our study are in line with other studies that reveal that cystatin C may parallel the degree of albuminuria, even in high-to-normal albuminuria.[45 46] Our data, which show that the urinary miRNAs studied and the biomarkers of PT dysfunction are independent of the level of renal function, are in keeping with studies that reveal a progressive decline in renal function in normoalbuminuric patients with type 2 diabetes.[47] miRNA21 and miRNA 124 showed increased levels the more the stage of DN progressed, a fact that might suggest a deleterious intervention of these miRNAs within the glomerulus, as well as the tubulointerstitium. By contrast, miRNA192 presented with decreased expression and correlated directly with eGFR, more accurately assessed in our study by means of serum creatinine and cystatin C.[30] The correlation of decreased urinary miRNA192 with eGFR could imply that the decreased levels of eGFR may be associated to loss of miRNA192 expression within the tubulointerstitial compartment, thus pleading in favor of a protective effect of miRNA192, a fact supported by several studies.[23 38] These results, however, are in contrast to other studies in which renal function declined progressively with the increasing levels of miRNA192, which is believed to promote fibrosis.[22 24] These pleiotropic roles of miRNA192 in the kidney, either anti-fibrotic or pro-fibrotic, may be cell-type dependent.[48]

There are several limitations of our study to be mentioned. First, the small size of the study groups interferes with the statistical significance of the study. Second, this is a cross-sectional study that requires validation by a prospective study in order to prove that podocyte injury and PT dysfunction are associated with a particular miRNA profile, phenomenon that holds true in the long term in patients with type 2 diabetes and predicts evolution from normoalbuminuria to microalbuminuria. Third, the differences in urinary miRNAs expression among the glomerular and tubulointerstitial compartments may be explained by the large variability of miRNAs excretion into the urine in the renal segments and across the groups studied. Finally, urinary miRNAs' excretion variability should be taken into account in relation to cell-specific type and the particular models studied.

The strength of our study relies on the documentation of a relation between urinary miRNAs and biomarkers of podocyte injury and PT dysfunction in patients with type 2 diabetes, even in the normoalbuminuria stage. This observation points to the clinical importance of urinary miRNAs in the assessment of early renal involvement in normoalbuminuric patients with type 2 diabetes, often perceived as a group of patients at low risk of developing DN.

CONCLUSION

In conclusion, in patients with type 2 DM there is an association between podocyte injury and PT dysfunction, and miRNA excretion, even in the normoalbuminuric patients. This observation documents a potential role of the miRNA profiles of miRNA21, miRNA124, and miRNA192 in early DN.

Urinary miRNAs excretion and their deregulated expression parallels PT dysfunction, a process that is independent of albuminuria and renal function. Despite their variability across the segments of the nephron, urinary miRNAs may be considered as a reliable tool for the identification of novel biomarkers in order to characterize the genetic pattern of podocyte damage and PT dysfunction in early DN of type 2 DM. Further research on larger cohorts is required with regard to a translational approach of these results in the clinical practice.

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Contributors OM and AV made substantial contributions to conception and design, analysis and interpretation of data. FG, AIS, and AnS did acquisition of data, drafting of the article, and IT technical assistance. VD did biochemical and ELISA assessments, analysis and interpretation of data. CG and FB did acquisition of data. GG revised the manuscript critically for important intellectual content. RP and DV did biochemical and ELISA assessments, and miRNAs assessments. SV did analysis and interpretation of data. PM did acquisition of data, analysis and interpretation of data, and drafting of the article. A-MP and OMC did design, analysis, and interpretation of data. SU did statistical analysis and interpretation of data. LP made substantial contributions to conception, design, analysis and interpretation of data, drafting of the article, and final approval of the version to be published. RP and DV contributed equally to the article. All authors are in agreement with the content of the manuscript.

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