BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Association of Elevated Urinary miR-126, miR-155, and miR-29b with Diabetic Kidney Disease

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Effective diabetic kidney disease (DKD) biomarkers remain elusive, and urinary miRNAs represent a potential source of novel noninvasive disease sentinels. We profiled 754 miRNAs in pooled urine samples from DKD patients ($n = 20$), detecting significantly increased miR-126, miR-155, and miR-29b compared with controls ($n = 20$). These results were confirmed in an independent cohort of 89 DKD patients, 62 diabetic patients without DKD, and 41 controls: miR-126 (2.8-fold increase; $P < 0.0001$), miR-155 (1.8-fold increase; $P < 0.001$), and miR-29b (4.6-fold increase; $P = 0.024$). Combined receiver operating characteristic curve analysis resulted in an area under the curve of 0.8. A relative quantification threshold equivalent to 80% sensitivity for each miRNA gave a positive signal for 48% of DKD patients compared with 3.6% of diabetic patients without DKD. Laser-capture microdissection of renal biopsy specimens, followed by quantitative RT-PCR, detected miR-155 in glomeruli and proximal and distal tubules, whereas miR-126 and miR-29b were most abundant in glomerular extracts. Subsequent experiments showed miR-126 and miR-29b enrichment in glomerular endothelial cells (GEnCs) compared with podocytes, proximal tubular epithelial cells, and fibroblasts. Significantly increased miR-126 and miR-29b were detected in GEnC conditioned medium in response to tumor necrosis factor- α and transforming growth factor- β 1, respectively. Our data reveal an altered urinary miRNA profile associated with DKD and link these variations to miRNA release from GEnCs. (*Am J Pathol* 2018; 188: 1982–1992; <https://doi.org/10.1016/j.ajpath.2018.06.006>)

Recent estimates suggest that 1 in 12 of the global population experiences diabetes mellitus, and approximately 40% of those affected will go on to develop diabetic kidney disease (DKD).¹ DKD is the leading

cause of end-stage renal disease, and predisposing factors include genetic causes, ethnicity, hyperglycemia, insulin resistance, intraglomerular hypertension, and hyperfiltration.^{2,3}

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Hyperglycemia results in numerous deleterious consequences, including up-regulated cytokine synthesis, renin-angiotensin system activation, generation of advanced glycation end products and reactive oxygen species, and increased protein kinase C activity.^{4,5} Nitric oxide and NF- κ B pathway—driven loss of endothelial and vascular modulation have been implicated in insulin resistance, and early DKD may be associated with insulin signaling defects specific to the podocyte.⁶ These insults result in loss of glomerular filtration rate and ultimately renal failure from mesangial hyperexpansion, nodular glomerulosclerosis, and tubulointerstitial fibrosis.⁷

Detection of urinary microalbuminuria currently forms the basis of DKD progression monitoring, varying from normal mean albuminuria values of approximately 10 mg/day to a diagnosis of microalbuminuria at 30 to 300 mg/day and macroalbuminuria >300 mg/day.⁸ Prognosis is complicated, because not all microalbuminuric patients progress to overt nephropathy. Several novel biomarkers have been assessed for utility in DKD, but none are being used as routine clinical markers, and they may lack specificity and sensitivity to predict individual DKD patient outcomes. In light of the above, novel markers that can discriminate etiology, progression, and/or response to treatment remain highly desirable.

miRNAs are ubiquitously expressed short noncoding RNAs that regulate the expression of most protein coding genes in the human genome, and detection of miR-192, miR-194, miR-215, miR-216, miR-146a, miR-204, and miR-886 is elevated in the kidney.⁹ Urinary miRNAs represent a highly promising novel source of noninvasive biomarkers that are stabilized via argonaute 2 protein/

exosome association and are rapidly and precisely detected by quantitative RT-PCR (RT-qPCR).¹⁰

Reports have suggested a role for miRNAs in the pathology of DKD,^{11,12} including previous work from this laboratory showing decreased miR-192 in biopsy specimens from late-stage DKD patients with diminished renal function.¹³ However, comparatively little is known about the abundance of urinary miRNAs in DKD patients.

We hypothesized that alterations in urinary miRNA profiles would be associated with DKD. Candidate DKD biomarkers were identified by comparing miRNA profiles in urine samples from a patient discovery cohort with those from unaffected controls. Selected candidates were then measured in a larger independent cohort. Subsequently, laser-capture microdissection (LCM) of renal biopsy specimens and *in vitro* cell culture were used to investigate the sources of our candidate urinary miRNA DKD biomarkers with respect to nephron domain and cell type.

Materials and Methods

Study Participants

DKD was defined in accordance with the National Kidney Foundation Kidney Disease Outcomes Quality Initiative Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease (CKD).¹⁴ Accordingly, CKD should be attributable to diabetes in the presence of macroalbuminuria (in the absence of urinary infection), in the presence of microalbuminuria with concomitant diabetic retinopathy, or in type 1 diabetes of at least 10 years' duration.¹⁴ The initial profiling study cohort

Table 1 Demographic and Clinical Parameters of Patients Recruited from Two Centers

Feature	Patients (n = 151)		
	Diabetic (n = 62)	DKD (n = 89)	Controls (n = 41)
Male sex, n (%)	37 (58)	55 (62)	18 (44)
Nonwhite, n (%)	13 (21)	33 (37)	
Age in years, means $\pm$ SD	52 $\pm$ 16.1	62 $\pm$ 13.6	55 $\pm$ 15.4
eGFR, mL/minute per 1.73 m ²			
Means $\pm$ SD	78 $\pm$ 16.3	30 $\pm$ 20.9	
Median (IQR)	84 (72–90)	22 (17–38)	
CKD stage, n (%)			
No CKD/CKD G1 (eGFR $\geq$ 90 mL/minute per 1.73 m ²)	23 (37)	2 (2)	
CKD G2 (eGFR = 60–89 mL/minute per 1.73 m ²)	32 (52)	10 (11)	
CKD G3 (eGFR = 30–59 mL/minute per 1.73 m ²)	5 (8)	17 (19)	
CKD G4 (eGFR = 15–29 mL/minute per 1.73 m ²)	2 (3)	45 (51)	
CKD G5 (eGFR < 15 mL/minute per 1.73 m ²)	0	15 (17)	
ACR, n (%) [*]			
A1: normal-high normal (ACR < 3 mg/mmol)	54 (87.1)	15 (16.9)	
A2: moderately increased (ACR = 3–30 mg/mmol)	8 (12.9)	25 (28.1)	
A3: severely increased (ACR > 30 mg/mmol)	0 (0)	49 (55.0)	

The two UK centers were as follows: Wales Kidney Research Tissue Bank, Cardiff (University Hospital Wales) and Birmingham (University Hospital Birmingham, Renal Impairment in Secondary Care Study Cohort).

^{*}ACR group cutoffs and nomenclature derived from Kidney Disease: Improving Global Outcomes 2012 recommendations.¹⁴

ACR, albumin/creatinine ratio; CKD, chronic kidney disease; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; IQR, interquartile range.

of 20 DKD patients and 20 healthy controls was obtained from the Wales Kidney Research Tissue Bank, University Hospital of Wales (Cardiff, UK). The DKD group was predominantly male (85%), and their mean age was 72 years (SD, ± 8.7 years). DKD patients were CKD stage 3 to 5 (predialysis), with a mean estimated glomerular filtration rate (eGFR) of 29 mL/minute per 1.73 m² (SD, ± 8.5 mL/minute per 1.73 m²) and a mean urinary albumin/creatinine ratio (ACR) of 13.5 mg/mmol (SD, ± 14.5 mg/mmol). The control group ($n = 20$) in the profiling cohort was 50% male, and their mean age was 47 years (SD, ± 11.0 years); they had no microalbuminuria (ACR, < 3 mg/mmol). For further details on ACR categories, see [Table 1](#).

The confirmation cohort was drawn from two secondary care facilities: the Wales Kidney Research Tissue Bank (as above) and the Renal Impairment in Secondary Care study, University Hospital of Birmingham (Birmingham, UK).¹⁵ Eighty-nine patients with DKD, including three patients with type 1 diabetes, and 41 healthy controls were recruited across the two sites. An additional control group of 62 diabetic patients without DKD were recruited from Cardiff, including 17 patients with type 1 diabetes. Ethical approval was granted by the Wales Kidney Research Tissue Bank Governance Committee and the South Birmingham Local Research Ethics Committee, respectively.

Patient demographics and clinical parameters are shown in [Table 1](#). All patients were recruited from specialist nephrology and diabetes care services at the two sites during the period spanning autumn 2010 to autumn 2013. DKD patients from the Renal Impairment in Secondary Care study cohort were predominantly advanced nephropaths, as per Renal Impairment in Secondary Care protocol inclusion criteria: briefly, patients with CKD stages 4 to 5 (predialysis) or CKD stage 3 and accelerated progression and/or proteinuria, as defined by the UK National Institute for Health and Care Excellence 2008 CKD guideline for secondary care review. The diabetic patient control group all had a diagnosis of diabetes by standard American Diabetes Association criteria,¹⁶ but without evidence of DKD (ie, not fulfilling the Kidney Disease Outcomes Quality Initiative criteria).

At the initial clinic visit, renal function was recorded using eGFR, calculated using the modification of diet in renal disease equation.¹⁷ Urine samples were aliquoted for ACR assessment and for RNA extraction (see below). ACR cutoffs for disease severity were defined as per Kidney Disease: Improving Global Outcomes 2012 guidelines.¹⁸

Urine Collection, RNA Isolation, and RT-qPCR Analysis

Urine samples were collected, and RNA extraction from 350 μ L of urine, generation of cDNA from equal volumes of RNA extracts, and RT-qPCR were then performed, as described in detail elsewhere.¹⁰ TaqMan assays (Thermo Fisher Scientific, Paisley, UK) used in this study were as follows: hsa-miR-29b-3p (identification number 000413), hsa-miR-126-3p (identification number 002228), hsa-miR-

155-5p (identification number 002623), and hsa-miR-191-5p (identification number 002299). Relative quantities were calculated using the $2^{-\Delta\Delta C_t}$ method, and miRNA expression was normalized to hsa-miR-191-5p.¹⁰

miRNA Profiling by TaqManArray Human MicroRNA Cards

Urinary miRNAs were reverse transcribed using the Megaplex Primer Pools (Human Pools A version 2.1 and B version 3.0; Thermo Fisher Scientific) with a predefined pool of 381 reverse transcription primers for each Megaplex Primer Pool. A fixed volume of 3 μ L of RNA solution was used as input in each reverse transcription reaction, and reverse transcription reactions were performed according to the manufacturer's recommendations. Reverse transcription reaction products were amplified using Megaplex PreAmp Primers (Primers A version 2.1 and B version 3.0; Thermo Fisher Scientific), the samples were then diluted to a final volume of 100 μ L, and control subject and DKD patient products were pooled as follows.

To exclude the possibility that sex, age, and eGFR status had extreme effects on miRNA expression profiles, the following pooling strategy was followed: control pool 1, urine samples from 5 females of average age 44.8 years; control pool 2, 5 females, with average age of 57.6 years; control pool 3, 5 males, with average age of 35.2 years; and control pool 4, 5 males, with average of 53.2 years. The following patient pool (PPs) were used: PP1, urine samples from five CKD3 patients with an eGFR between 43.3 and 36 mL/minute per 1.73 m²; PP2, five stage 3 patients, with an eGFR from 35 to 31 mL/minute per 1.73 m²; PP3, five stage 4/5 patients, with an eGFR from 27.3 to 23 mL/minute per 1.73 m²; and PP4, five stage 4/5 patients, with eGFR from 22 to 12.9 mL/minute per 1.73 m².

TaqManArray Human MicroRNA Cards A version 2.1 and B version 3.0 (Thermo Fisher Scientific) were used to quantify 754 human miRNAs. Each array included 377 test miRNAs, three endogenous controls, and a negative control. Quantitative PCR was performed on an Applied Biosystems 7900HT thermocycler (Thermo Fisher Scientific) using the manufacturer's recommended program.

LCM from Renal Biopsy Samples

Glomeruli and proximal tubular and distal tubular profiles were microdissected from sections (6 μ m thick) obtained from five formalin-fixed, paraffin-embedded archived renal biopsy samples from unaffected individuals using the Arcturus Pixcell Iie infrared laser enabled LCM system (Thermo Fisher Scientific).

Cell Culture

Human conditionally immortalized glomerular endothelial cell (GEnC) and human podocyte cell lines were propagated

at 33°C, as described previously.^{19,20} After 5 (GEnC) and 14 (podocyte) days, cells were transferred to 37°C incubation to inactivate the SV40 T antigen and permit differentiation, before experimental use. Where stated, GEnCs were growth arrested for 24 hours and then treated with 10 ng/mL tumor necrosis factor (TNF)- α or 1 ng/mL transforming growth factor (TGF)- β 1 at either 5 mmol/L normoglycemic or 25 mmol/L hyperglycemic D-glucose concentrations for 24 hours. Proximal tubular epithelial cell line HK-2²¹ and fibroblast²² cultures were maintained as described elsewhere. Cells and culture medium obtained from each well were used for RNA extraction, as described above.

Statistical Analysis

miRNA profiling data were analyzed using Thermo Fisher Scientific's DataAssist Software version 3.01, NormFinder Software version 0.953 (<http://moma.dk/normfinder-software>, last accessed February 21, 2018), and GraphPad Prism 6 version 6.0d (GraphPad Software, La Jolla, CA). Pearson correlation coefficients were used to detect clusters of similarity in miRNA threshold cycle values between each pool group in patients and between each pool group in controls. To identify a suitable reference gene for the normalization of miRNA expression in this study, the NormFinder algorithm was applied to the expression data obtained from the Human TaqMan miRNA Arrays. Analysis comparing miRNA levels between subjects with DKD and controls was performed using GraphPad Prism 6 version 6.0d. $P < 0.05$ was considered statistically significant. miRNA profiling data sets can be found in Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE114477).

Results

Altered Urinary miRNA Detection in DKD Patients

To select candidate miRNAs that may act as DKD biomarkers, data from unbiased expression profiling of 754 miRNAs in urine samples from 20 DKD patients and 20 unaffected controls were compared. Analyses were performed on four patient and four control pools, each composed of urine samples from five individuals, as recommended by Zhang and Gant.²³ Samples were pooled before profiling to minimize the contribution of subject-to-subject variation and to make substantive features easier to find, and thereby identify biomarkers common across individuals.²⁴ Previous analysis suggested that 40 individuals might optimally be pooled across eight arrays,²³ which was our chosen pooling approach.

miRNAs for which statistically significant fold-change increases were detected in patient urine compared with control samples (12 data points) and the corresponding down-regulated miRNAs (35 data points) are shown in Figure 1A. The fold-change data for these 47 miRNAs are

summarized in Figure 1B, and the 8 miRNAs exhibiting greater than fivefold change were subsequently selected as potential candidate biomarkers for further analysis.

Specific RT-qPCR assays were then used to analyze these miRNAs in each component urine sample pooled for profiling analysis. Statistically significant differences in miRNA detection between DKD patient and control urine samples were replicated for miR-126 (4.3-fold increase; $P = 0.0087$), miR-155 (22.9-fold increase; $P = 0.0024$), and miR-29b (4.9-fold increase; $P = 0.0002$) (Figure 1, C–E).

Elevated Urinary miR-126, miR-155, and miR-29b Detection in an Independent DKD Patient Cohort

To test the above findings, miR-126, miR-155, and miR-29b were quantified in samples from an independent cohort of patients with established DKD from the Renal Impairment in Secondary Care study.¹⁵ Samples from 89 patients meeting the criteria established in the UK National Institute for Health and Care Excellence 2008 criteria were available. An additional cohort of 62 patients with diabetes mellitus, but without proteinuria or other evidence of DKD, were included, as were samples from 41 individuals without evidence of diabetes or DKD (Table 1). Diabetes patients without DKD were included as a third group in this analysis to identify DKD-specific miRNA detection changes and not purely hyperglycemia-driven effects from our profiling comparison of DKD patients with control individuals.

Significant differences were again seen between DKD patients and controls for miR-126 (2.8-fold increase; $P < 0.0001$) (Figure 2A), miR-155 (1.8-fold increase; $P < 0.001$) (Figure 2B), and miR-29b (4.6-fold increase; $P = 0.024$) (Figure 2C). Comparison of DKD patients with diabetic patients without DKD was statistically significant for miR-126 (3.1-fold increase; $P < 0.0001$) and miR-155 (1.6-fold increase; $P = 0.024$), with a trend to increased miR-29b (4.1-fold increase; $P = 0.121$) (Figure 2, A–C).

RT-qPCR data for all three miRNAs were used to compare DKD patients and diabetic patients without DKD in the combined receiver operating characteristic curve analysis shown in Figure 2D, giving an area under the curve of 0.80. To analyze the contributions of each miRNA to the above receiver operating characteristic curve, individual specificity and likelihood ratios were calculated for relative expression values equivalent to a sensitivity of 80%.^{26,27} Data displayed in Table 2 illustrate the magnitude of corresponding specificity values was miR-126 > miR-155 > miR-29b, and that combined miRNA data resulted in a $\geq 6.5\%$ increase in specificity and likelihood ratio compared with individual miRNAs. These relative expression data were then used as consecutive threshold values to discriminate between DKD and diabetic patients without DKD (Table 3) from the independent cohort. The discriminatory order was miR-29b (DKD/diabetic patients without DKD = 5.62) > miR-126 (DKD/diabetic patients without

DKD = 3.48) > miR-155 (DKD/diabetic patients without DKD = 2.23), and relative expression values exceeding all three thresholds were obtained for 48.0% of DKD patients compared with 3.6% of diabetic patients without DKD (Table 3).

LCM Shows Increased Glomerular Abundance of miR-126 and miR-29b That Is Replicated in GEnC Culture

Previous reports have linked changes in miRNA expression to DKD pathology, but have focused on whole tissue studies. For example, association of decreased miR-192 expression with disease progression in DKD biopsy specimens by *in situ* hybridization has been reported.¹³

Herein, LCM was used to isolate glomeruli and proximal and distal tubules (Figure 3A) from histologically normal formalin-fixed, paraffin-embedded renal biopsy samples, and miR-126, miR-155, and miR-29b expression was analyzed by RT-qPCR. In Figure 3B, a typical CD10-stained formalin-fixed, paraffin-embedded biopsy section is seen before and after LCM to isolate glomeruli and proximal and distal renal tubules. miR-126, miR-155, and miR-29b were detected in extracts from all three nephron regions (Figure 3, C–E). Increased glomerular abundances were observed for miR-126 (Figure 3C) and miR-29b (Figure 3E), whereas miR-155 was most abundant in the distal tubule (Figure 3D).

Conclusions regarding nephron region-specific miRNA expression from the above analyses are inherently limited, however, because tissue extracts are subject to trace contamination by cells from other nephron domains. Therefore, cellular miRNA localization within each nephron region was subsequently investigated by RT-qPCR analysis of podocyte and endothelial cell (GEnC) cultures from the glomerulus, renal proximal tubular epithelial cells, and fibroblasts. Detection of miR-126 was significantly higher in GEnCs compared with other cell types (Figure 3F). Most miR-155 was detected in proximal tubular epithelial cells and least miR-155 was detected in GEnCs (Figure 3G), whereas miR-29b was most abundant in GEnCs (Figure 3H).

GEnC Release of miR-126 and miR-29b in an *in Vitro* Model of Hyperglycemia Is Driven by TNF- α and TGF- β 1, Respectively

The above data localized the majority of miR-126 and miR-29b expression to the GEnC. Stimuli by which miRNAs are

released into the glomerular ultrafiltrate, and hence the urine, were investigated next. Data from animal models of diabetes show increased glomerular and proximal tubular epithelial cell TNF- α expression, and renoprotective effects of TGF- β inhibitors have also been reported.^{28,29} GEnC expression of our candidate miRNAs was thus analyzed *in vitro* in response to TNF- α and TGF- β 1 in normoglycemia and hyperglycemia (Figure 4).

The presence of TNF- α led to significantly increased miR-126 detection in GEnC conditioned medium at 5 and 25 mmol/L D-glucose (Figure 4B), a pattern also seen for miR-29b after TGF- β 1 addition (Figure 4D). These cytokines did not increase GEnC expression of miR-126 (Figure 4A) or miR-29b (Figure 4C), a pattern consistent with increased release, but not expression, of miRNAs.

No significant changes in miR-155 were detected in response to elevated D-glucose with either cytokine, and data for TNF- α are shown (Figure 4, E and F). Similarly, changes in miR-126 after TGF- β 1 addition, and for miR-29b in the presence of TNF- α , were not observed (data not shown). Elevated D-glucose alone did not change miRNA expression in GEnCs or conditioned medium (Figure 4).

Discussion

DKD is the leading cause of kidney failure, requiring renal replacement therapy worldwide, but effective methods to identify and halt progression of disease-specific pathophysiological changes remain elusive. Current effective interventions, such as control of blood glucose and blood pressure, are challenging to achieve, costly, and time intensive. Existing tests track DKD from diabetic diagnosis to kidney failure, but do not allow accurate prognosis for the individual patient. In addition, the absence of treatment response biomarkers hinders development of emerging DKD therapies. There is, thus, an unmet need for additional DKD biomarkers to target intervention and follow response to therapy.

In this study, urinary miRNA DKD biomarkers were identified. Increased detection of miR-126, miR-155, and miR-29b was observed in the urine of DKD patients in comparison with both unaffected individuals and diabetic patients without DKD. miRNA localization and release studies further suggested specific release of miR-126 and miR-29b from GEnCs. This raised the possibility that

Figure 1 Urinary miRNA detection in urine samples from diabetic kidney disease (DKD) patients and control subjects. **A:** Volcano plot showing the detection profile of the 377 urinary miRNAs in TaqManArray Human MicroRNA Card A in DKD patients and controls. The dotted horizontal line represents a *P*-value boundary of 0.05. **B:** Fold change of miRNA detection between DKD patients and controls. DataAssist Software was used to perform relative quantification for sample comparison, to perform *t*-test sample group comparisons, and to produce the graphic output shown. **C–E:** Quantitative RT-PCR (RT-qPCR) analysis shows significant differences in detection of miR-126 (C), miR-155 (D), and miR-29b (E) between patients and control urine in the component urine samples pooled for profiling analyses (A and B). **C–E:** DKD patients versus controls for miR-126 (C), miR-155 (D), and miR-29b (E). Analysis was performed by unpaired two-tailed *t*-test with Welch's correction. Profiling data analysis using the NormFinder algorithm identified miR-191 as optimal for normalization of RT-qPCR data. Data were normalized to endogenous control miR-191 and are expressed as means $\pm$ SEM (C–E). *n* = 20 (A, DKD patients and controls, four pools of five patients, and C–E, each group). ***P* < 0.01, ****P* < 0.001.

Figure 2 Quantitative RT-PCR detection of selected miRNAs in patients and control subjects. **A–C:** Relative expression was significantly different in 89 diabetic kidney disease (DKD) patients compared with 62 diabetic patients without DKD and 41 controls for miR-126 (**A**) and miR-155 (**B**), and significantly different in DKD patients compared with controls for miR-29b (**C**). **A:** DKD patients versus diabetic patients without DKD and controls. **B:** DKD patients and diabetic patients without DKD; DKD patients versus controls. **C:** DKD patients versus controls. Analysis was performed by unpaired one-tailed *t*-test with Welch’s correction. Data were normalized to endogenous control miR-191. **D:** Combined receiver operating characteristic curve analysis for miR-126, miR-155, and miR-29b, with an area under the curve (AUC) of 0.80. Data were generated using the pROC package (<http://expasy.org/tools/pROC>)²⁵ in R-3.2.3. Data are expressed as means ± SEM (**A–C**). *n* = 89 (**A–C**, DKD patients); *n* = 62 (**A–C**, diabetic patients without DKD); *n* = 41 (**A–C**, controls). **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001.

urinary miRNA quantification might provide data on ongoing pathologic processes, and so aid patient stratification and measurement of response to therapy.

Urinary miRNA biomarkers have several potential significant advantages over circulating miRNAs for adoption into existing treatment pathways alongside current biomarkers, including speed and cost of noninvasive sample access.³⁰ However, few urinary miRNA DKD biomarker data have so far been reported. Previous studies have focused on circulating miRNAs and have generated conflicting data with respect to association of miR-126 with diabetes mellitus and/or DKD. A recent cross-sectional analysis of type 2 diabetes mellitus patients found a negative association with plasma miR-126,³¹ and similar findings have been reported for type 1 diabetes mellitus and all complications.³² By contrast, miR-126 detection did not change in whole blood from type 2 diabetes mellitus patients and control subjects, but decreased in DKD patient samples.³³ Furthermore, no change in plasma miR-126 was observed in a study of pediatric type 1

diabetic patients.³⁴ These analyses provide inconsistent data for the biomarker utility of circulating miR-126, in contrast to the significant and reproducible increases detected in miR-126, miR-155, and miR-29b in DKD patient urine in the present study.

The DKD-specific alterations in urinary miRNA profiles detected in this study may have functional significance. *In vitro* analyses localized miR-126 and miR-29b principally to the GEnC, with miR-155 expression distributed evenly across the nephron. Glomerular endothelial localization of miR-126 may reflect the role of this transcript in vascular regulation. Targeted mouse miR-126 deletion resulted in vascular abnormalities by removing inhibition of sprouty-related EVH1 domain-containing protein 1 expression, thereby enhancing vascular endothelial growth factor function.³⁵ A role in DKD pathology for vascular endothelial growth factor-A signaling between GEnCs and podocytes has been proposed.³⁶ In addition, miR-126 repression of vascular cell adhesion molecule 1 expression in human umbilical vein endothelial cells regulates

Table 2 Sensitivity and Specificity Values, Likelihood Ratios, and RQ Thresholds for miR-126, miR-155, miR-29b, and All Three miRNAs >80% ROC Curve Sensitivity Threshold

Group	Sensitivity, %	Specificity, %	Likelihood ratio	RQ threshold
All three miRNAs	80.21	63.64	2.206	>1.148
miR-126	80.41	57.14	1.876	>0.6762
miR-155	80.61	52.00	1.679	>0.9110
miR-29b	80.61	40.00	1.344	>0.8058

ROC, receiver operating characteristic; RQ, relative expression.

Table 3 DKD and D Patient Numbers and Percentages >80% ROC Curve Sensitivity Threshold for miR-126, miR-155, miR-29b, and All Three miRNAs

Group	miR-126	miR-155	miR-29b	All three miRNAs	Total
Patients >80% sensitivity threshold, <i>n</i> (%)					
D	23 (41.8)	30 (54.6)	13 (23.6)	2 (3.6)	55
DKD	80 (81.6)	67 (68.4)	73 (74.5)	47 (48.0)	98

D, diabetic patients without DKD; DKD, diabetic kidney disease; ROC, receiver operating characteristic.

their response to proinflammatory adhesion molecules.³⁷ miR-126 has also been implicated in the heterogenic inflammatory response of renal microvascular endothelial cells.³⁸

Increased expression of miR-155 has been observed in DKD patient renal biopsy specimens, in close correlation

with increased serum creatinine.³⁹ Furthermore, miR-155 deficiency attenuated renal damage and IL-17 expression was down-regulated in streptozotocin-induced DKD mice.⁴⁰ Together with miR-126, miR-155 has been implicated in multiple forms of vascular remodeling and associated with cardiovascular disease.⁴¹

Figure 3 Localization of miRNA expression by laser-capture microdissection (LCM) and cell culture. **A:** Key functional nephron domains include the glomerulus (G), the proximal tubule (PT), and the distal tubule (DT). **B:** A CD10-stained formalin-fixed, paraffin-embedded renal biopsy sample before and after excision of glomeruli by LCM. **C–E:** Relative expression of miR-126, miR-155, and miR-29b, respectively, in LCM-isolated Gs, PTs, and DTs from five renal biopsy specimens of healthy individuals. **C:** Statistically significant differences in miRNA expression were observed in G versus PT, and G versus DT. **F–H:** Relative expression of miR-126, miR-155, and miR-29b, respectively, in *in vitro* cultured HK-2 renal proximal tubular epithelial cells (PTCs), fibroblasts, podocytes, and conditionally immortalized glomerular endothelial cells (GENCs). **F:** Statistically significant differences in miRNA expression were observed in GENCs versus fibroblasts, and GENCs versus PTCs and podocytes. **G:** PTCs versus GENCs. **H:** GENCs versus podocytes. Analysis was performed by one-way analysis of variance with Tukey's multiple comparison test. Data were normalized to endogenous control miR-191 and are presented as means $\pm$ SEM (**C–H**). *n* = 5 (**C**, biopsy specimens); *n* = 4 (**H**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars = 100 μ m (**B**).

Figure 4 miRNA expression in glomerular endothelial cells (GENCs) and GENC conditioned medium in response to hyperglycemia and diabetic kidney disease–related cytokines. After 24 hours of culture in 5 or 25 mmol/L D-glucose, relative expression in GENCs and GENC conditioned medium, respectively, of miR-126 in response to 10 ng/mL tumor necrosis factor (TNF)- α (A and B), miR-29b in response to 1 ng/mL transforming growth factor (TGF)- β 1 (C and D), and miR-155 in response to 10 ng/mL TNF- α (E and F), and in untreated cells. **B:** Statistically significant differences in miRNA expression were observed in study of 5 mmol/L D-glucose versus 5 mmol/L D-glucose plus TNF- α , and 25 mmol/L D-glucose versus 25 mmol/L D-glucose plus TNF- α . **C:** Study of 5 mmol/L D-glucose versus 5 mmol/L D-glucose plus TGF- β 1, and 25 mmol/L D-glucose versus 25 mmol/L D-glucose plus TGF- β 1. **D:** Study of 5 mmol/L D-glucose versus 5 mmol/L D-glucose plus TGF- β 1, and 25 mmol/L D-glucose versus 25 mmol/L D-glucose plus TGF- β 1. Analysis was performed by one-way analysis of variance with Tukey's multiple comparison test. Data were normalized to endogenous control miR-191 and are presented as means $\pm$ SEM (A–F). $n = 4$ (D). * $P < 0.05$, ** $P < 0.01$.

Decreased miR-29b has been reported in early and advanced animal models of diabetic renal fibrosis.⁴² Chen and colleagues⁴³ found that loss of renal miR-29b in db/db mice led to increased albuminuria, TGF- β –mediated fibrosis, and immune injury, whereas restored miR-29b expression inhibited renal injury. Indeed, although up-regulated miRNAs were specifically studied, the importance of down-regulated miRNAs cannot be ignored.

miR-29b localized to the glomerular endothelium in this study. Reduction of collagen and laminin synthesis has been reported after forced miR-29b expression in human corneal endothelial cells.⁴⁴ In apolipoprotein E knockout mice, miR-29b induced aortic endothelial permeability in response to a high-fat diet and brought about aortic apoptosis by direct targeting of melatonin receptor mt1.⁴⁵ In addition, up-regulated miR-29b expression has been observed in human umbilical vein endothelial cells exposed to hyperglycemia.⁴⁶

The cytokine-driven release from GENCs observed for miR-126 (TNF- α) and miR-29b (TGF- β 1) reported herein suggests that these cells may be the principal source of elevated urinary miR-126 and miR-29b detected in DKD.

We speculate that this constitutes evidence for disease-related signaling down the nephron that will be interesting to test in future studies. Indeed, there is a reported association of urinary miRNAs with exosomes¹⁰ and exosomal transport, which might facilitate passage of miRNAs through the nephron, for all three candidate biomarker miRNAs.

Exosome-mediated release of miR-126 from CD34⁺ peripheral blood mononuclear cells is proangiogenic, and decreased miR-126 was detected in elevated glucose cell culture and diabetic patients.⁴⁷ miR-155 is depleted in urinary exosomes from microalbuminuric type 1 diabetes mellitus patients.⁴⁸ Endogenous miR-29b, spontaneously released from beta cells within exosomes, stimulates TNF- α secretion from spleen cells isolated from diabetes-prone nonobese diabetic mice *in vitro*.⁴⁹

In summary, we have used unbiased profiling approaches to identify a urinary miRNA signature associated with DKD and have subsequently confirmed increased miR-126, miR-155, and miR-29b in an independent patient cohort. miR-126 and miR-29b were identified as enriched in GENCs, and

released from these cells in response to DKD-related cytokines. Urinary miR-126, miR-155, and miR-29b are, therefore, promising DKD biomarkers, and the potential pathologic significance of miR-126 and miR-29b release from GEnCs merits further evaluation.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.06.006>.

References

- Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, Saran R, Wang AY, Yang CW: Chronic kidney disease: global dimension and perspectives. *Lancet* 2013, 382:260–272
- Cowie CC, Port FK, Wolfe RA, Savage PJ, Moll PP, Hawthorne VM: Disparities in incidence of diabetic end-stage renal disease according to race and type of diabetes. *N Engl J Med* 1989, 321:1074–1079
- Regele F, Jelencsics K, Shiffman D, Pare G, McQueen MJ, Mann JF, Oberbauer R: Genome-wide studies to identify risk factors for kidney disease with a focus on patients with diabetes. *Nephrol Dial Transplant* 2015, 30:26–34
- Makita Z, Radoff S, Rayfield EJ, Yang Z, Skolnik E, Delaney V, Friedman EA, Cerami A, Viassara H: Advanced glycosylation end products in patients with diabetic nephropathy. *N Engl J Med* 1991, 325:836–842
- Schrijvers BF, De Vriese AS, Flyvbjerg A: From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines. *Endocr Rev* 2004, 25: 971–1010
- Coward RJ, Saleem MA: Podocytes as a target of insulin. *Curr Diabetes Rev* 2011, 7:22–27
- Rodriguez-Iturbe B, Johnson RJ, Herrera-Acosta J: Tubulointerstitial damage and progression of renal failure. *Kidney Int Suppl* 2005, 99: S82–S86
- Parving H-H, Persson F, Rossing P: Microalbuminuria: a parameter that has changed diabetes care. *Diabetes Res Clin Pract* 2015, 107:1–8
- Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al: A mammalian microRNA expression atlas based on small microRNA sequencing. *Cell* 2007, 129:1401–1414
- Beltrami C, Clayton A, Newbury LJ, Corish C, Jenkins RH, Phillips AO, Fraser DJ, Bowen T: Stabilization of urinary microRNAs by association with exosomes and argonaute 2 protein. *Noncoding RNA* 2015, 1:151–165
- Trionfini P, Benigni A, Remuzzi G: MicroRNAs in kidney physiology and disease. *Nat Rev Nephrol* 2015, 11:23–33
- Simpson K, Wonnacott A, Fraser DJ, Bowen T: MicroRNAs in diabetic nephropathy: from biomarkers to therapy. *Curr Diab Rep* 2016, 16:35
- Krupa A, Jenkins R, Luo DD, Lewis A, Phillips AO, Fraser DJ: Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. *J Am Soc Nephrol* 2010, 21:438–447
- KDOQI clinical practice guideline for diabetes and CKD: 2012 update. *Am J Kidney Dis* 2012, 60:850–886
- Stringer S, Sharma P, Dutton M, Jesky M, Ng K, Kaur O, Chapple I, Dietrich T, Ferro C, Cockwell P: The natural history of, and risk factors for, progressive chronic kidney disease (CKD): the Renal Impairment in Secondary Care (RIISC) study: rationale and protocol. *BMC Nephrol* 2013, 14:95
- American Diabetes Association: Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010, 33 Suppl 1:S62–S69
- Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D; Modification of Diet in Renal Disease Study Group: A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. *Ann Intern Med* 1999, 130:461–470
- Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group (2013): KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Int Suppl* 2013, 3:S1–S150
- Satchell SC, Tasman CH, Singh A, Ni L, Geelen J, von Rhuland CJ, O'Hare MJ, Saleem MA, van den Heuvel LP, Mathieson PW: Conditionally immortalized human glomerular endothelial cells expressing fenestration in response to VEGF. *Kidney Int* 2006, 69:1633–1640
- Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P: A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 2002, 13:630–638
- Jenkins RH, Davies LC, Taylor PR, Akiyama H, Cumbers B, Beltrami C, Carrington CP, Phillips AO, Bowen T, Fraser DJ: miR-192 induces G₂/M growth arrest in aristolochic acid nephropathy. *Am J Pathol* 2014, 184:996–1009
- Midgley AC, Bowen T, Phillips AO, Steadman R: MicroRNA-7 inhibition rescues loss of epidermal growth factor receptor hyaluronan-dependent differentiation in fibroblasts. *Aging Cell* 2014, 13:235–244
- Zhang SD, Gant TW: Effect of pooling samples on the efficiency of comparative studies using microarrays. *Bioinformatics* 2005, 21: 4378–4383
- Kendziorzi C, Irizarry RA, Chen KS, Haag JD, Gould MN: On the utility of pooling biological samples in microarray experiments. *Proc Natl Acad Sci U S A* 2005, 102:4252–4257
- Robin X, Turck N, Hainard N, Tiberti N, Lisacek F, Sanchez JC, Müller M: pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011, 12:77
- Prowle JR, Calzavacca P, Licari E, Ligabo EV, Echeverri JE, Bagshaw SM, Haase-Fielitz A, Haase M, Ostland V, Noiri E, Westerman M, Devarajan P, Bellomo R: Combination of biomarkers for diagnosis of acute kidney injury after cardiopulmonary bypass. *Ren Fail* 2015, 37:408–416
- Schley G, Köberle C, Manuilova E, Rutz S, Forster F, Weyand M, Formentini I, Kientsch-Engel R, Kai-Eckardt U, Willam C: Comparison of plasma and urine biomarker performance in acute kidney injury. *PLoS One* 2015, 10:e0145042
- Navarro J, Milena F, Mora C, Leon C, Claverie F, Flores C, Garcia J: Tumor necrosis factor- α gene expression in diabetic nephropathy: relationship with urinary albumin excretion and effect of angiotensin-converting enzyme inhibition. *Kidney Int Suppl* 2005, 99:S98–S102
- Ohga S, Shikata K, Yozai K, Okada S, Ogawa D, Usui H, Wada J, Shikata Y, Makino H: Thiazolidinedione ameliorates renal injury in experimental diabetic rats through anti-inflammatory effects mediated by inhibition of NF- κ B activation. *Am J Physiol Renal Physiol* 2007, 292:F1141–F1150

30. Smith DA, Newbury LJ, Drago G, Bowen T, Redman JE: Electrochemical detection of urinary microRNAs via sulfonamide-bound antisense hybridisation. *Sens Actuators B Chem* 2017, 253: 335–341
31. Olivieri F, Spazzafumo L, Bonafè M, Recchioni R, Prattichizzo F, Marcheselli M, Micolucci L, Mensà E, Giuliani A, Santini G, Gobbi M, Lazzarini R, Boemi M, Testa R, Antonicelli R, Antonio Domenico Procopio AD, Bonfigli AR: miR-21-5p and miR-126a-3p levels in plasma and circulating angiogenic cells: relationship with type 2 diabetes complications. *Oncotarget* 2015, 6:35372–35382
32. Barutta F, Bruno G, Matullo G, Chaturvedi N, Grimaldi S, Schalkwijk C, Stehouwer CD, Fuller JH, Gruden G: MicroRNA-126 and micro-/macrovascular complications of type 1 diabetes in the EURODIAB prospective complications study. *Acta Diabetol* 2017, 54:133–139
33. Al-Kafaji G, Al-Mahroos G, Al-Muhtareh HA, Skrypnik C, Sabry MA, Ramadan AR: Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: a potential blood-based biomarker. *Exp Ther Med* 2016, 12:815–822
34. Osipova J, Fischer D-C, Dangwal S, Volkmann I, Wiedera C, Schwarz K, Lorenzen JM, Schreiver C, Jacoby U, Heimhalt M, Thum T, Haffner D: Diabetes-associated MicroRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. *J Clin Endocrinol Metab* 2014, 99:E1661–E1665
35. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN: The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell* 2008, 15:261–271
36. Brosius FC, Coward RJ: Podocytes, signaling pathways, and vascular factors in diabetic kidney disease. *Adv Chronic Kidney Dis* 2014, 21: 304–310
37. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ: MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proc Natl Acad Sci U S A* 2008, 105:1516–1521
38. Ásgeirsdóttir SA, van Solingen C, Kurniati NF, Zwiers PJ, Heeringa P, van Meurs M, Satchell SC, Saleem MA, Mathieson PW, Banas B, Kamps JA, Rabelink TJ, van Zonneveld AJ, Molema G: MicroRNA-126 contributes to renal microvascular heterogeneity of VCAM-1 protein expression in acute inflammation. *Am J Physiol Renal Physiol* 2012, 302:F1630–F1639
39. Huang Y, Liu Y, Li L, Su B, Yang L, Fan W, Yin Q, Chen L, Cui T, Zhang J, Lu Y, Cheng J, Fu P, Liu F: Involvement of inflammation-related miR-155 and miR-146a in diabetic nephropathy: implications for glomerular endothelial injury. *BMC Nephrol* 2014, 15:142
40. Lin X, You Y, Wang J, Qin Y, Huang P, Yang F: MicroRNA-155 deficiency promotes nephrin acetylation and attenuates renal damage in hyperglycemia-induced nephropathy. *Inflammation* 2015, 38: 546–554
41. Welten SMJ, Goossens EAC, Quax PHA, Nossent AY: The multifactorial nature of microRNAs in vascular remodeling. *Cardiovasc Res* 2016, 110:6–22
42. Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, Koh P, Thomas M, Jandeleit-Dahm K, Gregorevic P, Cooper ME, Kantharidis P: Suppression of microRNA-29 expression by TGF-β1 promotes collagen expression and renal fibrosis. *J Am Soc Nephrol* 2012, 23:252–265
43. Chen H-Y, Zhong X, Huang XR, Meng X-M, You Y, Chung ACK, Lan HY: MicroRNA-29b inhibits diabetic nephropathy in db/db mice. *Mol Ther* 2014, 22:842–853
44. Toyono T, Usui T, Villarreal G, Kallay L, Matthaai M, Vianna LMM, Zhu AY, Kuroda M, Amano S, Jun AS: MicroRNA-29b overexpression decreases extracellular matrix mRNA and protein production in human corneal endothelial cells. *Cornea* 2016, 35:1466–1470
45. Zhu HQ, Li Q, Dong LY, Zhou Q, Wang H, Wang Y: MicroRNA-29b promotes high-fat diet-stimulated endothelial permeability and apoptosis in apoE knock-out mice by down-regulating MT1 expression. *Int J Cardiol* 2014, 176:764–770
46. Silambarasan M, Tan JR, Karolina DS, Armugam A, Kaur C, Jeyaseelan K: MicroRNAs in hyperglycemia induced endothelial cell dysfunction. *Int J Mol Sci* 2016, 17:518
47. Mocharla P, Briand S, Giannotti G, Dörries C, Jakob P, Paneni F, Lüscher T, Landmesser U: AngiomiR-126 expression and secretion from circulating CD34⁺ and CD14⁺ PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. *Blood* 2013, 121: 226–236
48. Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, Bruno G, Cimino D, Taverna D, Derigibus MC, Rastaldi MP, Perin PC, Gruden G: Urinary exosomal microRNAs in incipient diabetic nephropathy. *PLoS One* 2013, 8:e73798
49. Salama A, Fichou N, Allard M, Dubreil L, De Beaurepaire L, Viel A, Jégou D, Bösch S, Bach J-M: MicroRNA-29b modulates innate and antigen-specific immune responses in mouse models of autoimmunity. *PLoS One* 2014, 9:e106153