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Genome-wide Profiling of Urinary Extracellular Vesicle microRNAs Associated With Diabetic Nephropathy in Type 1 Diabetes

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Introduction: Diabetic nephropathy (DN) is a form of progressive kidney disease that often leads to end-stage renal disease (ESRD). It is initiated by microvascular complications due to diabetes. Although microalbuminuria (MA) is the earliest clinical indication of DN among patients with type 1 diabetes (T1D), it lacks the sensitivity and specificity to detect the early onset of DN. Recently, microRNAs (miRNAs) have emerged as critical regulators in diabetes as well as various forms of kidney disease, including renal fibrosis, acute kidney injury, and progressive kidney disease. Additionally, circulating extracellular miRNAs, especially miRNAs packaged in extracellular vesicles (EVs), have garnered significant attention as potential noninvasive biomarkers for various diseases and health conditions.

Methods: As part of the University of Pittsburgh Epidemiology of Diabetes Complications (EDC) study, urine was collected from individuals with T1D with various grades of DN or MA (normal, overt, intermittent, and persistent) over a decade at prespecified intervals. We isolated EVs from urine and analyzed the small-RNA using NextGen sequencing.

Results: We identified a set of miRNAs that are enriched in urinary EVs compared with EV-depleted samples, and identified a number of miRNAs showing concentration changes associated with DN occurrence, MA status, and other variables, such as hemoglobin A1c levels.

Conclusion: Many of the miRNAs associated with DN occurrence or MA status directly target pathways associated with renal fibrosis (including transforming growth factor-β and phosphatase and tensin homolog), which is one of the major contributors to the pathology of DN. These miRNAs are potential biomarkers for DN and MA.

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More than 2 million people worldwide currently suffer from end-stage renal disease (ESRD). The United States has the highest prevalence rate, and accounts for more than 30% of individuals with ESRD worldwide.[1] One of the largest contributors to ESRD is diabetic nephropathy (DN), particularly in the western world where incidences of diabetes are higher than in developing countries, and diabetic patients live long enough to progress from DN to ESRD. However, as rates of diabetes in third-world countries continue to increase, so may the risk of developing DN, and consequently ESRD, worldwide.

DN is a microvascular complication associated with poor glycemic control. Early in the disease process, hyperglycemia induces glomerular hyperfiltration, which is followed by increased glomerular permeability to macromolecules and thickening of the glomerular basement membrane and eventually glomerular sclerosis and interstitial fibrosis. This disruption of glomerulus function leads to a decrease in the glomerular filtration rate and an increase in protein in the urine (proteinuria). Microalbuminuria (MA) has been used as an early biomarker of DN, but it lacks the specificity and sensitivity to detect early onset of DN: clinical factors unrelated to DN can affect MA status,[2] and recently it has been observed that up to 30% of DN cases may occur in

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the absence of obvious MA.[3,4] Nonetheless, the exact relationship between MA, DN, and progression to ESRD remains an area of active research.

Over the past 2 decades, much work has demonstrated that a class of short (~20 bp) noncoding RNAs, microRNAs (miRNAs), play an important role in shaping the cellular transcriptome,[5,6] are involved in a diverse set of cellular processes,[7] and are often perturbed in many disease states, including type 1 diabetes (T1D) and type 2 diabetes.[8,9] Recently, the discovery of miRNAs in the extracellular environment[10–12] and circulating in various biological fluids[13] has suggested that miRNAs may be functioning as paracrine or endocrine signals between cells. Some of these circulating miRNAs are encapsulated in various extracellular vesicles (EVs), including microvesicles, exosomes, and apoptotic bodies.[14] A number of studies have profiled these EV molecular contents and demonstrated their functional activities.[12,15–18] It has been proposed that specific circulating miRNAs can act as noninvasive diagnostic biomarkers for a wide variety of diseases and conditions.[19,20]

Several groups have looked at circulating miRNAs in patients with pre-T1D or patients with newly diagnosed T1D,[21] in juveniles with T1D,[22] and in urinary EVs of patients with incipient DN.[23] We previously reported a quantitative polymerase chain reaction (qPCR)-based profiling study of the urinary miRNA spectra of patients with T1D with MA who would eventually develop DN, identifying miRNAs associated with biological pathways, such as transforming growth factor-β/bone morphogenetic protein signaling, which are perturbed in DN and other renal and kidney diseases.[24]

Recently, advances have been made in methods to isolate and characterize EVs, and in next generation sequencing approaches to profile miRNAs.[20] To complement our previous qPCR-based miRNA study, we isolated and characterized EVs from the urine of patients with T1D with overt DN or MA, and used small-RNA sequencing (small-RNAseq) to comprehensively profile miRNAs in urine and urinary EVs. We detect miRNAs showing significant concentration changes in urine and urinary EVs that differ not only between MA and DN status, but also by factors such as hemoglobin A1c (HbA1c) levels. Follow-up validation using qPCR confirmed many of these findings.

METHODS

Sample and Study Collection

Urine samples were collected from participants of the Pittsburgh Epidemiology of Diabetes Complications (EDC) study, as described previously.[24] We analyzed urine from matched samples of 2 cohorts: (i) normoalbuminuric (N) diabetic patients who never developed microalbuminuria or nephropathy after prolonged (25-year) follow-up versus those who developed overt nephropathy (O), and (ii) patients who developed *intermittent* microalbuminuria (IMA) matched against EDC participants who developed *persistent* microalbuminuria (PMA), with 2 samples corresponding to a baseline urine sample from the last visit that tested negative for albumin and the subsequent follow-up, albuminuric sample that was collected 2 years after the first visit. A urine sample from healthy individuals (used as controls for the qPCR assays) was obtained from Innovative Research (Novi, MI). Samples were stored at −80°C until used for analysis. Before analysis, samples were thawed on ice and spun at 2000*g* at 4°C for 30 minutes to remove cellular debris. The resulting supernatant was then used for protein analysis, EV isolation, or RNA isolation as described later in this article. See supplementary data for detailed sample and study information.

Sodium Dodecyl Sulfate Protein Gel Electrophoresis

Individual urine samples were diluted in NuPAGE LDS sample buffer and NuPAGE Reducing reagent, and heated at 70°C for 10 minutes. The samples were then run on precast NuPAGE Novex Bis-Tris Mini gels with Novex Sharp prestained protein ladder and stained with SimplyBlue SafeStain, according to the manufacture's protocol (Thermo Fisher, Waltham, MA).

EV Isolation

EVs were isolated from 250 μl of urine using qEV size-exclusion column (Izon Science, Cambridge, MA) with de-gassed 1X phosphate-buffered saline. Eluate fractions (~500 μl) containing microvesicles (fractions 7–10) were collected individually. The subsequent fractions depleted of microvesicles (11–35) were collected into a single 15-ml tube. The vesicle fractions were pooled and concentrated to ~100 μl using Amicon 10K centrifugation filters (EMD Millipore, Billerica, MA) spun in a swing-bucket rotor at 4000*g* at 4°C for 20 minutes. To confirm the purification of EVs from samples, the qEV-purified EVs were examined with transmission electron microscopy at the Fred Hutchinson Cancer Center, as previously described.[25]

miRNA Isolation

miRNA was isolated from 250 μl of urine, or concentrated EV/EV-depleted fractions from the same amount of urine of each patient using the miRNeasy Micro kit (QAIGEN, Germantown, MD). The RNA was eluted

with 14 μl of nuclease-free H_20 and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with a RNA (Pico) chip; 5 μl RNA was used as input for library construction.

Small-RNA Library Construction and Sequencing

We used an in-house small-RNAseq library construction method that uses adapters with 4 degenerated bases to reduce adapter-RNA ligation bias (see Supplementary Methods for the detailed protocol). Individual library concentrations were measured using the NEBNext Library Quant Kit for Illumina (New England Biolabs, Ipswich, MA) and adjusted to a final pooled concentration of 2 nM and run on a NextSeq sequencer (Illumina, San Diego, CA).

Data Analysis of Small-RNAseq Results

We used an in-house small-RNAseq data analysis pipeline (sRNAnalyzer[26]) to identify and compare miRNA levels for each sample (including urine, EV, and EV-depleted fractions). The quantity of miRNA is indicated by read counts that were normalized by counts per mapped million. To be considered as expressed miRNA, the raw read count of the miRNA has to be more than 10 reads and more than 10% of the mean of individual sample in 70% of the samples. Samples were compared using *t*-test, with a $P < 0.05$, and a log2 fold change cutoff of ±0.60. For determination of linear correlation between miRNAs and Hb1Ac levels, the coefficient of determination (R^2), was calculated between these variables. All statistical analysis was done in Microsoft Excel (Redmond, WA).

qPCR

qPCR validation of miRNAs that showed concentration changes in the small-RNAseq datasets was performed using TaqMan Advanced miRNA assays (Thermo Fisher). Based on our small-RNAseq data, hsa-miR-16-5p was identified as invariant (did not show significant concentration changes across samples) and therefore was used as a normalization control for each assay. qPCR data were analyzed using Microsoft Excel. Relative mRNA or miRNA values are represented as the inverse of ΔC_t values (maximum number of cycles − ΔC_t) to give the linear range, which is directly proportional to the concentration of each mRNA in each sample, as previously described.[24,27] For qPCR assessment of specific kidney mRNA concentration, the QuantiTect SYBR Green PCR kit (QAIGEN) was used. The following PCR primers (forward and reverse, respectively) were used to detect mRNA present in urine, EV, or EV-depleted fractions: Aquaporin 2 (AQP2): GCTCCGCTCCATAGCCTTC, GGGTGCCAATACCCAAGCC; Nephrin (NPHS1): CTGCC TGAAAACCTGACGGT, GACCTGGCACTCATACTCCG; Podocin (NPHS2): ACCAAATCCTCCGGCTTAGG, CAA CCTTTACGCAGAACCAGA; and β-actin: CGTCCACCG CAAATGCTT, TCTGCGCAAGTTAGGTTTTGTC. The level of β-actin was used to normalize the results because it has been previously used as a control for mRNA concentration measurements in urine and urinary EVs.[28]

RESULTS

From the urine that was collected from participants of the Pittsburgh EDC study (see Supplementary Table S1), EVs were prepared using a size-exclusion chromatography (SEC)-based method that allowed for the separation of EVs, and EV-depleted fractions from each sample. We confirmed elevated protein levels (including a 60-to 80-kDa protein that likely represents albumin) in urine samples from patients with MA compared with patients without MA (Supplementary Figure S1A), and found their respective EV fractions isolated by SEC had reduced protein levels based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (Supplementary Figure S1B).

Isolated EVs were confirmed using electron microscopy, which revealed the presence of vesicles 30 to 100 nm in size that showed the typical morphological features of exosomes as described (Figure 1a and b).[25] Previous studies have shown the urine-derived EVs contain mRNA transcripts of kidney origin, including AQP2, NPHS1, and NPHS2.[28] We were able to detect these mRNAs in urine and EV, and EV-depleted samples, and interestingly found elevated levels of AQP2 and NPHS2 in patients with MA with overt nephropathy (overt) when compared with matched normoalbuminuric (non-MA) patients (normal) (Figure 1c) in urine and EV fractions (urine $P = 0.005693$ and 0.006899, respectively; EVs $P = 0.002592$ and 0.005345, respectively). Conversely, we did not see significant concentration differences of these transcripts between normal and overt patients in their respective EV-depleted fractions (Figure 1c), suggesting most of these mRNAs are present in EVs. The urine and EV ratio of NPHS1 and NPHS2 to AQP2 mRNA has been previously shown to be representative of MA status and can mark the progression of DN and ESRD.[29,30] Both urine and EV fractions of normal patients had higher NPHS1/AQP2 ratios compared with NPHS2/AQP2 ratios, whereas overt patients had higher NPHS2/AQP2 ratios compared with NPHS1/AQP2 ratios (urine $P = 0.025409$ and $P = 0.042664$, respectively; and EV $P = 0.045542$ and 0.009604, respectively; Figure 1d). We did not see significant normal versus overt differences in the NPHS1/AQP2 or NPHS2/AQP2 ratios in EV-depleted fractions (Figure 1d).

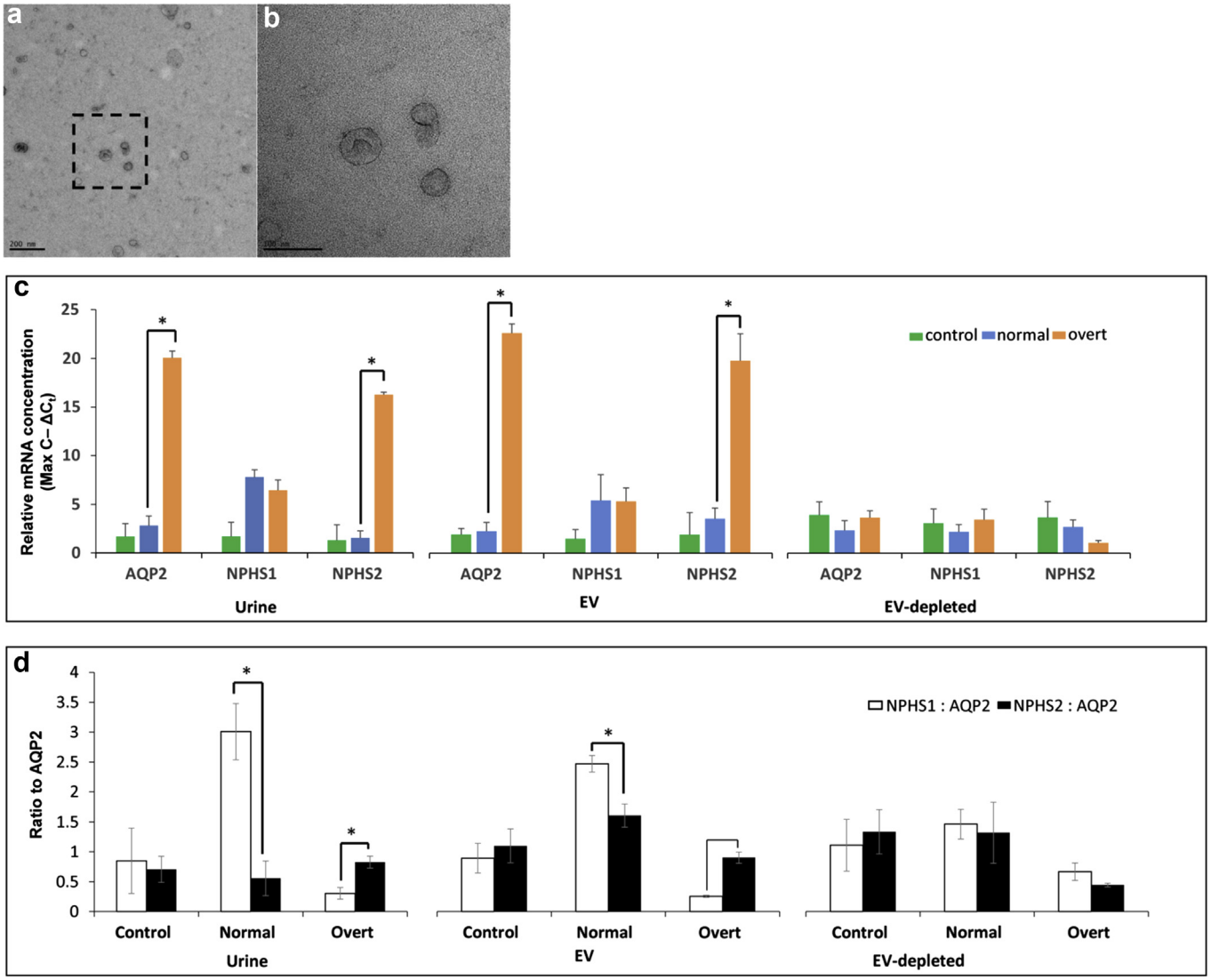

Figure 1. Isolation of extracellular vesicles (EVs) from urine of patients with type 1 diabetes (T1D). (a) Scanning electron micrograph of EVs isolated using size-exclusion chromatography (SEC) from urine. Bar in lower left indicates 200 nm for reference. (b) Higher-resolution image from boxed region in (a). Bar in lower left indicates 100 nm for reference. (c) Quantitative polymerase chain reaction results for mRNA kidney markers Aquaporin2 (AQP2), Nephrin (NPSH1), and Podocin (NPHS2) in urine, EV, and EV-depleted (EV-dep) samples for control (green bars, non-T1D), normal (blue bars, T1D nonmicroalbuminuria), and overt (orange bars, T1D overt diabetic nephropathy status) patient samples. Values are represented as the inverse of ΔC_t values (maximum number of cycles – ΔC_t) to give the linear range, which is directly proportional to the concentration of each mRNA in each sample. (d) The ratio of NPHS1 (white bars) and NPHS2 (solid bars) to AQP2 (NPHS1: AQP2, NPHS2: AQP2) from linear range values in (c) is shown for control, normal, and overt patient samples in urine, EV, and EV-dep samples. Statistically significant ($P \leq 0.05$) comparisons are indicated by an asterisk.

To identify miRNAs that exhibit specific concentration changes in the EVs, we made small-RNA libraries from each patient listed in Supplementary Table S1 from (i) total urine, (ii) EVs, and (iii) EV-depleted fractions. After sequencing, we obtained 7,250,689, 6,934,825, and 2,625,170 processed input reads from urine, EV, and EV-depleted fractions on average, respectively (Supplementary Table S2). Of those, 182,127, 270,688, and 187,920 reads mapped to miRNAs from these respective fractions (Supplementary Table S2).

We analyzed the data using $\log_2$-transformed, counts per mapped million normalized read counts (Supplementary Table S3). When comparing normal with overt patients, we observed a number of miRNAs that showed statistically significant concentration changes in urine and corresponding EV fraction (Figure 2a; Table 1). In urine, the concentrations of the 5 affected miRNAs (miR-130a-3p, miR-142-3p, miR-223-3p, miR-22-3p, and miR-320a-3p) were all elevated in overt samples. The EV fraction showed more affected miRNAs (Figure 2a; Table 1), and we validated the changes of miR-144-3p, miR-26a-5p, and miR-30c-5p in the EV fraction using qPCR (Figure 3a).

When analyzing the miRNA profiles of intermittent and persistent patients, we compared patients who

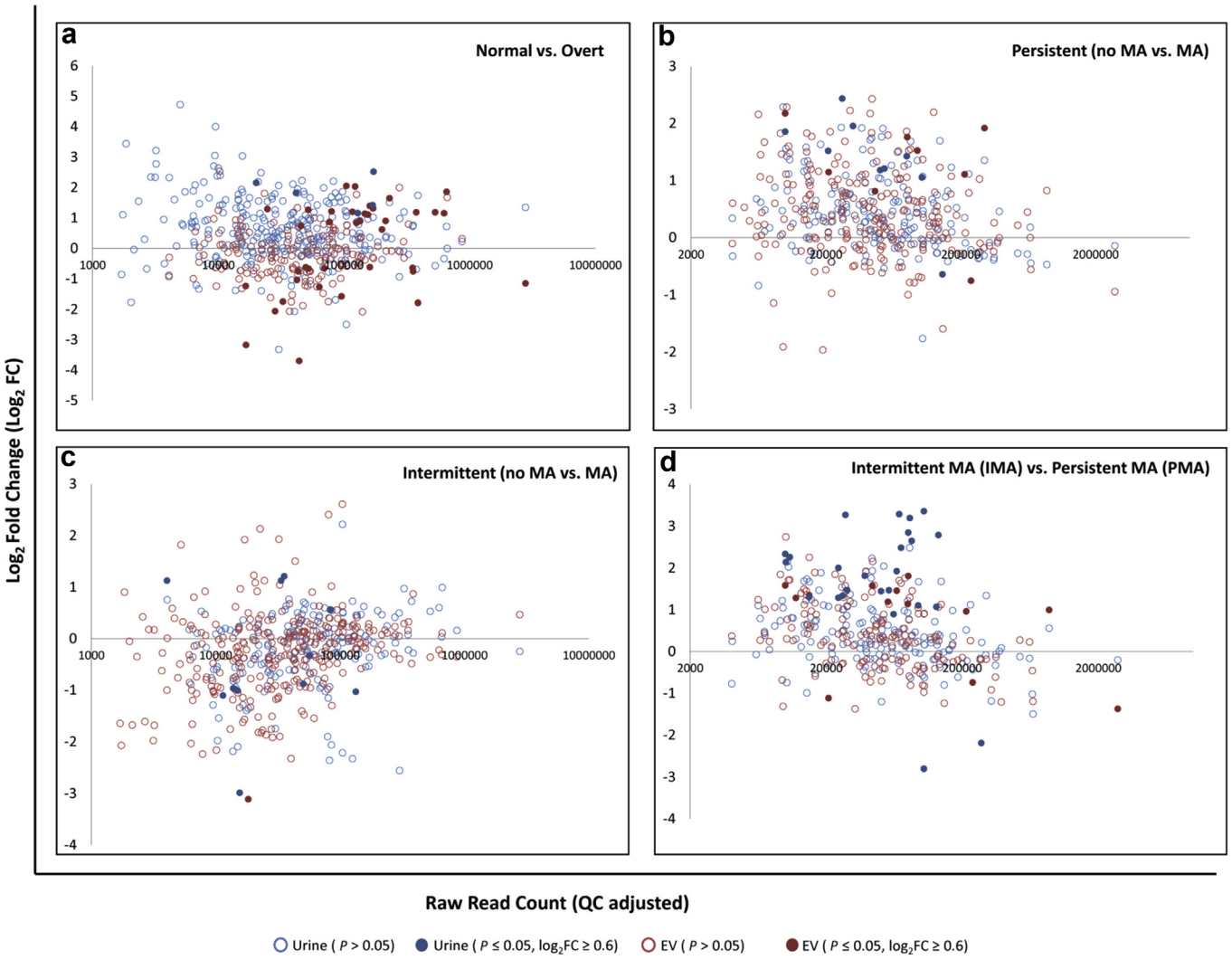

Figure 2. miRNA concentration changes from small-RNA sequencing across different type 1 diabetes (T1D) patient cohorts. Microalbuminuria (MA) plot of log_2FC (fold change) of counts per million (CPM) versus raw read count for (a) normal versus overt, (b) persistent (no MA vs. MA), (c) intermittent (no MA vs. MA), and (d) intermittent MA (IMA) versus persistent MA (PMA) T1D patient cohorts. Light blue dots represent individual miRNAs from urine that do not have statistically significant ($P \leq 0.05$) log_2FC values, whereas dark blue dots indicate miRNAs from urine showing statistically significant log_2FC values ≥ 0.6. Light red dots represent individual miRNAs from EVs that do not have statistically significant log_2FC values, whereas dark red dots indicate miRNAs from EVs showing statistically significant log_2FC values ≥ 0.6. EV, extracellular vesicle.

progressed to MA with their previous non-MA cycle within each cohort. Within the persistent cohort, where progression to MA status persisted for several disease cycles, we identified several urinary and EV miRNAs that were elevated in persistent patients' MA (PMA) cycle compared with the previous non-MA cycle (Figure 2b; Table 2). qPCR validation of miR-31-5p and miR-200c-3p confirmed concentration changes between non-MA and PMA in EVs, but not in urine (Figure 3b).

Within the intermittent patient cohort, where progression to MA status was intermittent for several disease cycles, we identified some urinary and EV miRNAs that showed concentration changes in patient MA (IMA) cycles compared with the previous non-MA cycles (Figure 2c), but only 1 miRNA, miR-671-5p, was identified that showed a statically significant concentration change in EVs (Table 3). The smaller number of affected miRNAs in this cohort may reflect the more irregular and mild nature of MA seen in these intermittent patients. An overall reduction of protein in intermittent MA patient urine compared with persistent and overt patients was observed (Supplementary Figure S1A).

When IMA transitions to PMA, it marks a critical clinical transition for patients with T1D, as comparison of albumin excretion rates and other clinical features between these 2 phases can be used to calculate risk of developing DN.[31] We compared miRNA profiles between patients with PMA and those with IMA

Table 1. Selected miRNAs showing differential concentration changes between Normal and Overt patients

Starting fraction	miRNA	NormAVE (log_2CPM)	OvertAVE (log_2CPM)	Log_2FC	*P*
Urine	hsa-miR-130a-3p ♂	8.80	10.62	1.82	0.0238
	hsa-miR-142-3p	8.20	10.35	2.16	0.0223
	hsa-miR-223-3p	11.33	13.85	2.52	0.0498
	hsa-miR-22-3p	11.87	13.29	1.42	0.0289
	hsa-miR-320a-3p	11.81	12.97	1.16	0.0332
EV (selected)	hsa-miR-941-1-3p	9.71	6.01	−3.70	0.0083
	hsa-miR-9-1-3p	7.53	9.59	2.05	0.0479
	hsa-let-7c-5p	9.31	11.33	2.03	0.0050
	hsa-miR-125b-1-5p	11.48	13.34	1.86	0.0067
	hsa-miR-486-1-5p	15.05	13.26	−1.79	0.0066
	hsa-miR-144-3p	11.98	10.24	−1.75	0.0108
	hsa-miR-30a-5p	12.82	14.46	1.65	0.0400
	hsa-miR-125a-5p	10.97	12.36	1.39	0.0146
	hsa-miR-30c-1-5p	12.25	13.56	1.31	0.0450
	hsa-miR-29b-1-3p ♂	9.99	11.28	1.29	0.0048
	hsa-miR-99b-5p	10.01	11.22	1.21	0.0246
	hsa-miR-23b-3p	11.10	12.30	1.20	0.0047
	hsa-let-7a-1-5p	13.37	14.55	1.18	0.0179
	hsa-miR-26a-1-5p	13.95	15.10	1.15	0.0023
	hsa-miR-451a-5p	17.85	16.70	−1.15	0.0036
	hsa-miR-27b-3p	12.42	13.55	1.14	0.0164
	hsa-miR-99a-5p	10.60	11.70	1.10	0.0431
	hsa-miR-26b-5p	12.36	13.25	0.90	0.0002
	hsa-miR-29c-3p	12.20	13.07	0.86	0.0066
	hsa-miR-363-3p	10.30	9.54	−0.76	0.0233
	hsa-miR-185-5p	7.93	5.40	−0.67	0.0299

CPM, counts per million; EV, extracellular vesicle; miRNA, microRNA.
♂ (male) or ♀ (female) designates gender-specific enrichment for a given miRNAs. See Supplementary Table S3 for additional details.

(patients at their MA disease cycle) and identified several miRNAs that showed concentration changes (Figure 2d; Table 4). In urine, miR-10a-5p was elevated in patients with IMA, and miR-200a-3p was elevated in patients with PMA; both were validated by qPCR. We also observed miR-200c-3p to be elevated in the EV fraction from patients with PMA compared to their previous non-MA cycle, and subsequently between patients with PMA and those with IMA in EV and urine. We were able to validate this result by qPCR; however, in only the EV fraction of patients with PMA and not in urine (Figure 3b).

Exosomal miRNAs Showing Correlation With Plasma Glucose Concentrations/HbA1c Levels in Patients With T1D

To determine if any miRNAs in EVs correlated with patient HbA1c level, we examined the linear correlation between total number of mapped reads and HbA1c levels (ranging from 5.2% to 14.3%; Supplementary Table S1). We did not observe any correlation between overall miRNA read counts and HbA1c levels (Figure 4a and b). Interestingly, several individual EV-associated miRNAs (miR-941-5p, miR-34c-5p, and miR-208a-3p) showed a significant correlation with the HbA1c level (Figure 4c, e, and g), whereas their EV-depleted equivalents did not (Figure 4d, f, and h). Because of the role for miR-34c-3p in glucose regulation in the kidney,[32] we confirmed its association with HbA1c levels in EV but not in EV-depleted samples using qPCR (Figure 4i and j).

To identify miRNAs in our dataset that showed preferential enrichment in EVs, we compared miRNA profiles between EV and EV-depleted fractions across all of our patients with T1D, and identified 60 miRNAs that showed significant enrichment in EV (Supplementary Table S4). Using the MEME software suite,[33] we identified an enriched motif present in the seed sequences of urinary EV-enriched miRNAs (Figure 5a), suggesting that some factors may be involved in sorting these miRNAs into EVs. Some of these EV-enriched miRNAs showed concentration differences in various comparisons (normal vs. overt, persistent non-MA vs. PMA, intermittent non-MA vs. IMA, and IMA vs. PMA) (Figure 5b).

DISCUSSION

We report the results of the first comprehensive next generation sequencing–based analysis of the changes of miRNA profiles in urine, urinary EV, and EV-depleted urine fractions from patients with T1D with various grades of DN and MA. Urinary EVs have been proposed as a potential source of biomarkers for various forms of kidney disease, including DN.[34] Although urinary EVs have been studied by various proteomics approaches,[35,36] little progress has been made in studying urinary EV-associated miRNAs during the development of T1D-associated DN. EV miRNA profiling is limited by several factors, including how the samples are collected, processed, and stored; the methods used for EV and RNA isolation; and a platform used for miRNA profiling that is both sensitive and free of technical artifacts. The Pittsburgh EDC study has allowed for urine samples to be collected and processed in a uniform manner, including optimal storage conditions, and minimal freeze-thaws. We have adapted an SEC-based method that provides consistent and reliable isolation of EVs based on their characteristic size range, minimally altering their characteristics and properties, and avoiding protein contamination. This SEC-based method performs as well if not better than ultracentrifugation and commercial kits in these regards.[37,38] We show that SEC-purified EVs contain kidney-enriched protein transcripts (AQP2, NPHS1, NPHS2), and their concentration changes are consistent with prior reports on their association with the onset of DN and ESRD.[28,29] Although SEC has many advantages over other methods, there are still some limitations that should be

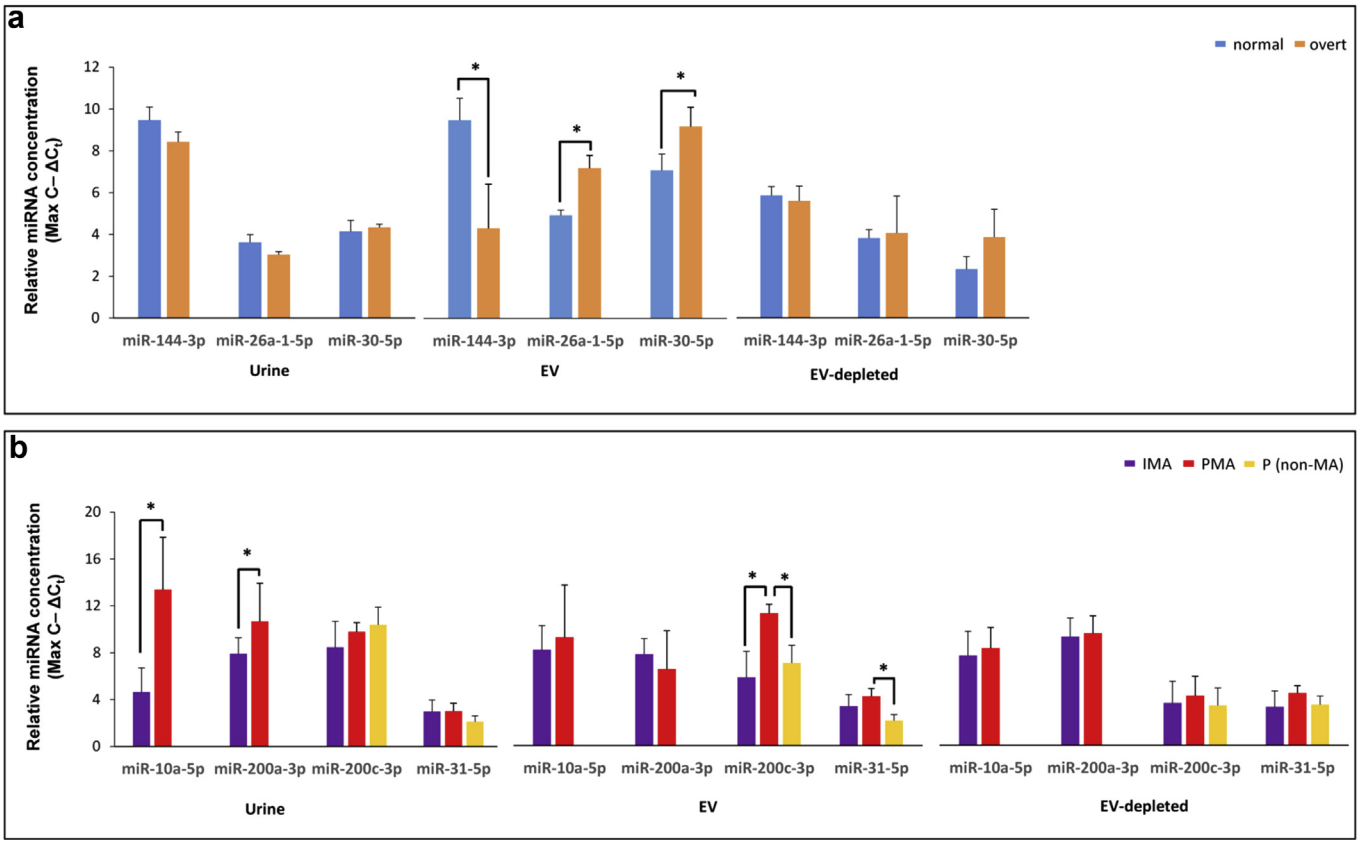

Figure 3. Quantitative polymerase chain reaction (qPCR) validation of microRNAs (miRNAs) from small-RNA sequencing of patients with type 1 diabetes (T1D). (a) qPCR results for miR-144-3p, miR-26a-5p, and miR-30c-5p in, urine, EV, and EV-depleted samples for normal (blue bars, non-MA), and overt (orange bars, overt MA status) patients. (b) qPCR results for miR-10a-5p, miR-200a-3p, miR-200c-3p, and miR-31c-5p in urine, EV, and EV-depleted samples for intermittent MA (purple bars), persistent MA (red bars), and persistent non-MA (yellow bars) patients. Values are represented as the inverse of ΔC_t values (maximum number of cycles – ΔC_t) to give the linear range, which is directly proportional to the concentration of each miRNA in each sample. Statistically significant ($P \leq 0.05$) comparisons are indicated by an asterisk. EV, extracellular vesicle fraction; EV-dep, EV-depleted fraction; IMA, intermittent (microalbuminuria); MA, microalbuminuria; PMA, persistent (microalbuminuria); P (nonmicroalbuminuria), persistent (nonmicroalbuminuria).

considered. Ultracentrifugation has been the preferred method for the isolation of EVs, due to the ability to isolate large amounts of EVs from large volumes of starting material. Current SEC columns that have been developed for EV isolation can isolate only 1 to 2 ml biofluid or cell culture media in a single use, whereas ultracentrifugation can scale up to the tens or hundreds of milliliters in a single setting. Careful considerations of the trade-offs between these methods should be considered before selecting the appropriate technique. Moreover, it has been shown that various factors that occur during RNA isolation and library preparation, including the isolation method being used in day-to-day batch effects, and RNA ligation bias can influence the miRNA profile from biofluids. Here, we have also revised and streamlined the existing small-RNA isolation and profiling method to reduce the bias that has been reported between RNA isolation and sequencing library construction kits commonly in use.[39,40]

From our small-RNAseq results, we identified miRNAs that showed significant concentration changes in patients when compared with non-DN controls (for patients with overt DN) or when compared with their previous non-MA urine collection cycle (for patients with IMA or PMA). Many of these miRNAs have been previously associated with kidney function, and have roles in the pathophysiology of diabetes[23,24,41–145] (Table 5). In DN patient EV fractions, 2 of these miRNAs, miR-486-1-5p and miR-363-3p, were identified in our previous study as being predictive for patients who will eventually develop MA during DN.[130] Several other miRNAs showing concentration changes in the EVs, including miR-125a-5p, miR-30c-1-5p, let-7a-1-5p, miR-26a-5p, and miR-451a-5p, also have been shown to be involved in DN in other studies.[43,60,122,146,147] In addition, several elevated miRNAs (miR-26a-5p, miR-26b-5p, and miR-27b-3p) are also involved in kidney glomerular function and injury.[110,113,148]

In both urine and EVs, we found miR-31-5p to be elevated in patients with PMA. miR-31-5p has been previously shown to be upregulated in polycystic kidney disease, and elevated in the serum of patients

Table 2. Selected miRNAs showing differential concentration changes between non-MA and MA persistent patients

Starting fraction	miRNA	PMA:NO-AVE (log_2CPM)	PMA:MA-AVE (log_2CPM)	Log_2FC	*P*
Urine	hsa-miR-133a-1-3p	5.97	8.40	2.43	0.0254
	hsa-miR-31-5p	7.95	9.81	1.85	0.0498
	hsa-miR-122-5p	8.96	10.48	1.52	0.0097
	hsa-miR-99b-5p	10.57	11.99	1.43	0.0324
	hsa-miR-92b-3p	8.29	9.49	1.21	0.0282
	hsa-miR-181b-1-5p	9.44	10.49	1.05	0.0077
EV	hsa-miR-182-5p	9.80	11.32	1.52	0.0446
	hsa-miR-200c-3p	11.83	13.58	1.76	0.0483
	hsa-miR-30d-5p	13.48	14.59	1.11	0.0414
	hsa-miR-31-5p	8.74	10.92	2.17	0.0457
	hsa-miR-335-5p	10.40	11.21	0.81	0.0142
	hsa-miR-96-5p	7.75	8.90	1.15	0.0298

AVE, average; CPM, counts per million; EV, extracellular vesicle; FC, fold change; MA, microalbuminuria; miRNA, microRNA; PMA:NO, Persistent MA: non-MA cycle; PMA:MA, Persistent MA:MA cycle.
See Supplementary Table S3 for additional details.

with T1D or type 2 diabetes with microvascular complications.[126–128] Additionally, miR-200c-3p, which we observed to be elevated in PMA patient EV fractions, plays an important role in glomerular cell function.[94] Although both of these miRNAs showed concentration changes in urine and EVs, qPCR validation suggests that the main source of the signal in the urine is likely from the EVs that are present. Conversely, miR-200a-3p, which has a well-characterized role during renal fibrosis induced by DN,[93] is also elevated in PMA urine (but not in EVs). miR-10a-5p and miR-10b-5p, shown to be enriched in kidney and involved in acute kidney injury,[47,48] are both elevated in the urine of patients with PMA compared with patients with IMA, but not the corresponding EV fractions. These results suggest that the miRNA spectra of urine and EVs are mostly unique, and the miRNA concentration changes present in urine mostly derive from non-EV miRNAs.

Table 3. Selected miRNAs showing differential concentration between non-MA and MA intermittent patients

Starting fraction	miRNA	IM:NO-AVE (log_2CPM)	IM:MA-AVE (log_2CPM)	Log_2FC	*P*
Urine	hsa-miR-3168-5p	10.23	7.24	-2.99	0.0153
	hsa-miR-342-3p	9.06	10.27	1.21	0.0487
	hsa-miR-152-3p	8.28	9.41	1.13	0.0164
	hsa-miR-339-3p	6.87	8.00	1.13	0.0392
	hsa-miR-4286-5p	9.58	8.47	-1.11	0.0270
	hsa-miR-192-5p	10.17	9.13	-1.03	0.0320
	hsa-miR-362-5p	8.43	7.42	-1.01	0.0458
	hsa-miR-197-3p	9.53	8.56	-0.97	0.0296
	hsa-miR-1307-3p	9.67	8.78	-0.88	0.0159
	hsa-miR-188-5p	6.82	5.23	-1.58	0.0128
	hsa-miR-424-3p	7.16	5.25	-1.91	0.0192
EV	hsa-miR-671-5p	6.43	3.32	-3.12	0.0451

AVE, average; CPM, counts per million; EV, extracellular vesicle; FC, fold change; MA, microalbuminuria; miRNA, microRNA; IMA:NO, Intermittent MA: non-MA cycle; IMA:MA, Intermittent MA:MA cycle.
See Supplementary Table S3 for additional details.

Table 4. Selected miRNAs showing differential concentration changes between IMA and PMA patients

Starting fraction	miRNA	I(MA)-AVE (log_2CPM)	P(MA)-AVE (log_2CPM)	Log_2FC	*P*
Urine	hsa-miR-10a-5p	10.16	13.52	3.36	0.0048
	hsa-miR-10b-5p	10.33	12.97	2.65	0.0408
	hsa-miR-124-1-3p	11.16	8.97	-2.19	0.0448
	hsa-miR-141-3p	7.61	10.88	3.27	0.0129
	hsa-miR-148a-3p	11.72	12.79	1.07	0.0428
	hsa-miR-183-5p	7.10	9.58	2.48	0.0428
	hsa-miR-192-5p	9.13	11.92	2.79	0.0222
	hsa-miR-200a-3p	9.16	12.44	3.29	0.0069
	hsa-miR-200c-3p	10.19	13.04	2.85	0.0202
	hsa-miR-29b-1-3p ♂	9.29	10.57	1.28	0.0017
	hsa-miR-30b-5p	10.80	12.27	1.46	0.0169
	hsa-miR-31-5p	7.47	9.81	2.33	0.0005
EV	hsa-miR-200c-3p	11.78	13.58	1.80	0.0351
	hsa-miR-31-5p	9.34	10.92	1.58	0.0343
	hsa-miR-373-3p	10.35	11.92	1.57	0.0495
	hsa-miR-451a-5p	17.32	15.95	-1.38	0.0166
	hsa-miR-362-5p	8.63	9.98	1.34	0.0195
	hsa-miR-28-3p	8.20	9.48	1.28	0.0116
	hsa-miR-660-5p	9.95	11.14	1.19	0.0173
	hsa-miR-99b-5p	10.38	11.52	1.14	0.0250
	hsa-miR-122-5p	10.60	9.48	-1.12	0.0284
	hsa-miR-21-5p	15.29	16.29	0.99	0.0291
	hsa-miR-30d-5p	13.63	14.59	0.96	0.0468
	hsa-miR-101-1-3p	13.57	12.82	-0.75	0.0008

AVE, average; CPM, counts per million; EV, extracellular vesicle; FC, fold change; I(MA) = intermittent MA: MA cycle; MA, microalbuminuria; miRNA, microRNA; P(MA), persistent MA:MA cycle.
♂ (male) or ♀ (female) designates gender-specific enrichment for a given miRNAs. See Supplementary Table S3 for additional details.

In addition, when looking at experimentally validated gene targets and pathways, many of these EV-associated miRNAs regulate pathways associated with renal fibrosis, including the transforming growth factor beta (TGF-β) signaling pathway and phosphatase and tensin homolog signaling (Tables 1–4). We used our miRNA profiling results to explore mirPath[149] and identified many additional T1D- and renal fibrosis–associated pathways, including the regulation of focal and cell adhesion molecules, tight/gap/adherens junctions, and ECM-receptor interactions, among many more (Figure 6).[150–164]

Although many studies provide *in vivo* and *in vitro* experimental support for the intracellular roles of miRNAs in kidney diseases and function, only a few studies have analyzed circulating miRNAs in patients with T1D.[20] In both plasma and urine of patients with T1D, miR-21 has been observed to be elevated in multiple cases.[22,140] We also observed such a change of miR-21 in patients with PMA. In addition, miR-148a-5p, miR-26a-5p, and miR-30a-5p have been reported to be elevated in the serum of patients with T1D.[21,140] Here we find these concentrations are also increased in

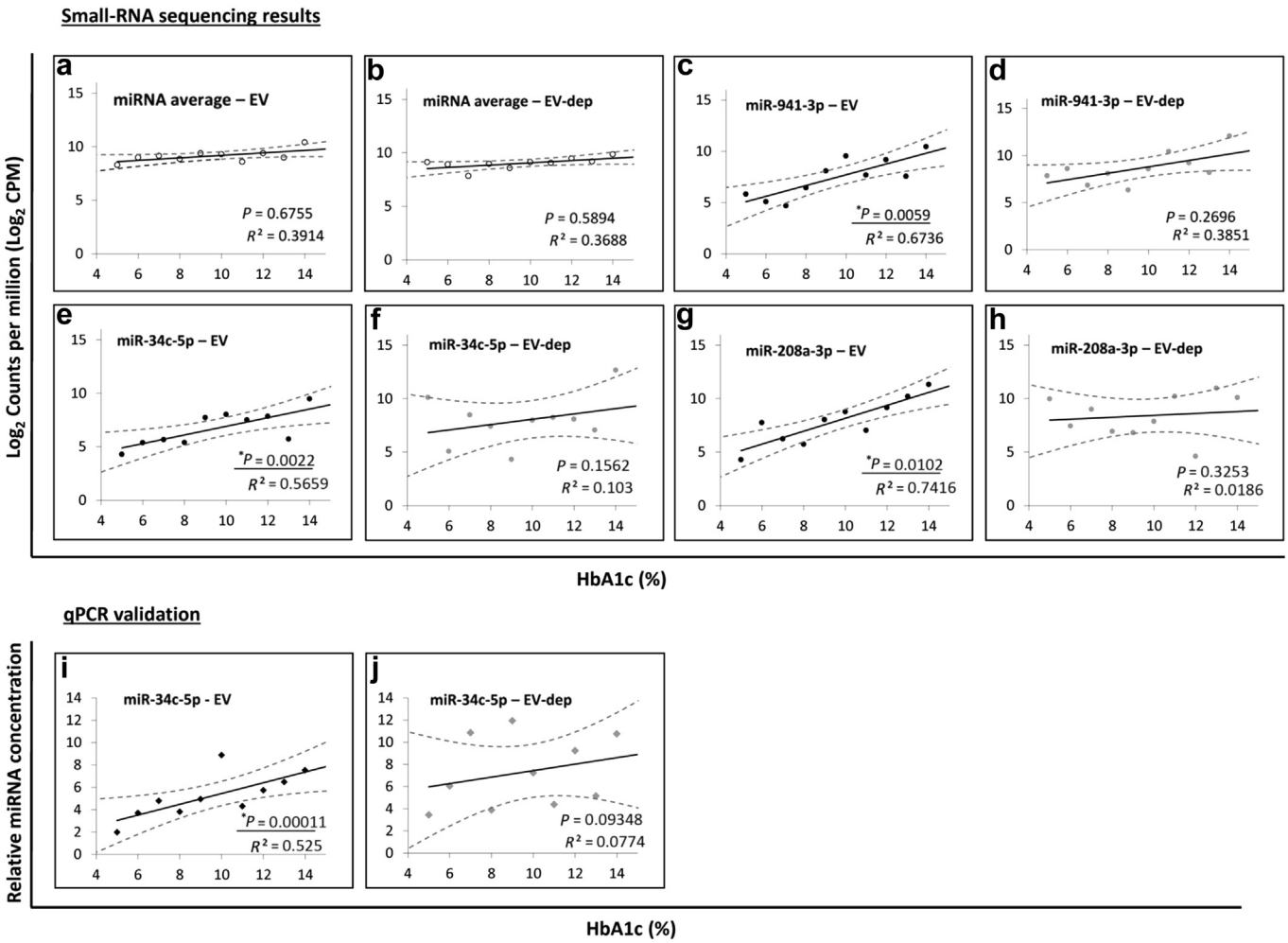

Figure 4. Extracellular vesicle (EV) microRNAs (miRNAs) that show linear correlation with type 1 diabetes patient plasma glucose (hemoglobin A1c [HbA1c]). Plots of $\log_2$ counts per million (CPM) versus patient HbA1c (%) for (a) average of all miRNAs in patient EV samples, (b) average of all miRNAs in patient EV-depleted samples, (c) miR-941-3p in patient EV samples, (d) miR-941-3p in patient EV-depleted samples, (e) miR-34c-5p in patient EV samples, (f) miR-34c-5p in patient EV-depleted samples, (g) miR-208a-3p in patient EV samples, and (h) miR-208a-3p in patient EV-depleted samples. Quantitative polymerase chain reaction (qPCR) results for miR-34c-5p in patient (i) EV and (j) EV-depleted samples. qPCR values are represented as the inverse of ΔC_t values (maximum number of cycles – ΔC_t) to give the linear range, which is directly proportional to the concentration of each miRNA in each sample. Correlation coefficients (R^2 values) and P values are reported in bottom right corners. Statistically significant ($P \leq 0.05$) linear correlations are underlined.

overt DN patient EVs (miR-26a-5p and miR-30a-5p) and PMA patient urine (miR-148a-5p).

We compared our results with other published studies of circulating (i.e., in serum and/or plasma) miRNAs in DN. miR-21-5p, miR-29c-5p, miR-31-5p, and miR-660-5p, have been reported to be elevated in patients with DN[139,165,166] compared with non-DN controls; we found 3 of 4 to be elevated in urinary EVs of PMA (miR-21-5p, miR-31-5p, miR-660-5p) and the fourth to be elevated in patients with overt DN (miR-29c-5p). As with serum and plasma, profiling of urinary miRNAs has been done previously in patients with T1D and DN. Our own previous work using quantitative reverse-transcriptase PCR to profile urinary miRNAs in individuals from the same patient cohort identified miR-92-5p, miR-141-3p, miR-335-5p, miR-486-5p, miR-28-3p, and miR-373-3p as being elevated in patients with DN or MA.[24,130] We found these miRNAs exhibited similar concentration changes in the data reported here, but with some being elevated exclusively in urine (miR-141-3p), or urine EVs (miR-28-3p, miR-373-3p, miR-335-5p, miR-486-5p). Limited profiling of miRNAs in urinary EVs of patients with DN or MA has also been previously reported, with miR-130a-5p and miR-192-5p levels being elevated in patients with early-stage DN.[23] In our data, however, we found these miRs elevated only in the urine of patients with overt DN (miR-130a-5p) and PMA (miR-192-5p), but not in their respective EV samples. The method of EV isolation used by Barutta *et al.*[23] (ultracentrifugation of pooled urine samples) may possibly account for the discrepancies between our results.

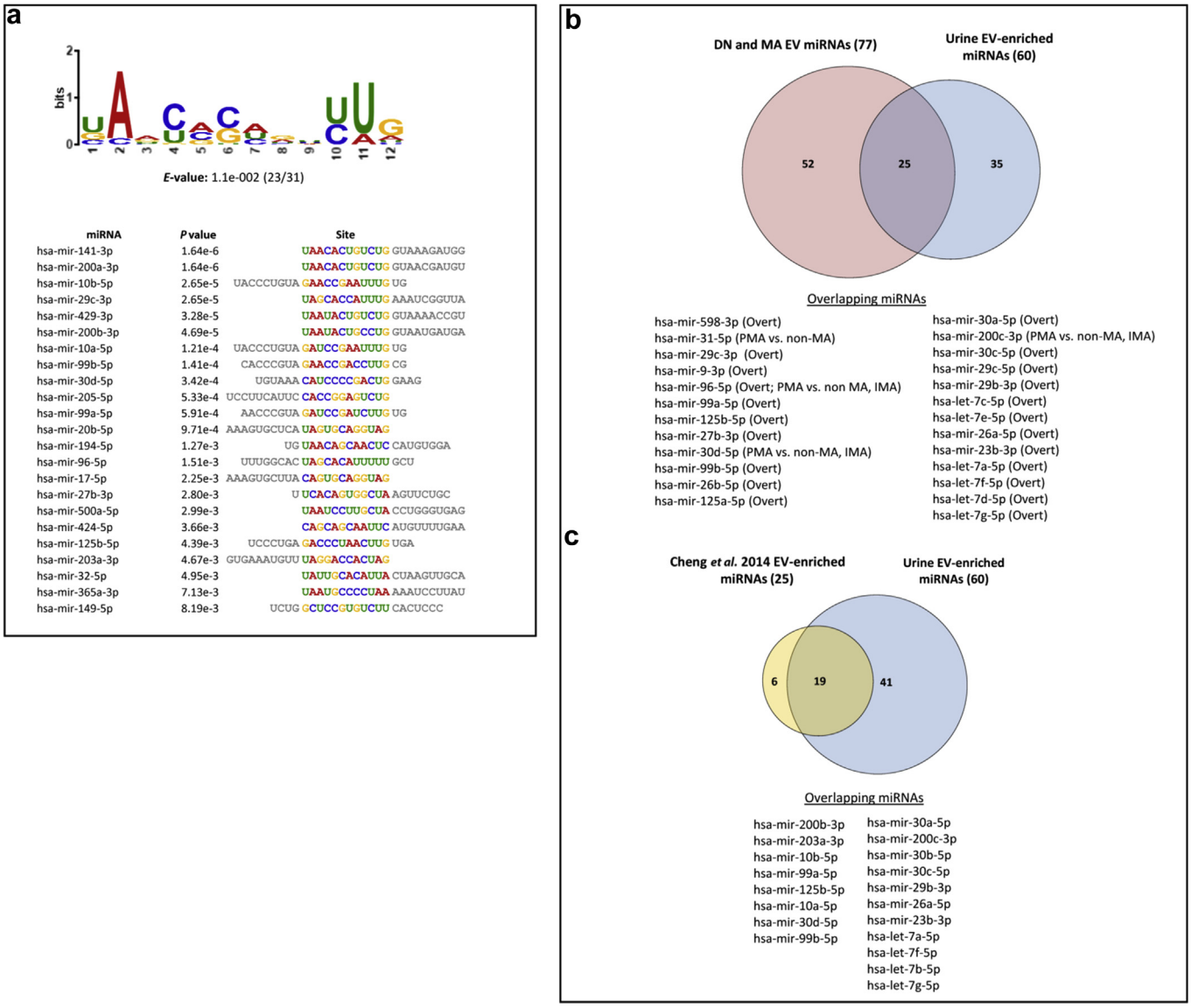

Figure 5. Identification of extracellular vesicle (EV)–enriched microRNAs (miRNAs) from patients with type 1 diabetes (T1D). (a) Novel sequence motif identified from the 31 urine-specific EV-enriched miRNAs (see Supplementary Table S3) with an E-value of 1.1e-002 in 23 of 31 samples. Each individual miRNA site alignment with the given *P* value is displayed. (b) Venn diagram showing overlap (25) between 77 miRNAs from patients with T1D who showed differential concentration changes in EVs (red) with 60 EV-enriched miRNAs (blue). (c) Venn diagram showing overlap (19) of top 25 urine EV-enriched miRNAs reported from Cheng *et al.* 2014[180] (yellow) with urine-specific EV-enriched miRNAs identified in this work (blue).

Indicators of diabetes progression, such as HbA1c (%) level, provide a somewhat quantitative assessment and correlate reasonably well with β-cell function. Although several miRNAs circulating in serum and plasma have been shown to have good linear correlation with HbA1c, no studies have examined correlations between urine or urinary EV miRNAs and HbA1c. The patients in this study had wide-ranging HbA1c levels (5.2% to 14.3%) that correlated well with increasing urinary EV-associated miR-941-5p, miR-34c-5p, and miR-208a-3p concentrations. Prior reports have shown that miR-34c-5p has a role in regulating glucose levels in the podocytes, where it inhibits glucose-induced apoptosis through the Notch pathway,[34] and in attenuating the epithelial–mesenchymal transition required for fibrosis through the transforming growth factor-β pathway.[167] Although an exact role for miR-941-3p remains elusive, it has been shown to preferentially target the insulin-signaling pathway, suggesting a possible role in regulating glucose levels.[168] It is interesting that elevated circulating miR-208a-3p has been shown to be strongly associated with cardiovascular disease,[169–172] and elevated HbA1c is a risk factor for

Table 5. miRNAs identified in this work that are associated with kidney function and/or diabetes

miRNA	Kidney function	Diabetes function	Targeted pathways (validated)
hsa-let-7a-1-5p	LN, RCC, DN	DN	TGF-β (TGFBRI)
hsa-let-7c-5p	Rfib, ESRD	T1D (ESRD)	TGF-β (TGF-βRI)
hsa-miR-101-1-3p	AKI	–	–
hsa-miR-10a-5p	AKI,AKR	T2D (Glucose regulation)	cAMP (CREB1)
hsa-miR-10b-5p	RCC, AKR, AKI	Insulin resistance	Apoptosis (BCL2L11)
hsa-miR-122-5p	RCC	T2D (insulin resistance, obesity)	–
hsa-miR-124-1-3p	RCC	T2D (Glucagon, gastric bypass treatment)	–
hsa-miR-125a-5p	PCKD, DN	T2D,DN	IL-6R
hsa-miR-125b-1-5p	CKD, AKI	T1D (β-cell)	–
hsa-miR-130a-3p	DN	T1D	–
hsa-miR-133a-1-3p	–	T1D (β-cell)	FoxO/AMPK
hsa-miR-141-3p	RCC, DN	DN, Obesity-induced DM	PTEN (PTEN), TGF-β (ZEB1/2), IGF2
hsa-miR-142-3p	AKI, AKR	T2D	TGF-β (TGFβRI)
hsa-miR-144-3p	IgA-N	T2D (Micro-vascular)	–
hsa-miR-148a-3p	LN-RD	T2D (β-cell)	PTEN (PTEN)
hsa-miR-152-3p	–	T2D (Insulin)	PTEN (PTEN)
hsa-miR-181b-1-5p	AKR, Nephron development	Glucose homeostasis, insulin resistance	Six2
hsa-miR-182-5p	AKI, PKD	T2D (Insulin)	–
hsa-miR-183-5p	–	T2D (β-cell)	–
hsa-miR-185-5p	AKR	β-cell	Cytokine/IGF (SOCS3)
hsa-miR-188-5p	AKI, RIRI	–	–
hsa-miR-192-5p	AKI, RIRI, DN	T2D (β-cell), DN	–
hsa-miR-197-3p	–	Glycemic impairment	–
hsa-miR-200a-3p	Rfib, DN	DN, insulin	TGF-β (TGFβRI), IGF
hsa-miR-200c-3p	Glomerular cell function	T2D (Endo)	TGF-β (ZEB1/2)
hsa-miR-21-5p	Rfib, DN, IgA-N	T1D, T2D	TGF-β (SMAD7), PTEN (PTEN)
hsa-miR-22-3p	Rfib	T1D	TGF-β (BMP-7,6)
hsa-miR-23b-3p	RCC	T1D (β-cell)	PTEN (PTEN), Apoptosis (DP5)
hsa-miR-26a-1-5p	Podocyte injury, LN-RD, DN	T2D (Glucose/Insulin)	IGF (GSK3B), TGF-β (CTGF)
hsa-miR-26b-5p	AKI	T2D (β-cell- Insulin)	PTEN (PTEN)
hsa-miR-27b-3p	Glomerular injury	T2D (Islet)	TGF-B (gremlin 1)
hsa-miR-28-3p	RCC, DN	T1D (DN), T2D	–
hsa-miR-29b-1-3p	Rfib	T2D (Hyperglycemia)	–
hsa-miR-29c-3p	ESRD	T1D (ESRD)	TGF-β (targeted by)
hsa-miR-30a-5p	AKI, NS	T2D (Glucose, β-cell)	Notch (Notch1)
hsa-miR-30b-5p	LN-RD	β-cell dysfunction	–
hsa-miR-30c-1-5p	AKI, DN	DN	TGF-β (CTGF)
hsa-miR-30d-5p	AKI, PKD	T2D, β-cells (insulin)	Insulin (MAP4K4)
hsa-miR-31-5p	PCKD	T1D (serum), T2D (micro-vascular)	–
hsa-miR-335-5p	Renal senescence, DN	T1D (DN)	SOD2
hsa-miR-342-3p	AKI	T1D,T2D (PBMCs)	–
hsa-miR-362-5p	RIRI	GDM	–
hsa-miR-363-3p	DN	T1D (DN)	–
hsa-miR-373-3p	DN	T1D (DN)	–
hsa-miR-451a-5p	DN	DN	–
hsa-miR-486-1-5p	DN, CKD, AKI, IgA-N	T2D, T1D (DN)	–
hsa-miR-660-5p	DN	T1D (DN)	–
hsa-miR-9-1-3p	–	T1D (serum)	–
hsa-miR-941-1-3p	–	–	Insulin (9)
hsa-miR-96-5p	PKD, RCC	T2D, β-cells (insulin)	IGF (synaptotagmin-like 4)
hsa-miR-99a-5p	AKR,RCC	Glucose/insulin regulation	mTOR
hsa-miR-99b-5p	AKR	T2D (IGT)	TGF-β (EMT)

AKR, Acute Kidney Rejection/Renal Graft Rejection; AKI, acute kidney injury; CKD, chronic kidney disease; DIKD, drug induced kidney damage, DN, diabetic nephropathy; Endo, endothelial dysfunction; EMT, epithelial–mesenchymal transition; EndMT, endothelial-mesenchymal transition; ESRD, end-stage renal disease; GD, gestational diabetes mellitus; IgA-N, IgA nephropathy; IGF, insulin-like growth factor; IGT, impaired glucose tolerance; LN-RD, lupus nephritis induced renal damage; MA, microalbuminuria; miRNA, microRNA; NS, nephrotic syndrome; PCKD, poly-cystic kidney disease; PKD, progressive kidney disease; PTEN, phosphatase and tensin homolog; RCC, renal cell carcinoma; Rfib, renal fibrosis: RIRI, renal ischemia-reperfusion injury; TGF, transforming growth factor; T1D, type 1 diabetes mellitus; T2D), type 2 diabetes mellitus.
See references 23,24,40–144.

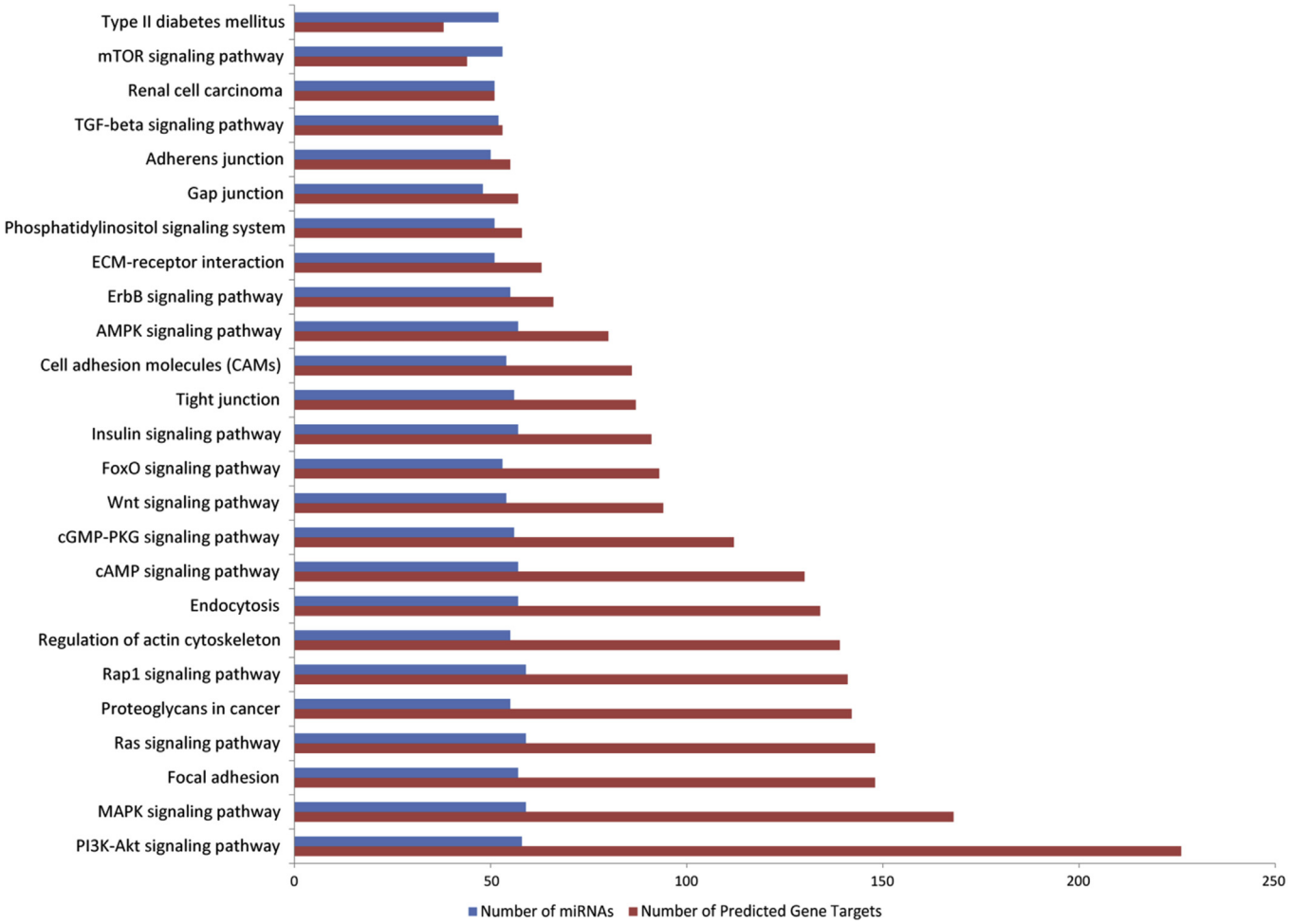

Figure 6. Identification of pathways associated with microRNAs (miRNAs) from patients with type 1 diabetes (T1D). Seventy-seven miRNAs from patients with T1D who showed differential concentration changes in extracellular vesicles was run through the mirPath program (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath) to identify predicted targeted pathways. Pathways associated with T1D and renal fibrosis are highlighted. Blue bars represent the number of miRNAs identified, and the red bars represent the number of predicted gene targets belonging to the associated pathway. AMPK, AMP-activated protein kinase; cGMP-PKG, cyclic GMP–protein kinase G; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; TGF, transforming growth factor.

cardiovascular disease across various diabetic populations.[173–177]

Our analysis of global miRNA concentration changes in urine, EV, and EV-depleted urine samples revealed a set of miRNAs that show significant enrichment in the EVs (Supplementary Table S4). Of the urine EV-enriched miRNAs, many contain a conserved sequence motif that could possibly direct selective transport of miRNAs from kidney cells into urinary EVs. Previous work has identified several unique sequence motifs in miRNAs isolated from EVs derived from different cell types.[178,179] Further experimental follow-up will be needed to determine if this motif is involved in miRNA sorting of this kind. In addition, many of the miRNAs that exhibit concentration changes in patient EV fractions are also EV-enriched, suggesting that these miRNAs (Figure 5b) might be promising EV-specific biomarker candidates for DN and/or MA. A previous study used a similar small-RNAseq approach to identify miRNAs enriched in EVs from the urine of healthy individuals.[180] Our cross-comparison of datasets found significant overlap of EV-enriched miRNAs between our results and this study (Figure 5c).

Although we observed several miRNAs with concentration changes similar to those reported in other prior studies, we also observed a number of miRNAs that either showed no change or showed a different direction of change. These differences are likely due at least in part to methodological differences, as these studies differ in biofluid type (serum, plasma, or urine), EV isolation methods (ultracentrifugation or commercial kits compared to SEC), approach to miRNA profiling (microarrays or quantitative reverse-transcriptase PCR compared with small-RNAseq), and by geographical and ethnic differences in patient populations, all of which can contribute to poor consistency among studies. Most circulating miRNA

studies use qPCR-based techniques for measurement, which are low throughput and cannot detect novel miRNAs. Although small-RNAseq overcomes these issues, inconsistencies between qPCR and small-RNA-seq–based platforms have been noted in urine samples in kidney injury,[131] and this might explain the minor inconsistencies with our previously published work.[24,47] For a more detailed discussion on the issues that limit miRNA quantitation and the challenges facing standardization, please see 2 of our recent reviews.[20,181] Nonetheless, this current approach using revised EV isolation and miRNA-profiling methods has allowed us to identify a number of miRNAs showing consistent concentration changes in urine or EVs from urine, which can be validated with qPCR. These miRNAs may be further developed as biomarkers to assess the disease status of T1D-associated DN.

DISCLOSURE

JFB and TO were paid consultants for Astute Medical and Sanofi, respectively, during the duration of this research project. The views and opinions in this research project are solely those of the contributing authors and do not necessarily reflect those of Astute Medical and Sanofi. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Patient information and demographics.

Table S2. Average RNA input concentration and read counts for urine, exosome, and exosome-depleted fractions.

Table S3. Expanded microRNA profiling data for urine, and exosome fractions ($\geq$0.6 $\log_2$FC, $P \geq 0.05$).

Table S4. Extracellular vesicle–specific enrichment in urine samples of patients with type 1 diabetes mellitus.

Figure S1. Protein gels from patient urine and extracellular vesicle (EV) fractions. (A) Patient urine samples run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis stained with Coomassie blue. (B) Corresponding EV fractions run under the same conditions. I, intermittent microalbinuria (MA) status; L, ladder; N, normal; O, overt nephrology; P, persistent MA status.

Supplementary Methods.

Supplementary material is linked to the online version of the paper at http://www.kireports.org/.

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