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Original article

MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype'

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ABSTRACT

Aims. – MicroRNAs (miRNAs) from extracellular vesicles (EVs) have been proposed as promising biomarkers for a number of diseases. In this study, their potential as urine-based biomarkers of diabetic nephropathy (DN) was assessed.
Methods. – MiRNAs were profiled in urinary EVs from 160 fasting subjects with normal glucose tolerance (NGT) and in T2DM patients with either microalbumininuria (MIC) or macroalbuminuria (MAC).
Results. – A total of 73 miRNAs detected in urinary EVs (NGT) were predicted to target important functions for kidney homoeostasis, thereby validating their use as indicators of kidney dysfunction. Indeed, a urinary EV miRNA signature was found to comprise increased levels of let-7i-3p, miR-24-3p and miR-27b-3p, and decreased levels of miR-15b-5p, to identify patients with MIC. ROC curve analysis confirmed this ability to identify MIC in normo-albuminuria T2DM (T2DM-NA) patients and to differentiate between MAC and T2DM patients. These miRNAs were also predicted to target protein networks involved in the Wnt/β-catenin signalling cascade, activin receptor signalling and cell differentiation/proliferation, and correlated with eGRF, HbA_{1c}, serum creatinine, urea, albumin and blood pressure. Concentrations of miR-30a-5p were specifically modified in urinary EVs from patients with MAC, but not MIC, suggesting that miR-30a-5p could be related to severe kidney damage.
Conclusion. – Urinary EV miRNAs correlate with the degree of MIC. As they are also thought to regulate pathways that are targets of pharmacological agents to prevent DN (reticulum stress, activin receptors), they may also serve as non-invasive 'liquid biopsies' to stratify patients at risk of developing MAC and to monitor treatment efficacy.

Introduction

Diabetes is a leading cause of chronic kidney disease worldwide, and accounts for 31–40% of end-stage renal disease cases in India [1,2]. While longstanding type 2 diabetes mellitus (T2DM) and poor glycaemic control are risk factors for diabetic nephropathy (DN), South Asian ethnicity has also been linked to a relatively greater susceptibility to this complication; in fact, there are significant differences in the epidemiology of microvascular complications between South Asians and people of other races [3,4]. Hyperglycaemia, hypertension and genetic predisposition are among the significant risk factors that affect kidney glomeruli, arterioles, tubules and interstitium. Micro-albuminuria (MIC) is considered the gold standard indicator of DN, yet it poses certain specificity and sensitivity concerns. Its predictive powers are limited, as structural changes in the glomerular basement membrane and renal vasculature may appear before the onset of MIC [5,6]. Moreover, clinical factors unrelated to DN can affect MIC status [7] and, recently, it was observed that ≤ 30% of DN cases can arise in the absence of obvious MIC [8,9]. Therefore, there

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is an unmet clinical need to identify novel, reliable biomarkers reflecting early-stage DN and progressive renal dysfunction in diabetes patients to allow for appropriate therapy that might prevent or slow DN evolution.

Significant efforts have been made worldwide to identify serum and urine biomarkers, including microRNAs (miRNAs), which can clinically detect early stages of DN and progressive kidney function decline in diabetes patients [10–12]. MiRNAs are small, non-coding RNAs involved in negative post-transcriptional regulation, and significant amounts of miRNAs have been found in human extracellular bodily fluids [13]. They are remarkably stable despite high extracellular RNase activities, as they are either enclosed in extracellular vesicles (EVs) such as exosomes, shedding vesicles and apoptotic bodies, or associated with or packaged within high-density lipoprotein (HDL), or associated with RNA-binding proteins [14]. For miRNAs exported in EVs, compelling evidence supports their role in a broad range of physiological and pathological processes, including mediation of cell-to-cell communication [15]. Interestingly, numerous studies have demonstrated that the level and composition of these circulating miRNAs correlate well with several pathologies, such as cancers, cardiovascular diseases, inflammation and metabolic disorders [16]; in the context of kidney disease, it has been shown that blood levels of miR-21 and miR-216a correlate with rates of kidney function decline and the risk of progression to dialysis-dependent kidney failure [17]. In addition, miRNA profiling of 12 human bodily fluids (amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, peritoneal fluid, plasma, pleural fluid, saliva, seminal fluid, tears and urine) found distinctive compositions in different fluid types [13]. Most notably, the miRNA spectrum in urine differs from that of most other bodily fluids, suggesting that extracellular miRNAs are not just passively released outside of cells. Indeed, several studies have shown that EV miRNA content does not simply reflect the miRNA repertoire of cells of origin, but also that some miRNAs are in fact selectively exported or retained within cells [18]. Therefore, alterations of miRNA composition in urinary EVs could provide information on disease pathophysiology as well as diagnostic endpoints for studies of renal disease [19,20]. In addition, large quantities of EVs are released from all nephron segments [21], and modification of their protein composition was found in diabetes patients with DN [22]. Moreover, focusing on EV miRNAs, a recent study revealed that urinary EV levels of miR-192, miR-194 and miR-215 were increased in Asian T2DM patients with MIC (T2DM-MIC) compared with non-albuminuria T2DM (T2DM-NA), and that this increase correlated with blood transforming growth factor-beta 1 (TGF-β1) levels [23]. Two other studies confirmed that miRNA levels from urinary exosomes were altered in DN, thereby reinforcing the concept that the miRNA profile is altered in urinary EVs from T2DM patients with DN [24,25].

Previously, our team was able to identify specific circulating miRNAs in Asians with a specific Indian phenotype that are differentially expressed in non-obese individuals with prediabetes and patients with T2DM compared with healthy subjects [26], thereby demonstrating the potential of circulating miRNAs to stratify T2DM patients according to their disease severity. In the context of the diabetes epidemic in the Indian population and their high susceptibilty, thereby escalating DN prevalence in India, there is nevertheless a major research gap in the identification of robust biomarkers. To correct this lack, it was necessary to determine whether miRNAs from urinary EVs could be used to identify potential biomarkers of DN pathogenesis.

Materials and methods

Study participants and their characteristics

In the present cross-sectional study, participants were randomly recruited (from September 2014 to December 2015) from ongoing epidemiological studies at the Madras Diabetes Research Foundation and Dr. Mohan's Diabetes Specialities Centre at Chennai, South India. The entire study group included subjects (n = 40 each) with normal glucose tolerance (NGT), patients with T2DM and normoalbuminuria (T2DM-NA), T2DM-MIC patients and T2DM patients with macroalbuminuria (T2DM-MAC) (Table 1). All subjects were identified based on World Health Organization (WHO) criteria for diagnosing diabetes. MIC (30–299 mg/dL) and MAC (> 300 mg/dL) were defined based on albuminuria detected in patients' urine over at least two or three consecutive determinations, each separated by 3 months. Estimated glomerular filtration rates (eGFRs) were calculated from serum creatinine to quantify kidney dysfunction in T2DM patients.

Institutional ethics committee approval was obtained for the study protocol, and written informed consent was given by all participants. The study was conducted as per the Declaration of

Table 1
Clinical and biochemical characteristics of study subjectsData are expressed as means ± standard deviation (SD).

Variables	Normal glucose tolerance (NGT) (n = 40)	Type 2 diabetes mellitus (T2DM-NA) (n = 40)	T2DM with micro-albuminuria (MIC) (n = 40)	T2DM with macro-albuminuria (MAC) (n = 40)
Age (years)	44 ± 6.3	46 ± 7.7	51 ± 6.0[a,b]	54 ± 6.2[a,b]
Diabetes duration (years)	–	4.8 ± 2.9	12.5 ± 4.6	17.7 ± 4.5
Fasting plasma glucose (mg/dL)	87 ± 12.1	137 ± 37.3[a]	166 ± 75[a]	172 ± 73.7[a,b]
Postprandial blood glucose (mg/dL)	100 ± 20.9	225 ± 67.9[a]	229 ± 91.3[a]	245 ± 78.7[a]
HbA_{1c} (mmol/mol)	36.7 ± 4.0	62.8 ± 20.5[a]	66.9 ± 20.8[a]	72.4 ± 24.3[a]
HbA_{1c} (%)	5.5 ± 0.4	7.9 ± 1.9[a]	8.3 ± 1.9[a]	8.8 ± 2.2[a]
Serum cholesterol (mg/dL)	163 ± 26.0	179 ± 32.4	163 ± 38	174 ± 58.4
Serum triglycerides (mg/dL)	110 ± 40.9	156 ± 72.1	154 ± 78.5	200 ± 155.3[a]
HDL cholesterol (mg/dL)	39 ± 7.9	41 ± 6.1	39 ± 8.9	38 ± 10.2
LDL cholesterol (mg/dL)	102 ± 23.1	107 ± 32.1	94 ± 29.7	96 ± 49.5
VLDL (mg/dL)	22 ± 8.2	31 ± 14.4	31 ± 15.7	40 ± 31.1
Blood urea (mg/dL)	20 ± 5.8	20 ± 5.3	25 ± 8.7	36 ± 20[a,b]
Serum creatinine (mg/dL)	0.75 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	1.3 ± 0.7[a,b]
Albuminuria (mg/dL)	5.4 ± 6.0	8.0 ± 5.5	86.3 ± 45.2[a,b]	350.0 ± 0.0[a,b]
eGFR (mL/min/1.73 m^2)	111 ± 23	113 ± 24	100 ± 31	67 ± 32[a,b]
Body mass index (kg/m^2)	25.43 ± 3.4	27 ± 5.0	26 ± 3.6	26 ± 4.6
Systolic blood pressure (mmHg)	121 ± 11.9	130 ± 16.2	133 ± 21.8[a]	135 ± 16.1[a]
Diastolic blood pressure (mmHg)	77 ± 10.2	80 ± 7.8	83 ± 9.4[a]	84 ± 7.7[a]

HDL/LDL/VLDL: high-/low-/very low-density lipoprotein; eGFR: estimated glomerular infiltration rate.
[a] $P < 0.05$ vs. NGT.
[b] P vs. T2DM-NA.

Helsinki. Fasting blood and urine samples were collected and handled separately for biochemical and molecular investigations.

Anthropometric measurements

Height, weight and blood pressure were measured using standardized methods. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Insulin resistance was calculated using homoeostasis model assessment for insulin resistance (HOMA-IR), using the following formula: [fasting insulin (μIU/mL) × fasting glucose (mmol/L)]/22.5.

Biochemical parameters

Fasting plasma glucose was assessed by the glucose oxidase–peroxidase (GOD–POD) method, and serum cholesterol was measured by the cholesterol oxidase–phenol-4-aminophenazone-peroxidase (CHOD–PAP) method. In addition, serum triglycerides were determined by the glycerol phosphate oxidase (GPO)–PAP method, HDL cholesterol by a direct method using polyethylene glycol (PEG)-pretreated enzymes, and blood urea and serum creatinine by Jaffe's method, using a Hitachi 912 chemistry analyzer and kits supplied by Roche Diagnostics (Hoffmann-La Roche, Basel, Switzerland). Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula. Glycated haemoglobin (HbA_{1c}) was estimated by high-performance liquid chromatography using a Variant testing system (Bio-Rad Laboratories, Hercules, CA, USA), and eGFR was estimated using the standard Modification of Diet in Renal Disease (MDRD) study equation: $186 \times (\text{serum creatinine}) - 1.154 \times \text{age} - 0.203 \times 0.742$ (if female).

Urinary EV isolation and total RNA extraction

Fasting urine samples (15 mL) were collected in sterile containers and centrifuged at 10,000 × g for 10 min to remove large particles (apoptotic bodies, microparticles), cell debris, organelles and protein aggregates. EVs were then precipitated from 5 mL of urine using miRCURY™ Exosome Isolation Kits (Exiqon A/S, Vedbaek, Denmark), according to the manufacturer's protocol (www.exiqon.com/ls/Documents/Scientific/exosome-kit-cells-urine-csf-manual.pdf). The EV purification method is based on capturing water molecules, which would otherwise form the hydrate envelope of particles in suspension. This allows the precipitation of particles < 100 nm in diameter using a low-speed centrifugation step. However, as this method is not specific for a given subpopulation of vesicles (exosomes or small microparticles), the generic term 'extracellular vesicles' has been used in this study.

Total RNA was isolated from EV pellets using the miRCURY kits (Exiqon), then quantified with a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). However, the workflow management software used in this study did not permit EV sizes to be measured, as total RNA was extracted directly from EV pellets to minimize EV loss. The result is an indirect validation that the total RNA contained mRNAs known to be associated with urinary EVs [aquaporin 2 (AQP2) and ALG-2-interacting protein X (ALIX). As shown in Fig. S1 (see supplementary material associated with this article online), there were no significant differences in AQP2 and ALIX mRNA levels in our three groups of T2DM patients (T2DM-NA, T2DM-MIC and T2DM-MAC).

Urinary EV miRNA profiling

For the discovery pilot study, 87 mature miRNAs were quantified in urinary EVs from a subset of individuals (n = 4 each) from the entire study group (Table 1); 8 μL of total RNA was then reverse-transcribed in a 40-μL reaction volume using miRCURY LNA™ Universal RT microRNA PCR cDNA synthesis kits (Exiqon). These miRNA quantifications were performed in 96-well plates using the human Urine Exosome Focus microRNA PCR Panel (Exiqon) and an Applied Biosystems ABI 7500 Real-Time PCR System (ThermoFisher Scientific). Quantitative polymerase chain reaction (qPCR) analyses were performed using Exiqon GenEx version 3.0 software as per the provider's instructions. The results for each plate were calibrated within the plate by performing PCR using a common miRNA [interplate calibrator (IPC)] to compensate for biases introduced in different runs. As recommended by Exiqon GenEx, the cut-off value of the threshold cycle (Ct) was set at 39. Amplification of the RNA spike-in (synthetic cel-miR-39-3p from *Caenorhabditis elegans*) was used to monitor RNA extraction, and internal standards provided by the kits (IPC) were then used to calculate the biases introduced in different runs (PCR efficiency of the 12 plates). All data were normalized against the mean Ct value of all samples. Comparisons between groups were made with Student's t-test ($P < 0.05$) for normalized data to select the differentially expressed miRNAs.

Validation of candidate miRNAs by individual qPCR

All miRNAs differentially expressed by urinary EVs in the pilot study were validated in the entire study group (n = 120; Table 1) by individual assays. Total RNA from urinary EVs was used for real-time (RT) qPCR using miRCURY LNA Universal RT microRNA PCR cDNA synthesis kits, followed by the microRNA PCR system, and ExiLENT SYBR® Green Master Mix Kit (Exiqon) in an ABI 7500 Applied Biosystems thermocycler. All data were individually normalized using Ct values of the synthetic cel-miR-39-3p used as a spike for RNA extraction efficiency.

Hierarchical clustering

This was calculated using pairwise average-link clustering and drawn using GenePattern version 3.2.3 (http://pwbc.garvan.unsw.edu.au/gp) [27].

Bio-informatic analysis

MiRNA target gene predictions were made with TargetScan 7.0 (www.targetscan.org/vert_70/) [28], which predicts biological targets of miRNAs by searching for the presence of 8mer, 7mer and 6mer sites matching the seed region of each miRNA. Predictions of miRNA functions were determined using the DIANA-miRPath v3.0 tool (www.microrna.gr/miRPathv3) [29].

Statistical analysis

These were performed with SAS software (SAS Institute Inc., Cary, NC, USA). Based on our pilot study (n = 4 patients/group) and the use of PASS 11.0 software (www.ncss.com/download/pass/updates/pass11/), the minimum sample size required was calculated as 32 for each group, taking into account a level of significance set at 0.05 and a statistical power of 0.95. Thus, it was considered safe to have 40 patients in each group to limit individual variations. Analysis of variance (ANOVA) was used to evaluate whether there was any evidence that the means of the population differed, then an ANOVA test was used to compare any differences between each pair of means. Only P-values < 0.05 were considered statistically significant. Corrected P-values were also calculated using Bonferroni's method for multiple comparisons.

In addition, receiver operating characteristic (ROC) analysis was performed for urinary EV miRNAs, which could distinguish T2DM-NA and T2DM-MIC or -MAC patients. Area under the curve (AUC) analysis and the Youden index were also applied to determine the optimal cut-off point, sensitivity and specificity of urinary EV miRNAs [30].

Results

All 120 T2DM non-obese patients (Table 1) were hyperinsulinaemic, hyperglycaemic and insulin-resistant compared with control subjects (n = 40). T2DM-MIC or -MAC patients (n = 80) were also hypertensive compared with controls, indicating

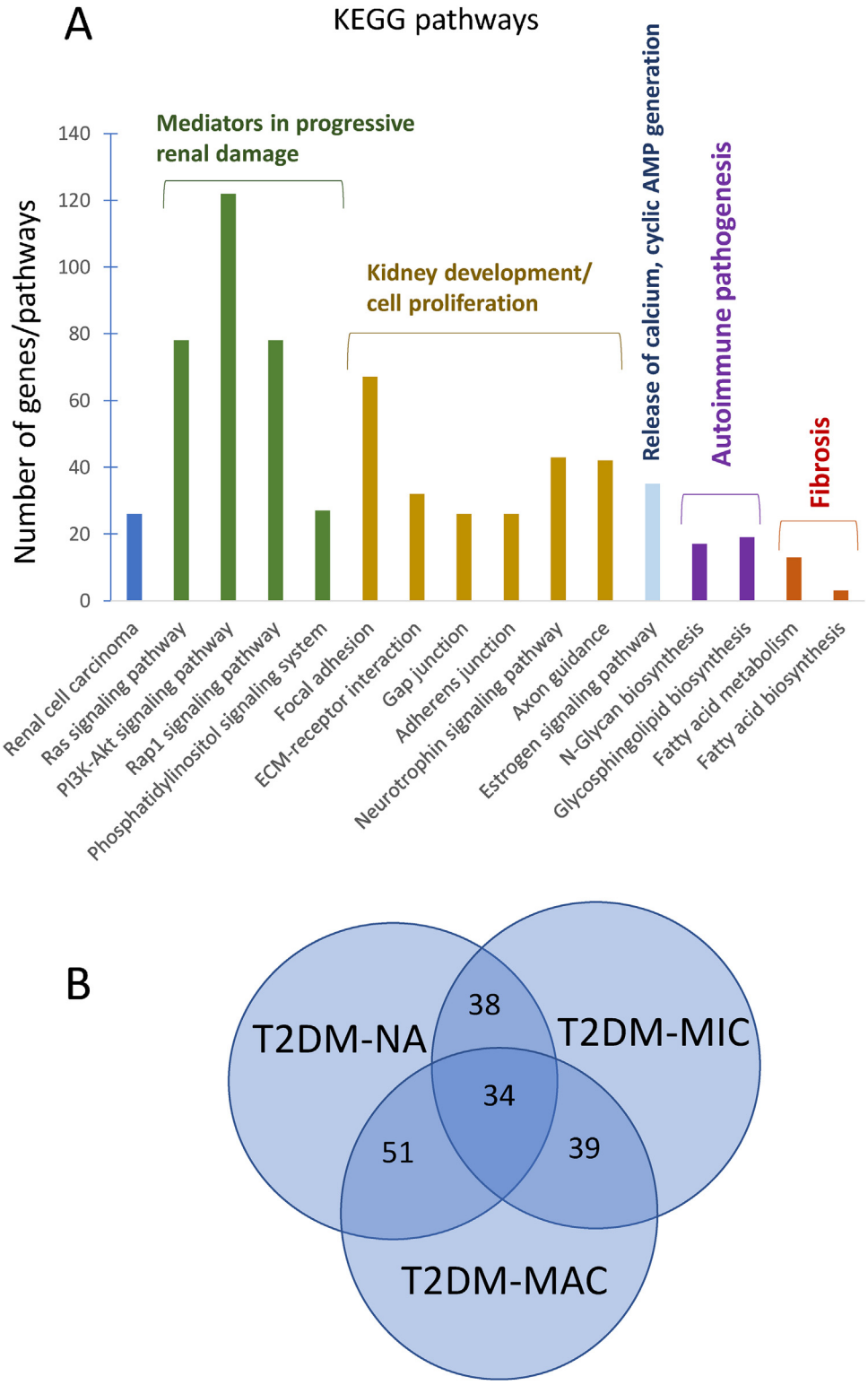

Fig. 1. A. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly enriched by genes collectively targeted by the 73 microRNAs (miRNAs) contained in urinary extracellular vesicles (EVs) from three control subjects with normal glucose tolerance (NGT; see also Table S2; see supplementary material associated with this article online). Target predictions are based on TargetScan and KEGG pathway identifications using DIANA-miRPath version 3.0 (www.microrna.gr/miRPathv3). B. Number of miRNAs identified in urinary EVs from three groups of type 2 diabetes mellitus (T2DM) patients. NA: non-albuminuria; MIC: micro-albuminuria; MAC: macro-albuminuria.

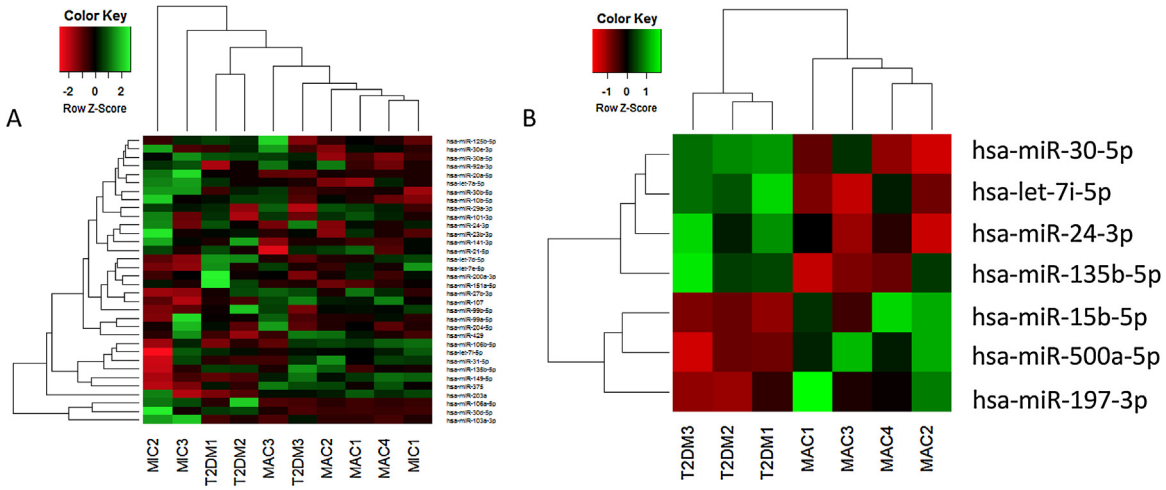

Fig. 2. Heat maps of (A) miRNA concentrations of urinary extracellular vesicles (EVs) in T2DM (non-albuminuria, NA) patients; and (B) only candidate miRNAs discriminating T2DM-NA from T2DM-MAC (macro-albuminuria) patients. Concentration levels were normalized against all mean signal intensities from all assays.

glomerular hyperfiltration and endothelial dysfunction [31]. Patients with MAC (n = 40) had significantly higher levels of serum triglycerides compared with controls, and significantly higher levels of serum creatinine and blood urea, both associated with abnormal renal function [lower eGFR compared with T2DM-NA patients ($P < 0.05$)]. Urinary EVs were isolated from fasting urine samples from all 120 T2DM patients and all 40 (NGT) controls.

Urinary EV miRNA profiling

These profiles were first determined in the discovery set of four subjects per group, using the Exiqon Pick-&-Mix microRNA PCR Panel, which allowed for amplification of the miRNAs typically found in EVs from human urine samples. Metabolic parameters in this subgroup of patients did not differ significantly from those of the entire group (data not shown). Of the 87 mature miRNAs simultaneously amplified, 73 were detected in urinary EVs from all control subjects (Table S1; see supplementary material associated with this article online). In addition, miRNA target gene predictions and analyses of their biological functions indicated that these 73 miRNAs collectively targeted important functions related to kidney development and homoeostasis (Fig. 1A, Table S2; see supplementary material associated with this article online), thereby validating their use as indicators of kidney dysfunction. This led to the hypothesis that variations of miRNA levels in urine EVs might indicate progressive renal damage associated with diabetes (fibrosis, inflammation, glomerular hyperfiltration, renal hypertrophy, extracellular matrix accumulation). Moreover, levels of the 34 miRNAs commonly found in urinary EVs from T2DM-NA, T2DM-MIC or T2DM-MAC patients (Fig. 1B) were closely similar (Fig. 2A). Indeed, few urinary EV miRNAs were differentially concentrated according to each group (Table 2) and, as expected, T2DM-NA and T2DM-MAC patients differed the most, with seven miRNAs differentially concentrated in their respective EVs (Fig. 2B). Conversely, only two miRNAs differed between T2DM-NA and T2DM-MIC patients and two of them (miR-93-5p and miR-375) were able to discriminate T2DM-MIC from T2DM-MAC patients.

Revalidation of urinary EV miRNAs

The candidate miRNAs differentially concentrated in EVs from T2DM patients were further confirmed in the entire study group (Table 1, n = 40 in each group). Let-7i-5p, miR-135b-5p, miR-15b-3p, miR-197-3p, miR-24-3p and miR-27b-3p were revalidated, and were able to discriminate T2DM-NA patients from those with early changes in renal function (T2DM-MIC) as well as those with more advanced renal alterations (T2DM-MAC; Fig. 3, Fig. S2; see supplementary material associated with this article online). In fact, miR-135b-5p and miR-197-3p were also modified in urinary EVs from T2DM-NA patients compared with control subjects, thereby suggesting they were more related to diabetes than to alterations of renal function. Thus, in our present study, EV

Table 2
Differentially concentrated miRNAs in urinary extra-celular vesicles (EVs) in three groups of T2DM patients: $P < 0.05$ considered significant by Student's t-test; miRNAs (In Bold) are those commonly identified in different subgroups; biological functions by DIANA-miRPath version 3.0 tool; only pathways with the largest number of target genes considered.

Groups	miRNAs	Fold changes	*P*-value	Biological function
NGT vs. T2DM-NA	**hsa-miR-135b-5p**	0.13	0.03	Hippo signalling pathway (hsa04390)
T2DM-NA vs. -MIC	**hsa-miR-27b-3p**	1.79	0.044	Protein processing in endoplasmic reticulum (hsa04141)
	hsa-miR-135b-5p	3.88	0.071	Hippo signalling pathway (hsa04390)
T2DM-NA vs. -MAC	**hsa-miR-135b-5p**	2.29	0.041	Hippo signalling pathway (hsa04390)
	hsa-miR-500a-5p	0.56	0.007	Fatty acid metabolism (hsa01212)
	hsa-miR-15b-5p	0.41	0.039	Protein processing in endoplasmic reticulum (hsa04141)
	hsa-miR-197-3p	0.34	0.049	Protein processing in endoplasmic reticulum (hsa04141)
	hsa-let-7i-5p	1.35	0.012	Proteoglycans in cancer (hsa05205)
	hsa-miR-30a-5	1.48	0.02	Ubiquitin-mediated proteolysis (hsa04120)
	hsa-miR-24-3p	1.88	0.027	No enriched pathway
T2DM-MIC vs. -MAC	**hsa-miR-27b-3p**	0.61	0.015	Protein processing in endoplasmic reticulum (hsa04141)
	hsa-miR-93-5p	2.09	0.043	MAPK signalling pathway (hsa04010)
	hsa-miR-375	0.17	0.049	Protein processing in endoplasmic reticulum (hsa04141)

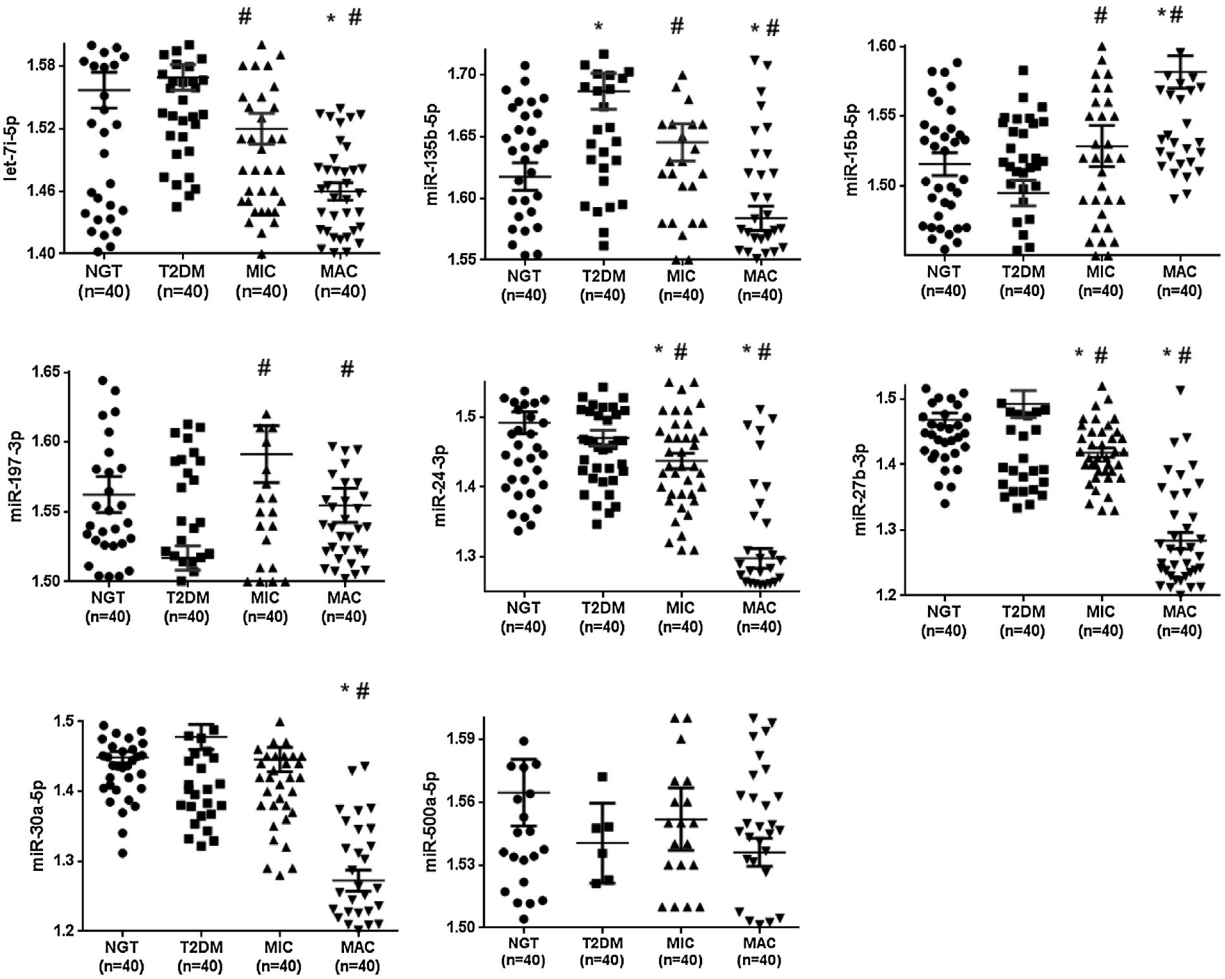

Fig. 3. Validation of urinary EV levels of miRNAs let-7i-5p, miR-135b-5p, miR-15b-5p, miR-197-3p, miR-24-3p, miR-27b-3p, miR-30a-5p and miR-500a-5p, using quantitative real-time (qRT) polymerase chain reaction (PCR). All data are threshold cycle (Ct) values inversely correlated with miRNA concentrations (expressed as means ± SEM; arbitrary units). * $P < 0.001$ vs. NGT; # $P < 0.001$ vs. T2DM; *P*-values adjusted by Bonferroni corrections (see also Fig. S2; see supplementary materials associated with this article online). NGT: normal glucose tolerance; T2DM: type 2 diabetes mellitus (no albuminuria); MIC: micro-albuminuria; MAC: macro-albuminuria.

concentrations of let-7i-5p, miR-15b-3p, miR-24-3p and miR-27b-3p were associated with MIC, and two of them (miR-15b-3p and miR-24-3p) were positively correlated with blood pressure. On the other hand, levels of miR-30a-5p were modified only in EVs from T2DM-MAC patients, suggesting that concentrations of this miRNA were associated with the degree of DN severity in this Indian patient population. Altered EV concentrations of miR-93-5p and miR-375 in T2DM-MIC and -MAC patients were not revalidated in the entire study group (data not shown). However, as depicted in Fig. S2, Bonferroni correction for multiple comparisons reinforced the conclusion that let-7i-5p, miR-135b-5p, miR-15b-3p, miR-24-3p, miR-27b-3p and miR-30a-5p were altered in T2DM-MAC vs. T2DM-NA patients, and that miR-27b-3p was strongly associated with early renal dysfunction.

As let-7i-5p, miR-15b-3p, miR-24-3p and miR-27b-3p were altered in pre-MAC states, our aim was then to predict the cellular pathways targeted by these four miRNAs to determine whether their alterations were related to renal dysfunction. There was thus a focus on the genes collectively targeted by these four miRNAs (n = 17 genes, TargetScan predictions) to determine whether they were involved in protein–protein association networks (using the STRING 10.5 database) [32]. Thus, our study unravelled three protein networks involving the Wnt/β-catenin signalling cascade, activin receptor signalling, and cell differentiation and proliferation (Fig. 4).

Correlations between miRNA concentrations in urinary EVs and metabolic parameters

EV levels of six (let-7i-5p, miR-135b-5p, miR-15b-5p, miR-24-3p, miR-27b-3p, miR-30a-5p) out of 10 miRNAs (Table 2) correlated with clinically relevant metabolic parameters associated with DN (Table 3), such as levels of HbA_{1c}, serum creatinine, blood urea and albumin, as well as with kidney dysfunction (eGFR). In addition, levels of let-7i-5p, miR-24-3p, miR-27b-3p and miR-30a-5p correlated with fasting and postprandial glucose levels, whereas miR-15b-5p and miR-24-3p correlated with systolic and diastolic blood pressure, respectively. Thus, these data confirmed that the selected miRNAs indeed correlated with abnormal clinical indicators predictive of or associated with DN.

ROC analysis

This was performed to evaluate let-7i-5p, miR-15b-5p, miR-24-3p, miR-27b-3p and miR-30a-5p as potential urinary EV-based biomarkers of DN in T2DM patients. First, combined EV levels of let-7i-5p, miR-15b-5p, miR-24-3p and miR-27b-3p were used to discriminate T2DM-NA from T2DM-MIC patients: ROC analysis yielded an AUC (AUROC) of 0.739 (Fig. 5A), confirming that EV concentrations of these four miRNAs could correctly identify the

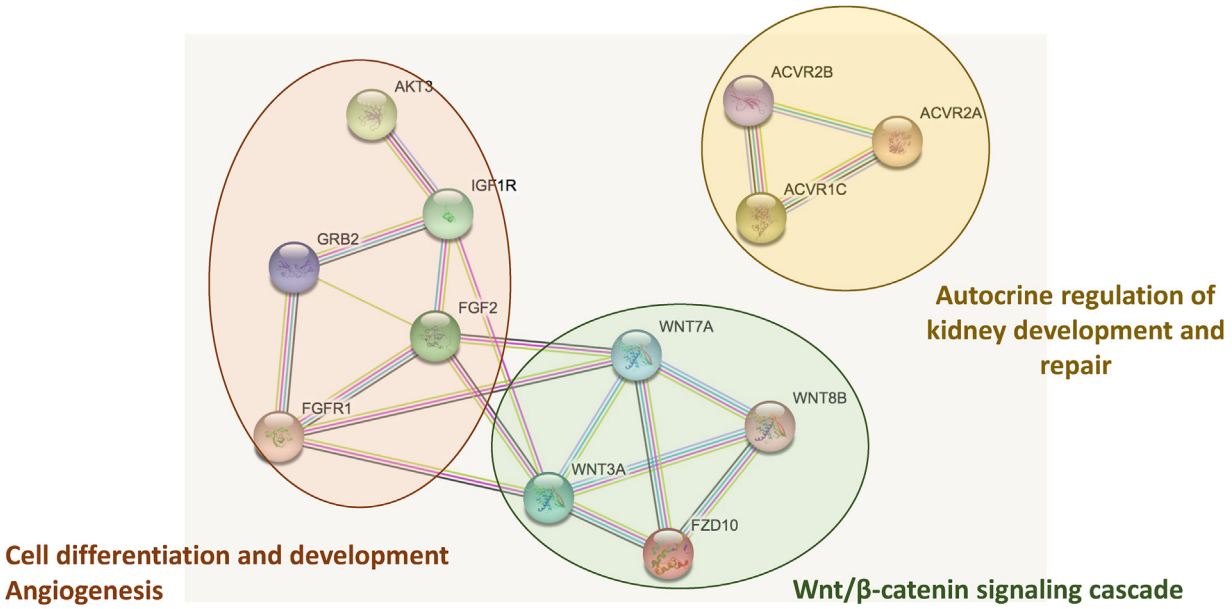

Fig. 4. Protein–protein association networks collectively targeted by four miRNAs (let-7i-5p, miR-15b-5p, miR-24-3p, miR-27b-3p) altered in urinary EVs from T2DM-MIC patients. Target gene predictions are by TargetScan, using DIANA-miRPath version 3.0 (www.microrna.gr/miRPathv3); functional protein association networks were identified by STRING 10.5 (https://string-db.org/).

Table 3
Significant correlations between miRNA concentrations in urinary EVs and metabolic parameter: as miRNA concentrations are expressed as Ct values, negative correlations with miRNA Ct values correspond to positive correlations with miRNA concentrations.

MiRNAs	Metabolic parameters	Estimate	*r*	r^2	Probt
let-7i-5p	Serum creatinine	−0.033739305	−0.16144	0.0260628736	0.0414
mIR-135b-5p		−0.034172002	−0.17383	0.0302168689	0.0279
miR-15b-5p		0.033049879	0.19446	0.0378146916	0.0137
miR-24-3p		−0.095855013	−0.38882	0.1511809924	<0.0001
mIr-27b-3p		−0.104870489	−0.4062	0.16499844	<0.0001
miR-30a-5p		−0.121432763	−0.43956	0.1932129936	<0.0001
let-7i-5p	Blood urea	−0.001399702	−0.19491	0.0379899081	0.0135
mIR-135b-5p		−0.001334545	−0.19756	0.0390299536	0.0123
miR-15b-5p		0.001021556	0.17492	0.0305970064	0.0269
miR-24-3p		−0.003566273	−0.42098	0.1772241604	<0.0001
mIr-27b-3p		−0.003784056	−0.42654	0.1819363716	<0.0001
miR-30a-5p		−0.00408366	−0.43018	0.1850548324	<0.0001
let-7i-5p	Albuminuria	−0.00028936	−0.43755	0.1914500025	<0.0001
mIR-135b-5p		−0.000211493	−0.33999	0.1155932001	<0.0001
miR-15b-5p		0.000215205	0.40014	0.1601120196	<0.0001
miR-24-3p		−0.000523185	−0.67065	0.4497714225	<0.0001
mIr-27b-3p		−0.000552062	−0.67575	0.4566380625	<0.0001
miR-30a-5p		−0.000552811	−0.63237	0.3998918169	<0.0001
miR-24-3p	Diastolic blood pressure	−0.001938452	−0.15796	0.0249513616	0.0461
miR-15b-5p	Systolic blood pressure	0.000934837	0.21286	0.0453093796	0.0069
let-7i-5p	Fasting plasma glucose	−0.000364725	−0.25067	0.0628354489	0.0014
miR-24-3p		−0.000482548	−0.28114	0.0790396996	0.0003
mIr-27b-3p		−0.000443592	−0.24679	0.0609053041	0.0017
miR-30a-5p		−0.000365974	−0.19028	0.0362064784	0.016
let-7i-5p	HbA1C	−0.009992343	−0.22559	0.0508908481	0.0041
miR-15b-5p		0.00578053	0.16047	0.0257506209	0.0427
miR-24-3p		−0.015149019	−0.28992	0.0840536064	0.0002
mIr-27b-3p		−0.013134085	−0.24003	0.0576144009	0.0022
miR-30a-5p		−0.010869919	−0.18564	0.0344622096	0.0188
let-7i-5p	Post-prandial blood glucose	−0.00021661	−0.20636	0.0425844496	0.0091
miR-24-3p		−0.00036686	−0.29955	0.0897302025	0.0001
mIr-27b-3p		−0.00032415	−0.25238	0.0636956644	0.0013
miR-30a-5p		−0.000216728	−0.15764	0.0248503696	0.0472
mIr-27b-3p	eGFR	0.0014667191	0.4097272183	0.1678763934	<0.0001
miR-24-3p		0.0015074749	0.4419542323	0.1953235434	<0.0001
let-7i-5p		0.0005389615	0.1861047522	0.0346349788	0.0188
miR-15b-5p		−0.0006106189	−0.2595369142	0.0673594098	0.0010
mIR-135b-5p		0.0004470837	0.1645375096	0.0270725920	0.0382
miR-30a-5p		0.0018043804	0.4709746177	0.2218170905	<0.0001

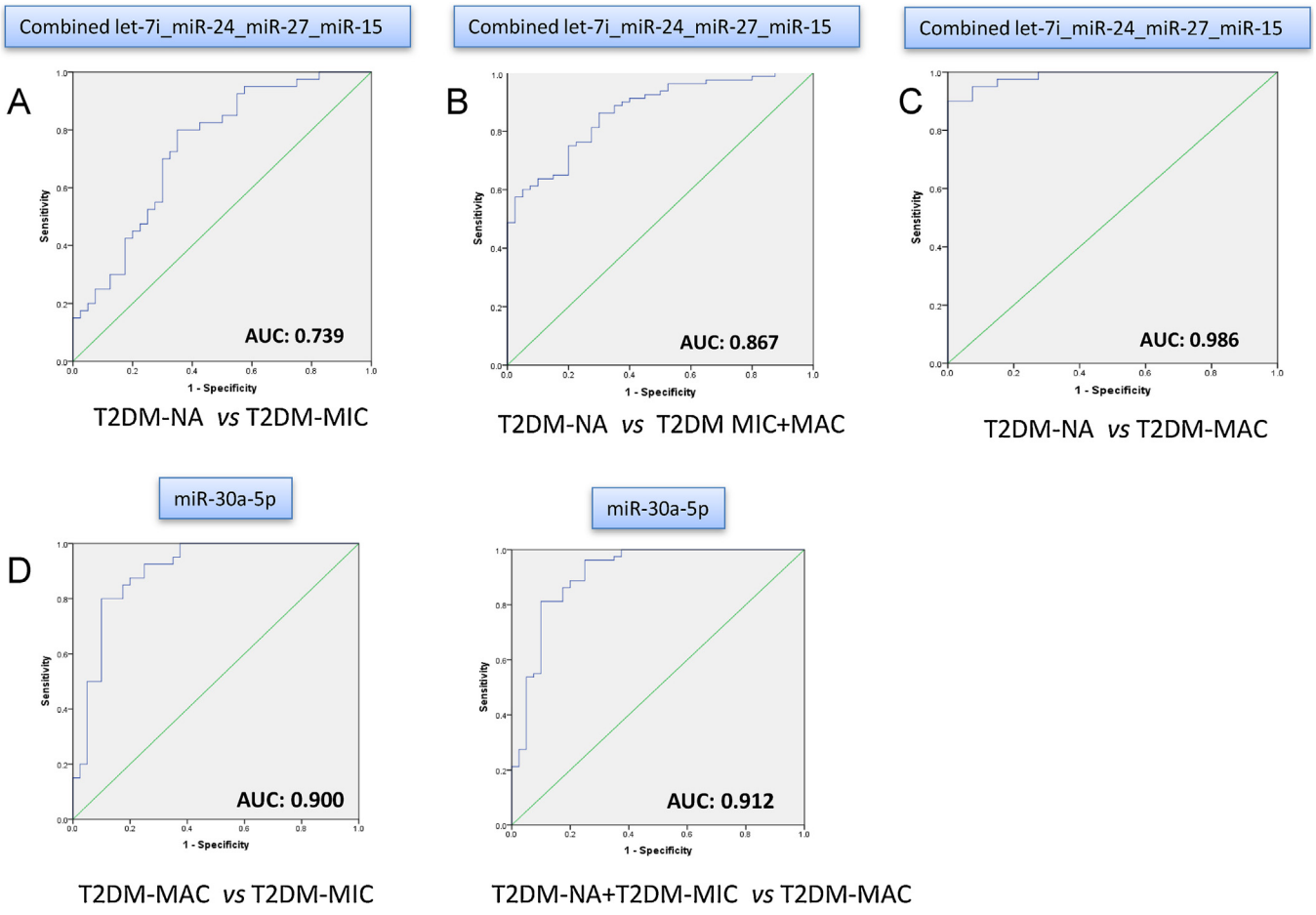

Fig. 5. Receiver operating characteristic (ROC) analysis was used to assess biomarker potential of urinary EV miRNAs (let-7i-5p, miR-15b-5p, miR-24-3p, miR-27b-3p, miR-30a-5p), including calculation of area under the curve (AUC).

majority of T2DM patients with early-stage kidney alterations related to diabetes. When considering T2DM-MIC and -MAC patients together, the AUROC was higher at 0.867 (Fig. 5B), whereas the AUROC curve was 0.986 for differentiating DN patients from T2DM-NA patients (Fig. 5C). Next, when EV levels of miR-30a-5p were used to distinguish T2DM-MIC from -MAC patients, the AUROC curve was 0.9 (Fig. 5D), and 0.912 when considering T2DM-NA and T2DM-MIC together vs. T2DM-MAC (Fig. 5E), thus confirming that miR-30a-5p could accurately identify T2DM patients with advanced-stage renal dysfunction.

Discussion

DN develops secondarily to diabetes and is the single most common disorder leading to renal failure. In India, 30% of T2DM patients also have DN [33] and, given the present prevalence of 73 million people with diabetes in India (International Diabetes Federation, 2017 data), DN is expected to become a huge health burden. Yet, DN can be avoided or impeded considerably if interventions start in the early stages [34]. However, in the context of DN risk prediction, there are certain limitations with MIC, including a lack of biomarkers to accurately identify diabetes patients at early risk of developing DN.

Recently, levels of circulating miRNAs associated with glomerular injury proved to be altered in the blood of patients with kidney fibrosis, suggesting that extracellular miRNAs in bodily fluids might be used to detect kidney changes [35–37]. For this reason, our present study evaluated a large number of well-phenotyped T2DM patients (n = 40/group) and stratified them according to their severity of renal dysfunction to determine whether miRNAs from urinary EVs could also be used to detect early renal modifications. In fact, our analysis identified four miRNAs that were differentially concentrated in EVs from T2DM-MIC compared with T2DM-NA patients (let-7i-3p, miR-24-3p and miR-27b-3p were increased, while miR-15b-5p was decreased) and also correlated with metabolic parameters associated with kidney dysfunction.

Bio-informatic analyses have predicted that the main intracellular pathway targeted by miR-15b-5p and miR-27b-3p was the protein-processing pathway (hsa04141) in endoplasmic reticulum (ER). This pathway is related to ER stress, and chronic ER stress has previously been identified in peripheral blood mononuclear cells (PBMCs) from T2DM patients [38]. Interestingly, ER is perturbed in various renal diseases [39] and is now considered a target for pharmacological agents to prevent or arrest the progression of DN [39]. Induced autophagy is considered the key homoeostasis mechanism to maintain podocyte integrity, as disruption of the unfolded protein response has led to podocyte injury and albuminuria in a mouse model [40].

In our present study, miR-15b-5p levels were associated with serum creatinine, blood urea, albuminuria and HbA_{1c}, but also with systolic blood pressure, known to induce kidney damage, thereby suggesting that miR-15b-5p might be associated with vascular dysfunction and ER stress in the development of DN. Target genes of let-7i-5p were significantly involved in proteoglycans metabolism and, interestingly, impaired metabolism of proteoglycans is thought to be involved in the pathogenesis of DN in association with glomerular endothelial injury. In fact, there are considerable

data reporting an increased excretion of urinary glycosaminoglycans/proteoglycans in both type 1 diabetes (T1D) and T2DM in association with DN [41]. Thus, our present data indicate that a urinary EV miRNA signature that combines increased levels of let-7i-3p, miR-24-3p and miR-27b-3p with a decreased level of miR-15b-5p might be used to identify patients at risk of developing MAC, as these miRNAs are thought to regulate renal function altered by DN. On collectively considering these four miRNAs, AUROC curve analysis confirmed their ability to distinguish both T2DM-MIC and -MAC patients from T2DM-NA patients. Furthermore, these four miRNAs were believed to regulate protein–protein association networks involved in the Wnt/β-catenin signalling cascade, activin receptor signalling, and cell differentiation and proliferation, all of which are pathways involved in kidney injury and repair after a wide variety of insults [42–44]. It has also been suggested that activins might be promising targets for therapeutic interventions in certain renal pathologies, such as polycystic kidney disease [42]. As such, besides their ability to identify T2DM patients at risk of developing major kidney complications, quantification of let-7i-3p, miR-24-3p, miR-27b-3p and miR-15b-5p might also serve as a signature for monitoring the effects of treatments and lifestyle interventions to reduce or prevent MAC development.

Our present analysis has also demonstrated that levels of miR-30a-5p were higher in the urinary EVs of MAC patients, but not T2DM-NA or T2DM-MIC patients. Therefore, changes in miR-30a-5p might be associated with severe kidney damage in this Indian patient population. Using different algorithms to predict its target genes, miR-30a-5p could be involved in both ubiquitin-mediated proteolysis (hsa04120; P = 0.013 with microT-CDS; P = 6.082e-06 with TarBase) and purine metabolism (hsa00230; P = 3.244e-05 with TargetScan). The ubiquitin-proteasome system is involved in protein homoeostasis and quality control, and is strongly suspected to be involved in DN progression [45], as it is modulated by hyperglycaemia and oxidative stress in the kidney [46]. In agreement with this, miR-30a-5p EV levels correlated positively with fasting and postprandial blood glucose as well as with eGFR (kidney damage).

Nevertheless, it should be mentioned that our study considered the same previously mentioned miRNAs as relevant for DN [23–25,47] and, thus, our results may have been influenced by the usually low number of patients in previous studies, mode of EV extraction and subtypes considered, subsets of analyzed miRNAs (which differed in all studies), type of diabetes (T1D vs. T2DM), presence of associated pathologies (such as retinopathy [47]), stage of pathology (MIC or MAC), and the fact that our study involved a specific population of T2DM patients from South India with the so-called ‘Asian Indian phenotype’, which has specific clinical characteristics [26].

However, although beyond the scope of our study, the important issue is whether urinary EV miRNA signatures correlate with miRNA expression in kidney tissue and, thus, whether they are direct reflections of kidney homoeostasis. Previous studies have demonstrated the crucial role of miRNAs in kidney homoeostasis [48], and their modified expression is associated with kidney dysfunction (such as fibrosis development [49] and accumulation of extracellular matrix proteins [50]). It was also previously found in an animal model of early experimental DN that miR-145 levels were increased in both urinary EVs and glomeruli [51]. In addition, increases in miR-145 content in both mesangial cells and mesangial cell-derived EVs were found when cultured mesangial cells were exposed to high glucose levels [51], thereby suggesting a potential mechanism for miR-145 overexpression in DN. Similar conclusions have been found in humans [23].

In conclusion, our study has identified, for the first time in an Indian population, altered miRNA signatures in urinary EVs associated with DN development in T2DM patients. Our results suggest that urinary EV miRNAs are potential ‘liquid biopsies’, which are urgently needed to stratify patients with different levels of renal changes in DN. Moreover, further prospective studies are now needed to validate these candidate miRNAs in the development of simple assay systems for point-of-care approaches to adapt their usefulness for precision medicine to prevent and manage DN.

Ethics approval and consent to participate

All patients’ information was anonymized at source, and unique ID codes were used to identify cases. The study protocol was approved by the Institutional Review Board and conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from all study participants before enrolment.

Contribution statement

Acquisition of data (PP, CS, VM, MB); analysis of data (PP, CS, SR, CG, EM); interpretation of data (SR, PP, MB); drafting the paper (PP, SR, MB); revising the paper critically for important intellectual content (SR, MB); and final approval of the final version to be published (PP, CS, CG, EM, VM, MB).

Disclosure of interest

The authors declare that they have no competing interest.
Paramasivam Prabu and Muthuswamy Balasubramanyam are responsible for the integrity of the work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.diabet.2018.08.004.

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