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ORIGINAL ARTICLE

Serum microRNAs are altered in various stages of chronic kidney disease: a preliminary study

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Abstract

Background: MicroRNAs (miRNAs) are innovative and informative blood-based biomarkers involved in numerous pathophysiological processes. In this study and based on our previous experimental data, we investigated miR-126, miR-143, miR-145, miR-155 and miR-223 as potential circulating biomarkers for the diagnosis and prognosis of patients with chronic kidney disease (CKD). The primary objective of this study was to assess the levels of miRNA expression at various stages of CKD.

Methods: RNA was extracted from serum, and RT-qPCR was performed for the five miRNAs and *cel-miR-39* (internal control).

Results: Serum levels of miR-143, -145 and -223 were elevated in patients with CKD compared with healthy controls. They were further increased in chronic haemodialysis patients, but were below control levels in renal transplant recipients. In contrast, circulating levels of miR-126 and miR-155 levels, which were also elevated in CKD patients, were lower in the haemodialysis group and even lower in the transplant group. Four of the five miRNA species were correlated with estimated glomerular filtration rate, and three were correlated with circulating uraemic toxins.

Conclusions: This exploratory study suggests that specific miRNAs could be biomarkers for complications of CKD, justifying further studies to link changes of miRNA levels with outcomes in CKD patients.

Key words: CKD, haemodialysis, microRNAs, renal transplantation, serum biomarkers

* These authors contributed equally to this work.

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Introduction

Chronic kidney disease (CKD) progression leads to end-stage renal disease, requiring either dialysis or transplantation [1]. During CKD progression, the number of functional nephrons decreases, resulting in accumulation of uraemic toxins, including indoxyl sulphate (IS), indole-3-acetic acid (IAA) and p-cresyl sulphate (PCS), which are poorly removed by dialysis [2] and are associated with cardiovascular morbidity and mortality [3–5]. New biomarkers are needed to more accurately stratify CKD progression and the risk of CKD-related complications. The ideal biomarker must be non-invasive, reliably measured in easily accessible sources such as blood, and have a high sensitivity and a high specificity [6].

In this regard, microRNAs (miRNAs) present in peripheral blood may constitute valuable new biomarkers [7]. miRNAs are defined as single-stranded RNA of 21–25 nucleotides, regulating more than half of the genome by repressing protein expression. Serum miRNAs are highly stable in blood and represent a novel class of diagnostic and prognostic biomarkers for numerous diseases [7]. miRNA levels have been associated with CKD in previous studies by our team and by other authors, in both cellular and animal models [8–10]. In a murine CKD model, we have shown that miR-126, miR-143, miR-145 and miR-223 are implicated in the vascular complications that occur during the later stages of CKD [9]. miR-126 is endothelial-specific and implicated in endothelial dysfunction [11]. miR-143 and miR-145, arising from the same precursor, are specifically and highly expressed in vascular smooth muscle cells and play important roles in vascular disease [12]. The inflammatory miR-223 is modulated by the uraemic toxin inorganic phosphate (Pi), as shown in *in vitro* vascular calcification models [10, 13], and its expression is increased *in vivo* in the aorta of CKD mice [9]. Importantly, the expression of all of these miRNAs was deregulated in the serum of CKD mice, suggesting similar regulation to that observed in human patients [9]. However, only limited data on miRNA expression are available in the course of human illness. To our knowledge, only two studies have assessed miRNA expression in CKD patients. Neal *et al.* [14] showed that some circulating miRNAs are decreased in the serum of CKD patients, but they did not use any reference gene in their qPCR technique, which makes the results difficult to interpret. Chen *et al.* showed a decrease of circulating levels of miR-125b, miR-145 and miR-155 in a group of 90 Stage III–IV CKD patients [8]. However, they used U6 as reference, and this small RNA from the splicing complex has subsequently been shown to be a low performance endogenous control for quantification of circulating miRNAs, as it fluctuates widely in human serum [15]. Roberts *et al.* demonstrated that the optimal strategy to accurately quantify extracellular miRNAs in serum is to normalize values to a synthetic spike-in control oligonucleotide [16], which is why we chose to use this strategy in the present study. To further investigate the connections between miRNA expression and uraemic toxicity, we decided to test correlations of the above-mentioned miRNAs with serum concentrations of IS, IAA and PCS, and several biochemical parameters known to be important in the assessment of CKD-related complications, particularly cardiovascular complications.

In this exploratory study, we describe the status of miRNA levels during the course of CKD including dialysis and renal transplantation and evaluate possible associations with other CKD-related parameters such as uraemic toxins. Our study raises the hope that miRNA levels could be used in the future as diagnostic tools to evaluate CKD progression and complications.

Materials and methods

Enrolled study subjects

This study was performed in 113 patients with CKD enrolled by two recruitment centres, namely Marseille and Amiens University Hospitals, in France. Chronic haemodialysis patients and kidney transplant patients were prospectively enrolled in the study from November 2008 to November 2011 in the Centre de Néphrologie et Transplantation Rénale, Hôpital de la Conception, Marseille. CKD patients were prospectively enrolled in the study from November 2008 to November 2011 in CHU-Amiens-Picardie. Healthy volunteers were recruited from November 2008 to November 2011 by the Centre d'Investigation Clinique of the Assistance Publique Hôpitaux de Marseille, France. Patients were selected according to the following inclusion criteria: age >18 years; no cardiovascular event, infection or surgical intervention (except for vascular access angioplasty) during the previous 3 months; no pregnancy; and no recent history of malignancy. Patients with CKD treated by haemodialysis had a dialysis session at least three times a week for a minimum of 6 months. Patients with CKD Stages III–V and renal transplant recipients had stable renal function for at least 3 months. Standard laboratory procedures were used for blood biochemistry evaluations. Blood samples were drawn before the second dialysis session of the week, on a Wednesday or a Thursday. Transplant recipients had undergone surgery at least 6 years before blood sampling. We employed no matching procedure for the selection of patients and healthy volunteers.

CKD Stages 3 and 4 were determined by estimated glomerular filtration rate (eGFR) as calculated by $eGFR (mL/min/1.73 m^2) = 175 \times (S_{cr})^{-1.154} \times (Age)^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if African American})$. CKD Stage 3 was defined as $30 mL/min/1.73 m^2 < eGFR < 60 mL/min/1.73 m^2$; Stage 4 as $15 mL/min/1.73 m^2 < eGFR < 30 mL/min/1.73 m^2$; and Stage 5 as $eGFR < 15 mL/min/1.73 m^2$.

Results obtained in patients were compared with those of 41 healthy male and female control subjects with normal renal function, no diabetes, no cardiovascular event and no chronic medication.

All participants gave their written informed consent. The study was approved by the local ethics committee and conforms to the principles outlined in the Declaration of Helsinki.

RNA isolation and qPCR

The selection of miRNAs was based on our previous *in vitro* data and data obtained from CKD animals [9, 10, 17]. miR-126, miR-143, miR-145, miR-155 and miR-223 were therefore investigated. Total RNA was isolated from serum and RT-qPCR was performed as previously described [9]. Briefly, serum RNA was isolated using the miRNeasy Serum/Plasma Kit (Qiagen). *Syn-cel-miR-39* miScript miRNA Mimic (Qiagen) was spiked in the RNA samples and used as exogenous control for data analysis, as previously described [18]. For all miRNAs, Taqman assays (Applied Biosystems) were used for cDNA synthesis and qPCR. *Syn-cel-miR-39* Mimic was used as endogenous control. qPCR was performed in triplicate on a CFX Connect™ qPCR detection system (BIORAD). Each patient was attributed a $2^{-\Delta Cq}$ value, where Cq is the difference between the Cq of the study sample and the Cq of the exogenous control. An extended version of our protocols is available in Supplementary data, Materials and Methods.

Measurement of uraemic toxins

Blood samples for miRNA and uraemic toxin measurements were collected at the same time. In haemodialysis patients, sera were

collected just before the beginning of the haemodialysis session. Serum samples from patients and controls were stored at -80°C . IS, IAA and PCS were measured in serum by high-performance liquid chromatography as previously described [19]. Briefly, 300 μL of ethanol containing 5 nmol p-ethylphenol were added to 100 μL of serum. Serum was saturated with 100 mg of NaCl. After 10 min, 700 μL of mobile phase A were added and the sample was centrifuged at 10 000g for 10 min. Serum samples were treated with ethanol. Uraemic solutes and IS were quantified with fluorescence detection monitored at specific excitation (E_x) and emission (E_m) wavelengths (Pol: E_x 272, E_m 319 nm; 3-IAA and 3-INDS: E_x 278, E_m 348 nm; p-C and p-CS, IS: E_x 285, E_m 310 nm) according to retention times. Concentrations of uraemic solutes were calculated using standard calibration curves (C_{N-C_U} ; C_{U-C_M}) by Shimadzu LC solution software (version 1.21). All samples were run in duplicate, and two reference samples were included in each run.

Statistics

Data are expressed as mean \pm SEM, number or frequency, as appropriate.

In 113 CKD patients, univariate linear regressions were performed to evaluate parameters linked to each miRNA. The characteristics of the study population are presented as mean \pm SEM or percentages. The Agosturo–Pearson test was performed to study distribution of values. Intergroup comparisons were performed with a χ^2 test (for categorical variables), Student's t test or a Wilcoxon–Mann–Whitney test (for continuous variables) and a Mann–Whitney test and Kruskal–Wallis test (for non-continuous variables). Spearman correlation tests were used to compare miRNA expressions with uraemic toxin concentrations and other parameters.

Univariate analyses were performed to identify parameters linked to each miRNA. For each miRNA linked to parameters, multivariable linear regression was performed to identify independent parameters linked to miRNA levels. Parameters entered in the models were linked to miRNA in univariate analysis. As CKD stages and uraemic toxins were highly correlated, only CKD stages were included in the model. The 'age' variable was systematically added to the model.

Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS software (version 18.0, SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp, Redmond, WA, USA). The limit of statistical significance was $P < 0.05$ for all tests.

Results

Patients and healthy subjects

The study was conducted in 154 adults from two French clinical research centres, including 41 healthy volunteers, 32 patients with CKD Stages III–V, 42 chronic haemodialysis patients and 39 renal transplant patients. The characteristics of the three patient groups and healthy controls are presented in Table 1. No significant difference in terms of gender was observed between groups, but there was a significant age difference between subgroups. This difference was, however, independent of miRNA levels. (Serum creatinine was not taken into account in the haemodialysis group because of its marked variability due to dialysis.)

Uraemic toxin concentrations in patients

Serum PCS and IS levels exhibited a 3-fold increase in CKD Stage III–V patients compared with control levels, and were further

elevated in the haemodialysis group (6.6-fold increase of PCS and 16-fold increase of IS; Figure 1A). The concentrations of these two uraemic toxins were significantly lower in kidney transplant patients than in CKD or haemodialysis patients, although still above control levels (1.8-fold and 2-fold, respectively). IAA levels were not significantly different between CKD patients and healthy controls and were increased only in the haemodialysis group (3.5-fold). IAA levels were lower in the renal transplant group than in the haemodialysis group, but were 1.6-fold higher than control values.

Differences of serum miRNA levels according to CKD stage

Serum miR-126, miR-143, miR-145, miR-155 and miR-223 levels differed according to CKD stage (Figure 1B).

Endothelial-specific miR-126 levels were not affected in the CKD Stage III–V group. However, they were significantly decreased in the haemodialysis group (0.77-fold) compared with control values, and decreased by one-half in the transplant group.

miR-143 and miR-145 are co-transcribed as a bicistronic unit [20]. However, differences between their profiles were detected. Compared with control levels, miR-143 levels showed a 6-fold increase in the CKD Stage III–V group and a 4-fold increase in the haemodialysis group, while they were decreased by one-half in the renal transplant group. In patients with CKD Stages III–V, miR-145 expression was increased 2-fold, although the difference was not statistically significant, possibly due to marked inter-individual variations. At the stage requiring haemodialysis, CKD induces a significant increase of miR-145 compared with controls (2.25-fold control levels). In contrast, a marked decrease of miR-145 expression (0.33-fold control levels) was observed in renal transplant recipients.

miR-155 expression was markedly and significantly enhanced in the CKD Stage III–V group (5-fold control levels) and miR-155 expression was significantly decreased in the haemodialysis group to 0.57-fold control levels. Finally, in renal transplant recipients, miR-155 expression was below the limit of detection.

In the serum of Stage III–V CKD patients, miR-223 expression was 2-fold higher than in controls, with marked inter-individual variations, certainly due to the heterogeneity of the patients in this group, ranging from mild to more severe CKD, leading to non-significant results. At the stage requiring haemodialysis, the CKD induced a significant increase of miR-223 (2.5-fold control levels). Finally, renal transplantation induced a marked decrease in miR-223 expression (0.4-fold control levels). The absolute Cq values of the miRNAs and the detectability of all miRNAs across patient groups are given in Supplementary data, Table S1.

In univariate analysis, all miRNAs were linked to CKD stages. To further confirm that, we also did a Kruskal–Wallis test to perform a global comparison of the four groups. This test indicated that there was a significant difference between the four groups for each microARN (data not shown). This result was confirmed by multivariable analysis, in which all miRNAs were independently associated with CKD stages.

U6 expression was measured in the healthy volunteers, haemodialysis patients and renal transplant recipients to test its value as an alternative reference (Supplementary data, Figure S1A). U6 levels varied widely according to patient status, with a marked 2.5-fold increase in the haemodialysis group and a >3-fold decrease in the renal transplant recipient group. On the other hand, cel-miR-39 levels did not vary at all in the various groups (Supplementary data, Figure S1B). This clearly indicates

Table 1. Clinical characteristics and serum biochemistry of healthy volunteers and patients

| | Healthy controls (n = 41) | CKD Stage III–V patients (n = 32) | Haemodialysis patients (n = 42) | Kidney transplant patients (n = 39) | P-value |
|---|------------------------------|--------------------------------------|------------------------------------|--|---------|
| Age (years) | 57.3 ± 1.92 | 66.5 ± 1.84 | 61.3 ± 2.6 | 55.9 ± 1.66 | 0.035 |
| Male gender (n%) | 23/56 | 15/46.8 | 18/42.8 | 14/35.9 | NS |
| Body mass index (kg/m ²) | 26.1 ± 0.9 | 28.8 ± 1.1 | 24.3 ± 0.8 | 24.3 ± 0.9 | 0.009 |
| eGFR (mL/min/1.73 m ²) | 93.2 ± 3.39 | 34.8 ± 2.21 | – | 53.1 ± 2.97 | <0.0001 |
| Time on dialysis or after transplantation (months) | – | – | 61 ± 78 | 76 ± 3.98 | NS |
| Type of kidney disease (n%) | | | | | |
| GN | – | 2/6.25 | 6/14.3 | 14/35.9 | |
| ADPKD | – | 5/15.6 | 2/4.8 | 5/12.8 | |
| Vascular nephropathy | – | 5/15.6 | 10/23.8 | 6/15.4 | |
| Interstitial nephropathy | – | 4/12.5 | 8/19 | 4/10.3 | |
| Diabetes | – | 8/25 | 8/19 | 1/2.6 | |
| Unknown | – | 8/25 | 8/19 | 9/23.1 | |
| Systolic BP (mmHg) | 128 ± 18 | 131 ± 14.7 | 132 ± 27.4 | 138 ± 20 | NS |
| Diastolic BP (mmHg) | 78 ± 15 | 79 ± 10.9 | 73 ± 16.8 | 82 ± 11 | NS |
| Current smokers (n%) | 0/0 | 13/40.6 | 7/16.6 | 8/20.5 | NS |
| History of cardiovascular disease (n%) | 0/0 | 3/9.3 | 19/45.2 | 10/25.6 | NS |
| Antihypertensive drugs (n%) | 0 | 25/78.1 | 30/71 | 30/76.9 | 0.03 |
| Statins (n%) | 0 | 22/68.7 | 7/16.6 | 27/69.2 | 0.01 |
| Antiplatelet drugs (n%) | 0 | 11/34.3 | 19/45.2 | 17/43.6 | NS |
| Anticoagulants (n%) | 0 | 5/15.6 | 12/28.6 | 2/5.1 | NS |
| ESA therapy (n%) | 0 | 4/12.5 | 30/71.4 | 2/5.1 | <0.0001 |
| Haemoglobin (g/dL) | 14.2 ± 1.35 | 12.9 ± 0.25 | 10.6 ± 1.3 | 13 ± 1.45 | <0.0001 |
| Serum albumin (g/L) | 39.7 ± 1.18 | 41.4 ± 0.82 | 37.3 ± 7.0 | 41 ± 3.8 | NS |
| Serum total calcium (mmol/L) | 2.31 ± 0.01 | 2.35 ± 0.02 | 2.32 ± 0.03 | 2.47 ± 0.10 | NS |
| Serum phosphate (mmol/L) | 1.08 ± 0.03 | 1.10 ± 0.04 | 1.68 ± 0.12 | 1.03 ± 0.04 | <0.0001 |
| Serum total cholesterol (mmol/L) | 5.34 ± 0.19 | 4.36 ± 0.24 | 4.52 ± 0.24 | 4.80 ± 0.16 | 0.0009 |
| Serum HDL cholesterol (mmol/L) | 1.34 ± 0.07 | 1.44 ± 0.08 | 1.18 ± 0.06 | 1.18 ± 0.16 | 0.013 |
| Serum LDL cholesterol (mmol/L) | 3.47 ± 0.17 | 2.24 ± 0.19 | 2.51 ± 0.19 | 2.15 ± 0.21 | <0.0001 |
| Serum triglycerides (g/L) | 1.06 ± 0.09 | 1.89 ± 0.03 | 1.93 ± 0.22 | 1.24 ± 0.07 | 0.003 |
| PCS (µmol/L) | 20.3 ± 3.5 | 65.9 ± 9.5 | 151 ± 14.7 | 42.0 ± 9.1 | <0.0001 |
| IAA (µmol/L) | 1.78 ± 0.15 | 1.46 ± 0.16 | 5.86 ± 0.92 | 1.78 ± 0.15 | <0.0001 |
| IS (µmol/L) | 3.37 ± 1.09 | 18.2 ± 2.66 | 89.8 ± 8.28 | 9.76 ± 2.22 | <0.0001 |

Data are expressed as mean ± SEM for numerical variables and as number/frequency for binary variables. The four groups were compared globally. GN, glomerulonephritis; ADPKD, autosomal dominant polycystic kidney disease; BP, blood pressure; ESA, erythropoiesis-stimulating agents.

that, at least in our hands, cel-miR-39 is a much better reference gene than U6.

Correlation of miRNA levels with eGFR and biochemical parameters

In the overall study population, miR-126, miR-145 and miR-223 were significantly and positively correlated with eGFR, whereas miR-143 was negatively correlated with eGFR and miR-155 was not correlated with this parameter (Table 2). The study of eGFR correlations within the CKD group, after excluding transplant patients, showed that miR-143 and miR-155 were strongly correlated with eGFR, miR-223 remained weakly correlated with eGFR, whereas miR-126 and miR-145 were no longer correlated with eGFR (data not shown). We also performed linear regression analyses on miRNAs levels in the CKD and kidney transplant group, after having excluded the haemodialysis group. Most of the correlations were lost when using this patient population, certainly due to the more limited number of patients. Interestingly, however, we found a positive correlation between miR-126 and eGFR in the kidney transplant population (Supplementary data, Figure S2).

Parathyroid hormone (PTH) levels are associated with increased cardiovascular risk in CKD patients [21]. miR-223

presented a strong negative correlation with PTH, while miR-155 was positively correlated with PTH. Interestingly, miR-143 and miR-155 were negatively correlated with total cholesterol and miR-143 was also negatively correlated with low-density lipoprotein (LDL) cholesterol. On the other hand, miR-126 was positively correlated with triglyceride levels. When doing an additional set of analyses after exclusion of the transplant patients, we found that a number of the correlations were lost, certainly due to the now more limited number of patients in the CKD and haemodialysis cohorts. However, miR-155 remained correlated with serum albumin, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, PTH, triglycerides, IAA and IS. miR-126 remained correlated with Pi, miR-143 with IAA and miR-223 with PTH (data not shown).

Associations between serum levels of miRNAs and uraemic toxins

Serum concentrations of IS and PCS were significantly correlated with miR-126 levels (Table 2). Serum IS and PCS levels were also correlated with miR-143 ($P < 0.0002$ and 0.011 , respectively) and miR-155 was correlated with IS ($P < 0.0033$). However, IAA was not correlated with any of the miRNAs studied and miR-145 and miR-223 were not correlated with any of the uraemic toxins.

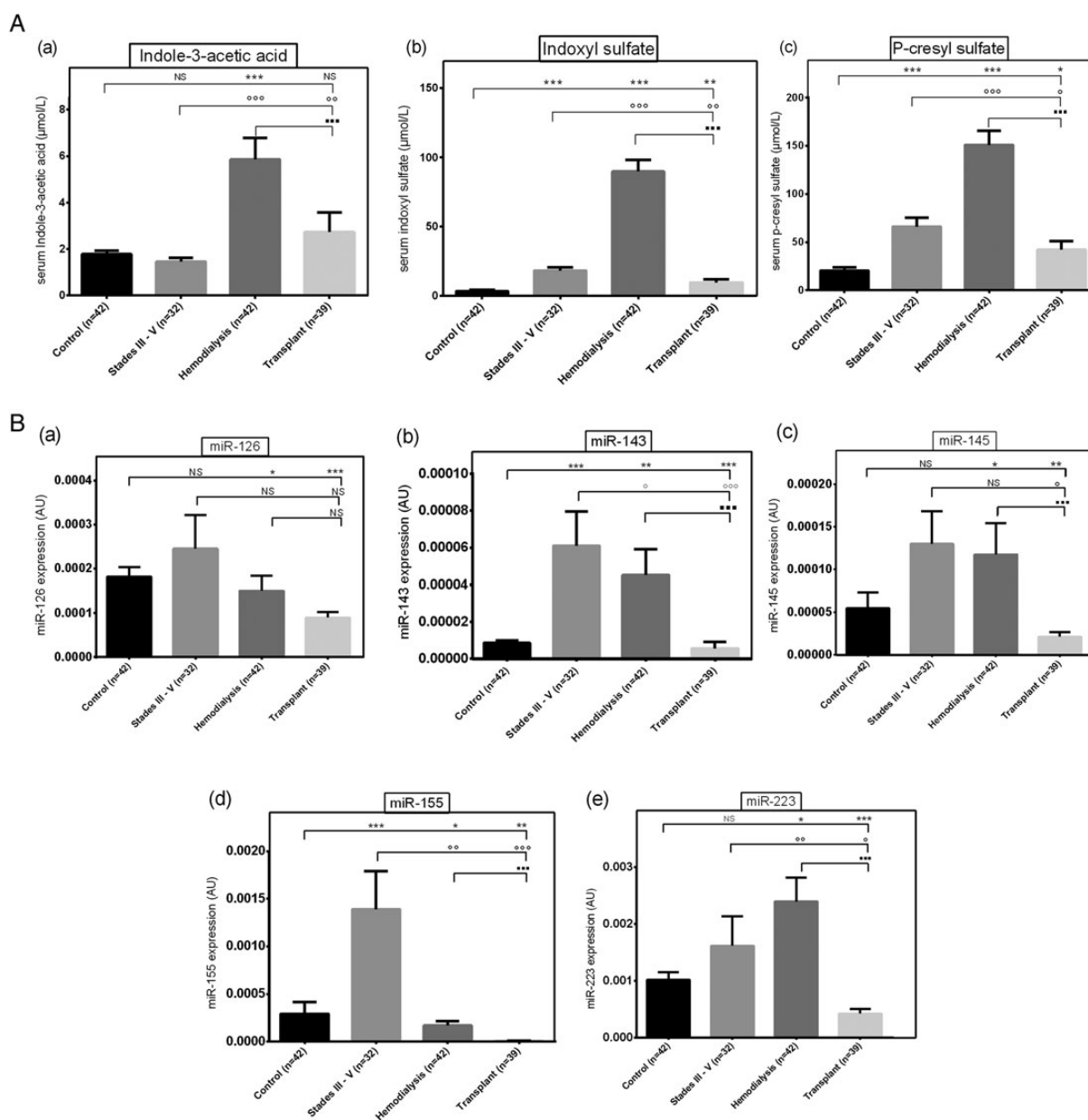


Fig. 1. Circulating uraemic toxin levels (A) and miRNAs levels (B) in healthy controls, CKD patients, chronic haemodialysis patients and renal transplant recipients. Sera were collected from patients with CKD Stage III–V ($n = 31$), haemodialysis patients ($n = 40$), healthy volunteers ($n = 38$) and renal transplant recipients ($n = 40$). miRNA levels were assessed using Taqman RT-qPCR. Each sample was assayed in triplicate. Data are expressed as mean \pm SEM. *, \$, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IP, another well-documented uraemic toxin [22], was negatively correlated with miR-126 and positively correlated with miR-143 and miR-155. Serum total calcium levels were not correlated with any of the miRNAs studied.

Discussion

Non-invasive biomarkers able to predict the progression of CKD and its complications need to be developed [23]. In the present exploratory study, we show that several miRNAs are altered at various stages of CKD, including haemodialysis and kidney transplantation. We have previously shown that miR-126, -143 and -223 levels are deregulated in murine serum during the course of experimental CKD [9]. The present study now shows that these miRNAs are also altered in patients with kidney

disease and extends the list of miRNAs concerned by adding miR-145 and miR-155. Schematically, miRNAs can be subdivided in two groups: (i) miRNAs that are increased in patients with CKD Stages III–V and haemodialysis and decreased in renal transplant recipients (miR-143, miR-145 and miR-223); and (ii) miRNAs that are increased in patients with CKD Stages III–V, decreased in haemodialysis patients and even more markedly decreased in renal transplant recipients (miR-126 and miR-155).

Elia et al. [24] and our group [20] have shown that miR-143 and -145 are decreased in damaged vessels, including in CKD mice. In the present study, we found that both miR-143 and miR-145 levels are increased in the serum of CKD patients, further increased in haemodialysis patients and decreased to below control levels in renal transplant recipients. A recent study has shown that miR-145 is decreased in the serum of patients with CKD [8].

Table 2. Correlations between serum miRNA levels and various clinical and serum biochemistry parameters (eGFR values of CKD and kidney transplant patients only)

| | miR-126 | miR-143 | miR-145 | miR-155 | miR-223 |
|---|-------------------------------|-------------------------------|-------------------------------|--|---|
| eGFR | $r = 0.2936$ $P = 0.0019$ | $r = -0.2266$ $P = 0.0195$ | $r = 0.2184$ $P = 0.0225$ | NS | $r = 0.3174$ $P = 0.0007$ |
| Serum creatinine ($\mu\text{mol/L}$) | $r = -0.3487$ $P = 0.0002$ | $r = 0.2244$ $P = 0.0195$ | $r = -0.2382$ $P = 0.0118$ | NS | $r = -0.3478$ $P = 0.0002$ |
| Body mass index (kg/m^2) | NS | NS | NS | NS | $r = -0.1793$ $P = 0.0298$ |
| Serum albumin (g/L) | NS | NS | NS | $r = 0.1981$ $P = 0.0208$ | NS |
| Serum total calcium (mmol/L) | NS | NS | NS | NS | NS |
| Serum phosphate (mmol/L) | $r = -0.2141$ $P = 0.0085$ | $r = 0.26$ $P = 0.0015$ | NS | $r = 0.1683$ $P = 0.043$ | NS |
| Serum glucose (mmol/L) | NS | $r = 0.2058$ $P = 0.037$ | NS | NS | NS |
| Serum total cholesterol (mmol/L) | NS | $r = -0.241$ $P = 0.003$ | NS | $r = -0.263$ $P = 0.001$ | NS |
| Serum HDL cholesterol (mmol/L) | NS | NS | NS | NS | NS |
| Serum LDL cholesterol (mmol/L) | NS | NS | NS | $r = -0.1686$ $P = 0.0083$ | NS |
| Serum iPTH | NS | NS | NS | $r = 0.4226$ $P = 0.0004$ ($n = 67$) | $r = -0.3044$ $P = 0.0093$ ($n = 72$) |
| Serum triglycerides (mmol/L) | $r = -0.2030$ $P = 0.0136$ | NS | NS | NS | NS |
| PCS ($\mu\text{mol/L}$) | NS | $r = 0.2116$ $P = 0.0112$ | NS | NS | NS |
| IAA ($\mu\text{mol/L}$) | NS | NS | NS | NS | NS |
| IS ($\mu\text{mol/L}$) | $r = -0.1723$ $P = 0.0363$ | $r = 0.3084$ $P = 0.0002$ | NS | $r = 0.2432$ $P = 0.0033$ | NS |

NS, not significant; iPTH, intact parathyroid hormone.

However, the authors used U6, a small RNA of the spliceosome, as their reference gene, which is now considered to be an unreliable endogenous control, as it fluctuates widely according to pathophysiological conditions [15]. We also found that miR-145 levels were decreased in the haemodialysis group compared with healthy volunteers when U6 was used as a reference gene (data not shown), as previously published [8]. However, in our hands, as also reported by other groups, U6 levels varied considerably according to the clinical state, indicating its unsuitability as a reliable reference gene. Roberts *et al.* have already shown that the best strategy when measuring serum miRNA levels is to normalize values to a synthetic spike-in oligonucleotide, cel-miR-39 [16], as cel-miR-39 levels did not vary in the various groups studied. We therefore adopted this strategy in the present study. The presence of miR-223 was enhanced in the serum of CKD Stage III–V and haemodialysis patients. In contrast, in renal transplant recipients, miR-223 levels were decreased. In a previous study, we found that miR-223 was increased in the aorta of CKD mice, but decreased in their serum [9]. According to Martino *et al.*, circulating miRNAs are not eliminated by haemodialysis [25], suggesting that haemodialysis is unlikely to influence miR-223 levels. C57BL/6 mice are particularly resistant to the development of glomerulosclerosis, and metabolic and inflammatory-mediated mechanisms [26, 27]. It is therefore conceivable that human patients regulate inflammatory miRNAs such as miR-223 in ways different from that of mice.

We found that miR-155 was increased in CKD patients. Zhang *et al.* [28] also reported miR-155 to be increased in a small group of haemodialysis patients. However, no control gene was used to

normalize miRNA expression in this study. In our hands, the expression of this miRNA was also altered when using a synthetic spike-in control gene. miR-155 levels were decreased in haemodialysis patients and renal transplant recipients. The same pattern was observed for miR-126 levels, one of the most abundant miRNAs in endothelial cells [29].

It is noteworthy that miR-143 and miR-155 were negatively correlated with total cholesterol, in accordance with recent reports showing that miR-143 and miR-155 overexpression is independently associated with the formation of atherosclerotic plaque in mice [30, 31].

Uraemic toxins, such as IAA, IS and PCS, are associated with cardiovascular mortality in CKD and dialysis patients [2, 5]. To the best of our knowledge, no previous study has tried to correlate the expression of these toxins with serum miRNA levels in CKD patients. Interestingly, miR-126 expression was correlated with PCS and IS, which could be related to the fact that miR-126 is an endothelial-specific miRNA, associated with endothelial dysfunction [32, 33]. On the other hand, IS was also correlated with miR-143 and miR-155, while IAA was not correlated with any miRNA. Our results show that three out of five miRNAs were correlated with uraemic toxin serum concentrations.

All miRNAs were markedly decreased in renal transplant recipients, which could be explained by immunosuppressive therapy, as Igaz *et al.* showed that corticosteroids can decrease the serum expression of certain miRNAs [34]. However, only one of the five miRNAs tested was significantly impacted by dexamethasone, suggesting that other mechanisms are involved. Our group has recently shown that uraemic toxin levels are

normalized after kidney transplantation [35]. So, the normalization of uraemic toxins after transplantation could explain, at least in part, why the correlation of IS and PCS with the levels of some miRNAs is altered in these patients. A particularly interesting set of miRNAs are those overexpressed in patients with CKD but decreased in haemodialysis patients. To our knowledge, these are the first markers to be shown to be increased in CKD but decreased in haemodialysis patients. This phenomenon was not observed for all miRNAs, suggesting that it is independent of the euration process.

A possible limitation of this study is the potential for selection bias due to the retrospective nature of the study. The patient groups are heterogeneous, as far as medication and comorbidities are concerned, and we cannot exclude that these factors may have played a role in determining miRNA levels irrespective of CKD stage. Larger multicentre prospective studies are clearly needed to confirm whether or not miRNAs can constitute useful biomarkers in CKD. The strengths of this study include the use of a large panel of well-characterized patients, reflecting the course of clinical CKD, and the fact that five miRNAs were quantified under identical state-of-the-art experimental conditions.

In conclusion, in this report we show that serum miRNA levels are altered in patients with CKD, patients receiving haemodialysis therapy and kidney transplant patients, suggesting that miRNAs could be useful biomarkers to identify specific complications of CKD. However, further studies are necessary to confirm the present data and to examine whether altered miRNA levels associate with CKD patient outcomes.

Supplementary data

Supplementary data are available online at <http://ckj.oxfordjournals.org>.

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Authors' contributions

L.M., V.M.-L.M., Z.A.M. and S.B. conceived and designed the experiments; B.B., E.M.-M., N.M. and P.P. performed the experiments; B.B., S.B., L.M., M.S., M.P. and S.L. analysed the data; and L.M., S.B., V.M.-L.M., Z.A.M. and T.B.D. contributed to the writing of the manuscript.

Conflict of interest statement

The authors have no conflicts of interest to report. The results of this research have not been published previously in whole or in part, except in abstract format.

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