British Journal of Clinical Pharmacology

Br J Clin Pharmacol (2017) **83** 584–592 584

HUMAN TOXICOLOGY

The effect of renal dysfunction and haemodialysis on circulating liver specific miR-122

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Received 21 March 2016; **Revised** 26 August 2016; **Accepted** 16 September 2016

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Keywords chronic kidney disease, dialysis, hepatotoxicity, microRNA, miR-122

AIMS

microRNA-122 (miR-122) is a hepatotoxicity biomarker with utility in the management of paracetamol overdose and in drug development. Renal dysfunction and haemodialysis have been associated with a reduction in circulating microRNA. The objective of this study was to determine their effect on miR-122.

METHODS

Blood samples were collected from 17 patients with end-stage renal disease (ESRD) on haemodialysis, 22 healthy controls, 30 patients with chronic kidney disease (CKD) and 15 patients post-kidney transplantation. All had normal standard liver function tests. Samples from ESRD patients were collected immediately pre- and post-haemodialysis. Serum alanine transaminase activity (ALT), miR-122 and miR-885 (liver enriched) were compared.

RESULTS

Circulating miR-122 was substantially reduced in ESRD patients pre-haemodialysis compared with the other groups (19.0-fold lower than healthy controls; 21.7-fold lower than CKD). Haemodialysis increased miR-122 from a median value of 6.7×10^3 (2.3×10^3–1.4×10^4) to 1.6×10^4 (5.4×10^3–3.2×10^4) copies ml^{-1}. The increase in miR-122 did not correlate with dialysis adequacy. miR-122 was reduced in the argonaute 2 bound fraction pre-haemodialysis; this fraction was increased post-dialysis. There was no change in miR-122 associated with extracellular vesicles. miR-885 was also reduced in ESRD patients (4-fold compared to healthy subjects) and increased by haemodialysis.

CONCLUSION

miR-122 is substantially lower in ESRD compared to healthy controls, patients with CKD and transplanted patients. Haemodialysis increases the concentration of miR-122. These data need to be considered when interpreting liver injury using miR-122 in patients with ESRD on dialysis, and specific reference ranges that define normal in this setting may need to be developed.

DOI:10.1111/bcp.13136

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- microRNA-122 (miR-122) is a sensitive and specific biomarker for drug-induced liver injury that is being qualified for use in clinical medicine and drug development.
- Renal dysfunction and haemodialysis can affect circulating microRNA concentrations.

WHAT THIS STUDY ADDS

- miR-122 is substantially lower in the circulation of patients with end-stage renal disease on haemodialysis.
- This decrease is specifically in the miR-122 fraction bound to the microRNA carrier protein argonaute 2.
- Haemodialysis increases the circulating concentration of miR-122.
- Reference ranges that define normality may need to take into account renal function.

Introduction

MicroRNAs are small (~22 nucleotide-long) non-protein coding RNA species involved in post-transcriptional gene-product regulation [1]. In blood, microRNAs are stable because they are protected from degradation by extracellular vesicles (ECVs), such as exosomes, RNA binding protein complexes, such as argonaute 2 (Ago2), and high-density lipoproteins [2, 3]. As they are amplifiable and some are tissue restricted, circulating microRNAs represent a reservoir for biomarker discovery. Liver-enriched miR-122 is released by injured hepatocytes, primarily bound to Ago2, and is a translational circulating biomarker for liver injury in zebrafish [4], rodents [5] and humans [6]. In patients with drug-induced liver injury (DILI), circulating miR-122 is increased around 100-fold [6, 7] and accurately predicts hepatotoxicity when all current hepatotoxicity biomarkers are still normal [7]. In the context of DILI there is a strong correlation between circulating concentrations of miR-122 and miR-885, another microRNA with a large fold increase following DILI. miR-885 is released directly from hepatocytes as reported by *in situ* hybridization studies of human liver DILI explants [8]. miR-122 is currently undergoing qualification as a clinical biomarker for stratification of patients at risk of paracetamol-induced liver injury and as a translational safety biomarker for use in preclinical and clinical drug development.

In the presence of liver injury, kidney function is one of the key predictors of death and need for urgent liver transplantation, with serum creatinine concentration being a component of the King's College Criteria [9] and the Model for End-Stage Liver Disease [10] scoring systems that are used for prognostic stratification. Circulating kidney injury molecule-1 (KIM-1) is a marker of kidney tubular injury that predicts outcome in patients with acute liver injury with higher sensitivity than creatinine and other common prognostic tools [11]. Renal dysfunction has been reported to affect the circulating concentration of microRNAs. Neal *et al.* reported significantly reduced total plasma microRNA and reductions in certain specific species (miR-16, -21, -210, -638) in patients with chronic kidney disease (CKD) [12]. We recently profiled the circulating miRNome in patients with paracetamol-induced acute liver injury and also demonstrated a global reduction in circulating microRNA with renal dysfunction [8]. In our profiling study there was no change in miR-122; however, an effect of renal dysfunction may have been undetectable given the high plasma miR-122 concentrations that accompany acute liver injury.

Patients with DILI may need short-term haemodialysis (HD), for example, as a bridge to possible liver transplantation. The effect of HD on circulating microRNAs depends on the species studied, with no effect being reported for some species (miR-21, -210) [13] but a significant decrease after HD being reported for miR-499 [14]. In the current study we measured circulating miR-122 concentrations in patients with end-stage renal disease (ESRD) before and after HD and compared them with healthy controls, patients with CKD and patients with a successful renal transplant. These data were compared with standard liver injury markers, dialysis parameters and miR-885.

Methods

Patients

In total, 84 subjects were recruited to this study, 17 with stable ESRD on maintenance HD from the outpatient dialysis unit at the Royal Infirmary of Edinburgh, UK (numbers as per power calculation), 22 healthy volunteers, 30 with CKD and 15 patients post-transplantation. Healthy controls and patients with ESRD and kidney transplantation were prospectively recruited for this study. Samples from patients with CKD were taken from a previously published study [15]. The study was approved by the local research ethics committee (Tayside Committee on Medical Research Ethics B) and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

ESRD patients

Inclusion criteria were: age 18 or over, treated with HD for over 3 months. Patients affected by liver disease or with a history of hepato-biliary surgery were excluded; other exclusion criteria were consumption of cytochrome P450-inducing medications, past medical history of epilepsy, cancer, alcoholism and/or psychiatric disease.

All patients were treated with HD for 4–5 hours per session, three times per week. Data collected included demographic characteristics, cause of ESRD, dialysis age and current medications. All patients were dialysed without heparin to prevent inhibition of polymerase chain reaction (PCR).

CKD patients

Patients with CKD stages I–V (estimated glomerular filtration rate 6–91 ml min^{-1}/1.73 m^2 calculated using the MDRD

equation) were matched with ESRD patients for age, gender, body mass index (BMI) and blood pressure (BP). In brief, subjects were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh. The inclusion criteria were: male or female CKD patients, 18–65 years old and clinic BP ≤ 160/100 mmHg, whether or not on anti-hypertensive medication. We excluded patients with a renal transplant or on dialysis, patients with systemic vasculitis or connective tissue disease, those with a history of established cardiovascular disease, peripheral vascular disease, diabetes mellitus, respiratory disease, or neurological disease, and those with current alcohol abuse or pregnancy.

Renal transplantation patients

Patients entered the study if their transplant was performed six or more months prior, their renal function was stable and their estimated glomerular filtration rate (eGFR) was greater than 60 ml $min^{-1}/1.73m^2$. Patients with abnormal liver functions tests or a history of liver disease were excluded.

Healthy volunteers

Age, gender, BMI and BP matched adults with no medical complaints and no medication use were recruited.

Blood collection

In healthy subjects blood was collected into three EDTA plasma tubes (2.7 ml) and processed without delay – centrifugation at 1200 *g* for 10 min at 4°C. Then one sample was immediately frozen at −80°C. To test miR-122 stability, the remaining plasma samples were left unprocessed at room temperature or 4°C for 24 h or 7 days.

Two blood plasma samples were collected from each HD patient, one immediately before and one after a single dialysis session, directly from dialysis needles. For CKD and transplant patients, samples were collected on the study day. ESRD, CKD and transplant patient blood plasma samples were immediately processed by centrifugation at 1200*g* for 10 min at 4°C and then supernatant frozen at −80°C.

Biochemical analysis

The following parameters were measured: full blood count, urea and electrolytes, creatinine, bilirubin, alanine aminotransferase (ALT), alkaline phosphatise (ALP) and gamma-glutamyl transferase (GGT).

MicroRNAs were measured by PCR using SYBR green-based detection as previously described [8]. RNA was isolated from 50 µl plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen, Venlo, Netherlands). RNA was eluted in a fixed volume of 14 µl, after which 2.5 µl of each eluate was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen, Venlo, Netherlands) following manufacturer's instructions. The synthesized cDNA was ten-fold diluted and used for cDNA template in combination with the miScript SYBR Green PCR Kit (Qiagen, Venlo, Netherlands) using the specific miScript assays (Qiagen, Venlo, Netherlands). Real-time PCR was performed on a Light Cycler 480 (Roche, Basel, Switzerland) using the recommended miScript cycling parameters.

Where indicated, results were confirmed by TaqMan-based PCR. The small RNA eluate was reverse transcribed using the Taqman assay containing specific stem-loop reverse-transcription RT primers (Applied Biosystems, Foster City, CA, USA) for each target miRNA species, following the manufacturer's instructions. In the reverse transcription reaction, 1 µl of RNA was used to produce the complementary DNA (cDNA) template. Then, 1 µl of cDNA was used in the PCR mixture with specific PCR primers (Applied Biosystems, Foster City, CA) in a total volume of 10 µl. Levels of miRNA were measured using the Light Cycler 480 (Roche, Basel, Switzerland).

Absolute quantification of microRNA was achieved by generating a standard curve using synthetic target. Standard curves were generated by reverse transcribing known concentrations of miScript miRNA mimics (Qiagen, Venlo, The Netherlands) in 0.1X TE buffer spiked with 10 ng μl^{-1} Poly-C (Sigma-Aldirch, Gillingham, UK). The resulting cDNA was measured using serial dilutions on three different plates to demonstrate minimal variability.

The Agilent 2100 Bioanalyzer Small RNA kit was used according to the manufacturer's instructions to quantify RNAs in the 6–150 nucleotide size range.

Extra-cellular vesicle isolation

Human plasma was fractionated by differential centrifugation to isolate microRNA containing ECVs. Plasma (1 ml) was centrifuged at 2000*g* for 30 min then 12 000*g* for 45 min. The supernatant was then ultracentrifuged at 110 000*g* for 1.5 h to pellet ECVs. The pellet was resuspended in 2 ml PBS, after which an additional ultracentrifugation step of 110 000*g* for 1.5 h was performed. The vesicles were re-suspended for miRNA analysis. ECV presence and number was quantified by nanoparticle tracking analysis as previously described [16].

Ago2 isolation

Magna Bind goat anti-mouse IgG magnetic bead slurry, 100 µl (Thermo Scientific, Waltham, USA), was incubated with 10 µg of mouse monoclonal anti-Ago2 (Abcam, Cambridge, UK) or mouse normal IgG (Santa Cruz Biotechnology, Dallas, US) antibodies for 2 h at 4°C. The antibody-coated beads were then added to plasma and incubated overnight at 4°C with rotation. Beads were washed three times and each sample then eluted in RNAse-free water before QIAzol was added for RNA isolation.

Statistical analysis

The primary outcome was difference in serum miR-122 before and after HD. Out of five microRNA species, the smallest difference between ESRD and control that was reported by Neal *et al.* was a two-fold reduction [12]. Therefore, this cutoff was used as the minimal effect size for the power calculation. We calculated the sample size as follows: alpha-level 0.05; beta-level 0.8; standard deviation 0.203; we estimated that to determine a minimal difference in miR-122 ΔCt of 0.165 (corresponding to a two-fold change in copy number/ml) we would require 17 participants. All data are presented as median with interquartile range as D'Agostino & Pearson omnibus normality test failed to demonstrate a normal

distribution for the data. Statistical analysis was performed using Graphpad Prism (GraphPad Software, La Jolla, California, USA). Nominal statistical significance was set at $P < 0.05$.

Results

Firstly, the stability of miR-122 was determined. After processing human blood into plasma, storage at room temperature or 4°C for 24 h or 7 days had no significant effect on miR-122 concentration (Figure 1). Subject demographics and medications are shown in Table 1. As expected, the patient groups had significantly lower haemoglobin concentrations and were prescribed multiple medications. Erythropoietin, iron, 1α-calcidol, phosphate binders and calcimimetics were more commonly used in the ESRD group. The aetiologies of CKD were polycystic kidney disease (n = 8), glomerulonephritis (n = 6), obstructive uropathy (n = 5), Alport Disease (n = 1), with one unknown cause. The aetiologies of ESRD were obstructive uropathy (n = 5), diabetic nephropathy (n = 4), glomerulonephritis (n = 3), polycystic kidney disease (n = 1), pyelonephritis (n = 1), hypertensive nephropathy (n = 1), interstitial nephritis (n = 1) and one unknown cause. The diseases that resulted in need for transplantation were IgA nephropathy (n = 3), hypertensive nephropathy (n = 1), interstitial nephritis (n = 1), nephrolithiasis (n = 2), adult polycystic kidney disease (n = 1), Alport syndrome (n = 2), reflux nephropathy (n = 3), diabetic nephropathy (n = 1) and focal segmental glomerulosclerosis (n = 1). In the transplanted patient group the median eGFR was 80 ml $min^{-1}/1.73m^2$ (IQR: 67–95; range: 60–97), as calculated by the MDRD equation.

Total circulating microRNA was lower in patients with CKD (Figure 2). Serum ALT activity was lower in CKD and ESRD compared with healthy controls and transplanted patients (healthy ALT: median 18 IU l^{-1} (IQR16–30); CKD 11 IU l^{-1} (6–13); ESRD 12 IU l^{-1} (9–19); transplant 23 IU l^{-1} (14–38)). (Figure 3A). Haemodialysis induced a statistically significant, but clinically insignificant, increase in ALT from 12 (9–19) to 13 (11–21) IU l^{-1}. By contrast with ALT, miR-122 was not different when healthy controls were compared with CKD (Figure 3B). Pre-HD miR-122 circulating concentration was 19-fold lower compared with healthy controls (pre-HD median value of 6.7×10^3 (2.3×10^3–1.4×10^4); healthy controls 1.3×10^5 (7.6×10^4–3.8×10^5) copies ml^{-1}; $P < 0.0001$). Compared with healthy subjects, miR-122 was 5.0-fold lower pre-HD when PCR was performed using Taqman (Supplementary Figure S1). HD induced a 2.4-fold increase in miR-122 (post-HD: 1.6×10^4 (5.4×10^3–3.2×10^4) copies ml^{-1}). In patients with renal transplantation, miR-122 was comparable to the healthy controls (post-transplant: 4.7×10^4 (2×10^4–2.3×10^5) copies ml^{-1}). miR-885 was also reduced in ESRD patients (4-fold compared to healthy subjects) and increased by haemodialysis (from a median value of 5 (2–14) to 10 (5–18) copies ml^{-1}) and renal transplantation (294 (224–436) copies ml^{-1}) (Figure 3C). In the 30 patients with CKD, there was no significant correlation between eGFR and miR-122 (Figure 4). In ESRD there was no correlation between change in miR-122 and fluid removed by HD, urea reduction ratio (URR) or change in ALT. There was a significant correlation with change in miR-885 (Figure 5).

miR-122 circulates bound to Ago2 and encapsulated in ECVs. ECVs were isolated from plasma (Figure 6); there was no difference in miR-122 when ESRD patient samples were compared to healthy controls (Figure 6). By contrast, when Ago2 was isolated from plasma, the miR-122 fraction bound to this protein was significantly lower in ESRD patients pre-HD compared to healthy controls and post-HD samples (Figure 6).

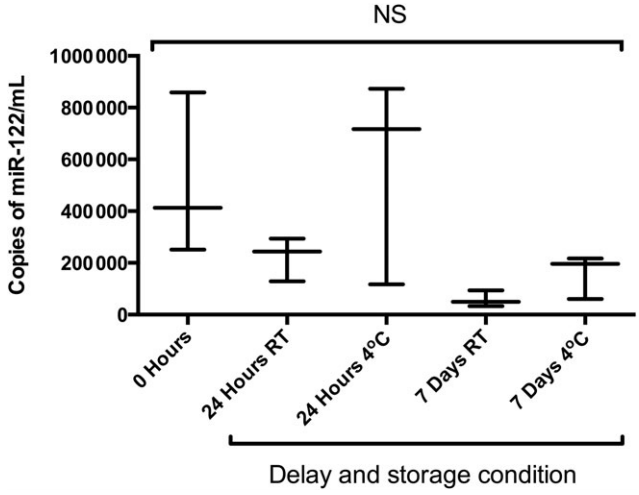

Figure 1

miR-122 is stable in plasma. Plasma samples were collected from healthy volunteers (n = 3) and immediately centrifuged to isolate plasma. One plasma sample was stored at −80°C without delay and the remaining tubes were left at room temperature (RT) or 4°C for 24 h or 7 days. Data presented as a Tukey plot

Discussion

An essential part of biomarker development is the definition of normal reference ranges that allows patients with disease to be identified with a level of accuracy that is fit for purpose given the biomarker's context of use. In this paper we demonstrate that Ago2-bound miR-122 is substantially reduced in patients with ESRD on HD who have standard liver function tests (such as ALT) within the normal range. Furthermore, HD increases miR-122, which may reflect hepatocyte injury given the concomitant increase in ALT and miR-885 and the lack of correlation with measures of dialysis adequacy. These data need to be considered when interpreting miR-122 concentrations in patients with ESRD on dialysis, and specific reference ranges that define normal in this setting may need to be developed.

Before commencing recruitment into this study, we confirmed that miR-122 was stable in plasma. This is consistent with the general view in the literature that microRNAs are stable in the circulation, a property that makes them attractive biomarker candidates. This stability is believed to be due to their binding to carrier proteins or encapsulation in vesicles. In the specific case of miR-122 we have recently

Table 1

Subject characteristics

	Healthy controls	CKD	Haemodialysis	Post-transplant
Number of patients	22	30	17	15
Age (years)	55 ± 10	48 ± 10	58 ± 13	43 ± 10
Sex M, % (*n*)	55 (12)	50 (15)	47 (8)	73 (11)
BMI (kg m^{-2})	26 ± 5	26 ± 6	27 ± 6	25 ± 4
SBP (mmHg)	128 ± 13	123 ± 14	129 ± 19	129 ± 15
ALT (U l^{-1})	22 ± 10	11 ± 7	15 ± 8	37 ± 44
Bil (μmol l^{-1})	9 ± 4	9 ± 3	9 ± 3	13 ± 6
CRP (mg dl^{-1})	1 ± 2	2 ± 2	10 ± 15	6 ± 6
Haemoglobin mg dl^{-1})	146 ± 13	134 ± 16	115 ± 12	135 ± 16
Medications, *n* (%)				
ACE-I		12 (40)	4 (20)	5 (33)
ARB		5 (17)	1 (6)	0 (0)
Beta-blockers		13 (43)	8 (47)	4 (27)
Statins		9 (30)	8 (47)	2 (13)
Antiplatelets		6 (20)	6 (35)	1 (7)
Warfarin		1 (3)	3 (18)	0 (0)
CCBs		14 (47)	4 (20)	7 (47)
Diuretics		1 (3)	0 (0)	0 (0)
Corticosteroids		0 (0)	0 (0)	10 (67)
Calcineurin inhibitor		0 (0)	0 (0)	15 (100)
Mycophenolate mofetil		0 (0)	0 (0)	12 (80)
Erythropoietin		2 (7)	17 (100)	0 (0)
Oral iron supplement		8 (27)	16 (99)	0 (0)
1-α calcidol		8 (27)	16 (99)	9 (60)
Phosphate binders		4 (13)	11(65)	4 (27)
Calcimimetics		0 (0)	3 (18)	0 (0)
alpha blockers		0 (0)	0 (0)	2 (13)

Age, gender; haemoglobin and medications of the 84 enrolled subjects (Healthy Controls, Chronic Kidney Disease (CKD) Haemodialysis and Post-transplant) are shown. ACE-I, ACE inhibitors; ARB, angiotensin receptor blockers; ALT, alanine aminotransferase; Bil, bilirubin; BMI, body mass index; CCB, calcium channel blockers; CRP, C-reactive protein; SBP, systolic blood pressure. Values are expressed as mean ± SD.

demonstrated that this microRNA species circulates predominantly bound to the carrier protein Ago2 [8].

The total circulating microRNA concentration was reduced in patients with CKD. This is consistent with data from Neal *et al*. who also reported a significant decrease [12]. Neal *et al*. presented data supporting a possible mechanism for this global reduction: patients with CKD have increased RNAse activity in their blood. The primary objective of our study was to explore miR-122 concentrations in patients with impaired kidney function. This liver-specific biomarker was reduced in the group with the worst kidney function, patients with ESRD. This is clinically important, as normal reference ranges may need to take into account the patient's GFR. Patients with CKD had miR-122 concentrations that were no different to controls. This is consistent with our previous published data that demonstrated no reduction in miR-122 in patients with CKD [6]. However, in the present study patients with CKD had lower serum ALT activity. This demonstrates a disconnect between ALT and miR-122 (which typically track each other, albeit with miR-122 having more rapid kinetics), and possibly reflects differences in the effect of renal dysfunction on mechanisms of ALT cellular release and clearance. Our published data demonstrate that miR-122 and miR-885 are related; both species are released from injured hepatocytes and their circulating concentrations are tightly positively correlated [8]. When compared with healthy controls, miR-885 was also substantially lower in patients with ESRD but not those patients with CKD.

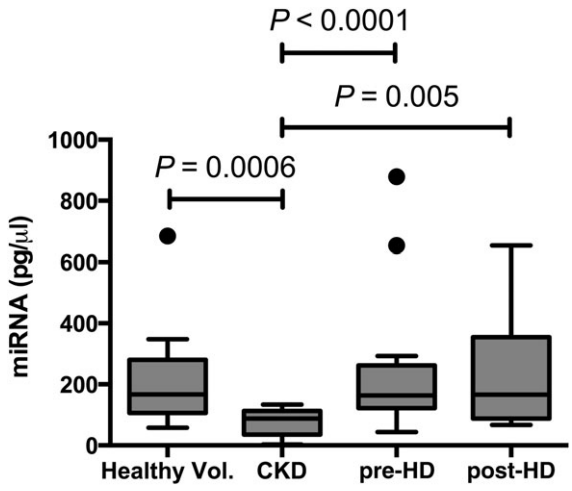

Figure 2

Total circulating microRNA is lower in chronic kidney disease (CKD) stage IV. microRNA in plasma was quantified by Agilent 2100 Bioanalyzer. Patient groups were healthy controls (n = 17), CKD (n = 20), and end-stage renal disease immediately before and after haemodialysis (pre-HD and post-HD, respectively, both n = 17)

HD has been reported to reduce the circulating concentration of certain microRNA species, which could relate to their being cleared in the dialysate fluid [14]. The data presented in this article are the first to demonstrate an increase in microRNA after HD. The increase in miR-122 did not correlate with parameters of dialysis adequacy or fluid removal, which argues against volume changes being the explanation for the increase. Serum ALT activity and miR-885 also increased after HD, which suggests that there is mild hepatocyte injury during the HD session.

In the field of drug-induced liver injury, miR-122 has significant promise as a new sensitive and specific biomarker of hepatocyte injury. More broadly, across a wide range of disease areas, circulating microRNAs are being proposed as biomarkers. However, their kinetics in the circulation are poorly defined, specifically their clearance. Following acute liver injury miR-122 is elevated in concentration earlier than ALT and decreases before ALT. However, this kinetic profile is not consistent across all microRNAs that change significantly following liver injury, with some changing later and having longer 'half-lives' in the circulation [8]. Future studies should investigate the clearance mechanisms of microRNA when combined with their different circulating carriers. Such studies would include defining the contribution of the kidney. The present study clearly demonstrates that the Ago2-bound form of miR-122 is substantially reduced in patients with ESRD. Mechanisms that underlie this are yet to be defined but may include reduced production of microRNA. However, if this were the case, the ECV-bound fraction would also be expected to fall. Neal *et al.* have reported that ESRD is associated with increased circulating RNAse activity [12]. The Ago2-bound form of miR-122 may be more sensitive to enzymatic digestion compared with ECV-encapsulated miR-122. *A priori* it could be hypothesized that the ECV fraction would differ between patients with ESRD and healthy controls because work by our group [17], and others [18], has clearly

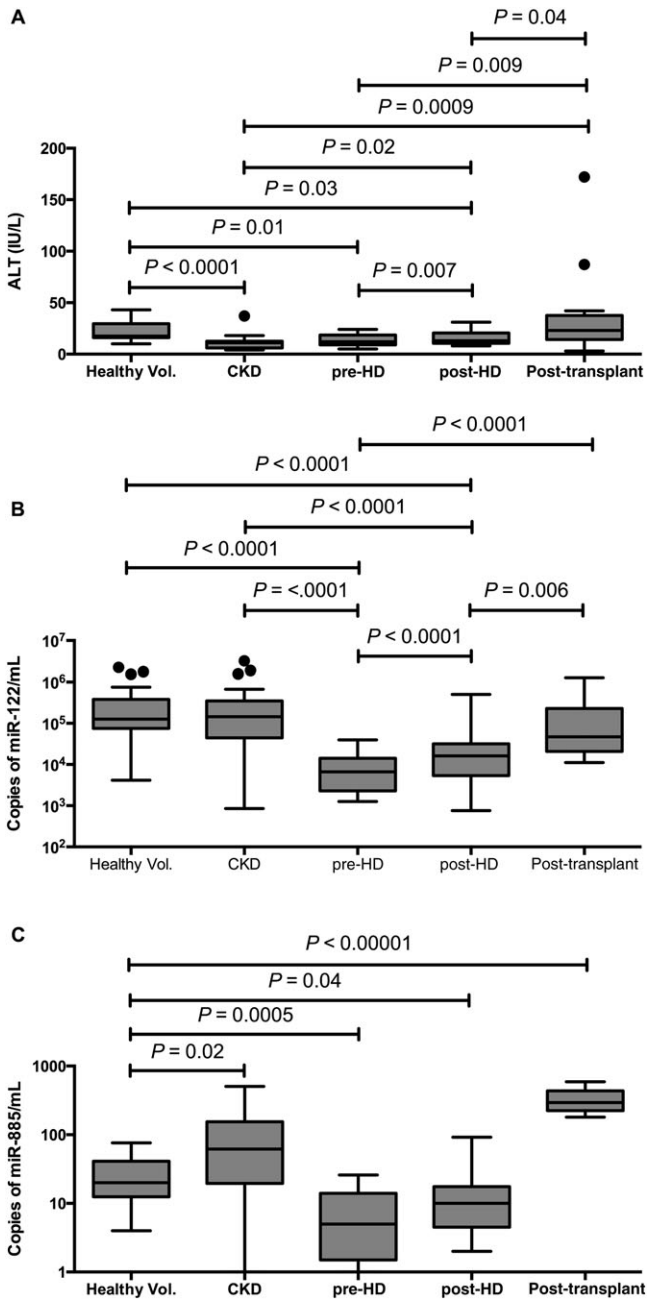

Figure 3

miR-122 is lower in end-stage renal disease and is increased by haemodialysis. Plasma was isolated from healthy controls (n = 22), chronic kidney disease (CKD) stage IV (n = 30), end-stage renal disease immediately before and after haemodialysis (pre-HD and post-HD, respectively, both n = 17) and patients with renal transplantation (n = 15). Serum ALT activity (A), miR-122 (B) and miR-885 (C) were measured. Data are presented as Tukey plots

demonstrated that systemically injected ECVs are excreted in urine. This would suggest that their clearance may be altered in ESRD. However, the miR-122 concentration in this fraction did not change. Defining the kinetics of miR-122 will be a priority if it is to be adopted into clinical practice and drug development.

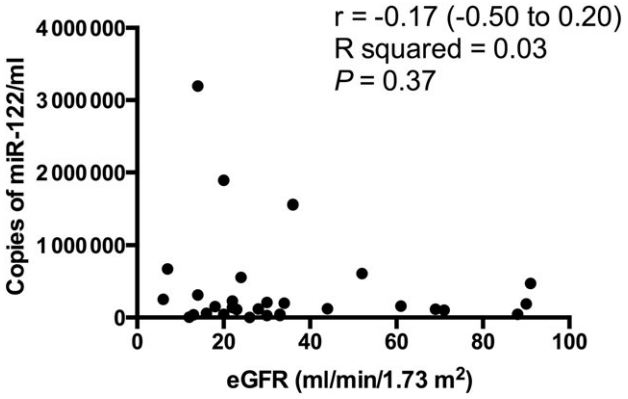

Figure 4

Circulating miR-122 is not related to the estimated glomerular filtration rate (eGFR) in patients with CKD. Each point represents a patient with CKD. eGFR calculated by the MDRD equation. Pearson correlation data are presented

miR-122 is being qualified as a biomarker for a range of diseases. In patients with established paracetamol-induced acute liver injury, miR-122 is increased on average around 100-fold [6]. In patients with ESRD, our study demonstrates a reduction in miR-122 of 19-fold compared with matched healthy controls. Therefore, the miR-122 signal associated with established ALI may still be appreciable even if the patient's baseline miR-122 concentration were lower than normal due to ESRD. An important future clinical application of miR-122 may be the stratification of patients at first presentation to hospital after paracetamol overdose with regard to their risk of subsequent ALI. In this 'pre-injury' context of use, the fold difference in miR-122 between high- and low-risk groups is smaller (around 17-fold) [7]. Therefore, pre-existing severe renal impairment may have a significant impact on the interpretation of miR-122. We propose that future qualification should include defining reference ranges in patients with reduced renal function.

There are some limitations to our study. Although recruitment was based on our pre-study power calculation, this is a discovery study that needs to be built on in larger patient cohorts. As is to be expected, patients with ESRD were prescribed multiple medications, which could potentially affect circulating microRNA concentrations. Such confounding by medication has been demonstrated for platelet-derived microRNAs and anti-platelet agents [19]. There are no data regarding the effect of anti-hypertensives on miR-122. Liver toxicity with ACE inhibitors, angiotensin receptor blockers and beta blockers is rare and all the patients in this study had normal standard liver function tests. Furthermore, our study included a CKD group who were prescribed similar

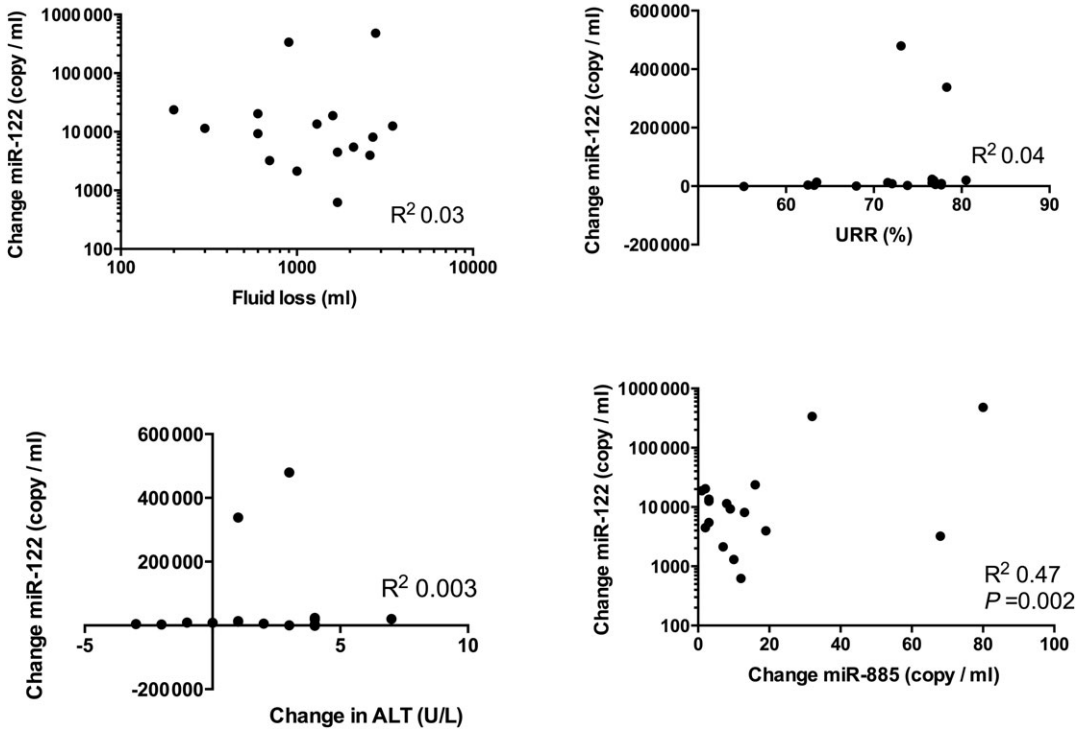

Figure 5

Haemodialysis-induced change in circulating miR-122 correlates with change in miR-885. In patients with end-stage renal disease, haemodialysis increased plasma miR-122. This increase did not have a significant relationship with volume of fluid removed during dialysis (A), urea reduction ratio (URR) (B) or change in ALT (C). There was a significant relationship with the dialysis-induced increase in plasma miR-885 (D)

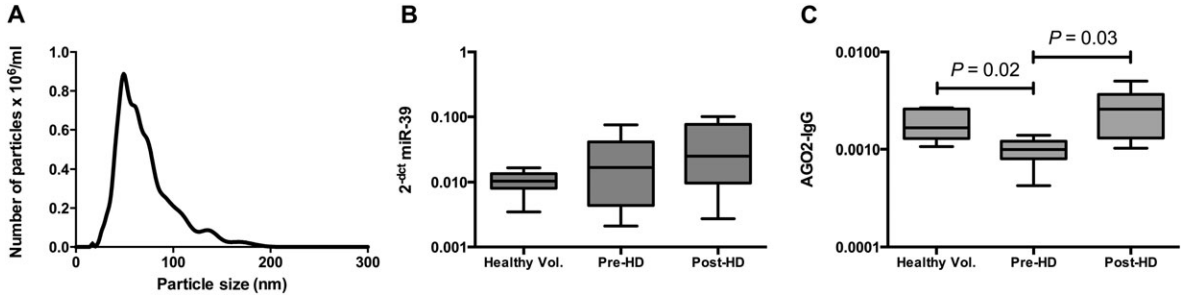

Figure 6

miR-122-5p in the extracellular vesicle fraction and Ago2 bound fraction. (A) size and number of particles measured by nanoparticle tracking analysis (NTA) following isolation of extracellular vesicles from human plasma by differential centrifugation. (B) Tukey boxplot of miR-122-5p measured in the extracellular vesicle pellet after ultracentrifugation of plasma samples from healthy volunteers and end-stage renal disease immediately before and after haemodialysis (pre-HD and post-HD). (C) Tukey boxplot of miR-122-5p measured in the antibody-isolated Ago2 fraction from healthy volunteers, pre-HD and post-HD. The *y*-axis represents the 2-Δ*Ct* value obtained from the Ago2 pull-down minus the 2-Δ*Ct* value obtained from IgG control pull-down from the same sample. All miR-122-5p measurements were normalized to spiked-in synthetic miR-39

cardiovascular medications but had substantially higher circulating miR-122 in comparison with ESRD patients. There is no evidence in the literature to suggest that the medications used more in the ESRD group (erythropoietin, iron, 1α-calcidol, phosphate binders and calcimimetics) have any effect on the liver or microRNA, at least in therapeutic doses.

In summary, miR-122 is lower in ESRD and haemodialysis restores the concentration of circulating miR-122 to healthy levels, which may reflect dialysis-induced hepatocyte injury given the concomitant increase in ALT and miR-885. These data need to be considered when interpreting liver injury using miR-122 in patients with ESRD on dialysis, and specific reference ranges that define normal in this setting may need to be developed.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

LR acknowledges the support of Società Italiana di Nefrologia (SIN). JWD acknowledges the support of NHS Research Scotland (NRS) through NHS Lothian and a BHF Centre of Research Excellence Award. ADBV was supported by an NC3Rs PhD Studentship (NC/K001485/1). ND is supported by a British Heart Foundation Intermediate Clinical Research Fellowship (FS/13/30/29994).

Contributors

L.R. and A.D.B.V. contributed equally to this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://onlinelibrary.wiley.com/doi/10.1111/bcp.13136/suppinfo.

Figure S1 Circulating miR-122 is lower in patients with end-stage renal disease pre-haemodialysis (pre-HD) compared with healthy volunteers. miR-122 was measured by TaqMan PCR. Data are presented as Tukey plots. n = 17 per group.