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Technical report (Original)

A folic acid-induced rat model of renal injury to identify biomarkers of tubulointerstitial fibrosis from urinary exosomes

Panthip Rattanasinganchan[a*], Kittipat Sopitthummakhun[b,c*], Kent Doi[d], Xuzhen Hu[d], D. Michael Payne[b], Trairak Pisitkun[b], Asada Leelahavanichkul[d,e]

[a]*Faculty of Medical Technology, Huachiew Chalermprakiet University, Samut Prakan 10540, Thailand*

[b]*Systems Biology Center, Research Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*

[c]*Faculty of Science and Technology, Huachiew Chalermprakiet University, Samut Prakan 10540, Thailand*

[d]*Kidney Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA*

[e] *Center of Excellence in Immunology and Immune-mediated Diseases, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*

Background: Chronic tubulointerstitial (TI) fibrosis is difficult to diagnose. Urinary exosomes contain many potential biomarkers, and may be useful to identify biomarkers of TI fibrosis. However, a suitable animal model to investigate urinary exosome biomarkers for TI fibrosis has not yet been specifically identified.

Objective: To test the suitability of a folic-acid induced rat model of renal injury for identifying potential biomarkers of fibrosis from urinary exosomes.

Methods: We employed a rat model of renal injury using a single intraperitoneal injection of folic acid. Urinary exosomes were isolated and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: We demonstrated severe acute kidney injury after folic acid injection by a 6-fold decrease in creatinine clearance at day 2, severe inflammatory cell infiltration in the TI area at day 7, and renal fibrosis at day 14. We collected 24 h urine samples from folic acid-injected rats and vehicle-injected control rats at days 7 and 14, then isolated urinary exosomes using a differential centrifugation method. We found that levels of exosomal marker proteins ALG-2-interacting protein 1 (ALIX; PDCD6IP) and tumor susceptibility gene 101 (TSG101) were higher in folic acid-injected rats than in control rats. LC-MS/MS analysis of urinary exosomal proteins from the folic acid-injected and control rats identified 372 proteins, including various potential TI fibrosis biomarkers.

Conclusion: We demonstrated the feasibility of using a folic acid-induced rat model of TI fibrosis for urinary proteomic analysis. These rats excreted sufficient urinary exosomes permitting the large-scale analysis and discovery of potential biomarkers of renal fibrosis.

Keywords: Biomarkers, chronic kidney disease, folic acid, interstitial fibrosis, proteomics, urine exosomes

Chronic kidney disease (CKD), a serious health problem worldwide arising from multiple causes, is a condition manifested by a progressive decline in renal function associated with irreversible renal pathologies that usually include glomerulosclerosis, vascular alterations, or tubulointerstitial (TI) fibrosis [1]. TI fibrosis, also arising from multiple causes (e.g., acute kidney injury), results from TI accumulation of excess extracellular matrix, primarily composed of collagen, leading to loss of normal renal function. TI fibrosis, also referred to as interstitial fibrosis with tubular atrophy, is an important prognostic factor in several forms of CKD, and is associated with deteriorating renal function [2]. Despite the high prevalence of TI fibrosis in CKD regardless of etiology, there are few biomarkers identified to detect TI fibrosis. Tubular proteinuria, a current method to determine TI injury, does not present at the early phase after tubular injury, and thus its use is not optimal for the early detection of and intervention in TI fibrosis [3].

Correspondence to: Asada Leelahavanichkul, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: asada.l@chula.ac.th

*These authors made equal contributions.

Identification of alternative biomarkers that would allow early detection of TI fibrosis using a noninvasive approach would offer a significant advantage in its diagnosis. One such approach involves the detection of disease biomarkers that have been released into body fluids as either intact cells or cellular components, a so-called liquid biopsy [4]. Cellular components called exosomes, 30-100 nm membrane vesicles secreted from several cell types, are a rich source of potential biomarkers in urine [5, 6]. Several types of molecules that are easily degraded in the extracellular environment, such as transcription factors and micro-RNAs [6, 7], are enriched and preserved inside exosomes. Therefore, urinary exosomes are a promising source of potential biomarkers of TI fibrosis.

Most current rodent models of CKD target the study of glomerulosclerosis [8]. However, we and others have described studies using a mouse model in which an acute kidney injury (induced by folic acid (FA) injection) results in predominantly TI fibrosis [9, 10] (and references therein). Unfortunately, only small volumes of mouse urine can be obtained, precluding a sufficient yield of urinary exosomes for biomarker discovery experiments. A FA-induced rat model of renal injury is also available and has been extensively characterized with respect to histopathology and renal function [11-15]. Here, we evaluated the FA-induced rat model of renal injury for its suitability in urinary exosome studies to identify biomarkers of TI fibrosis by proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and methods

Animals, model, and sample collection

Animal care followed the U.S. National Institutes of Health criteria for the care and use of laboratory animals in research, under protocols approved by the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee (ASP No. K087-KDB-11–14) following guidelines issued by the U.S. Department of Health and Human Services, the U.S. Department of Agriculture, the Association for the Assessment and Accreditation of Laboratory Animal Care, and Office of Laboratory of Animal Welfare (assurance No. A4149-01). Male Sprague Dawley rats (250-300 g) were purchased from Charles River Laboratories (Germantown, MD, USA) and had free access to water and standard laboratory rodent food. Rats were administered FA in 0.3 M $NaHCO_3$ (FA group, 250 mg/kg body weight) or 0.3 M $NaHCO_3$ alone (vehicle (Veh) group) using a single intraperitoneal injection (0.9 to 1.1 mL) (see Discussion for additional details). While there might be a difference in alkali administration between these groups, (i) the volume of the single injection was small, (ii) any effect on plasma pH would be quite transient, (iii) there was no obvious abnormality in mice after loading of this dose, and (iv) this is the standard protocol for many published studies using the FA-induced model of renal injury. Only FA-injected rats with blood urea nitrogen (BUN) ≥100 mg/dL at day 2 after injection were selected for further study (for animals with BUN <100 mg/dL, the development of TI fibrosis was inconsistent (data not shown).

In standard experiments using this FA-induced model of renal injury, samples of blood (drawn from the tail vein) and urine (24 h, collected using rat metabolic cages; Hatteras Instruments, Cary, NC, USA) were obtained for analysis and body weight was measured, before administration of either FA or Veh (day 0) and at days 2, 7, 14, and 28 after administration. We used 35 rats in the experiments: 20 rats were used to explore the model and 9 were used for the proteomic analysis. The remaining 6 rats failed to produce BUN >100 mg/dL and were excluded from the study.

For quantitative comparisons of putative biomarkers in urine and/or urinary exosomes, both specific protein measurements and normalization based on the total amount of protein in a single urine sample (i.e., a sample collected over a short time) are generally unsatisfactory [16]. First, normal physiological variations in water excretion can lead to relative dilution or concentration of urinary proteins in such samples, thereby affecting the total amount of protein present. Second, total protein excretion in urine can vary broadly among various pathophysiological states. Therefore, for accurate quantitative measurements from urine samples, it is necessary to measure the actual *rates of excretion* of both specific proteins (e.g., putative biomarkers) and total protein, and to ask whether the excretion rates differ between control and experimental populations. To minimize the effects of the physiological variables mentioned above, rates of excretion are generally measured over a 24 h urine collection period, and this analytical approach was termed “time-normalization”.

Evaluation of blood chemistry, proteinuria, and histology

All urine samples were collected (over 24 h) using metabolic cages, protease inhibitors were added, and samples were centrifuged at 1000 × *g* for 10 min to remove debris [5, 17, 18]. From the supernatant of each urine sample, two aliquots (100 μL) were reserved for creatinine and protein analyses, then the remainder of each urine sample was divided into two equal aliquots (for western blot and proteomic analyses) and stored at -80°C until used.

BUN, serum creatinine (SCr), and urine creatinine (UCr) were measured using colorimetric assays (QuantiChrom kits DIUR-500 for urea assay and DICT-500 for creatinine assay; BioAssay Systems, Haywood, CA, USA). Urine protein level (24 h urine albumin) was measured using enzyme-linked immunosorbent assay kits (Nephrat, Exocell, Philadelphia, PA, USA). Because SCr does not reach a steady state during acute kidney injury, we directly calculated creatinine clearance (CCr) from 24 h urine collection of UCr.

The CCr was calculated using the following equation: CCr = (UCr * 24 h urine volume)/SCr. For histology, kidneys were fixed in 10% neutral buffered formalin solution for paraffin embedding and sectioning (4 mm thickness), then stained using a Masson trichrome method. TI fibrosis was evaluated at 200 × magnification by the following semiquantitative criteria to estimate area of damage: 0, <5%; 1, 5%-10%; 2, 11%-25%; 3, 26%-50%; 4, >50%.

Isolation of urinary exosomes for western blot or proteomic analysis

For western blot analysis, exosomes from each urine sample were isolated separately; for proteomic analysis, all urine samples from each group were pooled before exosome preparation. Rat urinary exosomes were isolated by differential centrifugation (17,000 × *g* for 15 min then 200,000 × *g* for 1 h) as described previously [5, 17, 18]. Next, the isolated exosomes (i.e., the low-density membrane pellet after 200,000 × *g* centrifugation) were resuspended in an isolation solution (10 mM triethanolamine, 250 mM sucrose, pH 7.6) and depleted of Tamm–Horsfall protein by incubating with dithiothreitol (200 mg/ml) at 95°C for 2 min, followed by recentrifugation (200,000 × *g*, 1 h, 4°C). The final exosome pellets were dissolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for western blot and proteomic analysis.

Western blot analysis

After isolation of exosomes from individual rat urine samples, exosomal proteins were separated by one-dimensional SDS-PAGE (12% gel), transferred to nitrocellulose by electroblotting, and probed with the following primary antibodies: mouse monoclonal antibody to ALG-2-interacting protein 1 (ALIX; also known as programmed cell death 6-interacting protein or PDCD6IP) (BD Biosciences, Franklin Lakes, NJ, USA); rabbit polyclonal antibodies to tumor susceptibility gene 101 (TSG101) (Abcam, Cambridge, MA, USA), and clusterin (Sigma Aldrich, St. Louis, MO, USA). Immunoreactivity was visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich) and enhanced chemiluminescence substrate (Amersham Biosciences, Little Chalfont, Bucks, UK) using light-sensitive film (Kodak BioMax XAR, Rochester, NY, USA). Band intensities were determined by ImageJ version 1.36b (National Institutes of Health, Bethesda, MD, USA).

Proteomic analysis

Solubilized exosomes from pooled rat urine samples (from 3 rats in each group) were fractionated by SDS-PAGE (12%), digested with trypsin, and analyzed by nanospray LC–MS/MS as described previously [5]. Briefly, each sample lane was cut into eight pieces (from top to bottom), and each gel piece was minced and subjected to in-gel tryptic digestion as follows: washed (25 mM NH_4HCO_3 in 50% acetonitrile); rehydrated (10 mM dithiothreitol, 25 mM NH_4HCO_3) at 56°C for 1 h; alkylated (55 mM iodoacetamide in 25 mM NH_4HCO_3) at room temperature for 1 h; washed twice and rehydrated again at 4°C for 0.5 h; then finally incubated with the sequencing-grade trypsin (product No. V5111; Promega, Fitchburg, WI, USA) at 37°C overnight. Subsequently, peptides were extracted from the gel in 50% acetonitrile containing 5% formic acid, then desalted and concentrated using a ZipTip (Merck Millipore, Darmstadt, Germany), eluted with 0.1% FA and transferred to LC-MS/MS auto-sampler vials. The LC-MS/MS analysis was carried out using a ProteomeX workstation LTQ-MS (Thermo Finnigan, San Jose, CA, USA). The NCBI RefSeq protein database (*Rattus norvegicus*), appended with a list of common contaminants (pig and bovine trypsin, and serum albumin, and human isoforms of keratin), was searched using BioWorks version 3.1 (Thermo Finnigan) based on the SEQUEST algorithm. False

discovery rate (FDR) was calculated using the target-decoy search strategy described by Elias et al. [19]. Low quality matches were filtered using a FDR threshold of <1%. Protein was quantified by spectral counting of all corresponding peptides from a given protein at each experimental time point. For this study, normalized spectral counts (total spectral counts divided by the amino acid number of a given protein) were used as a rough approximation of absolute protein abundance.

Statistical analysis

All data are expressed as mean ± SEM. Differences between or among groups were analyzed for statistical significance by one-way ANOVA (SPSS for Windows, version 14.0, SPSS Inc., Chicago, IL, USA) $P < 0.05$ was accepted as significant.

Results

Characterization of renal function and histopathology in the folic acid-induced model of renal injury

An acute change in renal function, as determined by an increase in BUN concentration and a decrease in CCr, was detected at day 2 after FA injection (**Figure 1A and B**). By day 7 after injection, both BUN and CCr concentrations demonstrated spontaneous recovery of renal function to the control level, and there were no subsequent changes in either BUN or CCr observed after day 7. By contrast, urine volume was substantially elevated at day 7 (and returned to normal by days 14 and 28), indicating that at least some aspects of renal function remained (at least transiently) abnormal (**Figure 1C**). Nevertheless, there was no change in albuminuria or body weight (data not shown), suggesting a lack of glomerular involvement in this model of renal injury, which is consistent with findings of other studies [14, 15, 20]. The occurrence of acute tubular injury in the present model was demonstrated histologically, with interstitial infiltration by inflammatory cells observed at 1 week after FA injection, and significant interstitial fibrosis (as determined by Masson trichrome staining) detected at both 1 and 2 weeks after FA injection (**Figure 1D and E**); inflammatory cell infiltration appeared diminished at 2 weeks after FA injection. In addition, the histopathology at day 28 was not different from day 14 (data not shown). Despite histological differences between FA- and Veh-treated rats, proteinuria levels (as a TI injury indicator) were not different (**Figure 1F**). This supports the necessity for better biomarkers specific for TI injury and fibrosis, and a discovery of such new biomarkers by proteomic analysis could provide novel information compared to conventional proteinuria levels.

Next, we conducted a preliminary feasibility study to evaluate the use of urinary exosomes to identify novel protein biomarkers of TI fibrosis.

Urinary exosomes after folic acid-induced renal injury

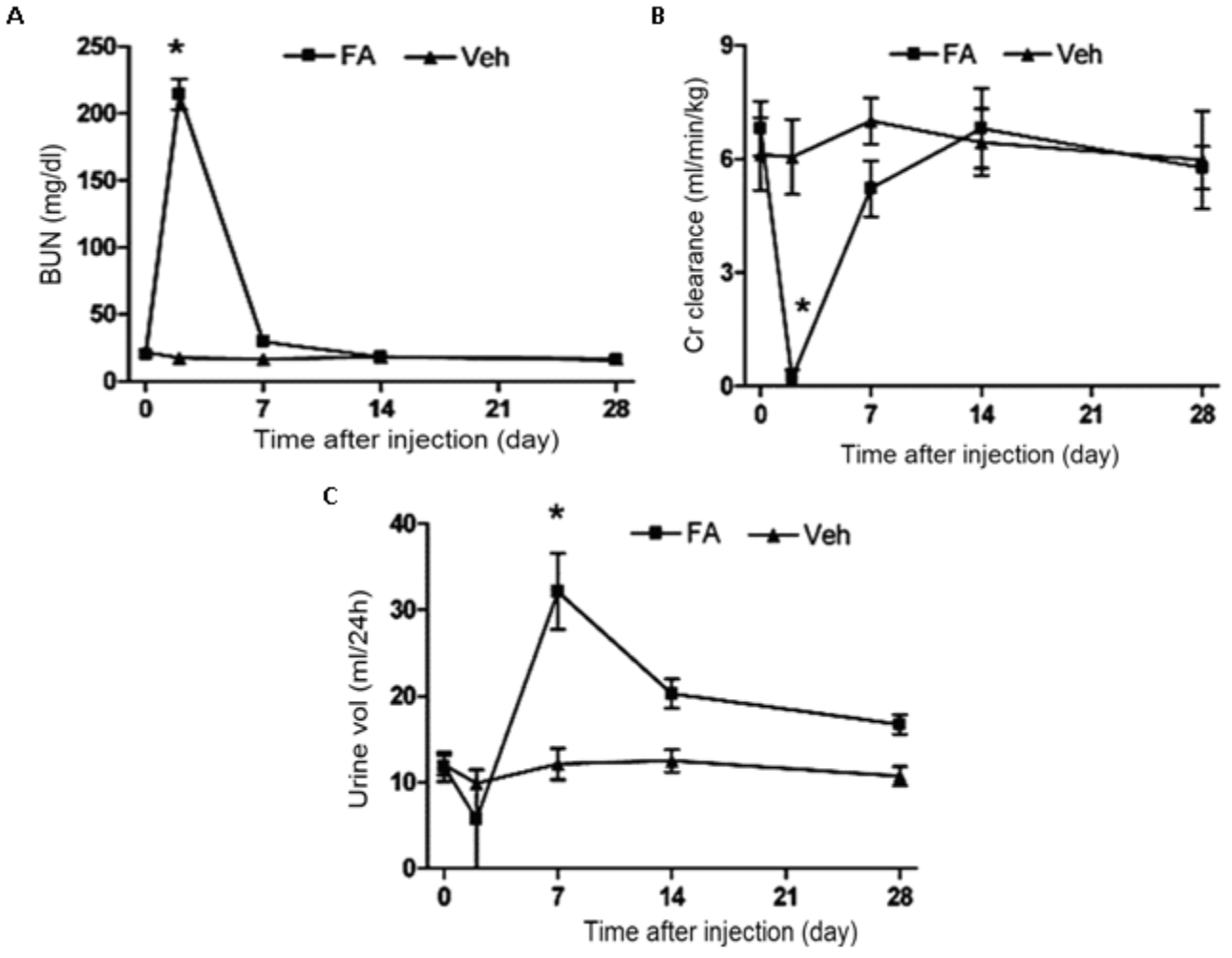
Isolation of exosomes from urine (24 h collection) was confirmed by immunoblot detection of ALIX and TSG101, marker proteins for urinary exosomes [5, 17] (**Figure 2A**). After injection of FA, the amounts of ALIX and TSG101 exosomal proteins in rat urine were increased compared with the Veh-injected controls, and were approximately 3-fold higher at week 2 after injection (**Figure 2B and C**). The samples analyzed by immunoblotting were time-normalized, representing exosomes produced by FA- and Veh-treated rats during the same 24 h (see "Materials and Methods: Animals, model, and sample collection" for a complete explanation). Because the amounts of urinary exosomal marker proteins increased 3-fold after FA-injection, then either (i) 3-fold more exosomes were produced by FA-treated rats (i.e., content of ALIX and TSG101 per exosome is constant) or (ii) the content of ALIX and TSG101 per exosome was 3-fold higher in FA-treated rats (i.e., number of exosomes produced is constant). To distinguish between these two possibilities, in future studies we would need to quantify the number of exosomes (e.g., using nanoparticle tracking analysis).

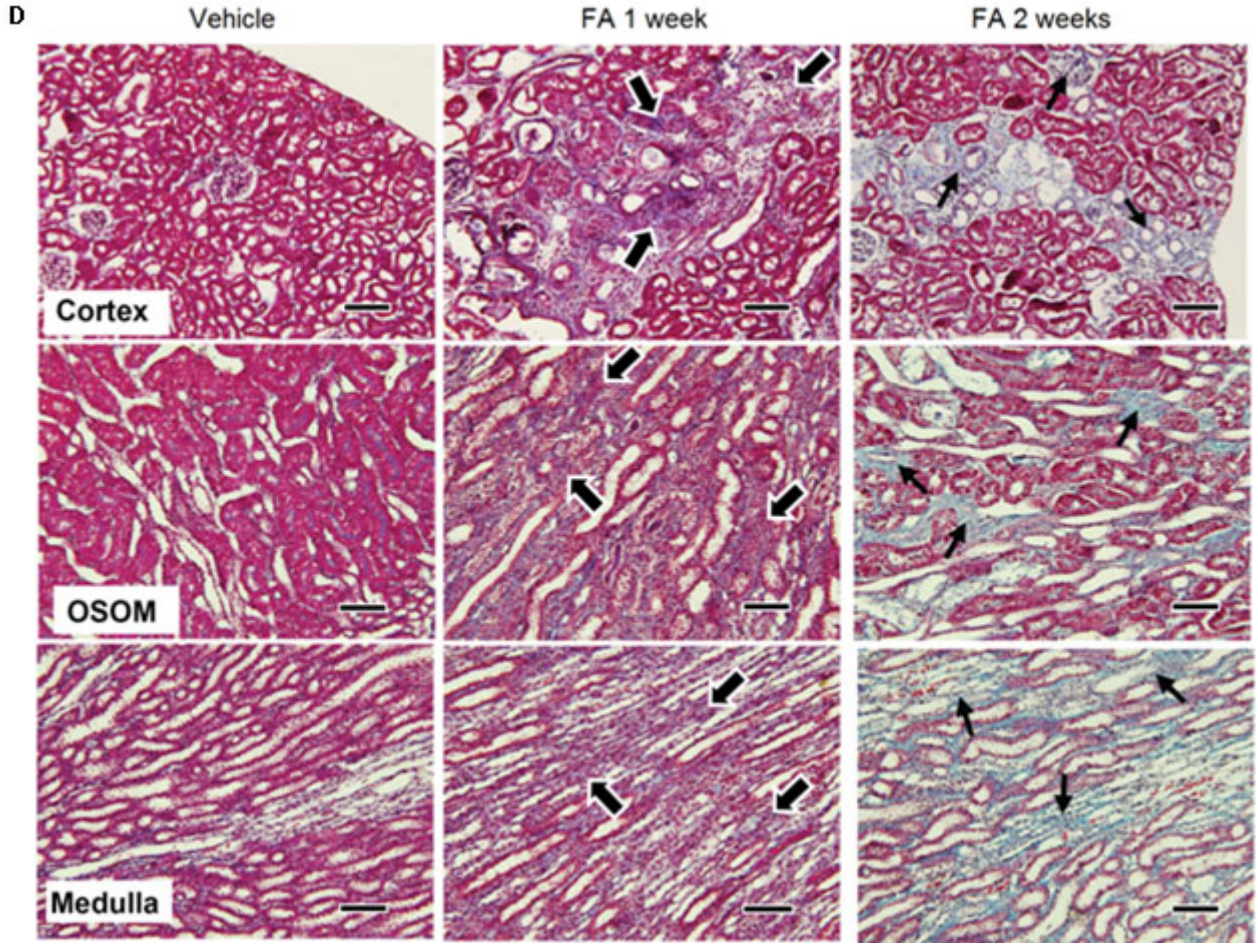
Proteomic analysis of urinary exosomes

We performed proteomic analysis of urinary exosomes from rats injected with Veh alone, and at 1 and 2 weeks after FA injection. We identified a combined total of 372 proteins from the exosomes of all three samples; approximately one-third of these proteins (134) were common to all three samples (**Figure 3**; see Supplemental Data at http://sysbio.chula.ac.th/Supplemental_Data/Folic_acid-induced_rat_kidney_fibrosis_2016/Supplemental_Data.xlsx for a complete list of the proteins identified and their associated information). In the present study, an average of approximately 250 proteins was identified in each exosome sample (range

about 200-320). The most abundant proteins identified in the exosome samples (as estimated from normalized spectral counts) are shown in **Table 1**. Among the 20 most abundant proteins identified in control exosomes, 19 were also found in both 1- and 2-week FA samples, and 5 are known to be membrane proteins predominantly or exclusively expressed in the kidney (**Table 1**). Furthermore, one of these proteins (aminopeptidase N) has been used previously as a urinary biomarker for renal damage [21]. Consistent with the immunoblotting results (**Figure 2**), the urine exosome marker protein ALIX was also detected exclusively in both exosome samples from FA-treated rats by proteomic analysis. TSG101, the other urine exosome marker protein confirmed by immunoblotting, was only detected at very low abundance in a single exosome sample (FA-treated, 1 week) by proteomic analysis (Supplemental Data). Finally, 10 of the 25 proteins most frequently identified in exosomes [22] were also detected in all three samples from this study, and 17 of 25 were found in at least two samples.

Among the most abundant proteins identified uniquely in urinary exosomes from FA-treated rats (i.e., not present in control exosomes), the top 2 most abundant proteins (-1-antiproteinase and annexin A5) were common to both 1- and 2-week FA samples (**Table 1**). In addition, increased abundance in urine following acute kidney injury has been demonstrated previously for both of these proteins [23, 24]. With respect to potential use of urinary exosomes for biomarker discovery, the results for one of these proteins, annexin A5, were particularly interesting. In all, proteomic analysis identified 8 different annexin proteins, which could be grouped into 3 categories: (i) those found in relatively moderate abundance in all 3 samples; (ii) those found in low abundance and only in some samples; and (iii) annexin A5, which was absent in control exosomes, but was found in moderate abundance in both exosome samples from FA-treated rats (**Table 2**). This result is consistent with previous identification of annexin A5 as a potential urine biomarker for diseases of the kidney [24].





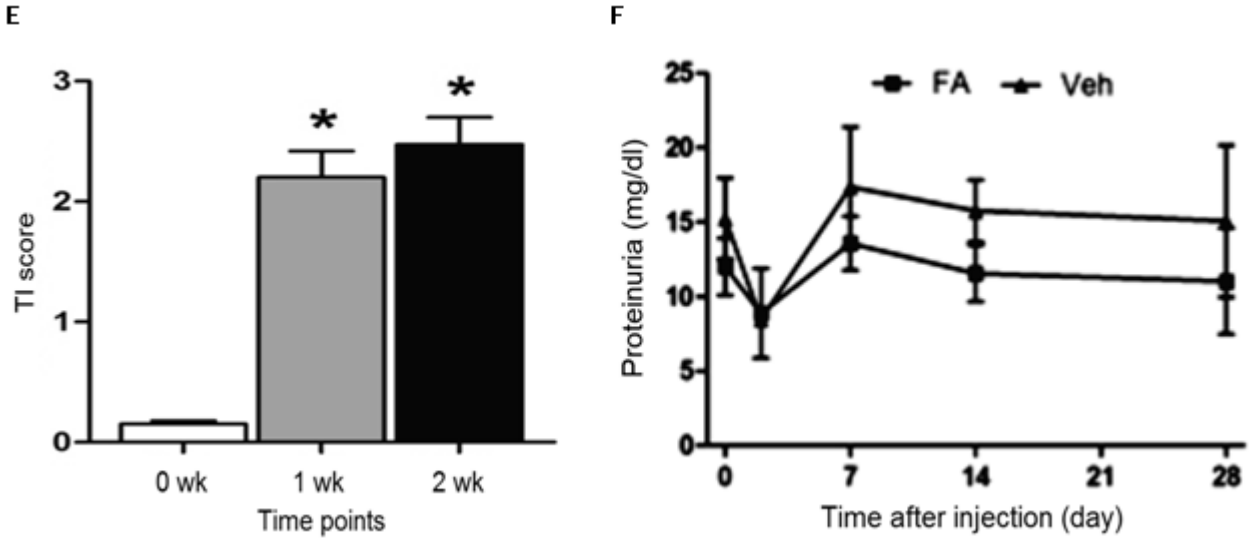


Figure 1. Characteristics of renal pathology and renal injury in rat model of tubulointerstitial (TI) fibrosis induced by a single injection of folic acid (FA) intraperitoneally. Renal injury was demonstrated by blood urea nitrogen (BUN) (**A**), creatinine clearance (Cr clearance) (**B**), and urine volume (**C**), n = 5–7/time point. *$P < 0.01$ for FA-treated vs. vehicle (Veh)-treated rats at the same time point. Representative Masson trichrome staining with the original magnification at 200 × of left kidney of a Veh-treated control (left column), 1 week after FA treatment (middle column) and 2 weeks after FA treatment (right column (**D**). In the sections from rats treated with FA after 1 week (middle panels), extensive infiltration by mononuclear cells demonstrated areas of tubular inflammation (indicated with thin arrows). The prominent blue stained areas in the sections from rats treated with FA after 2 weeks (right panels) revealed the presence of TI fibrosis (indicated with thick arrows). Scale bar 200 μm. Comparison of the semiquantitative estimation of TI injury (n = 3–5/group) (E). *$P < 0.01$ for FA-treated vs. Veh-treated rats at the same time point. Proteinuria levels between FA and Veh control rats were not different (**F**).

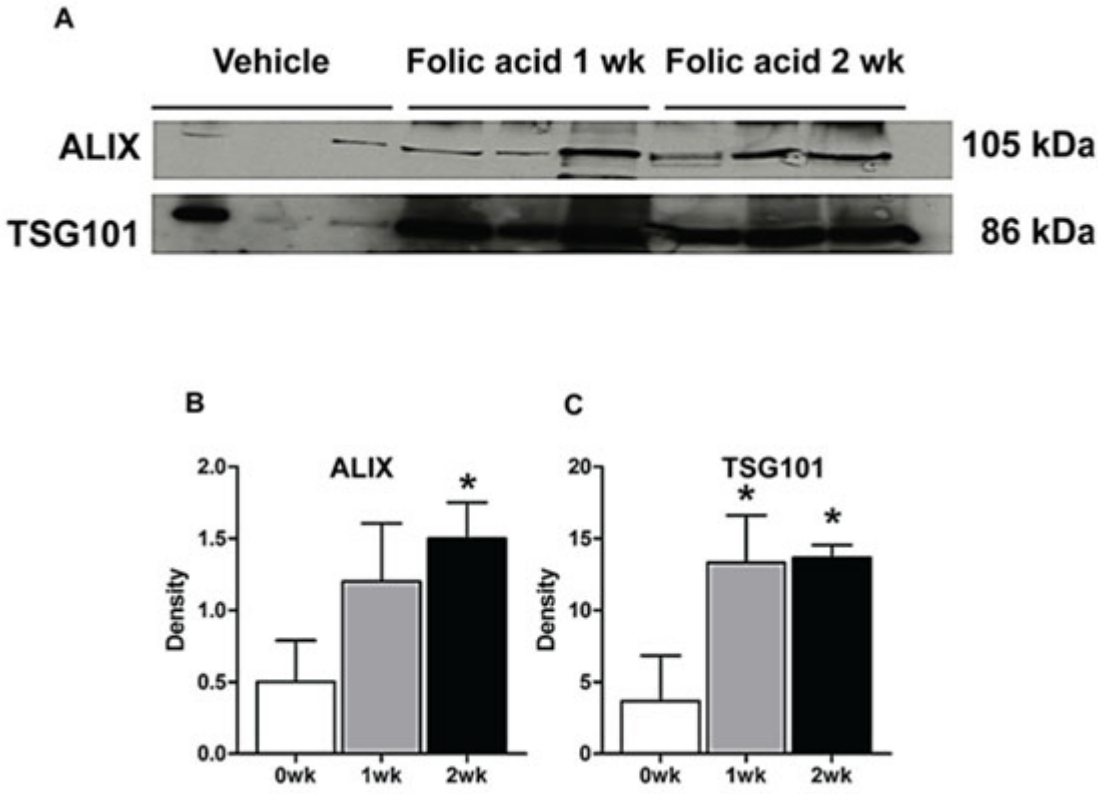


Figure 2. Western blot analysis of urinary exosomes from 24 h urine collection in a folic acid (FA) induced rat model of tubulointerstitial fibrosis. Exosomes were isolated from urine of rats at 1 and 2 weeks after treatment with either vehicle (Veh) or FA. Urinary exosome samples from individual rats were analyzed for exosome marker proteins, ALIX and TSG101, by western blotting (**A**) and subsequent densitometric analysis (**B** and **C**). Urinary exosome samples were loaded based on time-normalization (see Methods). **P* < 0.01 for FA-injected vs. Veh-iniected rats.

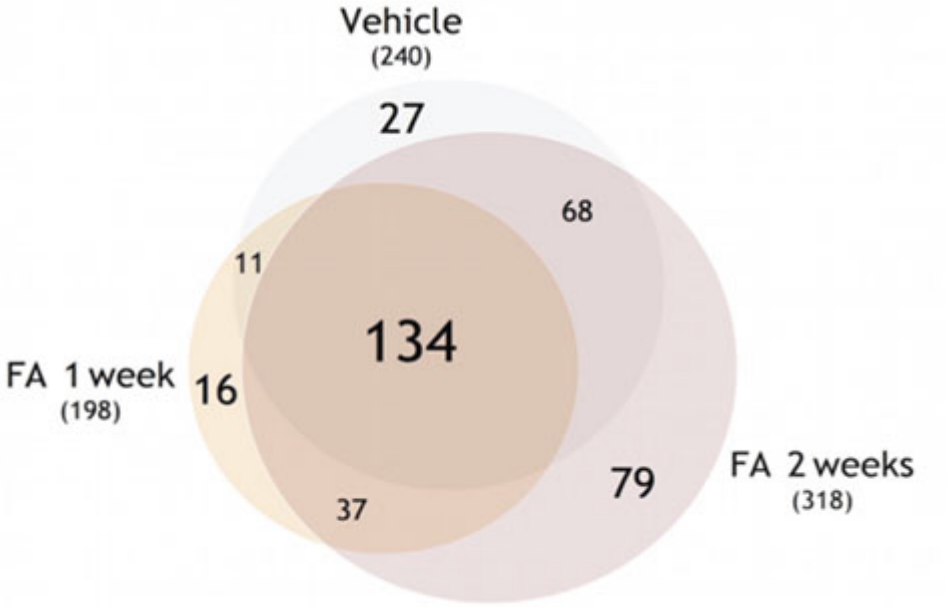


Figure 3. Venn diagram showing the proportional distribution of proteins identified among the three exosome samples. A total of 372 proteins were identified, including 134 proteins that were common to all three exosome samples, and 27, 16, and 79 proteins that were unique to exosomes from the individual samples (Veh-treated, and FA-treated at 1 and 2 weeks, respectively, and 3 rats in each group).

Another interesting protein identified uniquely in urinary exosomes from FA-treated rats was clusterin (**Table 1** and Supplemental Data, which has been previously reported as a biomarker for acute kidney injury [25] and renal fibrosis [26]. In the present study, we confirmed the proteomic result of significantly increased clusterin after FA administration by immunoblotting (**Figure 4**).

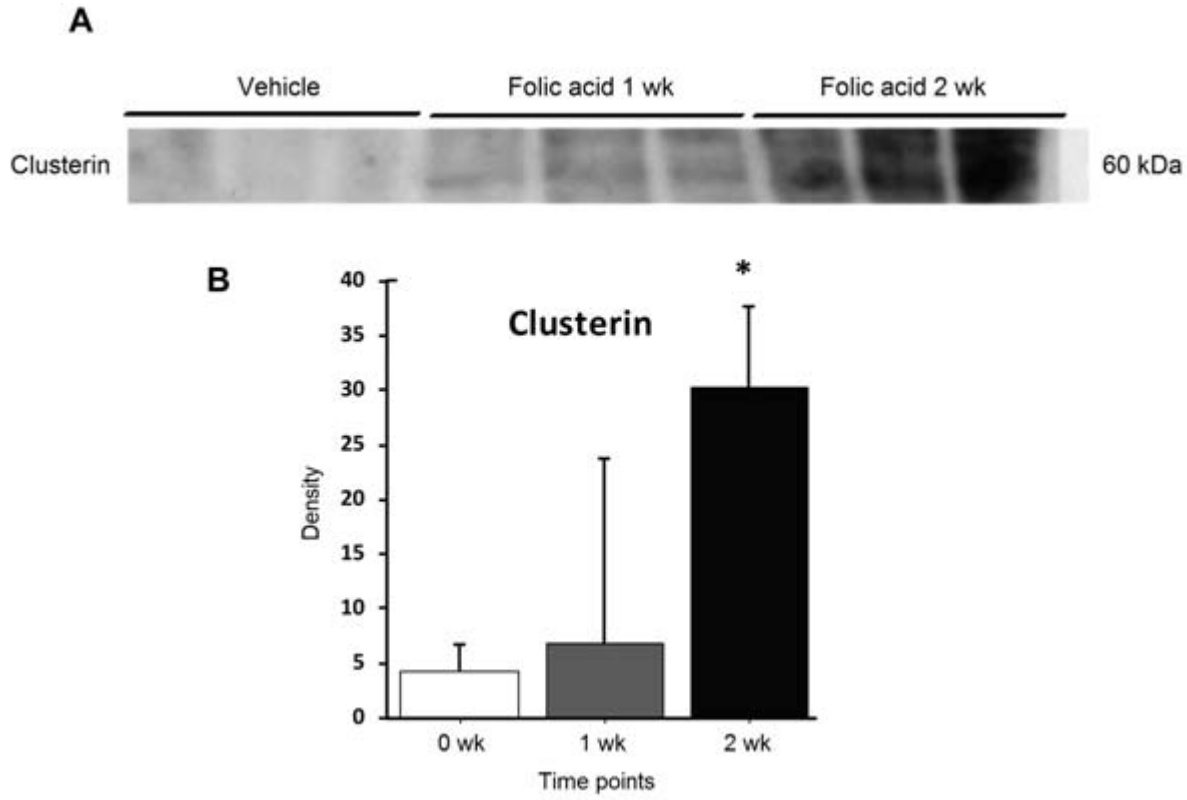


Figure 4. Western blot analysis confirming liquid chromatography-tandem mass spectrometry results for clusterin in urinary exosomes from a folic acid (FA) induced rat model of tubulointerstitial fibrosis. Exosomes were isolated from the urine of rats at 1 and 2 weeks after treatment with either vehicle or FA. Urinary exosome samples from individual rats were analyzed for clusterin by western blotting (**A**) and subsequent densitometric analysis (**B**). Urinary exosome samples were loaded based on time-normalization (see Methods). *$P < 0.01$ for FA-injected vs. vehicle-injected rats.

Table 1. Comparison of the most abundant proteins (estimated from normalized spectral counts) identified in urine exosomal samples from vehicle-treated rats and rats 1 and 2 weeks after folic acid (FA) treatment.

Ref. Sequence number	Protein name	Normalized spectral counts		
		Vehicle	1 week post-FA	2 weeks post-FA
Top 20 most abundant proteins in vehicle sample				
NP_954887.1	Cystatin-related protein 2 precursor	602	216	403
NP_061998.1	Probasin precursor	373	243	446
NP_446292.1	Gamma-glutamyltransferase 1**	282	308	192
NP_598306.1	Common salivary protein 1	195	132	333
NP_037025.1	Actin, gamma-enteric smooth muscle	191	226	314
NP_037347.1	Aflatoxin B1 aldehyde reductase member 3	187	3	24
NP_036740.1	Neprilysin**	181	101	269
NP_001002807.1	Chloride intracellular channel protein 1	178	62	75
NP_113902.1	Ribonuclease UK114	175	51	66
NP_036767.1	Anionic trypsin-1 precursor	175	362	443
NP_058778.1	Uromodulin precursor**	160	70	318

Table 1. (Con) Comparison of the most abundant proteins (estimated from normalized spectral counts) identified in urine exosomal samples from vehicle-treated rats and rats 1 and 2 weeks after folic acid (FA) treatment.

Ref. Sequence number	Protein name	Normalized spectral counts		
		Vehicle	1 week post-FA	2 weeks post-FA
NP_112274.1	Aminopeptidase N precursor**	153	109	218
NP_058912.1	Neutral and basic amino acid transport protein rBAT**	152	47	132
NP_037315.1	Meprin A subunit beta precursor	115	17	80
NP_997476.1	Prostatic steroid-binding protein C2	112	20	531
NP_036947.1	Glutamate–cysteine ligase catalytic subunit	105	0	17
NP_001005889.2	Radixin	101	39	84
NP_543180.1	ADP-ribosylation factor 3	99	50	39
NP_036850.1	Cystatin-related protein 1 precursor	97	97	608
NP_059001.1	Glutamate–cysteine ligase regulatory subunit	95	7	18
Top 20 most abundant proteins in sample 1 w post-FA treatment (unique to FA samples, absent from vehicle sample)				
NP_071964.2	Alpha-1-antiproteinase precursor*	0	75	178
NP_037264.1	Annexin A5*	0	69	47
XP_001055138.1	PREDICTED: similar to putative breast adenocarcinoma marker	0	32	0
NP_444180.2	Clusterin precursor*	0	31	22
XP_001055585.1	PREDICTED: similar to grancalcin	0	23	0
NP_001025081.1	Programmed cell death 6 interacting protein (ALIX)	0	21	10
NP_476487.1	Complement component C9 precursor	0	16	0
NP_068611.1	Histone H2A type 4	0	15	8
NP_599153.1	Albumin	0	15	13
NP_620796.1	Galectin-3-binding protein precursor	0	14	7
NP_058821.1	CD63 antigen	0	13	8
NP_059055.2	Ras-related protein Rab-10*	0	10	25
NP_001019475.1	Glutathione S-transferase Mu 4	0	9	9
NP_803435.1	Prostatic steroid-binding protein C1 precursor*	0	9	18
NP_001004236.1	Tetraspanin-1	0	8	4
NP_071605.2	Complement decay-accelerating factor precursor	0	8	5
NP_073204.1	Transglutaminase 4 (prostate)	0	7	6
NP_446145.1	Cyclin-D-binding Myb-like transcription factor 1	0	7	0
NP_036638.1	Sodium/potassium-transporting ATPase subunit alpha-3	0	7	3
NP_001013087.1	PCTP-like protein	0	7	0
Top 20 most abundant proteins in sample 2 w post-FA treatment (unique to FA samples, absent from vehicle sample)				
NP_071964.2	Alpha-1-antiproteinase precursor*	0	75	178
NP_037264.1	Annexin A5*	0	69	47
XP_001070423.1	PREDICTED: hypothetical protein	0	0	45
NP_788265.1	S100 calcium-binding protein, ventral prostate	0	0	33
XP_001055098.1	PREDICTED: similar to carbonyl reductase 3	0	4	29
XP_001068139.1	PREDICTED: hypothetical protein	0	0	27
NP_067599.1	Myoglobin	0	0	26
NP_059055.2	Ras-related protein Rab-10*	0	10	25
NP_445937.1	Protein S100-A6	0	0	22
NP_444180.2	Clusterin precursor*	0	31	22
NP_037245.1	ATPase, Na+/K+ transporting, beta 1 polypeptide	0	7	20
NP_803435.1	Prostatic steroid-binding protein C1 precursor*	0	9	18
NP_001020832.1	Protein crumbs homolog 3 precursor	0	0	18
NP_112313.1	Macrophage migration inhibitory factor	0	0	17
XP_001060853.1	PREDICTED: similar to ATPase, H+ transporting, lysosomal Accessory protein 2	0	0	16
NP_036792.2	Elongation factor 1-alpha 2	0	2	15
NP_001013169.1	Guanine nucleotide-binding protein subunit alpha-14	0	0	14
NP_446316.1	Transitional endoplasmic reticulum ATPase	0	2	14
NP_071636.1	Brain acid soluble protein 1	0	0	14
NP_068512.1	Monocyte differentiation antigen CD14 precursor	0	0	13

**Membrane protein abundantly/predominantly expressed in the kidney

*Absent in control exosome sample, and present in both 1-week (inflammation) and 2-week (fibrosis) exosome samples from FA-treated rats

Table 2. Annexin family proteins identified in rat urine exosomes

RefSeq number	Protein name	Normalized spectral counts		
		Vehicle	1 week post-FA	2 weeks post-FA
Group 1: found in relatively moderate abundance in all 3 samples				
NP_001011918.1	Annexin A11	56	91	111
NP_077069.3	Annexin A4	50	41	9
NP_063970.1	Annexin A2	18	74	29
Group 2: found in low abundance and only in some samples				
XP_001071546.1	PREDICTED: similar to Annexin A10	9	6	0
NP_037036.1	Annexin A1	6	6	0
XP_001068572.1	PREDICTED: similar to annexin A13	6	0	3
NP_569100.1	Annexin A7	2	13	2
Group 3: found in moderate abundance only in exosome samples from FA-treated rats				
NP_037264.1	Annexin A5	0	69	47

Discussion

Acute tubulointerstitial (TI) injury, leading to subsequent TI fibrosis, is responsible for a significant number of cases of acute and chronic kidney diseases encountered in clinical practice [27, 28]. At present, reliable early diagnosis of TI fibrosis by current clinical and laboratory techniques is not possible [29]. Urinary biomarkers that would allow early detection of TI fibrosis using a noninvasive approach would be a significant advance in clinical diagnosis and monitoring of CKD. While analysis of urinary exosomes using large-scale proteomics seemed to be an ideal strategy for discovery of the requisite biomarkers, this approach requires identifying a suitable animal model of TI fibrosis.

Rodent models of acute kidney injury and TI fibrosis, involving a single injection of a large dose of FA, were first described almost 50 years ago [11, 30] and then extensively characterized over the following 2 decades [12-15]. Major findings that have been reported after numerous studies are that the acute kidney injury after FA injection is (i) dose dependent, (ii) almost exclusively limited to renal collecting ducts, and (iii) no other tissues appear to be affected [9, 11, 14, 30].

Following the protocol described in the original report [11], virtually all of the subsequent studies employing the rat model have used administration of FA by intravenous injection. This often leads to accumulation of sharp, needle-like FA crystals in renal tubules, resulting in physical/obstructive damage to the tissue, which was originally thought to be the mechanism responsible for renal toxicity. However, Fink et al. [15] showed that the crystal formation could be markedly reduced/eliminated by intravenous injection of FA in rats pretreated with $NaHCO_3$ to induce alkalosis (increasing the solubility of FA), but the injury to the renal collecting ducts persisted, thereby demonstrating a direct nephrotoxic effect of FA on these cells. Furthermore, by contrast with studies using the rat model, intraperitoneal injection of FA is employed in studies using mice [9, 30] and FA crystal formation in renal tubules has never been reported, yet acute kidney injury and fibrosis still occurred exclusively in the collecting ducts. After nearly 30 years, the mechanism of this toxicity remains unknown, but is still under active investigation.

The majority of investigations using this rat model of renal injury have been short-term (2-4 days) focused on mechanisms of cell proliferation, cell death, tissue regeneration, or hypertrophy. In contrast, for the present study we used this model to focus specifically on TI injury and fibrosis over a much longer time period (up to 4 weeks). Another novel aspect of the present study using the FA-injected rat model is our adoption of the intraperitoneal route for injection of FA, to avoid crystal formation in the renal tubules.

We injected FA intraperitoneally in rats and characterized the model, identifying an early TI inflammatory phase (apparently maximal after 1 week) accompanied by the slightly delayed development of a fibrotic phase (reaching a maximum between 1-2 weeks). Most indicators of renal function returned to normal within 1-2 weeks. Next, as a proof of concept for biomarker discovery, we determined that a 24 h urine collection using this FA-induced rat

model of TI fibrosis was adequate for the urine exosome proteomic analysis. Using proteomic LC-MS/MS analysis in this preliminary evaluation study, we identified numerous exosome-associated and kidney-expressed proteins, confirming both the nature and origin of the proteins we had isolated from urine. As further confirmation, proteomic analysis of samples isolated from rats after renal injury and development of TI fibrosis revealed the presence of elevated levels of known exosome-associated proteins, and 132 other proteins that were not detected in the Veh-treated control rats (from a combined total of 372 proteins identified in all samples). Among this latter group of 132 proteins were at least two proteins that have been suggested previously as biomarkers of renal injury, including clusterin [25] and annexin A5 [24, 31]. Urinary exosomes could be secreted from all cell types lining the urinary tract system (e.g., podocytes, tubular cells, and ureter, and bladder epithelial cells). Based on cross-referencing with the rat transcriptomic databases from microdissected rat glomeruli and tubule segments [32], we found that most of the exosomal proteins found from urine of FA-treated rats at 1 week or 2 weeks cannot be confidently assigned to a particular cellular origin; however, Alas2, Kcnmb2, and Lrg1 were uniquely mapped to glomerular origin, and Serpina3n and Cubn were uniquely mapped to tubular origin.

Further studies are needed to confirm and explore these initial findings, and to identify a collection of biomarkers that exhibit variation in presence or abundance in urine exosomes at various times after renal injury and development of TI fibrosis. Dynamic profiling of such biomarkers may provide clinicians with useful tools with which to diagnose, monitor, and predict prognosis of TI fibrosis at different stages of kidney diseases. This rat model that produces such rapid TI fibrosis, occurring within 2 weeks after FA injection, should also be quite useful for future research in the field of renal injury and CKD.

Conflict of interest statement

The authors declare that there is no conflict of interest in this research.

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