# Urinary Tubular Protein-Based Biomarkers in the Rodent Model of Cisplatin Nephrotoxicity: A Comparative Analysis of Serum Creatinine, Renal Histology, and Urinary KIM-1, NGAL, and NAG in the Initiation, Maintenance, and Recovery Phases of Acute Kidney Injury

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**Background:** Several biomarkers are becoming available for the early detection of acute kidney injury (AKI), but few have been directly compared.

**Objective:** To compare urinary kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and N-acetyl glucosaminidase (NAG) against serum creatinine and renal histological score in the initiation, maintenance, and recovery phases of cisplatin (CP)-induced AKI.

**Methods:** Sprague-Dawley rats (300–350 g) were injected once through their tail veins with CP (CP group) at 5.5 mg/kg or with same volume of normal saline vehicle (Control group). Rats were euthanized at 2, 4, 6, 12, and 24 hours, and on days 2, 3, 6, and 10 (n = 12 in the CP group and n = 6 in the Control group at each time point), and urine, blood, and kidney samples were analyzed.

**Results:** A significant increase in serum creatinine was noted by day 3 in the CP group versus Control group [1.46 (0.12) vs 0.28 (0.03) mg/dL; mean (SE); $P < 0.05$]. The renal histology scores for brush border loss and tubular necrosis were significantly higher at 12 and 24 hours, respectively, in the CP group. Urinary kidney injury molecule-1 levels were significantly higher at 24 hours in the CP group than in the Control group [48.26 (13.13) vs 8.21 (3.31) pg/mg creatinine; $P < 0.05$] and remained elevated through day 10. Both urine NAG and NGAL levels were significantly higher by day 2 in the CP than in the Control group [NAG, 8.19 (0.82) vs 3.48 (0.40) pg/mg creatinine, P G 0.05; NGAL, 2911.80 (368.10) vs 1412.60 (250.20) pg/mg creatinine, $P < 0.05$]. Urinary NAG remained elevated for 6 days and NGAL for 3 days.

**Conclusions:** Our study suggests a temporal hierarchy in the ability of certain urinary protein-based biomarkers to detect AKI after a well-defined tubular injury. Comparative analyses of urinary biomarkers are warranted in clinical settings such as patients receiving CP to discern the time course and pattern of expression.

**Key Words:** acute kidney injury, acute renal failure, early AKI biomarkers, NGAL, KIM-1, NAG, acute tubular necrosis

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Acute kidney injury (AKI) is often characterized by an abrupt loss of renal function, detected primarily by measuring increase in traditional biomarkers such as serum creatinine and blood urea nitrogen (BUN).[1] Although these are simple to measure, using these markers to detect AKI poses many challenges.[2,3] First and foremost is the delay in diagnosing AKI. Serum creatinine and BUN have to accumulate due to reduced renal clearance during a period to become abnormal. Serum creatinine and BUN are not directly related to AKI but are surrogate markers of glomerular filtration rate (GFR). This means that a diagnosis of AKI can be missed if AKI is not associated with significant reduction in GFR.[4] Such misdiagnosis often occurs in the early stages of nephrotoxin-induced AKI, when GFR may not be depressed.[5] Moreover, renal recovery is difficult to discern using the traditional markers in patients on dialysis as these markers are removed by dialysis. AKI remains a significant clinical challenge as there are no effective treatments for it, and it is associated with a higher mortality and health care cost.[6] There is an urgent need for AKI biomarkers that are expressed earlier than the traditional ones. Early diagnosis of AKI in the clinical practice can enable early intervention with the potential for better outcomes, and in the development of new drug, to decide whether to proceed with the process. Several studies have suggested that tubular injury-based urinary biomarkers could be more useful than the traditional markers to predict and diagnose AKI.[7] However, there have been few studies to our knowledge that have directly compared the prominent urinary markers in animal or clinical studies. Here, we aimed at analyzing serially measured urinary biomarkers, such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and *N*-acetyl glucosaminidase (NAG) along with renal histology and serum creatinine. We used a bona-fide model of AKI based on cisplatin (CP) injection in the rats that in many ways mimic the clinical setting. We carried out measurements during the initiation, maintenance, and early recovery phases of AKI.

## MATERIALS AND METHODS

All animal studies conducted in this work had received full and prior approval from the Institutional Animal Care and Use Committee of University of Texas MD Anderson Cancer Center. From a preliminary dose-response study, CP at a single 5.5 mg/kg dose was chosen, as it produced a reproducible AKI with minimal systemic toxicity. Groups of male Sprague-Dawley rats (Harlan, IN) weighing 300 to 350 g were kept in standard plastic cages at room temperature (22°C−24°C) on a 12-hour light/dark cycle with access to food and water at the MD Anderson Cancer Center Animal Facility. Rats were injected through their tail vein with CP (1 mg/mL) at a dose of 5.5 mg/kg of body weight (CP group) and the control rats received the vehicle, 0.9% saline, IV through their tail vein at 5.5 mL/kg of body weight (Control group). To represent the initiation, maintenance, and recovery phases of AKI, the study consisted of 9 time points after injection (2, 4, 6, 12, and 24 hours as

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initiation phase, days 2 and 3 as maintenance phase, and days 6 and 10 as part of recovery phase). A total of 162 rats were injected, and, to make it manageable, they were injected in 2 batches at 4 weeks apart. In each batch, 6 rats were injected with CP and 3 rats with saline in the same sitting for each time point. Immediately after injection, the rats were housed back into their cages with access to food and water. Thus, each time point had 12 rats in the CP group and 6 rats in the Control group. The rats were anesthetized with intraperitoneal pentobarbital at the end of each time point for terminal experiment. Urine sample was collected using 24-gauge needle directly from the bladder exposed through an abdominal incision. The samples were centrifuged (3000 rpm) for 5 minutes at 24°C and the supernatants were stored immediately in labeled aliquots at −80°C. Blood samples collected from the inferior vena cava in heparinized syringes were centrifuged as previously mentioned to store plasma at −80°C for further analysis. Before euthanizing animals, kidneys were removed and stored in 10% neutral buffered formalin solution for histopathological examination.

In preparation for biochemical analyses, samples were thawed at 37°C in a water bath. Plasma and urine samples were analyzed for creatinine using the Beckman autoanalyzer based on Jeffe reaction. Urinary NAG was measured by an ELISA assay using a kit bought from Sigma-Aldrich, St Louis, MO, and the instructions contained in the brochure were strictly followed. All assays for urinary biomarkers were run in a single batch in duplicate or triplicate. For urinary NGAL and KIM-1, the multiplex assay kit called Kidney Injury Panel1 from Meso Scale Discovery (MSD), LLC, Gaithersburg, MD (http://www.mesoscale.com/CatalogSystemWeb/Documents/Kidney_Injury_Panel_1_rat.pdf) was purchased, and was used in our dedicated Clinical Research Immunology Laboratory under the technical supervision of a dedicated staff from MSD. Briefly, the multiplex assay provides a method for measuring the levels of multiple protein targets in a single sample. In this kit, lipocalin-2 or NGAL, TIM-1, and KIM-1 are measured simultaneously based on sandwich immunoassay. The assay plate is precoated with capture antibodies. The urine sample premixed with a solution containing albumin tracer and a solution containing NGAL and KIM-1 detection antibodies conjugated with SULFO-TAG labels were incubated. The analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. A buffer that provides the appropriate chemical environment for electrochemiluminescence is added in the final step. The plate is loaded into a SECTOR Imager 2400 where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample. This panel has been validated and has been used in other studies.[8–10] In our study, a 50-fold dilution of urine samples provided the best results. The urinary values of KIM-1, NAG, and NGAL were corrected for urine creatinine to adjust for variation in urine concentration.

To assess AKI at the histological level, digital images were obtained under light microscopy at 200× magnification of hematoxylin and eosin–stained coronal sections (5 sections, 2 from the middle and 1 each from upper and lower ends) of the left kidney. To assess injury, the tubular sections with brush border loss and necrosis (Fig. 1) were separately identified and scored as percentages of tubular sections with brush border loss or necrosis over the total tubular sections in the field (ranged from 30 to 40 tubular sections). To maintain consistency, fields were chosen for digital images by scanning around the cortical margin at 2.5 mm apart.

### Statistical Analysis

Statistical differences between the CP and saline-treated rats for serum creatinine values, renal histological scores, and urinary biomarker values were tested at each time point using the nonparametric Wilcoxon-Man-Whitney rank sum test. A $P$ value of <0.05 was considered statistically significant.

## RESULTS

Rats tolerated the CP and saline injections, and no death was noted during the 10 days of postinjection period. The serum creatinine level became significantly higher in the CP group 3 days after CP injection compared to saline-injected Control group [1.46 (0.12) mg/dL vs 0.28 (0.03); mean (SE), $P < 0.05$]. Although the serum creatinine level fell on days 6 and 10, it remained significantly higher on those days in the CP group than in the Control group (Fig. 2A).

A significantly higher histological score for the brush border loss (as the percentage of tubules with brush border loss over total tubules in the field) was seen in the CP group as early as 12 hours after CP injection compared to the Control group [12.50% (3.22%) vs 1.30% (0.40%), $P < 0.05$], and remained consistently higher through the rest of the time points including days 6 and 10 of the recovery phase (Fig. 1 and 2B). Increase in tubular necrosis (as the percentage of tubules with necrosis over total tubules in the field) after CP injection was discernible at 24 hours [4.50% (1.04%) vs 0.29% (0.18%), $P < 0.05$] with full expression evident on day 3 and remained persistent on days 6 and 10 (Fig. 2C).

Among the urinary protein-based biomarkers, urinary KIM-1 levels were significantly higher in the CP group compared to Control group as early as 24 hours time point [48.26 (13.13) vs 8.21 (3.31) pg/mg creatinine; $P < 0.05$] (Fig. 3A). The urinary level for KIM-1 peaked on day 3, but dropped off on days 6 and 10 but still remained significantly elevated than the Control group. Urinary NAG (Fig. 3B) and NGAL (Fig. 3C) levels were significantly higher in the CP group on day 2 compared to saline-injected Control group [NAG, 8.19 (0.82) vs 3.48 (0.4) pg/mg creatinine; $P < 0.05$ and NGAL, 2911.80 (368.10) vs 1412.6 (250.12) pg/mg creatinine]. The levels for urinary NAG remained significantly high through day 6. Peak urinary levels for NGAL and NAG were seen on day 3, similar to urinary KIM-1. Compared to saline control, urinary KIM-1 levels were significantly elevated from days 1 through 10, NAG levels from days 2 through 6 and NGAL from days 2 through 3.

## DISCUSSION

More than 20 different AKI biomarkers have been identified as new and possibly improved versions, but few have been directly compared either in the clinical or in the experimental settings.[7] Here, we used a well-established model CP-induced AKI in the rats to concomitantly analyze 3 prominent urinary protein-based biomarkers along with renal histology and serum creatinine. After CP injection, urinary KIM-1 was elevated as early as 24 hours, whereas the level of serum creatinine-the traditional marker-was noted to be elevated only on day 3. Urinary NAG and NGAL levels were elevated on day 2. Out of the 10 days of the study, urinary KIM-1 was significantly elevated for 9 days, serum creatinine for 7 days, NAG for 4 days, and urinary NGAL for 2 days. A discernible difference in histological score for brush border membrane loss was seen as

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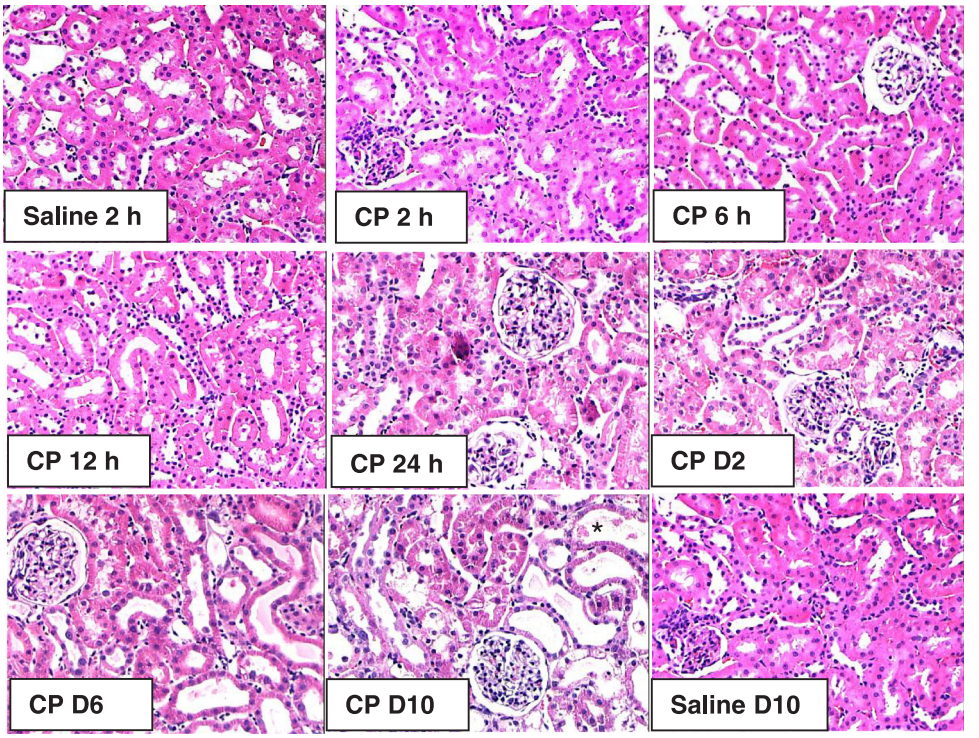


**FIGURE 1.** Composite of representative digital images of hematoxylin and eosin–stained renal histology obtained under light microscopy. All time points for CP injection and 2 time points for saline injection (saline 2 hours and saline day 10) are presented. To score injury, the tubular sections with brush border loss and necrosis were scored and the mean score is presented as graphs in Figure. 2. Stark contrast in histological injury (brush border loss and necrosis) is seen between kidneys of CP D10 and Saline D10: several tubules with brush border loss and necrosis are seen in CP D10 compared to Saline D10. The asterisk (*) shown in CP D10 shows one tubular section of several, with necrosis and brush border losses.

early as 12 hours of CP injection and persisted through day 10. Tubular necrosis was significantly higher in the CP-group starting from day 1 and persisting through day 10.

The need for a biomarker or a set of biomarkers for the early detection of AKI cannot be overemphasized. The tubular proteins released during tubular injury have garnered much attention as better biomarkers as they can potentially be true, early, real time, and proportionate to the injury. Among the 3 tubular protein-based urinary biomarkers we tested, KIM-1 (TIM-1/HAVCR-1) is a type I cell membrane glycoprotein, and its mRNA levels were noted to be markedly elevated after initiation of kidney injury.[11] The ectodomain of KIM-1 is shed from proximal tubular epithelial cells into urine in rodents and humans after injury.[11,12] Studies using well-established nephrotoxicant models showed that urinary KIM-1 significantly outperformed traditional markers of serum creatinine and BUN.[13,14] In a clinical study, urinary KIM-1 levels diagnosed AKI 12 hours after lengthy cardiac surgeries in children with an AUC value of 0.81 from ROC analyses, whereas rises of serum creatinine were observed only after 24 to 72 hours.[15] In our study, urinary KIM-1 elevation at 24 hours after CP injection in rats compared to serum creatinine elevation on day 3 is consistent with this clinical observation. That we found elevation in urinary KIM-1 24 hours after injury vs. 12 hours in the children undergoing surgery may be explained on the basis that renal injury in children undergoing cardiac surgery could have begun several hours before the end of the lengthy cardiac surgery.

NGAL, another well-studied and prominent protein-based urinary biomarker that we tested in our study, is a 25- kd protein initially identified bound to gelatinase in specific granules of the neutrophil. Although it is expressed in various tissues at low levels, it is induced in epithelial cells upon inflammation or other types of injury.[16] In mouse models, shortly after CP administration and renal ischemia, increased levels of NGAL mRNA and protein were seen in kidneys.[9,17] In one of the widely cited clinical studies in children undergoing cardiopulmonary bypass surgery, the development of AKI was identified in 2 hours after surgery with very high degree of sensitivity and specificity.[18] Urinary and plasma NGAL levels have also been proven to predict contrast-induced AKI with a high-degree diagnostic power as early as 2 hours after IV contrast administration.[19] In our study, however, statistically significant elevation of urinary NGAL was noted only on day 2 of CP injection and the levels were no longer significant after day 3.

NAG, the third AKI biomarker studied here, is a legacy biomarker that has reemerged as an important biomarker in recent studies.[20] Urinary NAG is a lysosomal brush-border enzyme of 140 kd that breaks down glycoproteins in the proximal tubules where it is mainly expressed. Plasma levels of NAG are normally not filtered by the glomeruli and its excretion into urine correlates with tubular cell injury. After the administration of nephrotoxic compounds, increased urinary NAG levels have been observed typically before increase in traditional serum markers as creatinine and BUN.[21] In our study, NAG performed well in relation to KIM-1 and NGAL in that its urinary excretion was significantly increased by day 2, which was a day before the rise in serum creatinine, and remained elevated for 6 days.

Although ours is an early attempt to measure a few prominent and well-studied urinary biomarkers in the same sample


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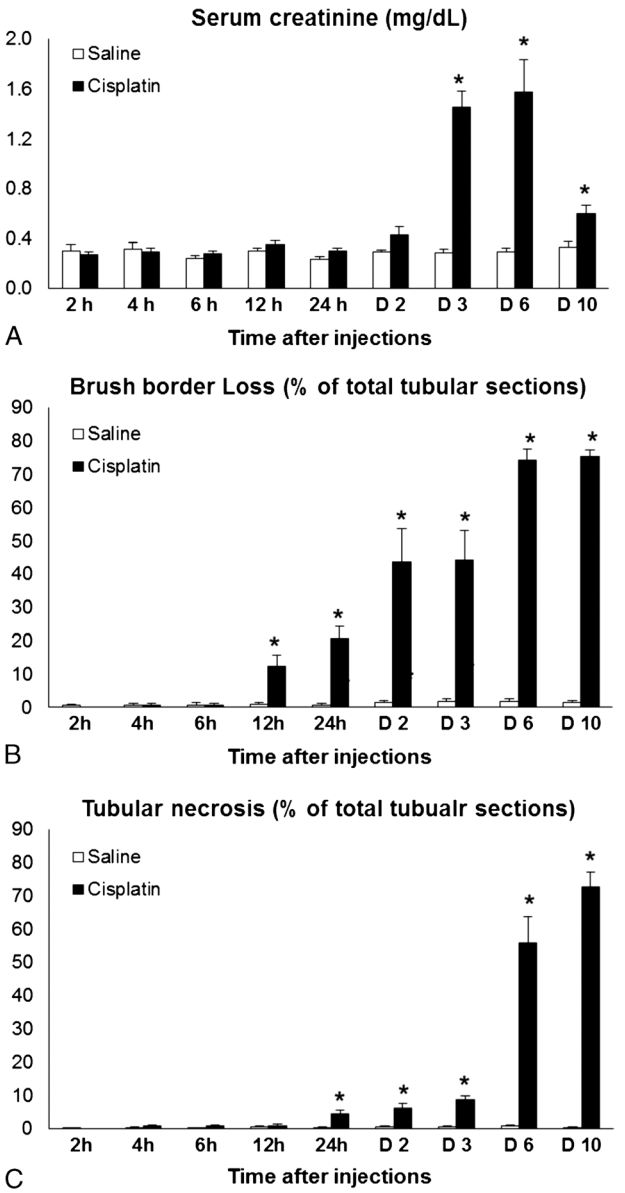


**FIGURE 2.** Time course of serum creatinine levels (upper panel, A) and renal histological scores (also see Fig. 1), that is, scores for tubular brush border loss (middle panel, B) and tubular necrosis (lower panel, C), in the CP group and saline-injected Control group. Separate groups of animals (n = 12 in CP group and n = 6 in Control group) were studied at each time point after CP or saline injection. *$P < 0.05$ between CP and Control at each time point.

simultaneously, our findings need to be verified, especially as it pertains to NGAL findings. Other studies in mice and humans have suggested that NGAL is an early riser after the initiation of tubular injury. NGAL is measured here using a validated electrochemiluminescence-based sandwich assay performed by an experienced operator with appropriate controls and standards. Moreover, NGAL was the paired analyte of KIM-1 in the assay plate. In a prospective clinical study in which urinary biomarkers were analyzed in urine samples obtained 2 hours after cardiopulmonary bypass, urinary KIM-1 achieved the highest area under-the-receiver-operator-characteristic curve followed by IL-18 and NAG.[22] We have not measured IL-8 in our study but the finding that KIM-1 and NAG have good clinical study performance is consistent with the findings we have in this study. Also, our study besides examining the urinary biomarkers in cohorts of rats at various time points had the added advantage of studying renal histology at each time point as well.

In conclusion, our study suggests a probable temporal hierarchy in the ability of certain urinary protein-based biomarkers to detect AKI after a well-defined injury of renal tubules. The pattern of expression or area under the curve for each biomarker in this study was also different. More comparative analyses of newly identified urinary biomarkers especially with time sequence are going to be helpful to fully maximize their use in AKI diagnosis.

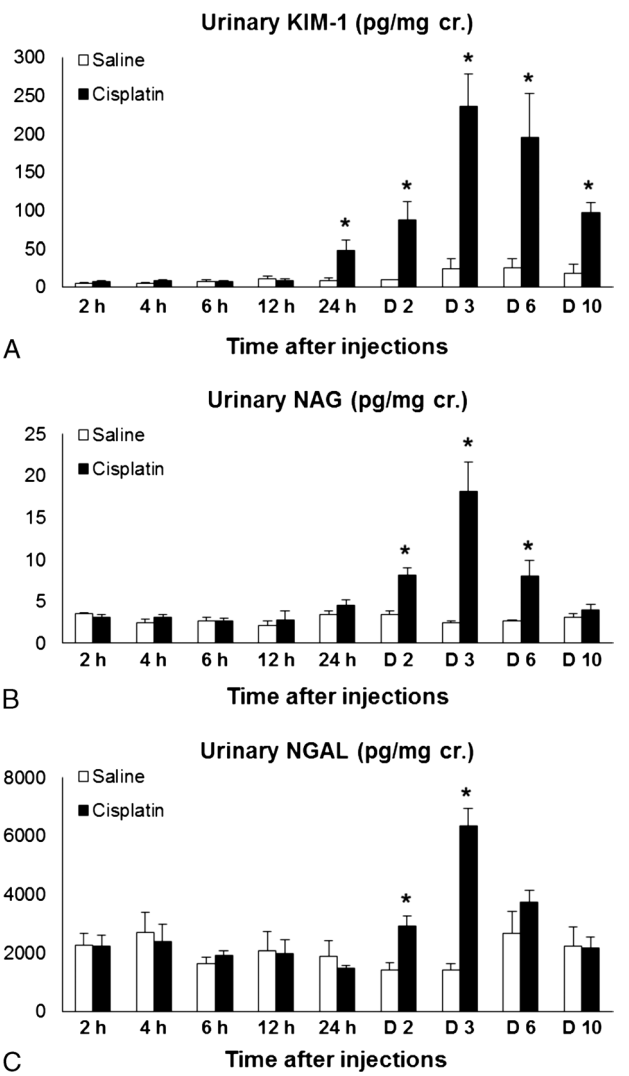


**FIGURE 3.** Time course of urinary tubular protein-based AKI biomarkers (KIM-1 upper panel, A; NAG middle panel, B; NGAL lower panel, C) after CP or saline injection. Separate groups of animals (n = 12 in CP and n = 6 in saline Control) were studied at each time point after CP or saline injection. *$P < 0.05$ between CP and Control at each time point.

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