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Urinary Neutrophil Gelatinase-associated Lipocalin as a Marker for Identification of Acute Kidney Injury and Recovery in Dogs with Gentamicin-induced Nephrotoxicity

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Background: Acute kidney injury (AKI) is associated with high mortality rates in dogs, which may be a consequence of late recognition using traditional diagnostic tests. Neutrophil gelatinase-associated lipocalin (NGAL) is a protein-induced during kidney injury that may identify AKI earlier than traditional tests.

Objectives/Hypothesis: To evaluate urinary NGAL (uNGAL) and uNGAL-to-urinary creatinine ratio (UNCR) as early markers of kidney injury and recovery in an AKI model in dogs. It was hypothesized that these markers would document AKI earlier than serum creatinine concentration.

Animals: Five purpose-bred dogs.

Methods: Prospective study. Acute kidney injury, defined as a > 50% increase in serum creatinine concentration above baseline, was induced in dogs by gentamicin administration (8–10 mg/kg SC q8h). Blood and urine collected for biochemical analyses and uNGAL and urinary creatinine concentrations, respectively, during AKI induction and recovery.

Results: Acute kidney injury was diagnosed significantly earlier based on a 7-fold increase in UNCR compared to a > 50% increase in serum creatinine concentration (day 8; range, 2–10 mg/dl vs day 16; range, 14–19 mg/dl; $P = .009$). During recovery, the initial decrease in UNCR preceded the decrease in serum creatinine concentration by a median of 2 days. The uNGAL changes paralleled UNCR changes, but the increase in uNGAL was triphasic; the initial peak occurred earlier than UNCR (median, day 11 versus median, day 19).

Conclusions and Clinical Importance: The UNCR was early marker of gentamicin-induced AKI and its decrease documented onset of renal recovery. Additional studies are needed to validate this marker in dogs with naturally occurring renal injury.

Key words: Acute kidney injury; Biomarker; Neutrophil gelatinase-associated lipocalin; uNGAL to urinary creatinine ratio.

Acute kidney injury is characterized by an abrupt and sustained decrease in glomerular filtration rate. It is common in dogs, and is associated with high treatment costs and high morbidity and mortality.[1–3] The overall mortality rate for human beings and dogs with AKI is approximately 50%, but outcome is highly dependent on etiology and available treatment options.[1–4] This high mortality rate has remained almost unchanged, despite advances in diagnostic testing and available treatments, including renal replacement treatment.[1–7] One of the speculated reasons for this high mortality is late recognition of disease and consequently a narrow window of opportunity for intervention. Early grades of AKI may go undetected when kidney function is assessed using traditional diagnostic tests, such as serum creatinine concentration.

Abbreviations:

AKI	acute kidney injury
uNGAL	urinary neutrophil gelatinase-associated lipocalin
UNCR	uNGAL to urinary creatinine ratio
USG	urine-specific gravity
VMTH	Veterinary Medical Teaching Hospital

Neutrophil gelatinase-associated lipocalin (NGAL) has been identified as 1 of the earliest and most robustly induced proteins in both ischemic kidney injury and in animal models of nephrotoxicity.[8,9] It belongs to the lipocalin superfamily and was identified as a component of neutrophil granules. It is expressed during inflammatory responses, but also is expressed in the epithelial cells of various tissues.[10] Normally, urinary NGAL (uNGAL) is expressed at low concentrations, but its expression is induced by renal tubular epithelial injury. Plasma NGAL was found to be an accurate marker of AKI in human patients undergoing cardiac surgery.[11] Urinary NGAL was reported as a marker for AKI in dogs undergoing surgical procedures as a model for AKI.[12] In another study, we reported that a > 7-fold increase in uNGAL-to-urinary creatinine ratio (UNCR) above baseline discriminated AKI from other types of urinary disease. Our previous study demonstrated UNCR to be a specific marker, showing an increase in dogs with naturally acquired AKI, but many

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of these dogs were affected by other comorbidities that could have affected assessment of NGAL concentrations. Furthermore, assessment of NGAL as a marker for renal recovery was not performed in that study.[13] In an experimental model evaluating 2 dogs with gentamicin-induced AKI, uNGAL increased before serum creatinine concentration, but dogs were euthanized before recovery and NGAL was not followed during this phase of disease.[14] These collective observations suggest NGAL has utility as an early biomarker of AKI in dogs, but further evaluation in an experimental model is needed to support these preliminary data and to determine if increases in uNGAL are specific for kidney damage and not other inflammatory comorbidities. In addition, changes in uNGAL have not been reported during the recovery phase of AKI, which may herald the onset of kidney repair.

The aims of this study were to evaluate uNGAL concentration and UNCR as potential early markers of both kidney injury and kidney recovery in normal dogs with gentamicin-induced AKI. We hypothesized that uNGAL concentration and UNCR would increase earlier than serum creatinine concentration after gentamicin administration and decrease earlier during the recovery phase.

Materials and Methods

This protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Davis. All dogs were adopted at the conclusion of the study.

Dogs

In this prospective study, 6 adult purpose-bred research dogs were evaluated for inclusion in the study. Dogs were deemed healthy based on normal physical examination findings and absence of abnormalities on a CBC, serum biochemical analysis, urinalysis, and urine culture.

Induction and definition of Acute Kidney Injury

Nephrotoxicity was induced by administration of gentamicin[a] (8 mg/kg SC) q8 h to each dog. If AKI (defined as a 50% increase in serum creatinine concentration from baseline [day 0]) was not established after 7 days, the dosage of gentamicin was increased to 10 mg/kg SC q8 h.[15,16] Administration of gentamicin was discontinued in individual dogs at the first documented increase in serum creatinine concentration ≥50% above baseline. During AKI induction, serum creatinine concentration was evaluated twice daily to ensure timely discontinuation of gentamicin as soon as AKI was established, thereby attempting to mitigate development of severe injury that would result in adverse clinical signs, irreversible kidney injury, or both. Recovery of AKI was documented as a persistent decrease in serum creatinine concentration from its peak after discontinuation of gentamicin. Lactated ringer solution[b] (1 L SC q12 – q24 h) was administered only during the recovery period at the investigator's discretion to any dog that appeared hypodipsic.

Sample Collection

At baseline (day 0), CBC, serum biochemistry analysis, urinalysis and urine culture were performed at the diagnostic laboratories of the William R. Pritchard Veterinary Medical Teaching Hospital using standard methodologies. Blood was collected for serum creatinine, BUN, potassium, sodium, chloride, phosphorus, calcium concentrations, and for packed cell volume and total solids determinations, at baseline and after administration of gentamicin at the following time points: twice daily during AKI induction, once daily from the establishment of AKI until day 24, and then on days 26, 28, 31, 35, 39, 46, and 60. Six milliliters of blood were obtained from the jugular or lateral saphenous veins using a 22-gauge needle. Urine was collected by antepubic cystocentesis whenever possible or occasionally by voiding (at the same time points as described for blood collections), because previous studies documented that uNGAL and UNCR did not differ based on method of urine collection.[13] Urine-specific gravity (USG) was measured using a standard refractometer. All blood and urine samples were placed on ice immediately after collection and centrifuged in a refrigerated centrifuge within 30 minutes of collection. Samples for biomarker measurement were stored at −80°C pending batched analysis.

NGAL Analysis

The NGAL concentrations were measured in canine urine using an ELISA kit[c] as previously described.[13] Briefly, the kit contained a plate with test wells that are precoated with a mouse monoclonal antibody against canine NGAL, an additional site-specific biotinylated canine NGAL monoclonal antibody, a reference standard, and proprietary reagents. A standard curve for NGAL was created using 8 dilutions (ranging from 0 to 400 pg/mL) of canine NGAL reference standard, which were evaluated on the same plate as the experimental samples. Both experimental samples and reference standards were diluted 1:100 using a proprietary diluent and then added to the anti-canine NGAL antibody-coated sample wells and incubated for 1 hour at room temperature with gentle agitation. After incubation, all wells were washed to remove unbound antibody, and the secondary biotinylated antibody was added and incubated with agitation at room temperature for 1 hour. After the second incubation, all wells were washed with a proprietary wash solution and incubated with a streptavidin-horse radish peroxidase reagent at room temperature with agitation for 1 hour. After this incubation, a tetramethylbenzidine-based peroxidase substrate was added for 10 minutes at room temperature in the dark for color development. At 10 minutes, a dilute sulfuric acid stop solution was added, and the optical density of the solution in the well was measured at 450 nm using a plate reader. The concentrations of the experimental samples were calculated from a standard curve of the optical densities using curve-fitting software.[d]

Statistical analysis

Continuous variables (eg, time between identification of AKI based on UNCR and serum creatinine concentration) were compared using the Mann–Whitney U-test. Analyses were performed using statistical software.[e] For all tests applied, $P < .05$ was considered statistically significant.

Results

One dog was excluded from the study because of the presence of an *E. coli* bacterial cystitis. The included dogs were 2-year old, female intact purpose-bred hound dogs. Median body weight was 22.3 kg (range, 20–22.7 kg).

The median serum creatinine concentration at baseline (day 0) before gentamicin administration was

1.0 mg/dL (range, 0.7–1.0 mg/dL). Median baseline uNGAL and UNCR were 507 pg/mL (range, 142–1,047 pg/mL) and 280 pg/mg (range, 185–608 pg/mg), respectively. All other biochemical variables were within the reference ranges before gentamicin administration.

Serum creatinine concentration remained unchanged during the first 2 weeks of gentamicin administration. All dogs required an increase in gentamicin dosage on day 8 to induce a > 50% increase in serum creatinine concentration. A > 50% increase in serum creatinine concentration from baseline was documented in all dogs on median day 16 (range, 14–19 days).

Urinary NGAL increased gradually until day 7 of gentamicin administration, from a median baseline of 507 pg/mL (range, 142–1,047 pg/mL) to a median uNGAL concentration of 1875 pg/mL (range, 285–11,785 pg/mL). After day 7, median uNGAL increased steeply, until it peaked at 166,000 pg/mL (range, 52,170–521,119 pg/mL) on day 19, which represented a 185-fold increase in median uNGAL concentration compared to baseline. The initial change in UNCR paralleled the change in uNGAL and reached a > 7-fold increase on median day 8 (range, 2–10 days) of gentamicin administration compared to median day 16 (range, 14–19 days; $P = .009$) to document a > 50% increase in serum creatinine concentration. In contrast to UNCR, the increase in uNGAL was triphasic, and the initial uNGAL peak occurred at an earlier time point (median day 11 versus median day 19 for UNCR). The maximal increase in UNCR was more pronounced compared to the increase in uNGAL (557- versus. 185-fold increase from baseline, respectively). All dogs experienced decreases in USG (ie, <1.012), and the decline in USG paralleled the increases in UNCR; the peak UNCR corresponded temporally to the nadir of USG.

The UNCR decreased progressively after its peak value on median day 19. During the recovery phase, the decrease in median UNCR preceded the decrease in serum creatinine concentration by 2 days (Fig 1F). In contrast, uNGAL decreased initially on median day 10 but fluctuated before a sustained and progressive decrease on median day 26; this sustained decrease in uNGAL was apparent only at 5 days after the decrease in serum creatinine concentration.

Discussion

The results of the present study indicate that uNGAL and UNCR are consistent and early markers of gentamicin-induced AKI in dogs. Previously, a >7-fold increase in UNCR above baseline concentration was shown to discriminate AKI from other types of urinary disease (eg, lower urinary tract disease, chronic kidney disease).[13] The present study indicates that a diagnosis of AKI using UNCR (based on a 7-fold increase from baseline) can be made earlier than a diagnosis of AKI using serum creatinine concentration (based on a 50% increase from baseline) by a median of 8 days. Although similar threshold values have not been established for uNGAL, the uNGAL paralleled the changes in UNCR, and these observations suggest both uNGAL and UNCR are earlier markers of AKI compared to serum creatinine concentration. Increases in UNCR paralleled decreases in USG in all dogs, but AKI may be missed if only monitoring for trends in USG, especially if animals are receiving fluid treatment or have other comorbidities that result in low USG at the time of evaluation.

One of the speculated reasons for the high and almost unchanged mortality rate with AKI is late recognition using current diagnostic markers, primarily serum creatinine concentration. With traditional testing, AKI is recognized most commonly when overt clinical manifestations occur or biochemical abnormalities suggestive of severe kidney dysfunction are identified. Once a substantial reduction in kidney function is present, management of AKI involves intensive supportive care, with the requirement for hospitalization and possibly renal replacement treatment. Despite intervention, severe AKI is still associated with high mortality.[3,4,17] To date, there are no sensitive and specific markers routinely used to identify AKI earlier than identified by serum creatinine concentration.

The utility of early predictive biomarkers has been demonstrated in human patients, where AKI is acquired most commonly during hospitalization for treatment of other conditions. In veterinary medicine, many dogs are presented for treatment of adverse clinical signs and are subsequently diagnosed with AKI at an advanced stage of disease. At this stage, routine markers of AKI (eg, serum creatinine and blood urea nitrogen concentrations) are sensitive enough to diagnose the disorder.[2] Nevertheless, a subset of dogs develop hospital-acquired AKI or are evaluated at earlier and less advanced stages of kidney injury (before overt failure is present), and for this patient population, more sensitive markers are required to diagnose the disease in a timely fashion. In addition, more sensitive markers are needed to monitor dogs that receive nephrotoxic drugs, to allow for identification of unknown factors that may lead to the initiation of kidney injury and to allow for critical evaluation of early interventions that may prevent development of overt renal failure. Recently, the incidence of hospital-acquired AKI in dogs, diagnosed based on >150% or >0.3 mg/dL increase in serum creatinine concentration from baseline, was shown to be as high as 12%.[18] The incidence of AKI may have been even higher if more rigorous diagnostic criteria had been used for all cases, as recommended by the International Renal Interest Society (IRIS) AKI grading system (>0.3 mg/dL increase in serum creatinine concentration from baseline). Also, the diagnosis of hospital-acquired AKI may have been documented more often if more sensitive biomarkers, such as uNGAL, had been utilized. Some dogs with undiagnosed mild forms of AKI might progress to overt kidney failure as a consequence of delayed treatment, and early diagnosis may be critical for improved outcomes.

In our study, changes from baseline for serum creatinine concentration and UNCR were used to document AKI. We selected a more rigorous increase in serum creatinine concentration to define AKI (eg, >50%

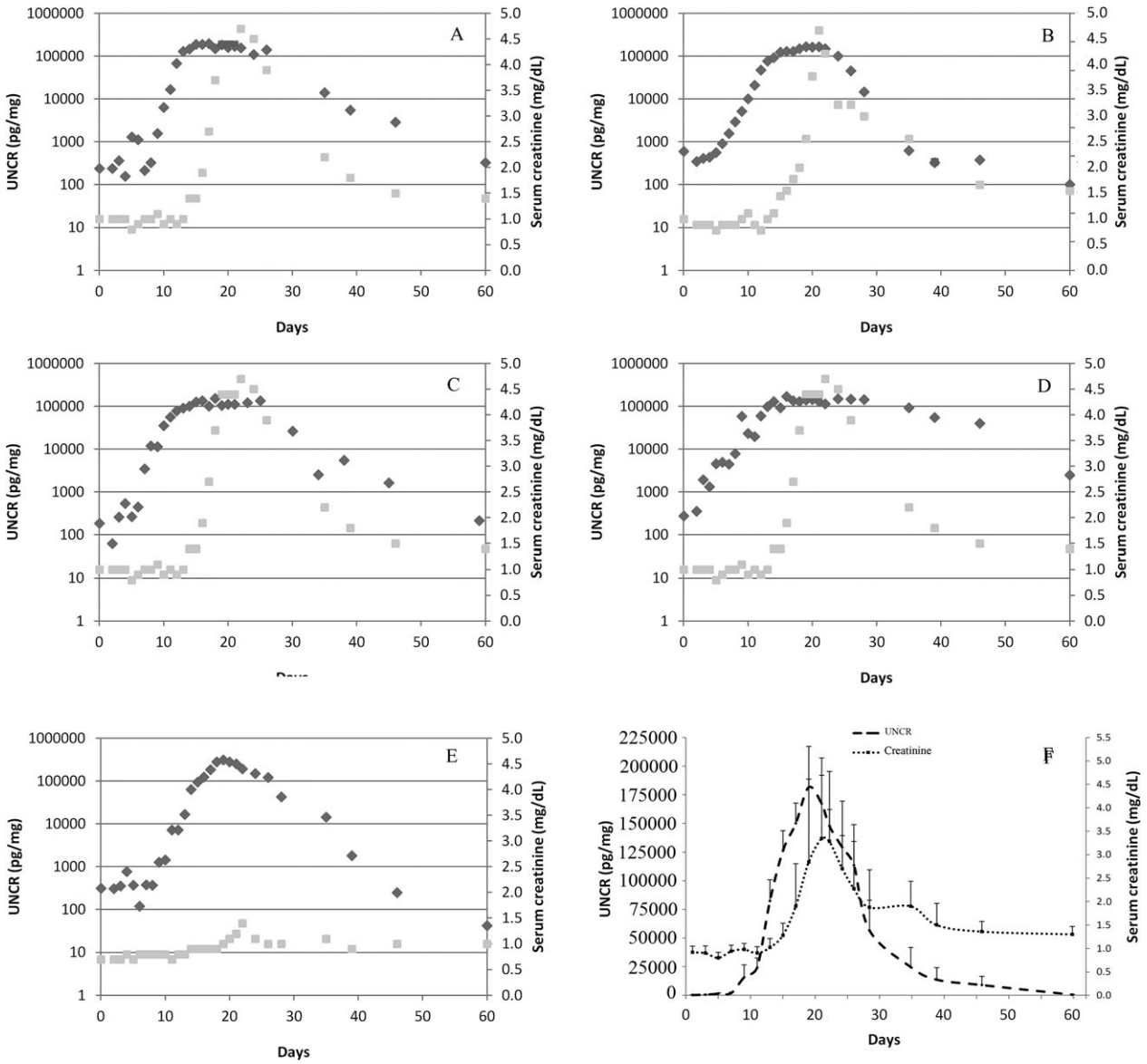

Figure 1. (A–E) Urinary NGAL/creatinine ratio (UNCR) and serum creatinine concentration after gentamicin administration in all 5 dogs. The day of development of acute kidney injury, based on a 7-fold increase in UNCR and 50% increase in serum creatinine concentration is depicted by arrows. (F) Sequential changes in mean and standard deviation of urinary NGAL/creatinine ratio (UNCR) and serum creatinine concentration after gentamicin administration during induction of acute kidney injury, and during recovery from acute kidney injury. Occasionally, there is an overlap in the standard deviation bars for the UNCR and creatinine lines. Note that each individual standard deviation line arises from the individual dataset that it represents and ends on the small horizontal standard deviation line.

increase from baseline) than proposed by recent IRIS AKI guidelines to ensure establishment of intrinsic renal injury and to avoid misclassification of AKI that could occur with creatinine assay variation or mild, undetectable dehydration and prerenal azotemia.[13] Nonetheless, even using the IRIS criteria, the diagnosis of AKI was made significantly earlier when changes in uNGAL or UNCR were used to document kidney injury, as compared to serum creatinine concentration, a finding that is in agreement with studies in human patients.[19]

We have reported previously that UNCR was increased in dogs with established AKI, chronic kidney disease and urinary tract infection.[13] In this previous study, a 7-fold increase in UNCR from baseline discriminated AKI from other urinary diseases.[13] The previous study also demonstrated that the increase in UNCR precedes the increase in serum creatinine concentration, but that study was not designed to assess how early the diagnosis of AKI can be made using UNCR compared to serum creatinine concentration. The present study further demonstrates that uNGAL and UNCR are early and consistent markers of AKI compared to serum creatinine concentration because all dogs experienced a > than 7-fold increase in UNCR after gentamicin administration, but also that the increase in UNCR preceded the prediction of AKI

based on serum creatinine concentration by a median of 8 days.

In previous studies evaluating NGAL in dogs with naturally occurring kidney disease, comorbidities (eg, sepsis, heatstroke) existed and might have affected serum NGAL and uNGAL concentrations.[13] This present study evaluated uNGAL and UNCR in an experimental population of dogs that did not have confounding comorbidities that could contribute to the documented increase in serum NGAL and uNGAL concentrations, supporting the conclusion that changes in NGAL were specific to kidney injury and not caused by other underlying inflammatory conditions.

We also evaluated the recovery phase of AKI, which has not been done in previous studies. We found that the recovery phase of AKI could be identified earlier based on UNCR compared to serum creatinine concentration. The UNCR started to decrease 2 days earlier than the decrease in serum creatinine concentration. This finding suggests the decrease in UNCR may be an earlier marker of renal repair compared to a decrease in serum creatinine concentration. A larger population of dogs would be required to determine if this decrease will be statistically significant. Although the 2-day difference might not seem pronounced, early detection of recovery is important to project hospitalization costs, and timely identification of renal recovery might influence therapeutic management, assessment of outcome, and decisions about euthanasia. Monitoring of uNGAL as a marker of active and potentially ongoing tubular injury also has the potential to provide information regarding active repair of the kidney. Creatinine as a filtration marker is unable to document this process of repair. In the current study, from approximately day 25 to day 60, UNCR progressed from markedly increased values to baseline, at a time when serum creatinine concentration was relatively static. This response suggests that tubular injury still was active but improving throughout this interval. The decrease in serum creatinine concentration reflects improvement in kidney function, and therefore is delayed as compared to NGAL. Measurement of NGAL provides insights into the status of the disease process, which has not been previously identified with traditional markers.

The cause of the triphasic response of uNGAL during the maintenance phase of kidney injury (between induction and recovery) is not readily apparent, nor is the difference in response between uNGAL and UNCR during this phase. The fluctuations in uNGAL may represent waves of parenchymal injury and NGAL induction and excretion throughout this phase that are not reflected when uNGAL is indexed to urinary creatinine concentration. The issue of indexing urinary biomarkers markers (eg, to creatinine) remains a controversial and unresolved subject, and it is conceivable the unindexed uNGAL provides predictions about the extent and timing of the kidney insult that are masked by creatinine indexing. Urinary creatinine (a filtration marker) and uNGAL (an active tubular injury marker) likely document independent processes occurring in different segments of the nephron, and indexing 1 process by the result of the other may mask their independent behaviors. We suggest that oscillations in uNGAL reflect waves of insult or active injury to the tubular epithelium ongoing in the kidney over time that likely are independent of the secondary or subsequent changes in the filtration of creatinine which could modify these observations. The indexing of urinary biomarkers should be further evaluated.

Our study has some limitations. The first limitation is the small number of dogs evaluated. We attempted to overcome this problem by extensive serial evaluation of the study animals to provide greater time resolution for the identified changes. Second, the study design evaluated the utility of UNCR and uNGAL in a single animal model of AKI and therefore might not represent the changes in kidney damage associated with other etiologies. Other studies have, however, indicated that NGAL is increased in several etiologies of AKI, and we believe these results will likely apply to dogs with a broad array of naturally acquired AKI, but further investigation is warranted.[13] In some cases, such as nephrotoxic drugs, baseline uNGAL measurements could be obtained before administration, allowing for evaluation of changes in uNGAL from baseline, as was done in our study. To more widely apply the use of AKI biomarkers in clinical practice, the upper limits of uNGAL and UNCR must be evaluated in large numbers of clinical patients so that cut-off values can be obtained for use when baseline values are not available.

In conclusion, this study documented that uNGAL and UNCR may be useful diagnostic tools for the early detection of gentamicin-induced AKI, and they also may serve as early markers for recovery from AKI. Additional studies are needed to validate the diagnostic utility of these markers in clinical settings associated with diverse causes of AKI and to define the most appropriate index to express changes in NGAL excretion.

Footnotes

[a] Wedgewood Pharmacy, Swedesboro, NJ, USA
[b] Braun Medical Inc., Irvine, CA, USA
[c] BioPorto, ALPCO, Salem, NH, USA
[d] Molecular Devices, SpectraMax190, Sunnyvale, CA, USA
[e] SPSS 17.0 for Windows, Chicago, IL, USA

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Conflict of Interest Declaration: Drs. LeRoy and Kowalkowski are employees of AbbVie Laboratories. The NGAL assay is not an AbbVie product and Drs. LeRoy and Kowalkowski did not have a conflict of interest in hypothesis generation and experimental design, organizing and conducting experiments, interpreting and analyzing the results, or writing and revising the manuscript.

Off-label Antimicrobial Declaration: Gentamicin was used for induction of acute kidney injury, and not for treatment of a susceptible bacterial infection.

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