J Vet Intern Med 2013;27:1362–1367

Evaluation of Neutrophil Gelatinase-Associated Lipocalin as a Marker of Kidney Injury in Dogs

G. Segev, C. Palm, B. LeRoy, L.D. Cowgill, and J.L. Westropp

Background: Acute kidney injury (AKI) is a common and often fatal disorder in dogs.

Hypothesis: Urine neutrophil gelatinase-associated lipocalin (NGAL)/creatinine ratio is a sensitive and specific biomarker of AKI in dogs.

Animals: Ninety-four dogs.

Methods: Prospective study. Dogs were classified as follows: (1) healthy dogs, (2) dogs with lower urinary tract disorders, (3) dogs with chronic kidney disease (CKD), (4) dogs with azotemic International Renal Interest Society (IRIS) AKI Grades II–V, and (5) dogs with IRIS AKI Grade I (nonazotemic). Urinary NGAL was quantitated in each dog using an ELISA assay and concentrations were expressed as a ratio to urinary creatinine concentration from the same specimen, and designated the urinary NGAL/creatinine ratio (UNCR).

Results: There was a significant difference in UNCR among the study groups ($P < .001$). Both the azotemic and nonazotemic AKI groups had higher UNCR when compared with all other groups ($P < .001$ for all pairs). There was a statistically significant difference in UNCR between dogs diagnosed with CKD compared with dogs with lower urinary tract diseases ($P = .005$) as well as between dogs with CKD and healthy dogs ($P = .001$). Receiver operator characteristics (ROC) analysis of UNCR as an indicator of azotemic and nonazotemic AKI had an area under the ROC curve of 0.94 and 0.96, respectively.

Conclusions and Clinical Relevance: NGAL/creatinine ratio is a sensitive and specific marker of AKI. It can be used to screen patients at risk for AKI and can be utilized to diagnose milder forms of AKI potentially earlier in the course of the disease.

Key words: Acute kidney injury; Canine; Chronic kidney disease; Survival; Urinary biomarker.

Acute kidney injury (AKI) represents a spectrum of diseases that are associated with a sudden onset of renal parenchymal injury.[1] The injury may be mild and go unnoticed, but more characteristically, AKI is recognized by failure of the kidneys to meet the excretory, metabolic, and endocrine demands of the body.[1,2] The term AKI has been developed to heighten emphasis on the necessity for early recognition of AKI and to sensitize clinicians to the presence of kidney injury early in its course, when therapeutic interventions may be more effective and outcomes more positive.[2] To facilitate this concept, the International Renal Interest Society (IRIS) has adopted a grading scheme to categorize and stratify the severity of AKI (Appendix).[2] IRIS AKI Grade I represents an early and mild degree of kidney injury when serum creatinine concentration is within the reference range, but the diagnosis of kidney injury at this stage using routine clinical parameters can be a diagnostic challenge. Strategic biomarkers that predict the presence of kidney injury before overt clinical abnormalities are present and before development of azotemia would greatly facilitate the detection of kidney injury when it might be most responsive to medical management and when prevention of progression may be possible.

Abbreviations:

AKI	acute kidney injury
CKD	chronic kidney disease
IRIS	International Renal Interest Society
NGAL	neutrophil gelatinase-associated lipocalin
ROC	receiver operator characteristics
UNCR	urinary NGAL/creatinine ratio
UTI	urinary tract infection

The overall mortality rate for human beings and animals with AKI is approximately 50%, but it is highly dependent on the etiology and available treatment options.[3–5] This high mortality rate has remained almost unchanged, despite advances in diagnostic test and available treatments, including renal replacement therapy.[3,4,6–9] One of the suggested reasons for the high mortality is the late recognition of AKI using traditional clinical diagnostic tests including serum creatinine concentration and urinalysis, leaving a narrow window of opportunity for therapeutic or preventive intervention before the development of kidney failure.[2,7] Therefore, there is a recognized need for sensitive and specific markers for early identification of AKI in both human and veterinary medicine, and there is active search and growing interest in the discovery of biomarkers for early recognition of AKI. Biomarkers potentially may be useful

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Submitted February 27, 2013; Revised June 7, 2013; Accepted July 30, 2013.

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10.1111/jvim.12180

for diagnosing, classifying, and grading the severity of disease.[10]

Biomarkers can be evaluated individually or in combination to maximize sensitivity, specificity, and phase of disease. Neutrophil gelatinase-associated lipocalin (NGAL) is a promising biomarker for early detection of AKI.[11–13] It is a 25 kDa protein covalently bound to neutrophil gelatinase.[14] Normally, it is expressed at low concentrations, but its expression is markedly induced by renal tubular epithelial injury.[11] Functionally, NGAL exerts a spectrum of iron-dependent biological activities, and its administration mitigates experimentally induced renal injury in mice, suggesting that it may have renoprotective properties.[11,15] NGAL has been identified as one of the earliest and most robustly induced proteins in both ischemic and nephrotoxic animal models of AKI.[16,17]

Neutrophil gelatinase-associated lipocalin can be detected in both blood and urine, and a specific ELISA test for canine NGAL is currently available.[a] In a study evaluating urinary NGAL as a marker of ongoing kidney injury in dogs with X-linked hereditary nephropathy, there was a gradual increase in urine NGAL/creatinine ratio (UNCR) as disease progression occurred.[18] The UNCR also was significantly higher in early (nonazotemic) stages of dogs with X-linked hereditary nephropathy compared with unaffected dogs, but this difference did not reach statistical significance.[18] Urinary NGAL also was found to be a sensitive predictor for AKI associated with surgical procedures in human patients.[19] The specificity of urinary NGAL for the detection of early kidney injury has not been reported previously in the dog.

We hypothesized that UNCR would differentiate patients with AKI from those with other renal or urinary diseases. The objectives of this study were (1) to evaluate the sensitivity and specificity of UNCR for AKI compared with other urinary diseases, (2) to assess UNCR as an early marker of AKI compared with clinical parameters currently used (eg, serum creatinine concentration), and (3) to correlate changes in UNCR with the severity and outcome of AKI.

Materials and Methods

Dogs and Definition of Medical Conditions

Dogs were enrolled into this prospective study and classified into the following groups: (1) healthy dogs, (2) dogs with lower urinary tract disorders, (3) dogs with chronic kidney disease (CKD), (4) dogs with azotemic AKI (IRIS AKI Grades II–V), and (5) dogs with nonazotemic AKI that presented with normal serum creatinine concentrations (IRIS AKI Grade I), but developed AKI during hospitalization. Dogs with multiple concurrent renal or urinary disorders (eg, CKD, UTI) as well as dogs with prerenal azotemia were excluded.

Dogs were deemed healthy based on unremarkable history and physical examination as well as absence of abnormalities on CBC, serum biochemistry profile, urinalysis, and urine culture. Dogs were classified as having lower urinary tract disease if any of the following were present as the sole clinical diagnosis: bacterial cystitis based on a positive bacterial culture of urine collected by cystocentesis, uroliths, or lower urinary tract neoplasia based on diagnostic imaging and cytology. Dogs with concurrent CKD or AKI were excluded from this group. The diagnosis and severity of CKD was based on IRIS CKD staging guidelines.[20] A diagnosis and stratification of the severity of azotemic AKI was based on IRIS grading (see Appendix), according to the following criteria: (a) acute onset of historical features, clinical signs, or diagnostic information consistent with AKI, (b) IRIS AKI Grade II or higher, and (c) lack of ultrasonographic evidence of chronic renal changes. Dogs were classified with IRIS AKI Grade I on the basis of criteria "a" and "c" above for azotemic AKI, and based on blood and urine samples obtained daily from dogs that were presented to the veterinary hospitals according to definitions in the Appendix.

Sample Collection

Complete blood count, serum biochemistry profile, and urinalysis were performed in the laboratories of the 2 participating hospitals. Urine was collected from all dogs by cystocentesis at presentation. In a subset of healthy dogs, urine samples were collected both by cystocentesis and by voiding to assess the potential influence of collection method on the urine NGAL results. All samples were stored at −80°C pending analysis. Dogs with IRIS AKI Grade I were not azotemic at presentation, but developed azotemic AKI in subsequent days during hospitalization. Urinary NGAL analyses were performed on samples collected at presentation when dogs were nonazotemic.

NGAL Analysis

Urinary NGAL UNCR was measured using a commercially available sandwich ELISA according to methods and instructions provided with the assay.[a] Briefly, the kit contains a plate precoated with a mouse monoclonal antibody against canine NGAL from the tested sample, as well as an additional site-specific biotinylated anti-canine NGAL monoclonal antibody and reference standards and reagents. A standard curve for NGAL was created using 8 dilutions (ranging from 0 to 400 pg/mL) of canine NGAL reference standard (included in kit), which were evaluated on the same plate as the clinical samples. Experimental samples and reference standards were diluted 1 : 100 using the included proprietary sample diluent, 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 supplied proprietary wash solution and a streptavidin-HRP reagent was incubated 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.[b] The concentrations of the experimental samples were calculated from a standard curve using curve fitting software.[b]

Statistical Analysis

The distribution of all continuous parameters (normal versus nonnormal) was assessed using the Shapiro–Wilk's test. Based on data distribution, nonparametric tests were used to compare continuous parameters among groups. The UNCR was compared among the study groups using the Kruskal–Wallis test. The

Mann–Whitney *U*-test was used for post hoc analyses with Bonferroni correction to compare differences between specific groups. The correlation between continuous parameters among study groups was assessed using the Pearson or Spearman rank correlation test. The receiver operator characteristics (ROC) procedure was used to assess UNCR as a predictor for AKI and as an outcome predictor. The ROC analysis also was used to select cutoff points and to calculate corresponding sensitivities and specificities of UNCR for the prediction of AKI or the outcome. The optimal cutoff point was defined as the point associated with the fewest misclassifications. For all tests applied, $P \leq .05$ was considered statistically significant. All calculations were performed using statistical software.[c]

Results

Signalment

Ninety-four dogs were included in the study. Twenty-eight dogs (12 males and 16 females) were classified as healthy. Seventeen dogs (8 males and 9 females) were diagnosed with lower urinary tract diseases. Of these, 14 had bacterial cystitis (8 dogs with *Escherichia coli*, 2 dogs with Proteus, 2 dogs with *Pseudomonas aeruginosa*, and 2 dogs with *Klebsiella pneumoniae*), 2 had transitional cell carcinoma, and 1 had calcium oxalate urolithiasis. Twenty dogs (11 males and 9 females) were diagnosed with CKD. Twenty-one dogs (13 males and 8 females) had IRIS AKI Grades II–V (azotemic AKI). Of these, 2 dogs developed AKI after anesthesia, 2 developed AKI after snake envenomation, 2 dogs had leptospirosis, 1 dog had ethylene glycol intoxication, and a cause for AKI was not determined for the remaining dogs. Eight dogs (5 males and 3 females) were classified as IRIS AKI Grade I (nonazotemic AKI). Of these, 7 dogs presented with heatstroke and 1 dog after anesthesia.

The median age of all dogs was 81 months (range, 12–181 months; Table 1) with no differences in age among groups except for healthy dogs, which were significantly younger than dogs with lower urinary tract disease ($P = .007$).

Serum Creatinine Concentration

Median serum creatinine concentrations are presented in Table 1. Dogs with IRIS AKI Grades II–V and CKD had higher serum creatinine concentration compared with all other groups ($P < .001$ for all pairs).

Urinalysis

Among dogs with azotemic categories of AKI (Grades II–V), 6/21 (29%) had evidence of glucosuria and 4/21 (19%) had evidence of granular casts. Among dogs with IRIS AKI Grade I classification, 2/8 (25%) had evidence of glucosuria and 1/8 (13%) had granular casts reported on the urine sediment examination. None of the dogs with CKD or lower urinary tract diseases had glucosuria or cylindruria.

NGAL

Median UNCR for healthy dogs was 520 pg/mg (range, 40–3,660 pg/mg). In 10 healthy dogs, urine samples were collected both by cystocentesis and by voiding. There was no statistical difference between the median UNCR of samples collected by cystocentesis and those collected by voiding (350 pg/mg; range, 110–1,480 pg/mg versus 520 pg/mg; range, 110–1,480 pg/mg, respectively; $P = .9$).

Median UNCRs for all groups are presented in Figure 1. Dogs diagnosed with AKI of any grade had the highest median UNCR. There was a statistically significant difference between the UNCR among the study groups (Fig 1, $P < .001$). Dogs with AKI (of any grade) had the highest UNCR when compared with all other groups ($P < .001$ for all pairs). There was a statistically significant difference in UNCR between dogs diagnosed with CKD and dogs with lower urinary tract diseases ($P = .005$) as well as between dogs with CKD and healthy dogs (Fig 1, $P = .001$). The UNCR also was significantly higher in dogs with lower urinary tract diseases compared with healthy dogs (Fig 1,

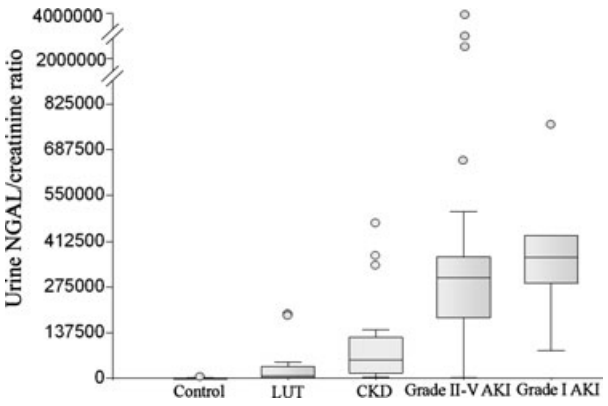

Fig 1. Urinary NGAL/creatinine ratio of healthy dogs and dogs with different renal and urinary tract disorders. Data are presented as boxes and whiskers. Each box includes the interquartile range, whereas the line within a box represents the median, and the whiskers represent the range, and extend to a maximum of 1.5 times the interquartile range. Outliers are depicted by circles.

Table 1. Age and serum creatinine concentration at presentation in 94 dogs with urinary tract disorders.

	Healthy	LUT Disorders	CKD	Azotemic AKI	AKI Grade I
Age (months)	60 (24–122)	120 (24–180)	87 (12–162)	102 (36–172)	64 (36–181)
Creatinine (mg/dL)	0.8 (0.4–1.3)	0.65 (0.46–1.35)	4.0 (1.3–11.7)	7.6 (1.7–18.2)	1.25 (1.04–1.32)

LUT, lower urinary tract disorders; CKD, chronic kidney disease; AKI, acute kidney injury.
Medians (ranges) are presented.

$P < .001$). Among the entire cohort of dogs, there was no correlation between UNCR and serum creatinine concentration.

The nonazotemic IRIS AKI Grade I group included dogs that progressed to more severe, azotemic AKI during hospitalization in which samples were obtained before the development of azotemic AKI. The median UNCR in this AKI group was not significantly different from the azotemic AKI group with higher IRIS AKI Grades (384,000 pg/mg; range, 84,100–1,440,000 pg/mg versus 322,000 pg/mg; range, 1,190–3,990,000 pg/mg, respectively; $P = .61$), but was significantly higher compared with all other groups ($P < .001$; Fig 1).

Sensitivity and Specificity

The ROC analysis of UNCR as a predictor of azotemic forms of AKI compared with related, non-AKI renal or urinary conditions had an area under the ROC curve of 0.94 (95% confidence interval [CI], 0.88–1.00). The optimal cutoff point was 120,000 pg/mg, corresponding to sensitivity and specificity of 95% and 89%, respectively. ROC analysis of UNCR ratio as an indicator of IRIS AKI Grade I disease compared with related renal or urinary conditions (excluding azotemic AKI) had an area under the ROC curve of 0.96 (95% CI, 0.89–1.00). The optimal UNCR cutoff point was 238,000 pg/mg corresponding to sensitivity and specificity of 100% and 85%, respectively.

Outcome

Seven of 21 dogs (33.3%) with azotemic grades of AKI survived. A significant difference in UNCR between survivors and nonsurvivors was not found (median, 307,000 pg/mg; range, 184,000–503,000 pg/mg versus median, 363,000 pg/mg; range, 1,190–3,990,000 pg/mg, respectively; $P = .83$). Three of 8 dogs (37.5%) with IRIS AKI Grade I survived. There was no significant difference in UNCR between survivors and nonsurvivors in the nonazotemic IRIS AKI Grade I dogs (median, 297,000 pg/mg; range, 287,000–368,000 pg/mg versus median, 431,000 pg/mg; range, 84,100–1,440,000 pg/mg, respectively; $P = .18$).

Discussion

This study demonstrates that UNCR is a sensitive and specific marker for azotemic and nonazotemic forms of AKI among samples of dogs with various renal and urinary disorders. Furthermore, UNCR was a marker of mild and potentially early stages of AKI and may serve as an important predictor of AKI before it can be identified using conventional clinical diagnostic tests (eg, serum creatinine concentration, glucosuria, cylindruria).

Acute kidney injury is associated with high morbidity and mortality.[2,3,4,21] One of the speculated reasons for this association is that AKI typically is not recognized until later stages of severity, when the clinical manifestations have progressed and conventional markers of AKI, such as serum creatinine and urea nitrogen concentrations, are increased. Early identification of the disease might help promote earlier treatment before injury is irreparable and therefore might decrease the high mortality rate associated with AKI. Many studies in human medicine have demonstrated the presence of specific and novel blood and urinary biomarkers that increase before increases of conventional diagnostic parameters (ie, serum creatinine concentration) outside of their reference ranges. These novel biomarkers have the potential to identify injury early in its pathologic course.[12,17,22,23] This study, in agreement with a recent publication,[19] demonstrates that UNCR is a promising urinary biomarker for all degrees and potentially early phases of AKI in dogs with naturally occurring disease.

NGAL belongs to the lipocalin superfamily. It was identified originally as a component of neutrophil granules, but is also expressed in epithelial cells of various tissues.[24] NGAL binds catecholate type siderophores and thereby prevents bacteria from acquisition of siderophore-bound iron.[25,26] NGAL is upregulated in response to inflammatory signals, including epithelial injury associated with AKI.[24] In a rat model of toxic AKI, NGAL was induced predominantly in proximal tubule cells.[13] Because of its early and substantial increase after kidney injury, it is considered one of the early and robust biomarkers of AKI. NGAL also was detected in the first urine output after renal ischemia was induced in a mouse model of AKI.[12] It has been shown to increase as early as 2 hours after cardiopulmonary bypass in human patients[12,23] and as early as after 1 hour in human patients undergoing cardiac surgery.[17] On the basis of these observations, NGAL has been proposed as an AKI screening test in human patients admitted to intensive care units after invasive cardiac procedures.[27,28]

This study also suggests that UNCR is a specific marker for AKI prediction in dogs when compared with other upper and lower urinary diseases such as CKD, bacterial cystitis, and urolithiasis. It is yet to be determined whether or not specificity is maintained when evaluating dogs with other nonurinary diseases (eg, sepsis). The increase in UNCR in dogs with all IRIS Grades of AKI was substantially and significantly higher compared with all other conditions that were evaluated, and only minimal overlap was noted between dogs with AKI and dogs with other (upper and lower) urinary tract diseases. The high area under the ROC curve also supports these findings. Given that no significant difference was noted between azotemic and nonazotemic AKI groups, and given the high area under the ROC curve in both AKI groups, the findings of this study support UNCR as a sensitive and specific diagnostic marker that can accurately predict the presence of kidney injury before serum creatinine concentration increases outside of the normal reference range. Of the dogs with nonazotemic IRIS AKI Grade I, only 33% of dogs had glucosuria

and only 19% manifested cylindruria, demonstrating its relative sensitivity compared with these traditional biomarkers of AKI.

Increases in the expression of biomarkers (including NGAL) have been shown to correlate with AKI severity and clinical outcomes in human patients, and have the potential to be used as prognostic indicators in dogs.[23] In this study, UNCR at presentation was not an accurate predictor for survival. Serial evaluation of UNCR may be a better prognostic indicator. Future studies are needed to evaluate if sequential changes in UNCR will better predict resolution or progression of renal injury.

NGAL/creatinine ratio was increased significantly in dogs with lower urinary tract diseases compared with healthy dogs. Neutrophils present attributable to inflammation in lower urinary tract diseases could be the origin of the increased NGAL concentration in the urine. UNCR also was increased in a rat model of upper and lower urinary tract infection.[29] Although increases in UNCR in lower urinary tract diseases slightly decreased the specificity of this marker for AKI, there was minimal overlap between dogs with AKI and dogs with lower urinary tract diseases. It is difficult to exclude completely the presence of subclinical kidney involvement in dogs with some of the diseases included in the lower urinary tract group. However, subclinical pyelonephritis in dogs in this group is unlikely given the lack of inflammatory leukograms and absence of clinical signs associated with pyelonephritis.

The high sensitivity and specificity of biomarkers to assess AKI in veterinary medicine may be compromised compared with their use in human medicine, because many dogs and cats present for medical care only when kidney injury is well established and readily identified by traditional clinical parameters. However, patients prone to develop AKI can be screened and identified in a timely fashion. In a study evaluating the presence of AKI based on increase in serum creatinine concentration in the critical care setting, 24 (15%) of 164 hospitalized dogs were diagnosed with AKI.[30] Hospital-acquired AKI maybe is under diagnosed in veterinary medicine. The use of biomarkers in veterinary medicine may identify higher proportions of hospital-acquired AKI than currently are recognized by traditional evaluation of serum creatinine concentration.

Despite the high specificity and sensitivity of UNCR for diagnosis of AKI, we recommend that it be used in the appropriate patient population, namely in nonazotemic dogs that are at risk for AKI, such as those undergoing general anesthesia, those with suspected heatstroke or those exposed to nephrotoxins. As a better understanding of NGAL develops, it also may prove to be useful in predicting recovery from AKI. Early identification of AKI in veterinary patients is crucial as the window of opportunity for treatment is relatively narrow, and because management by hemodialysis is not readily available and can be cost prohibitive.

There are several limitations to this study. Sample size was relatively small (eg, IRIS AKI Grade I). Only dogs with stable CKD were included in the CKD group; however, some dogs might have had a concurrent acute component to their azotemia. Likewise, dogs with bacterial cystitis may have had subclinical pyelonephritis, although this seems unlikely based on the diagnostic criteria utilized. Finally, it cannot be completely excluded that UNCR is not increased in nonrenal conditions, as such dogs were not included in the study.

In conclusion, UNCR was shown to be a sensitive and specific marker of naturally occurring AKI in dogs. It can be used to screen patients at risk for AKI and to diagnose AKI early in its course when the injury is mild (IRIS AKI Grade I). UNCR also is increased in CKD, but it is yet to be determined if this marker can be used as an early predictor of chronic disease. Additional studies also are warranted to determine if changes in UNCR will better predict resolution or progression of renal injury.

Footnotes

[a] BioPorto; ALPCO, Salem, NH
[b] Molecular Devices; SpectraMax190, Sunnyvale, CA
[c] SPSS 17.0 for Windows, Chicago, IL

Acknowledgments

Conflict of Interest Declaration: Authors disclose no conflict of interest.

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Appendix: IRIS AKI Grading Criteria (www.IRIS-Kidney.com)

AKI Grade	Serum Creatinine	Clinical Description
Grade I	<1.6 mg/dL (<140 μmol/L)	Nonazotemic AKI a. Documented AKI: (Historical, clinical, laboratory, or imaging evidence of acute kidn ey injury, clinical oliguria/anuria, volume responsiveness*)…and b. Progressive *nonazotemic* increase in serum creatinine; ≥0.3 mg/dL (≥26.4 μmol/L) within 48 hours c. Measured oliguria (<1 mL/kg/h) or anuria over 6 hours
Grade II	1.7–2.5 mg/dL (141–220 μmol/L)	Mild AKI a. Documented AKI and static or progressive azotemia b. Progressive azotemic increase in serum creatinine; ≥0.3 mg/dL (≥26.4 μmol/L) with in 48 hours, or volume responsiveness* c. Measured oliguria (<1 mL/kg/h) or anuria over 6 hours
Grade III	2.6–5.0 mg/dL (221–439 μmol/L)	Moderate to severe AKI a. Documented AKI and increasing severities of azotemia and functional renal failure
Grade IV	5.1–10.0 mg/dL (440–880 μmol/L)	
Grade V	>10.0 mg/dL (>880 μmol/L)	

AKI, acute kidney injury.

* Volume responsive is an increase in urine production to >1 mL/kg/h over 6 hours or decrease in serum creatinine to baseline over 48 hours.