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Urinary neutrophil gelatinase-associated lipocalin distinguishes pre-renal from intrinsic renal failure and predicts outcomes

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In established acute kidney injury (AKI), serum creatinine poorly differentiates prerenal from intrinsic AKI. In this study, we tested whether urinary neutrophil gelatinase-associated lipocalin (NGAL) distinguishes between intrinsic and prerenal AKI, and tested its performance in predicting a composite outcome that included progression to a higher RIFLE (Risk, Injury, Failure, Loss of function, End stage renal disease) class, dialysis, or death. Urinary NGAL was measured using a standardized clinical platform in 161 hospitalized patients with established AKI. Sixteen patients were excluded because of postrenal obstruction or insufficient clinical information. Of the remaining 145 patients, 75 had intrinsic AKI, 32 had prerenal AKI, and 38 patients could not be classified. Urinary NGAL levels effectively discriminated between intrinsic and prerenal AKI (area under the receiver-operating characteristic curve 0.87). An NGAL level over 104 μg/l indicated intrinsic AKI (likelihood ratio 5.97), whereas an NGAL level <47 μg/l made intrinsic AKI unlikely (likelihood ratio 0.2). Patients experiencing the composite outcome had significantly higher median urinary NGAL levels on inclusion. In logistic regression analysis, NGAL independently predicted the composite outcome when corrected for demographics, comorbidities, creatinine, and RIFLE class. Hence, urinary NGAL is useful in classifying and stratifying patients with established AKI.

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Acute kidney injury (AKI) is common in hospitalized patients and is increasing in incidence.[1,2] Both the 'Risk, Injury, Failure, Loss of function, End stage renal disease' (RIFLE) classification from the acute dialysis quality initiative and the acute kidney injury network (AKIN) definition of AKI are based on serum creatinine levels and urinary output to define the severity of AKI.[3,4] Both classifications are effective in risk stratifying patients, as the more advanced RIFLE or AKIN stages predict poor clinical outcomes. However, serum creatinine levels do not always reflect the severity of renal damage. First, during early AKI even a large decrease in glomerular filtration rate may cause only a modest increase in serum creatinine, because creatinine must first accumulate in the blood. Second, underlying chronic kidney disease (CKD) can cause a high serum creatinine level in the absence of AKI. Missing information about the baseline creatinine level often confounds interpretation of an elevated serum creatinine concentration. Third, substantial increases in serum creatinine can be observed in renal hypoperfusion even when the kidneys are structurally intact, resulting in prerenal AKI.[5] Patients with prerenal and postrenal AKI must be identified as soon as possible, as their treatment is decidedly different than that of patients with intrinsic AKI.[5,6] Importantly, prerenal AKI is associated with a lower mortality than intrinsic AKI.[2] Serum and urine diagnostic indices, including fractional excretion of sodium (FeNa), fractional excretion of urea (FeUrea), and urea creatinine (UC) ratio are commonly determined; however, these indices may be of limited utility in diagnosing intrinsic AKI.[5,7–9] Hence, the diagnostic workup and therapeutic management of patients with established AKI would greatly benefit from a clinical test that facilitates a differential diagnosis of intrinsic and prerenal AKI at an early time point and that helps in stratifying the patient at risk.

Novel biomarkers of renal tubular damage potentially fulfill the criteria of such a test. They include (but are not

limited to) neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1, liver-type fatty acid-binding protein, and interleukin 18.[10] These molecules are produced within the kidney in response to injury and can be detected in plasma or urine. In contrast to conventional markers, such as serum creatinine, blood urea nitrogen, or serum cystatin C, these markers do not reflect kidney function, but instead signify structural damage to cells of the kidney. Consequently, these markers are rapidly detectable in response to injury and their increased levels are independent of a functional deficit. Conversely, a purely functional drop of glomerular filtration rate, as is the case in pure prerenal AKI, is not expected to result in an upregulation of these markers. Hence, the detection of novel biomarkers in the setting of established AKI (by creatinine criteria) may facilitate a differential diagnosis between prerenal and intrinsic AKI and aid in risk-stratifying patients beyond currently available parameters (for example, creatinine or RIFLE class).

One of the most promising novel biomarkers is NGAL, a 25 kDa protein that is produced in renal tubules in response to structural kidney injury[11,12] and secreted into the urine.[13] In previous studies, NGAL was effective in the early diagnosis of AKI in several clinical settings, including perioperative AKI, contrast-induced AKI, sepsis-associated AKI, and AKI following kidney transplantation.[14–19] Importantly, NGAL effectively discriminated patients in the emergency room with intrinsic AKI from those with other diagnoses including prerenal AKI, CKD, and those who actually had normal renal function.[20] Furthermore, several studies suggested that NGAL could be useful in the prediction of poor clinical outcomes in AKI.[17,20–22] In this study, we addressed the performance of urinary NGAL levels in established AKI based on RIFLE criteria. Specifically, we asked whether NGAL would aid in distinguishing intrinsic from prerenal causes and predict an unfavorable clinical course.

RESULTS

Patient characteristics

Of 161 patients initially included into the study for established AKI (RIFLE-R, -I, or -F) 16 were excluded for postrenal obstruction ($n=6$) or insufficient clinical information ($n=10$). The remaining 145 patients were included into the outcome analysis and underwent diagnostic adjudication (Figure 1). At the time of inclusion into the study, 32 patients (22.1%) had RIFLE-R AKI, 65 (44.8%) had RIFLE-I AKI, and 48 (33.1%) had RIFLE-F AKI. In all, 49 patients (33.8%) experienced the composite outcome. Of these, 19 patients (13.1%) displayed a step-up in RIFLE severity class, 18 patients (12.4%) required renal replacement therapy, and 28 patients (19.3%) died.

Patients who experienced the composite outcome did not differ significantly from all other patients with respect to demographics or comorbidities, although a previous history of CKD (stage 3 or up) tended to be more frequent in patients with an unfavorable clinical course ($P=0.097$; Table 1). Serum creatinine and blood urea nitrogen levels at the time of inclusion were significantly higher in patients who experienced the composite outcome compared with all others, whereas RIFLE class, UC ratio, FeNa, and FeUrea were not significantly different.

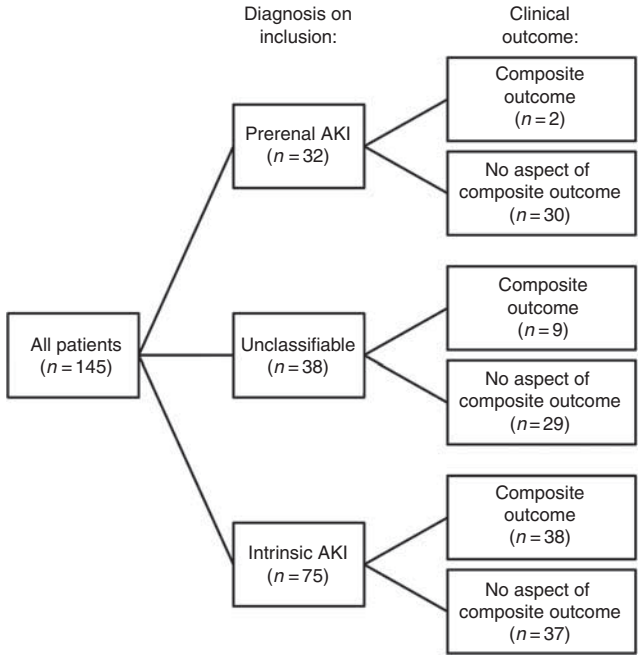

Figure 1 | Study flow diagram. AKI, acute kidney injury.

A total of 107 patients were ultimately diagnosed with prerenal AKI ($n=32$) or intrinsic AKI ($n=75$). Of patients with intrinsic AKI, 46 patients (61.3%) had a clinical diagnosis of acute tubular necrosis, whereas 29 patients (38.7%) had intrinsic AKI caused by other renal diseases, mainly nephrotoxic AKI ($n=9$) and acute glomerulonephritis ($n=7$). In all, 38 patients were unclassifiable, because they could not be clearly attributed to one of the two categories even after thorough inspection of all clinical information. In most of these cases, we could not differentiate prerenal AKI from acute tubular necrosis based on the clinical information ($n=24$). In other cases, a prerenal event coincided with contrast agent administration ($n=10$) or other potential nephron-damaging events ($n=4$).

Patients with prerenal AKI were significantly older than patients with intrinsic AKI (Table 1). In addition, congestive heart failure was significantly more frequent in patients with prerenal AKI. Serum creatinine and RIFLE severity class on inclusion were significantly higher in patients with intrinsic AKI when compared with prerenal AKI. The composite outcome (step-up in RIFLE severity class, dialysis initiation, or mortality) was experienced by 38 patients with intrinsic AKI (50.7%), but only by 2 patients with prerenal AKI (6.3%, $P<0.001$; Table 1). Of these two patients with prerenal AKI and adverse outcomes, one received a single session of hemodialysis in the setting of diuretics-induced prerenal AKI

Table 1 | Patient characteristics by clinical course and diagnosis

Characteristic	All patients (*n*=145)	Clinical course after inclusion		Diagnosis on inclusion		
		Composite outcome (*n*=49)	No aspect of composite outcome (*n*=94)	Intrinsic AKI (*n*=75)	Prerenal AKI (*n*=32)	Unclassifiable (*n*=38)
Demographics						
Mean age (s.d.), years	67.7 (14.4)	65.6 (14.4)	68.8 (14.4)	64.3 (15.5)§	71.1 (14.2)	71.5 (10.7)
Women, *n* (%)	59 (40.7)	19 (38.8)	40 (41.7)	27 (36.0)	14 (43.8)	18 (47.4)
Black race, *n* (%)	1 (0.7)	0 (0)	1 (1)	0 (0)	1 (3.1)	0 (0)
Comorbidities						
Congestive heart failure, *n* (%)	101 (69.7)	34 (69.4)	67 (69.8)	42 (56.0)§	26 (81.3)	33 (86.8)
Diabetes mellitus, *n* (%)	42 (29)	12 (24.5)	30 (31.3)	19 (25.3)	11 (34.4)	12 (31.6)
Hypertension, *n* (%)	118 (81.4)	40 (81.6)	78 (81.3)	54 (72.0)	28 (87.5)	36 (94.7)
Coronary artery disease, *n* (%)	44 (30.3)	17 (34.7)	27 (28.1)	18 (24.0)	11 (34.4)	15 (39.5)
Peripheral artery disease, *n* (%)	21 (14.5)	9 (18.4)	12 (12.5)	10 (13.3)	6 (18.8)	5 (13.2)
Cerebrovascular disease, *n* (%)	13 (9)	4 (8.2)	9 (9.4)	7 (9.3)	2 (6.3)	4 (10.5)
CKD stage at baseline (based on eGFR), *n* (%)[a]						
Stage 1: $\geqslant$90	43 (29.7)	13 (26.5)	30 (31.3)	28 (37.3)	7 (21.9)	8 (21.1)
Stage 2: 60–89	64 (44.1)	19 (38.8)	45 (46.9)	28 (37.3)	11 (34.4)	25 (65.8)
Stage 3: 30–59	33 (22.8)	15 (30.6)	18 (18.8)	14 (18.7)§§	14 (43.8)	5 (13.2)
Stage 4: 15–29	5 (3.4)	2 (4.1)	3 (3.1)	5 (6.7)	0 (0)	0 (0)
CKD stage 3 or up at baseline, *n* (%)	38 (26.2)	17 (34.7)	21 (21.9)	19 (25.3)	14 (43.8)	5 (13.2)
Kidney parameters on inclusion[b]						
Median serum creatinine baseline, μmol/l (IQR)	87 (69–105.5)	89 (68.5–121.5)	84.5 (69.3–100)	83 (69–108)	95.5 (77–115.5)	79 (66.8–95)
Median serum creatinine on inclusion, μmol/l (IQR)	202 (157.5–263.5)	225 (170–299)*	188.5 (154.3–245.5)	233 (190–357)§§§	175.5 (141.3–223)	168 (151.8–220.3)
Median serum creatinine inclusion/baseline, ratio (IQR)	2.3 (1.8–3.3)	2.4 (1.9–3.4)	2.2 (1.8–3.1)	2.9 (2.1–3.8)§§§	2 (1.7–2.4)	2 (1.7–2.7)
Median serum urea/serum creatinine ratio (IQR)	81.9 (57.2–100.4)	87.8 (57.4–102.2)	81.2 (56.3–100)	70 (47.6–91)	95.5 (68.8–122.7)	89.5 (75.4–107.5)
Median fractional excretion of sodium, % (IQR)	0.9 (0.3–2.2)	0.7 (0.2–2.1)	1 (0.3–2.6)	1 (0.4–2.7)	1 (0.3–2.1)	0.6 (0.2–2.2)
Median fractional excretion of urea, % (IQR)	24.9 (15.4–43.3)	23.9 (12.8–44.5)	25.1 (16.1–41)	33.5 (14.9–49.7)	23.8 (16.3–31.7)	20.3 (13.9–30.6)
Median NGAL on inclusion, μg/l (IQR)	95.4 (37.6–362.2)	248.2 (78–1010.6)***	68.3 (26.9–214)	255.6 (98.5–872.9)§§§	31.3 (15.9–75.5)	49.3 (29.8–112.1)
Median NGAL on inclusion, μg/g creatinine (IQR)	115.7 (35.9–370.9)	235.4 (103.8–900.6)**	71.8 (28–220.7)	273.5 (112.9–890.8)§§	36.5 (18.5–75.7)	51.4 (27–166.2)
Development of kidney parameters after inclusion						
Median serum creatinine 2 days after inclusion, μmol/l (IQR)	148 (102–242)	205 (149–366.5)**	121.5 (95–168.5)	199 (139–389)§§§	116.5 (87.5–152.3)	111 (84.3–164.3)
Median peak serum creatinine within 7 days, μmol/l (IQR)	190 (133.8–300.3)	273 (204.8–436.3)***	159 (127–233.3)	277 (191–422.8)§§§	128.5 (113.5–162.8)	154 (126.3–227.8)
Median NGAL 2 days after inclusion, μg/l (IQR)	102.2 (35.1–326.5)	474.2 (119.4–1250.4)***	64.1 (27.1–139.3)	187.2 (99.1–891.4)§	22.1 (10.2–91.8)	41.2 (28.6–136)
Median NGAL 2 days after inclusion, μg/g creatinine (IQR)	113.4 (39.2–482.2)	483.1 (113.4–1576.8)**	78.3 (27.9–260.6)	330.4 (103.1–1517.2)§	30.6 (11.4–81.7)	61.3 (32.2–184.4)
Outcomes						
Step-up in RIFLE class after inclusion, *n* (%)	19 (13.4)			14 (19.4)§§	0 (0)	5 (13.2)
Initiation of renal replacement therapy, *n* (%)	18 (12.4)			14 (18.7)§	1 (3.1)	3 (7.9)
In-hospital mortality, *n* (%)	28 (19.3)			23 (30.7)§§	1 (3.1)	4 (10.5)
Composite outcome (unfavorable clinical course), *n* (%)	49 (33.8)			38 (50.7)§§§	2 (6.3)	9 (23.7)

Abbreviations: AKI, acute kidney injury; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; IQR, interquartile range; NGAL, neutrophil gelatinase-associated lipocalin; RIFLE: Risk, Injury, Failure, Loss of function, End stage renal disease.
[a]eGFR was calculated using the Modification of Diet in Renal Disease formula.
[b]Units for serum creatinine are in μmol/l. To convert serum creatinine from μmol/l to mg/dl, divide by 88.4.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ between patients with and without outcomes using *t*-test or χ^2 test as appropriate.
$^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$ between the intrinsic and prerenal AKI groups using *t*-test or χ^2 test as appropriate.

in chronic congestive heart failure, but made a quick recovery of creatinine thereafter. The other patient had diarrhea-induced prerenal AKI and initially recovered from AKI, but died later during hospitalization from an unrelated event. Unclassifiable cases resembled prerenal AKI cases with respect to demographics and comorbidities, but displayed higher peak creatinine levels during follow-up and more unfavorable clinical outcomes, which reflects the fact that they represent neither purely prerenal nor purely intrinsic AKI by clinical criteria (Table 1).

Urinary NGAL levels in the diagnosis of intrinsic versus prerenal AKI

NGAL levels were significantly higher in patients with a clinical diagnosis of intrinsic AKI when compared with prerenal AKI ($P = 0.007$; Table 1, Figure 2). Unclassifiable patients displayed intermediate NGAL levels consistent with the fact that they represent marginal cases. To determine NGAL test characteristics in the diagnosis of intrinsic AKI versus prerenal AKI, we performed a receiver-operating characteristic (ROC) analysis. Table 2 shows the areas under the ROC curves (AUC-ROC), for different biomarkers of intrinsic AKI, including NGAL levels, serum creatinine at the time of inclusion, RIFLE class at the time of inclusion, UC ratio, FeNa, and FeUrea. The AUC of NGAL (0.87, CI 0.81–0.94) was significantly higher than that of any other marker tested ($P < 0.05$; Figure 3). We also determined the sensitivities, specificities, positive and negative predictive values, and positive and negative likelihood ratios for NGAL and other biomarkers at different cutoff levels (Table 3). We had recently conducted a large multicenter study of patients admitted to emergency rooms, which had indicated that, in the absence of additional clinical information, a urinary NGAL level >104 μg/l was suggestive of intrinsic AKI, whereas a urinary NGAL level <47 μg/l was suggestive of normal kidney function, stable CKD, or prerenal AKI.[23] To validate these results in our cohort with established AKI, we tested these same cutoffs on the current study population. An NGAL level cutoff level at 104 μg/l provided a high specificity (0.88) and a high positive likelihood ratio (5.97) for a diagnosis of intrinsic AKI. This finding indicates that a urinary NGAL >104 μg/l in a patient with established AKI is suggestive of intrinsic AKI. Conversely, an NGAL cutoff level

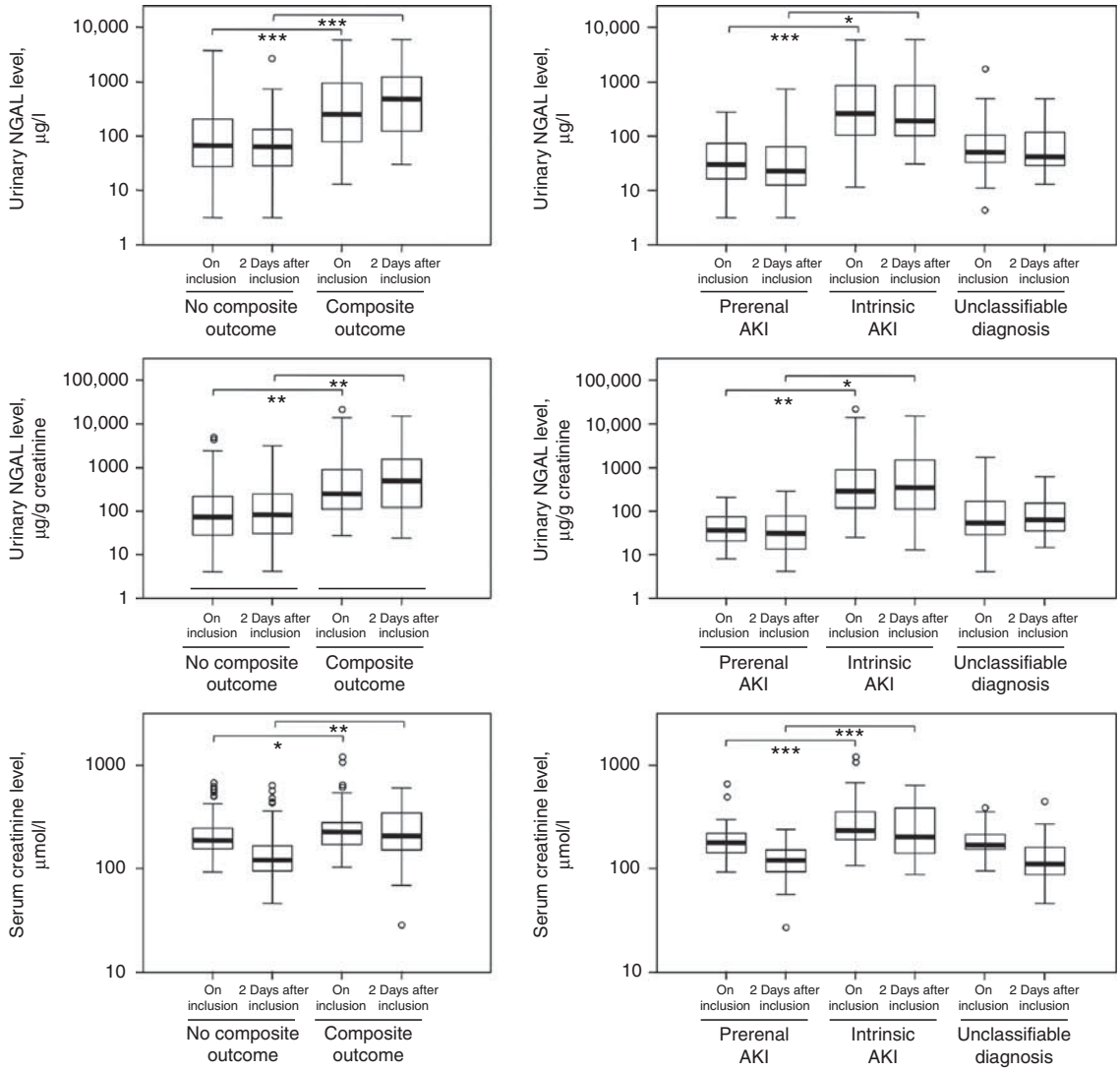

Figure 2 | Box plots of biomarker levels in differential diagnosis of acute kidney injury (AKI) and prediction of outcomes. Biomarker levels are presented on a log 10 scale. Boxes indicate median, lower, and upper quartiles. Lower and upper whiskers represent data within 1.5 interquartile ranges of the lower quartile and within 1.5 interquartile ranges of the upper quartile, respectively, of log 10-transformed biomarker levels. Circles represent outliers. NGAL, neutrophil gelatinase-associated lipocalin. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 2 | ROC analysis of different biomarkers in the differential diagnosis of AKI and in the prediction of outcomes

Test[a]	Diagnosis of intrinsic AKI (versus prerenal AKI)	Prediction of composite outcome (unfavorable clinical course)
Urinary NGAL	0.87 (0.81–0.94)***	0.71 (0.62–0.8)***
Urinary NGAL/urinary creatinine	0.89 (0.82–0.95)***	0.71 (0.62–0.8)***
Serum creatinine	0.74 (0.63–0.84)***,#	0.61 (0.51–0.71)*,§
RIFLE class	0.72 (0.62–0.82)***,#	0.56 (0.47–0.66)#
Fractional excretion of urea	0.59 (0.48–0.71)#	0.49 (0.38–0.6)#
Fractional excretion of sodium	0.54 (0.42–0.65)#	0.45 (0.34–0.55)#
Serum urea/serum creatinine ratio[b]	0.71 (0.59–0.82)**,#	0.48 (0.37–0.58)#

Abbreviations: AKI, acute kidney injury; NGAL, neutrophil gelatinase-associated lipocalin; RIFLE: Risk, Injury, Failure, Loss of function, End stage renal disease; ROC, receiver-operating characteristic.
[a]Data represent areas under the ROC curves (AUC-ROC) with 95% confidence intervals.
[b]Smaller test result indicates more positive test.
*$P<0.05$ versus null hypothesis: true area=0.5.
**$P<0.01$ versus null hypothesis: true area=0.5.
***$P<0.001$ versus null hypothesis: true area=0.5.
#$P<0.05$ versus urinary NGAL.
§$P=0.067$ versus urinary NGAL.

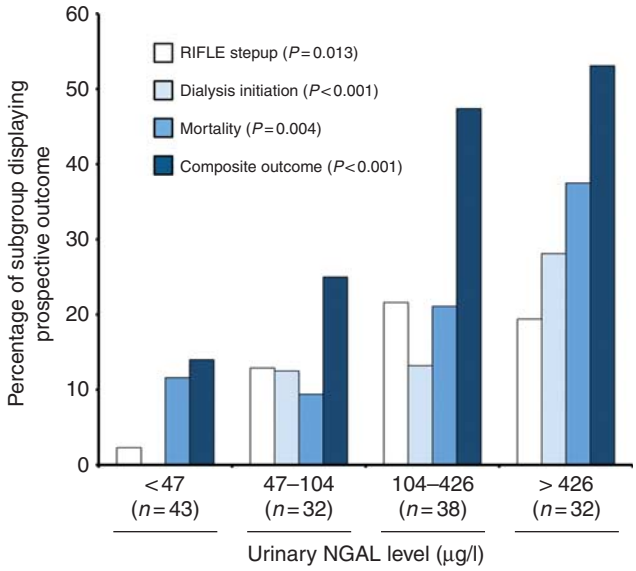

Figure 3 | Urinary neutrophil gelatinase-associated lipocalin (NGAL) levels and outcomes. Patients were stratified by NGAL level. Percentages of the subgroups that experience aspects of the composite outcome are shown. *P* for trend is significant for composite and individual clinical outcomes. RIFLE: Risk, Injury, Failure, Loss of function, End stage renal disease.

of 47 µg/l provided a high sensitivity (0.89) and a negative likelihood ratio of 0.2 indicating that an NGAL level below this cutoff is useful in excluding intrinsic AKI. Correction of the NGAL levels for urinary creatinine yielded similar test characteristics to NGAL alone (Tables 2 and 3). Test characteristics of the other markers at percentile-adjusted cutoffs are shown in Table 3. Together, these data indicate that NGAL levels can discriminate intrinsic from prerenal AKI and validate independently derived cutoff levels. Figure 2 shows box plots for urinary NGAL and serum creatinine on inclusion and 2 days after inclusion, stratified by the diagnostic category (intrinsic AKI versus prerenal AKI), demonstrating that NGAL levels displayed less overlap between the diagnostic groups when compared with serum creatinine.

NGAL levels in the prediction of an unfavorable clinical course

To determine the test characteristics of NGAL and other biomarkers in predicting the composite outcome (step-up in RIFLE severity class, dialysis initiation, or mortality), we analyzed biomarker levels, scatter, ROC curves, and performed logistic regression analysis. Median NGAL levels on inclusion and 2 days after inclusion were significantly higher in patients, who later experienced the composite clinical outcome, when compared with all others (Table 1, Figure 2). We stratified patients into four groups of increasing NGAL level (<47, 47–104, 104–426, and >426 µg/l). Cutoffs were determined based on the 60th, 75th, and 90th percentiles of NGAL levels in the above-mentioned emergency room cohort.[23] We examined the relationship between the NGAL level and clinical outcomes (step-up in RIFLE severity class, dialysis initiation, and mortality). We found a progressive increase in outcome frequency with increasing urinary NGAL levels. The associations between NGAL level and all outcomes (composite and individual AKI outcomes) were statistically significant (Figure 3). ROC analyses indicated that NGAL had an AUC-ROC of 0.71 in the prediction of the composite outcome, whereas serum creatinine and RIFLE severity class had AUC-ROCs of 0.61 and 0.56, respectively (Table 2).

To analyze the effect of a sustained versus transient elevation of NGAL on clinical outcomes, we analyzed the effect of NGAL levels on inclusion in relation to NGAL levels 2 days after inclusion. Within patients with an NGAL level >104 µg/l on inclusion, 38 patients still displayed an elevated NGAL >104 µg/l 2 days after inclusion (sustained elevation of urinary NGAL), whereas 12 patients had NGAL levels that normalized to <104 µg/l (transient elevation of urinary NGAL). Notably, only two patients with a transiently elevated NGAL excretion (16.7%), but 22 patients with a sustained elevation of NGAL (57.9%) experienced the composite outcome indicating an unfavorable clinical course ($P<0.05$). These data suggest that a quick normalization of an elevated NGAL level is predictive of a beneficial outcome, whereas a sustained high NGAL excretion is a predictor of a poor clinical course.

We used logistic regression analysis to analyze how urinary NGAL performed after an adjustment for other clinical predictors of an unfavorable clinical course or other established laboratory tests. We generated a conventional prediction model that corrected for demographics,

Table 3 | Test characteristics of biomarkers in the prediction of intrinsic AKI versus prerenal AKI at different cutoff levels

Biomarker	Cutoff level	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio
Urinary NGAL (absolute level)	>47 μg/l	0.89 (0.8–0.95)	0.53 (0.35–0.7)	0.82 (0.71–0.89)	0.68 (0.46–0.84)	1.91 (1.31–2.78)	0.2 (0.1–0.41)
	>104 μg/l	0.75 (0.63–0.84)	0.88 (0.7–0.96)	0.93 (0.83–0.98)	0.6 (0.44–0.73)	5.97 (2.37–15.08)	0.29 (0.19–0.43)
Urinary NGAL (per urinary creatinine)	>45 μg/g	0.88 (0.78–0.94)	0.55 (0.36–0.72)	0.82 (0.72–0.9)	0.65 (0.44–0.82)	1.94 (1.31–2.89)	0.22 (0.11–0.43)
	>128 μg/g	0.72 (0.6–0.81)	0.87 (0.69–0.96)	0.93 (0.82–0.98)	0.56 (0.41–0.7)	5.55 (2.2–14.01)	0.33 (0.22–0.47)
Serum creatinine	>167 μmol/l	0.85 (0.75–0.92)	0.44 (0.27–0.62)	0.78 (0.67–0.86)	0.56 (0.35–0.75)	1.52 (1.1–2.09)	0.34 (0.18–0.63)
	>205 μmol/l	0.63 (0.51–0.73)	0.66 (0.47–0.81)	0.81 (0.68–0.9)	0.43 (0.29–0.58)	1.82 (1.1–3.03)	0.57 (0.41–0.79)
RIFLE severity class (R=1, I=2, F=3)	2	0.88 (0.78–0.94)	0.34 (0.19–0.53)	0.76 (0.65–0.84)	0.55 (0.32–0.76)	1.34 (1.03–1.75)	0.35 (0.17–0.72)
	3	0.49 (0.38–0.61)	0.88 (0.7–0.96)	0.9 (0.76–0.97)	0.42 (0.31–0.55)	3.95 (1.53–10.15)	0.58 (0.46–0.73)
Serum urea/serum creatinine ratio (urea (mmol/l)/creatinine (mmol/l))	<63	0.45 (0.34–0.57)	0.89 (0.7–0.97)	0.92 (0.76–0.98)	0.38 (0.26–0.51)	4.07 (1.36–12.18)	0.62 (0.5–0.77)
	<84	0.63 (0.51–0.74)	0.63 (0.42–0.8)	0.82 (0.69–0.91)	0.39 (0.25–0.54)	1.7 (1.01–2.87)	0.59 (0.42–0.83)

Abbreviations: AKI, acute kidney injury; NGAL, neutrophil gelatinase-associated lipocalin; RIFLE: Risk, Injury, Failure, Loss of function, End stage renal disease.
Optimal cutoff levels for NGAL (μg/l) were derived from an independent patient cohort in the emergency room.[24] Cutoff levels of the remaining biomarkers represent adjusted percentiles in the current study population to ensure comparability of the results.

Table 4 | Multiple logistic regression models for the prediction of an unfavorable clinical course (step-up in RIFLE severity class, dialysis initiation, or mortality)[a]

Parameters	Wald score	*P*-value	Odds ratio[b]	R square	AUC-ROC[b]	Diagnostic accuracy
Biomarker model						
Age (>70 years)	3	0.083	0.5 (0.2–1.1)	0.215	0.74 (0.64–0.83)	74.50%
Serum creatinine (>205 μmol/l)	4.6	0.031	2.3 (1.1–4.9)			
Urinary NGAL (>104 μg/l)	13.3	<0.001	4.2 (1.9–9.0)			
Conventional model						
Age (>70 years)	3.3	0.068	0.5 (0.25–1.1)	0.093	0.65 (0.56–0.75)	68.30%
Serum creatinine (>205 μmol/l)	6.3	0.012	2.5 (1.2–5.2)			

Abbreviations: AKI, acute kidney injury; AUC-ROC, area under the receiver-operating characteristic curve; CKD, chronic kidney disease; NGAL, neutrophil gelatinase-associated lipocalin; RIFLE: Risk, Injury, Failure, Loss of function, End stage renal disease.
[a]Both models were adjusted for gender, histories of CKD, congestive heart failure, vascular disease, hypertension, diabetes, and RIFLE class.
[b]Data in parentheses are 95% confidence intervals.
Wald score, *P*-value, and odds ratio are parameters to describe the contribution of the individual covariate to the model. R square, AUC-ROC, and diagnostic accuracy are measures of the overall performance of the model.

comorbidities, serum creatinine, and RIFLE class on inclusion and then added NGAL to the model to test whether it significantly added to the prediction of the composite outcome (Table 4). We found that age and serum creatinine were individual parameters that contributed to the prediction of the composite outcome. NGAL excretion significantly added to the predictive performance of the model ($P<0.001$). Three measures of the overall performance of the regression model (R square, AUC-ROC, and diagnostic accuracy) increased when NGAL was added to the model (Table 4). The adjusted odds ratio of an NGAL >104 μg/l in the prediction of the composite outcome was 4.2. These data indicate that NGAL levels significantly add to the prediction of an unfavorable clinical course when combined with conventional predictors.

As these data suggested that urinary NGAL levels may add to the RIFLE severity classification in risk stratifying patients with established AKI, we conducted an exploratory analysis on our data set and stratified patients according to RIFLE severity class and NGAL level (Figure 4). The analysis revealed that, within the RIFLE-I class, patients with an NGAL >104 μg/l displayed a markedly higher percentage of poor clinical outcomes, compared with those with an NGAL ⩽104 μg/l ($P=0.001$; Figure 4). Within the RIFLE-R or RIFLE-F class, an NGAL level >104 μg/l was also associated with higher percentages of poor clinical outcomes, but these differences did not reach statistical significance (Figure 4). It should be noted that our study was originally not powered to analyze subsets of patients by RIFLE severity class. Yet, these data strongly suggest that urinary NGAL may be particularly useful in risk stratifying patients with RIFLE-I AKI.

As urinary NGAL levels diagnosed intrinsic versus prerenal AKI and were useful in the prediction of an unfavorable clinical course, we asked in a secondary outcome analysis whether urinary NGAL may also be useful in predicting whether established AKI will be transient or sustained. Therefore we constructed a logistic regression model that included conventional markers and NGAL in the prediction of sustained AKI, defined as RIFLE-AKI lasting more than 3 days. In a conventional prediction model that was adjusted for demographics, comorbidities and established laboratory tests, RIFLE severity class was the single significant predictor of sustained AKI. However, when urinary NGAL was added to RIFLE severity class in a biomarker-assisted model, it independently added to the predictive performance of the model ($P<0.001$). In addition, three measures of the overall performance of the regression model (R square, AUC-ROC, and diagnostic accuracy) increased when NGAL was added to the model (Supplementary Table 1 online). These data indicate that RIFLE severity

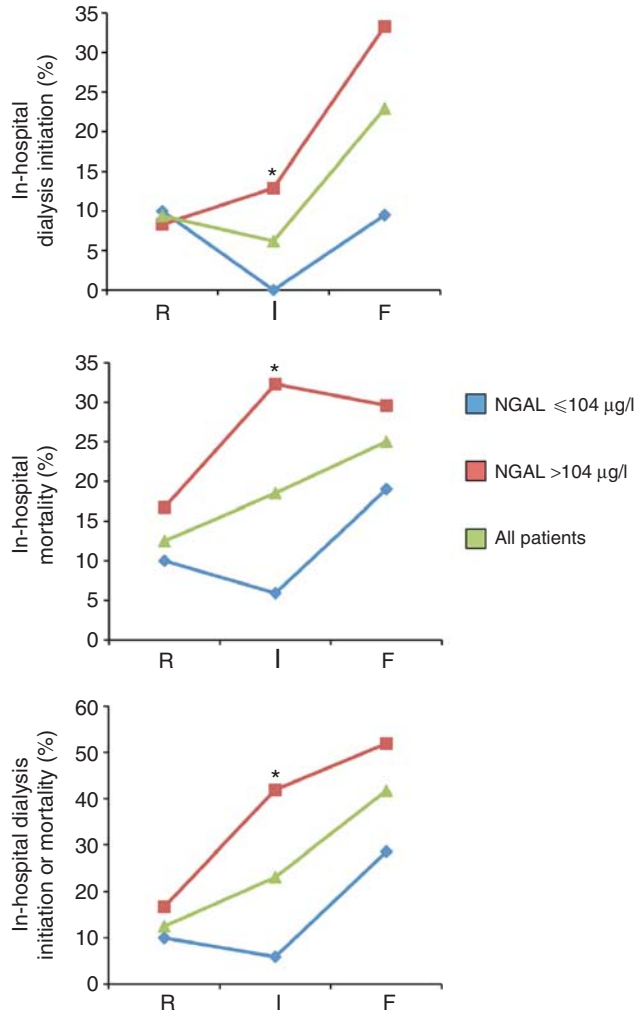

Figure 4 | Sub-stratification of RIFLE severity classes by neutrophil gelatinase-associated lipocalin (NGAL). Patients were stratified by RIFLE severity class (R, I, F) and by NGAL level (cutoff 104 μg/l). Percentages of in-hospital mortality, in-hospital dialysis initiation, or a composite outcome are plotted by RIFLE class and NGAL levels. *$P < 0.05$ versus NGAL ⩽104 μg/l. RIFLE: Risk, Injury, Failure, Loss of function, End stage renal disease.

class and NGAL level are independent predictors of sustained AKI. This further supports the notion that RIFLE class and biomarker levels can be used in a complementary manner to improve clinical risk prediction in established AKI.

DISCUSSION

In a patient cohort with established AKI as defined by RIFLE criteria, urinary NGAL at the time of presentation distinguished intrinsic AKI from prerenal AKI and predicted an unfavorable clinical course. ROC analyses indicated that NGAL performed significantly better than conventional laboratory tests in diagnosing intrinsic AKI. Logistic regression analysis indicated that NGAL was an independent predictor of an unfavorable clinical course and markedly improved the performance of the prediction model. In addition, our study suggests that NGAL may help in stratifying subclasses within the RIFLE severity classification, in particular, within the RIFLE-I severity class.

Although urinary NGAL is well established as a biomarker for the early diagnosis of AKI, our knowledge about its role in established AKI is limited. Our study is the first to systematically group patients with established AKI into distinct diagnostic categories, namely prerenal and intrinsic AKI, and link these categories with biomarker levels. To avoid bias in the diagnostic adjudications, study physicians were strictly blinded to the results of biomarker analyses and followed predefined guidelines to assign patients to diagnostic categories. To ensure a high quality dataset, patients with diagnostic ambivalence were excluded from the analysis. Our analysis of test characteristics of NGAL excretion indicated a good performance of NGAL in discriminating intrinsic AKI and prerenal AKI according to ROC analyses (AUC-ROC 0.87). NGAL performed significantly better in diagnosing intrinsic AKI than serum creatinine, RIFLE class, UC ratio, FeNa, and FeUrea. We also validated cutoffs derived from an independent emergency room study.[23] Analyses of these cutoffs on our patient cohort confirmed that an NGAL ⩽47 μg/l made intrinsic AKI decidedly unlikely, whereas an NGAL >104 μg/l was indicative of intrinsic AKI. These data are also consistent with a previous study carried out in emergency room patients, which showed that NGAL effectively discriminated patients with intrinsic AKI from those with prerenal azotemia, stable CKD, and normal renal function.[20] Notably, urinary NGAL levels had similar test characteristics whether or not they were corrected for urinary creatinine concentrations.

We were surprised to note the poor performance of several conventional indices used to differentiate intrinsic and prerenal AKI. Most remarkably, FeNa did not significantly discriminate prerenal and intrinsic AKI and FeUrea had only marginal performance. The limited diagnostic utility of these urinary indices had been previously acknowledged[24] and, in our cohort, may be explained in part by the high prevalence of congestive heart failure (69.7%) and hypertension (81.4%), as urinary indices are affected by diuretic therapy.

Our study confirmed the known association of AKI etiology and outcomes. Although 33.8% of all patients and 50.7% of patients in the intrinsic AKI group experienced the composite outcome indicating an unfavorable clinical course, only 6.3% of patients with prerenal AKI experienced aspects of the composite outcome. These data show that in our cohort intrinsic AKI is an unfavorable predictor.

We further explored the utility of urinary NGAL in predicting unfavorable clinical outcomes. Several studies have linked high NGAL levels with clinical outcomes, including hemodialysis initiation, progression of AKI, and mortality.[20–22,25,26] We defined a composite outcome for our study, consisting of a step-up in RIFLE severity class, dialysis initiation, or mortality after inclusion. NGAL performed well in predicting this outcome with an ROC of 0.71. This result is similar to the diagnostic performance of NGAL in the prediction of poor clinical outcomes in other cohorts.[27]

Importantly, logistic regression analysis indicated that a conventional prediction model that included age and serum creatinine was markedly improved when urinary NGAL level was added to the model. In a secondary endpoint analysis, NGAL was also a major predictor of sustained AKI (RIFLE-AKI lasting more than 3 days) and independently added to a multiple regression model that also included the main conventional predictor of sustained AKI, RIFLE severity class. These results indicate that the addition of NGAL significantly improves conventional prediction models of unfavorable clinical courses even after AKI has been established. This predictive utility of urinary NGAL may be due—at least in part—to its ability to identify intrinsic damage to the nephron.

One of the strengths of our study in comparison to earlier studies is that urinary NGAL levels were determined on a standardized clinical platform that is, or shortly will be, widely available to clinicians. This state-of-affairs overcomes one of the main limitations of earlier studies, which derived their results based on experimental NGAL assays. However, our study also has limitations. First, this study was carried out in a single center. Second, the adjudicators assigned the diagnostic categories intrinsic AKI versus prerenal AKI based on the clinical information they had available. Thus, these categories could not be based on gold standard measures such as renal biopsy results, but instead relied on creatinine dynamics and on the response to clinical intervention. This resulted in a group of 'unclassifiable' patients. Inspection of baseline criteria, outcomes, and biomarker levels indicates that this unclassifiable group represents an intermediate group of patients that displays neither 'purely prerenal' nor 'purely intrinsic AKI'. We also attempted to conduct an outcome analysis on unclassifiable patients only. However, we did not find a significant association of urinary NGAL or serum creatinine with composite or individual outcomes in these patients, which is likely explained by insufficient statistical power in this relatively small group ($n = 38$) with only few clinical outcomes ($n = 9$). Finally, the conclusion that patients of individual RIFLE severity classes can be sub-stratified using urinary NGAL is based on a *post hoc* analysis of our data set and clearly requires confirmation by additional studies. Nonetheless, our study clearly indicates a value of urinary NGAL in established AKI beyond that of currently used laboratory tests.

MATERIALS AND METHODS

Patient population

The Charité University Ethics Committee approved the study (EA3/011/08) and written informed consent was obtained. All hospitalized patients meeting the RIFLE classification (either a $>50\%$ increase in serum creatinine concentration or a $>25\%$ decrease in glomerular filtration rate compared with baseline)[3] were eligible for assessment and were generally enrolled at the time of nephrology consultation. Two study physicians collected urine and blood samples, and obtained a clinical follow-up from the medical records. The study physicians were not involved in managing the patients. Patients were initially included ($n = 161$) when the study physician confirmed AKI. Patients were excluded from the study when an assignment of the baseline creatinine level was not possible, when sufficient follow-up was not available, or when postrenal obstruction was identified as a cause of AKI ($n = 16$).

Sampling and measurement of renal biomarkers

We obtained initial urine and blood samples and a follow-up urine sample 2 days after enrollment. We centrifuged urine samples at 3000 r.p.m. for 10 min and stored the supernatants at $-80\,^{\circ}C$. Urinary NGAL was measured using ARCHITECT (Abbott Laboratories, Abbott Park, IL) technology, which uses a non-competitive, two anti-analyte antibody sandwich.[28] In this assay, the first antibody is covalently attached to paramagnetic microparticles and the second antibody is covalently attached to acridinium. NGAL measurement was performed using an automated sequence, wherein urine sample and microparticle reagents were incubated together for 18 min, then particles were washed, and the acridinium labeled antibody was added for 4 min. After a second wash, the acridinium label was triggered by peroxide and base. The chemiluminescent signal was calibrated using known quantities of recombinant NGAL. The maximum NGAL concentration of standards was 1500 ng/ml and specimens with greater concentrations were diluted to read within the calibration range. Creatinine, sodium, urea, FeNa, and FeUrea determinations were part of the routine clinical assessments of AKI patients at our institution and were determined by standard automated methods (Jaffé reaction for creatinine, ion selective electrode for sodium, urease/glutamate-based kinetic test for urea). Estimated glomerular filtration rate was calculated with the Modification of Diet in Renal Disease formula.

Diagnosis and outcomes

Diagnostic adjudication was performed by two clinical consultants, who were instructed to use all available data, including the precipitating events, the time course of serum creatinine concentration, the response to treatment including volume resuscitation, modification of diuretic dosage, the use of vasopressors, immunosuppressive drugs, and the result of renal biopsies when available. Consultants were blinded to NGAL levels, but not to other laboratory tests. Baseline serum creatinine was determined by review of the previous 12 months of the medical record, or if unavailable, baseline serum creatinine was assumed from the hospital course. The most likely causes for the increase in creatinine were recorded and patients were classified into the three categories: prerenal AKI, intrinsic AKI, or unclassifiable.

By the inclusion criterion, all patients displayed RIFLE-AKI on the day of enrollment. Patients were classified as 'prerenal AKI', when the increase in serum creatinine concentration had been caused by factors that compromise renal perfusion, and when creatinine rapidly improved to baseline with volume repletion or improvement in cardiac output within 3 days of directed therapy. In select cases ($n = 6$), patients with a slower normalization of serum creatinine (within 7 days) were scored as prerenal, when the slow course was clearly due to the fact that a more careful hydration regimen was warranted (for example, in congestive heart failure). In patients with prerenal AKI, there had to be no pre-enrollment exposure to insults that result in intrinsic kidney damage (for example, nephrotoxin exposure).

Patients were classified as 'intrinsic AKI' when the increase in serum creatinine concentration had been caused by a potential acute tubular necrosis-inducing event, such as prolonged hypotension, sepsis, systemic inflammatory response syndrome, or other insults

associated with structural damage to the kidney. The latter included glomerulonephritis, vasculitis, preeclampsia, interstitial nephritis, contrast nephropathy, multiple myeloma, and thrombotic microangiopathy. In patients with intrinsic AKI, serum creatinine levels did not respond appropriately to fluid resuscitation and/or hemodynamic optimization.

When disagreements between the two clinical consultants occurred, they convened to discuss the case. If that resolved the disagreement, the diagnosis was revised according to the outcome of the discussion. However, when a level of disagreement or uncertainty remained, the patient was placed into the 'unclassifiable' category. These cases also included patients that were exposed to additional clinical events after inclusion that may have affected the serum creatinine time course. Additional ambiguous cases occurred when a potential nephron-damaging event had preceded the onset of RIFLE-AKI, but when creatinine normalized quickly or when the timing of recovery was not clearly discriminatory between prerenal AKI and intrinsic AKI.

The prospective composite outcome of the study was an unfavorable clinical course defined by a step-up in RIFLE severity class (RIFLE-R to RIFLE-I or RIFLE-I to RIFLE-F) within 7 days from the time of inclusion, need for in-hospital renal replacement therapy, or in-hospital mortality. The secondary outcome of the study was 'sustained AKI' defined as RIFLE-AKI that lasted for more than 3 days.

NGAL cutoff levels

Urinary NGAL cutoff levels analyzed in this study were derived from the study on an independent multicentric cohort of 1677 patients admitted to emergency departments in the United States and in Germany.[23] In that study, we had tested whether urinary NGAL levels effectively discriminated patients with intrinsic AKI from those with prerenal AKI, CKD or normal kidney function. We had found that urinary NGAL levels below 47 μg/l (corresponding to the 60th percentile of urinary NGAL levels across the entire cohort) made a diagnosis of intrinsic AKI unlikely, whereas urinary NGAL levels higher than 104 μg/l (corresponding to the 75th percentile of urinary NGAL levels across the entire cohort) were indicative of intrinsic AKI.[23] To validate these cutoffs in an independent cohort, we analyzed test characteristics of these NGAL cutoffs in the current study population. Cutoff levels of the remaining tests (serum creatinine, RIFLE severity class, UC ratio) represent percentiles within the current cohort that correspond to these NGAL cutoffs to ensure comparability among different tests.

Statistical analysis

For statistical analyses, we used PASW Statistics Version 18.0 or 19.0 (SPSS, Chicago, Illinois) and SAS, version 9.1 (SAS Institute, Cary, North Carolina). We compared continuous variables between two groups using Student's *t*-test and categorical variables by using χ^2 tests, rejecting the null hypothesis at $P<0.05$. Data are presented as mean (s.d.) or median (interquartile range). We determined ROC curves and calculated area under the curve including 95% confidence intervals. We compared areas under the ROC curves using nonparametric tests.[29] We calculated sensitivity, specificity, positive predictive value, and negative predictive value, positive likelihood ratio and negative likelihood ratio including 95% confidence intervals using efficient-score method (corrected for continuity).[30] To determine the association of biomarkers (NGAL, FeNa, FeUrea, serum creatinine, and UC ratio), demographic variables (age >70 years, gender, race), comorbid conditions (diabetes, hypertension, congestive heart failure, coronary artery disease, peripheral artery disease, cerebrovascular disease, preexisting CKD), and RIFLE severity class (1 = R, 2 = I, 3 = F), with the composite outcome of unfavorable clinical course or with the secondary outcome of sustained AKI, we used univariate logistic regression analysis followed by multivariate logistic regression with stepwise entry (forward) or exit (backward) of variables to minimize the number of covariates in the model. We then constructed final models based on variables that remained associated with the prediction of the composite outcome (age >70 years, creatinine >205 μmol/l, and NGAL >104 μg/l) and with the prediction of sustained AKI (RIFLE severity class, NGAL >104 μg/l). The overall fit of the logistic regression models was tested using Hosmer and Lemeshow test.

DISCLOSURE

KMSO and TLN have consultation agreements with Abbott. KMSO and SE have participated in advisory board meetings for Tardis Medical Consultancy, Amsterdam, Netherlands. Columbia University has licensed the use of NGAL to Abbott Laboratories and to Biosite/Inverness.

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SUPPLEMENTARY MATERIAL

Table S1. Multiple logistic regression models for the prediction of sustained AKI (RIFLE-AKI lasting more than 3 days).
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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