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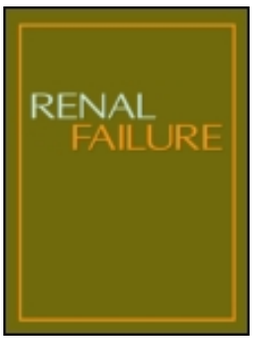

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CLINICAL STUDY

Combination of Renal Biomarkers Predicts Acute Kidney Injury in Critically Ill Adults

Stelios Kokkoris[1], Maria Parisi[1], Sofia Ioannidou[2], Evangelia Douka[1], Chrysoula Pipili[1], Theodoros Kyprianou[1], Anastasia Kotanidou[1] and Serafim Nanas[1]

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Abstract

Objective: Most studies so far have focused on the performance of individual biomarkers to detect early acute kidney injury (AKI) in the adult intensive care unit (ICU) patients; however, they have not determined the predictive ability of their combinations. The aim of this study was to compare the predictive abilities of plasma neutrophil gelatinase-associated lipocalin (pNGAL), urine neutrophil gelatinase-associated lipocalin (uNGAL), plasma cystatin C (pCysC), serum creatinine (sCr), and their combinations in detecting AKI in an adult general ICU population. *Methods*: A total of 100 consecutive ICU patients were included in the analysis. AKI was defined according to RIFLE criteria. Biomarker predictive abilities were evaluated by area under the curve (AUC), net reclassification improvement (NRI), and integrated discrimination improvement (IDI). *Results*: AKI occurred in 36% of patients 7 days post-admission. All three novel biomarkers as well as sCr had moderate predictive abilities for AKI occurrence. The most efficient combinations (pNGAL + sCr and pNGAL + uNGAL + sCr) were selected to participate in the subsequent analyses. Both combinations, when added to a reference clinical model, increased its AUC significantly (0.858, $p = 0.04$). Their NRI (0.78, $p = 0.0002$) was equal to that of pNGAL, but higher than that of the other three biomarkers, whereas their IDI was higher than that of any individual biomarker (0.23, $p = 0.0001$). Both combinations had better specificities, positive likelihood ratios, and positive predictive values than those of any individual biomarker. *Conclusion*: The biomarker combinations had better predictive characteristics compared with those of each biomarker alone.

Keywords: acute kidney injury, NGAL, cystatin C, biomarkers, ICU, acute renal failure

INTRODUCTION

The incidence of acute kidney injury (AKI), previously known as acute renal failure, in critically ill patients is currently rising,[1] with a mortality rate of 50% which has remained constant during the last decades.[2] The lack of an early biomarker is an obstacle for the development of new preventive strategies and timely interventions against AKI.[3] Recent research for the discovery of novel biomarkers with early diagnostic and/or prognostic value has revealed several candidates. A number of studies have investigated the prognostic abilities of neutrophil gelatinase-associated lipocalin (NGAL),[4–11] cystatin C (CysC),[11–16] kidney injury molecule-1,[11] interleukin-18,[10,11] L-type fatty acid-binding protein,[10] and *N*-acetyl-β-D-glucosaminidase[10] for early AKI development in the heterogeneous population of adult critically ill patients, having found only moderate predictive abilities so far. However, none of them has evaluated their combined use in adult intensive care unit (ICU) patients. Because AKI represents a complex, multifactorial, and heterogeneous clinical condition, we hypothesized that a panel of AKI biomarkers would be superior to each biomarker alone.

The main purpose of this study was to compare the predictive abilities of admission plasma neutrophil gelatinase-associated lipocalin (pNGAL), plasma cystatin C (pCysC), urine neutrophil gelatinase-associated lipocalin (uNGAL), serum creatinine (sCr), and their combinations for AKI development within 7 days post-ICU admission.

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MATERIALS AND METHODS

Study Design

This was a prospective observational study of adult patients admitted to a 30-bed general Critical Care Department of a tertiary hospital of Athens from October 2010 to March 2011. All consecutive patients admitted to the ICU were screened for eligibility. The exclusion criteria are shown in Figure 1. The lowest sCr level found within 12 months prior to ICU admission was used as baseline. If it was not available, then the lowest of the on-admission sCr or final ICU sCr was used. A similar approach has been shown to be more accurate than using the Modification of Diet in Renal Disease (MDRD) equation with the same assumed glomerular filtration rate (GFR) to back-calculate a baseline sCr.[18] Patients were enrolled within 12 h of ICU admission at the latest. The protocol was approved by the Institutional Ethics Committee (Scientific Committee of "Evangelismos" General Hospital) and informed consent was obtained from all patients' next-of-kin persons.

AKI was defined using the sCr and urine output criteria of the RIFLE classification,[19] and patients were scored daily for AKI development and RIFLE staging according to the above criteria. Demographics, comorbidities, admission diagnosis, recent exposure to nephrotoxic agents, disease severity scores, routine laboratory data, and outcome measures, such as ICU mortality, renal replacement therapy (RRT) initiation, and ICU length of stay, were also recorded.

Biomarker Measurements

Blood samples were collected within 1 h, at the latest, of enrolment. Arterial blood was sampled in ethylenediaminetetraacetic acid (EDTA) tubes from an arterial line and was centrifuged at 3000 rpm at 4°C for 10 min. The supernatants were stored at −70°C. Urine samples were

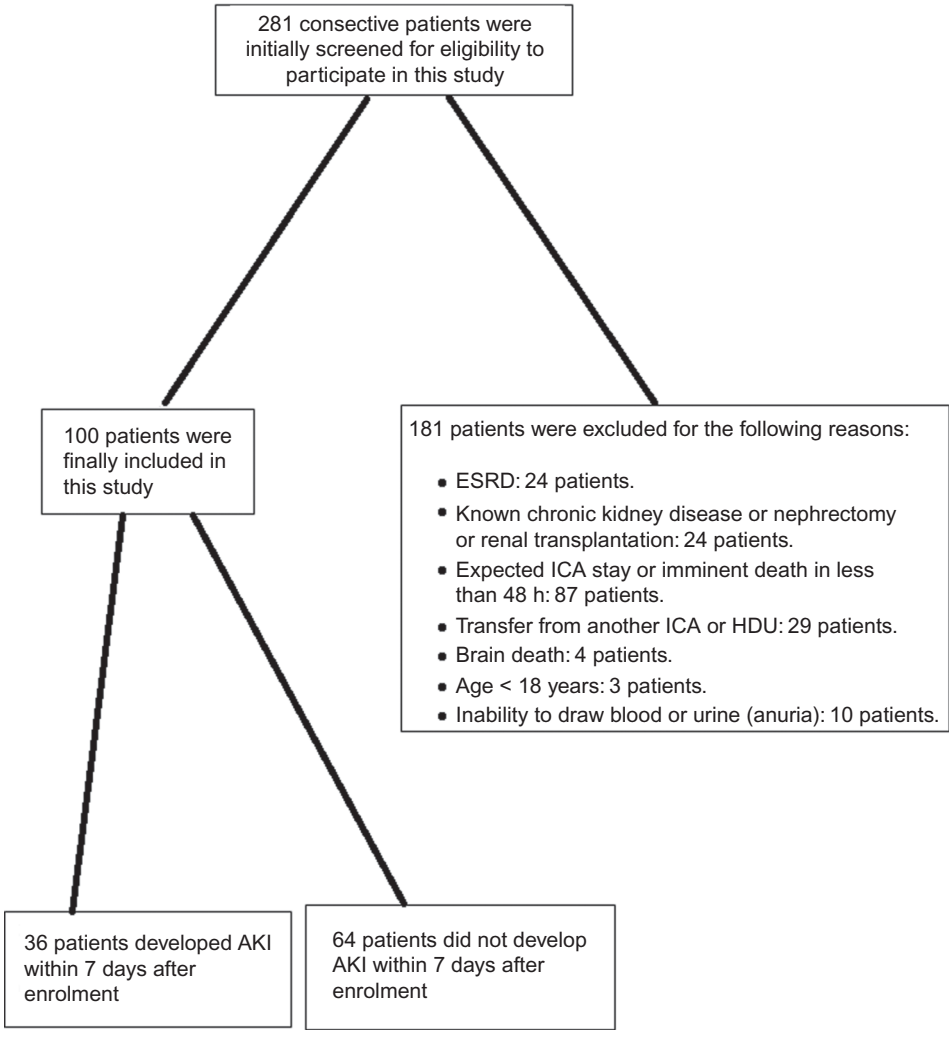

Figure 1. Study flowchart.
Notes: ESRD was defined according to KDIGO (CKD–MBD) Work Group definition: eGFR < 15 mL/h or hemodialysis.[17] Chronic kidney diseases included glomerulonephritis, interstitial renal disease, and obstructive uropathy. AKI, acute kidney injury; ESRD, end-stage renal disease; ICU, intensive care unit; HDU, high-dependency unit.

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also collected within 1 h after enrolment by the urine catheter reservoir and immediately stored at −70°C. pNGAL was measured using the Triage® NGAL test in conjunction with the Triage Meter (Biosite Inc., San Diego, CA, USA), a point-of-care, fluorescence-detected immunoassay. The Triage assay has a detection range of 60–1300 ng/mL with a coefficient of variation of 10–15%. Creatinine levels in both serum and urine specimens were determined by routine methods in the biochemistry laboratory of our hospital and pCysC levels were quantified by using an MNII nephelometer (Dade Behring GmbH, Marburg, Germany). uNGAL was measured by a chemiluminescent microparticle assay using the ARCHITECT platform (Abbott Diagnostics Inc., Abbott Park, IL, USA). Personnel performing the biomarker measurements were blinded to each patient's clinical information.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation, if normally distributed, or median (interquartile range), if the normality assumption did not hold, and their comparisons were made by *t*-test or Mann–Whitney *U*-test, respectively. Categorical variables were expressed as number (%) and compared with the χ^2 or Fischer's exact test. Biomarker values in all statistical analyses refer to time-of-admission samples.

In order to assess the discriminative ability for AKI prediction, univariate and multivariate logistic regressions were used for individual biomarkers and all their possible combinations, respectively. Receiver-operating characteristic (ROC) curves were generated for each biomarker and combination. The areas under the curve (AUC) were compared using the nonparametric method developed by DeLong et al.,[20] and the most efficient combinations (with AUCs higher than those of the individual biomarkers) were included in the subsequent analyses. Optimal cut-off values for AKI detection were determined for each biomarker and their combinations using the Youden's index,[21] based on which sensitivity (Sn), specificity (Sp), positive and negative predictive values, and positive and negative likelihood ratios were calculated.

We quantified the improvement of biomarkers and their combinations on AKI risk prediction with modern statistical methods: the category-free net reclassification improvement (NRI) and integrated discrimination improvement (IDI) indices.[22–24] First, the most efficient clinical model (containing simplified acute physiology score III (SAPS III) and international normalized ratio (INR)) was determined by stepwise backward elimination. Potential predictor variables for AKI development 7 days after ICU admission included age, gender, acute physiology and chronic health evaluation II (APACHE II), sequential organ failure assessment, SAPS III, hemodynamic instability, diabetes mellitus, white blood cell count, INR, lactate, and pH on admission. Multiple logistic regression analyses were conducted to assess the performance of the predictive models while combining individual biomarkers and their combinations with the reference clinical model. Improvements in the model were evaluated by AUC, NRI, and IDI. All variables were log-transformed before entering the logistic regressions because of their skewed distributions.

All tests were two-sided and significance was accepted at $p < 0.05$. MedCalc v.12.2.0 (MedCalc, Mariakerke, Belgium) software was used for AUC comparisons and the calculation of Sn, Sp, predictive values, and likelihood ratios. NRI and IDI were calculated using R v.2.10.1 (R Foundation, Vienna, Austria). All other statistical analyses were performed using SPSS v.12 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient Characteristics

The study flowchart is depicted in Figure 1. Of the 100 patients enrolled, 36 developed AKI within 7 days, 10 needed RRT during ICU stay, while their ICU mortality rate was 33%. Among AKI patients, maximum RIFLE classes were as follows: RIFLE-R 52%, RIFLE-I 36%, and RIFLE-F 12%. Patient's admission characteristics, renal parameters, and outcome measures are shown in Table 1. Patients who developed AKI were older, had higher APACHE II and SAPS III scores, higher baseline and admission renal parameters, and higher mortality and RRT need. uNGAL and pNGAL were moderately correlated on entry to the ICU (Spearman's $r = 0.52$, $p < 0.005$).

Predictive Abilities of Individual Biomarkers and Their Combinations for AKI Development within 7 Days Post-Admission

All three novel biomarkers and sCr had moderate predictive ability for AKI occurrence and none of them had significantly higher AUC over the others, as shown in Table 2. The vast majority of their combinations had higher AUCs than that of each biomarker alone, but only two of these biomarker combinations (pNGAL + sCr and pNGAL + uNGAL + sCr) reached statistical significance ($p < 0.05$ vs. sCr) and were selected to participate in the subsequent analyses (Table 2). Notably, in the multivariate logistic regression model that combined all four biomarkers, only pNGAL and sCr were independently associated with AKI development (Table 2). Table 3 shows the formula for the fitted multiple regression model of each combination, as first described by Han et al.[26,27] The ROC curves of the two combinations and the individual biomarkers are depicted in Figure 2. Table 4 demonstrates various predictive characteristics of each biomarker and their two best combinations based on the ideal cut-off value according to Youden's index. Both combinations had better Sp, positive likelihood ratios [(+) LRs], and positive predictive values (PPVs) than those of any individual biomarker. Of note, the combination of pNGAL with

Table 1. Patient's admission characteristics, renal parameters, and outcomes.

	No AKI ($n = 64$)		AKI ($n = 36$)		p-Value
Demographics					
Age (years)	49	[35.0–66.3]	63	[50.3–80.8]	0.004
Gender (male)	34	(53.1)	23	(63.9)	0.40
Comorbidities					
DM	9	(14.1)	6	(16.7)	0.77
Hypertension	20	(31.3)	10	(27.8)	0.82
CAD	5	(7.8)	7	(19.4)	0.11
COPD	4	(6.3)	3	(8.3)	0.70
HF	2	(3.1)	4	(11.1)	0.18
Cancer	16	(25.0)	9	(25.0)	1.0
Patient type					
Medical	28	(43.8)	19	(52.8)	0.41
Elective surgery	14	(21.9)	3	(8.3)	0.10
Emergency surgery	19	(29.7)	12	(33.3)	0.82
Admission diagnosis					
Neurologic	32	(50.0)	12	(33.3)	0.14
Respiratory	12	(18.8)	12	(33.3)	0.14
Cardiovascular	2	(3.1)	3	(8.3)	0.35
Polytrauma	8	(12.5)	6	(16.7)	0.56
Neurotrauma	9	(14.1)	3	(8.3)	0.53
Gastrointestinal	5	(7.8)	4	(11.1)	0.72
Admisson parameters					
APACHE II (mean SD)	13.9	(5.6)	16.8	(5.2)	0.012
SOFA (mean SD)	6.2	(2.6)	7.2	(3.2)	0.206
SAPS III (mean SD)	54.2	(12.1)	64.6	(14.2)	0.000
INR	1.1	[1.1–1.2]	1.3	[1.1–1.5]	0.01
Glucose (mg/dL)	130	[112.0–168.0]	160.5	[125.5–205.5]	0.004
CRP (mg/L)	5.7	[2.8–15.9]	13.0	[3.4–22.8]	0.117
Lactate (mg/L)	1.3	[0.9–2.3]	1.7	[1.0–2.7]	0.185
WBC ($\times 10^3/\mu L$)	11.8	[9.5–15.3]	13.8	[11.4–20.9]	0.017
Nephrotoxic agents (pre-admission exposure)					
Diuretics	3	(4.7)	2	(5.6)	1.0
Antibiotics	20	(31.3)	9	(25.0)	0.64
Contrasts	12	(18.8)	8	(22.2)	0.80
Chemotherapy	1	(1.6)	4	(11.1)	0.055
Shock	11	(17.2)	13	(36.1)	0.050
Baseline renal parameters					
sCr (mg/dL)	0.7	[0.6–0.8]	0.8	[0.6–1.0]	0.016
eGFR (mL/min/1.73 m^2)	116.7	[88.7–138.4]	88.2	[68.3–125.1]	0.02
Admission renal parameters					
sCr (mg/dL)	0.7	[0.5–0.8]	1.0	[0.7–1.3]	0.000
pUrea (mg/dL)	32.5	[22.0–44.5]	55.5	[40.0–90.8]	0.000
pNGAL (ng/mL)	60.0	[60.0–61.0]	152	[60.0–245.0]	0.000
uNGAL (ng/mL)	29.8	[7.3–80.7]	106.8	[61.0–343.6]	0.000
pCysC (mg/L)	0.8	[0.6–1.0]	1.3	[0.8–1.8]	0.000
Outcome					
ICU mortality	12	(18.8)	21	(58.3)	0.000
RRT need (during ICU stay)	0	(0.0)	10	(27.8)	0.000
Sepsis (within first week)	23	(35.9)	17	(47.2)	0.29
ICU-LOS (days)	15.0	[6.3–23.8]	16.0	[11.3–31.0]	0.052
MV duration (days)	12.5	[4.0–20.0]	15.0	[9.3–27.8]	0.093

Notes: Values are expressed as n (%) or median [IQR], unless otherwise noted. Baseline eGFR was calculated according to MDRD equation.[25] AKI, acute kidney injury; IQR, interquartile range; DM, diabetes mellitus; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; HF, heart failure; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; SAPS III, simplified acute physiology score; INR, international normalized ratio; CRP, C-reactive protein; WBC, white blood cell count; eGFR, estimated glomerular filtration rate; sCr, serum creatinine; pUrea, plasma urea; pNGAL, plasma neutrophil gelatinase-associated lipocalin; uNGAL, urine neutrophil gelatinase-associated lipocalin; pCysC, plasma cystatin C; RRT, renal replacement therapy; LOS, length of stay; MV, mechanical ventilation.

sCr had outstanding values for Sp (97%), (+) LR (20), and PPV (92%).

We then developed the most efficient reference clinical model for AKI prediction, which included SAPS III and INR, using multivariate logistic regression with stepwise backward elimination. This model could predict AKI with reasonable certainty (AUC = 0.756). Each biomarker was then added to the reference model to assess

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Table 2. Predictive performance of each individual biomarker and all their possible combinations for AKI development within 7 days post-admission.

Logistic regression model	p-Value in the model	AUC	95% CI
Univariate models			
pNGAL	0.0001	0.777	0.68–0.85
pCysC	0.0001	0.750	0.65–0.83
uNGAL	0.0003	0.743	0.64–0.82
sCr	0.0001	0.765	0.67–0.84
Multivariate models			
pNGAL	0.0005	0.799	0.71–0.87
+pCysC	0.034		
pNGAL	0.0002	0.814	0.72–0.88
+uNGAL	0.36		
pNGAL	0.0001	0.823	0.73–0.89*
+sCr	0.0016		
pCysC	0.0037	0.759	0.66–0.84
+uNGAL	0.13		
pCysC	0.043	0.776	0.68–0.85
+sCr	0.015		
uNGAL	0.014	0.805	0.71–0.87
+sCr	0.0005		
pNGAL	0.0013	0.801	0.71–0.87
+pCysC	0.053		
+uNGAL	0.85		
pNGAL	0.0004	0.821	0.73–0.89
+pCysC	0.76		
+sCr	0.012		
pNGAL	0.0012	0.835	0.75–0.90*
+uNGAL	0.54		
+sCr	0.0019		
pCysC	0.29	0.807	0.71–0.88
+uNGAL	0.065		
+sCr	0.008		
pNGAL	0.0016	0.822	0.74–0.90
+pCysC	0.89		
+uNGAL	0.58		
+sCr	0.010		

Notes: AKI, acute kidney injury; AUC, area under curve; CI, confidence interval; pNGAL, plasma neutrophil gelatinase-associated lipocalin; pCysC, plasma cystatin C; uNGAL, urine neutrophil gelatinase-associated lipocalin; sCr, serum creatinine. *$p < 0.05$ versus sCr_{AUC}, according to the test of DeLong et al.[20] for AUCs comparison.

improvement in the predictive ability of the model (Table 5). Of the individual biomarkers, only the addition of pNGAL to the model increased the AUC significantly from 0.756 to 0.852 ($p = 0.03$). Moreover, the addition of pNGAL to the reference model improved its predictive ability, as measured by the category-free NRI (0.78, $p = 0.0002$) and IDI (0.16, $p = 0.0004$) indices, more than each of the other three biomarkers did (Table 5). Next, we followed the same procedure with the two biomarker combinations. Both of them, when added to the reference model, increased AUC significantly and slightly more than pNGAL did (0.858, $p = 0.04$); their NRI was equal to that of pNGAL (0.78, $p = 0.0002$), but higher than that of the other three biomarkers, whereas their IDI was higher than that of each individual biomarker (0.23, $p = 0.0001$) (Table 5).

DISCUSSION

We performed a prospective observational study in a heterogeneous adult ICU population, the main finding of which was that biomarker combinations had better performance for AKI prediction compared with each biomarker alone. More specifically, we compared the predictive ability of four renal biomarkers (pNGAL, pCysC, uNGAL, and sCr) and their most efficient combinations for AKI development within 1 week after admission. Each biomarker combination had better AUC, higher IDI, and higher Sp, (+) LR, and PPV, compared with each biomarker alone. The NRI of each combination was equal to that of pNGAL, but higher than that of the other three biomarkers. Of individual biomarkers, pNGAL had higher NRI and IDI compared with those of pCysC, uNGAL, and sCr, although its AUC was not significantly higher than that of the aforementioned three biomarkers. Compared with the two combinations, pNGAL had the same NRI, but lower AUC, IDI, Sp, (+) LR, and PPV.

It is speculated that the increases in NGAL levels after renal tubular injury may serve to limit injury even at the beginning of the insult. In renal tubular injury, human pNGAL levels are increased on the order of 7- to 16-fold, and human uNGAL levels increased by 25- to 100-fold.[28] Synthesis of NGAL protein in the distal nephron and secretion into the urine appears to promote cell survival and proliferation. With respect to pNGAL, it is considered that it originates from distant organs (AKI results in a dramatically increased NGAL mRNA expression in liver and lungs)[29]; from neutrophils, macrophages, and other immune cells as an acute phase reactant, whereas any decrease in GFR could result in its accumulation in systemic circulation.[30] On the other hand, pCysC is primarily a sensitive marker of reduction in GFR[31] and appears to be a good biomarker in the prediction of AKI.[32]

Table 3. Formula for fitted multiple logistic regression model for combining biomarkers.

For AKI ($n = 36$) vs. no AKI (64) combined	
pNGAL, sCr	$-9.1131 + 4.5981 \times \log_{10}$ pNGAL $+ 4.8566 \times \log_{10}$ sCr
pNGAL, uNGAL, sCr	$-8.9421 + 4.2611 \times \log_{10}$ pNGAL $+ 0.2758 \times \log_{10}$ uNGAL $+ 4.7366 \times \log_{10}$ sCr

Note: AKI, acute kidney injury; pNGAL, plasma neutrophil gelatinase-associated lipocalin; pCysC, plasma cystatin C; uNGAL, urine neutrophil gelatinase-associated lipocalin; sCr, serum creatinine.

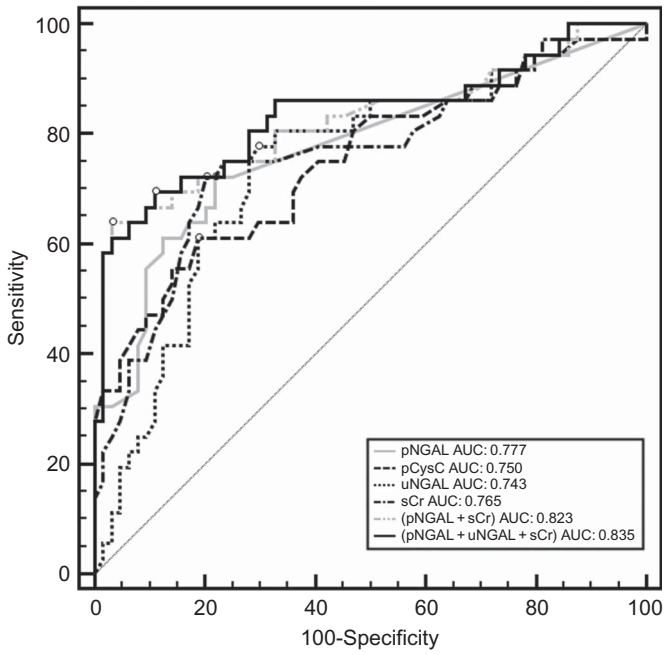

Figure 2. Receiver-operating characteristic (ROC) curves of the individual biomarkers and the two best combinations of them.
Notes: pNGAL, plasma neutrophil gelatinase-associated lipocalin; pCysC, plasma cystatin; uNGAL, urine neutrophil gelatinase-associated lipocalin; sCr, serum creatinine; AUC, area under curve. Open circle indicates the ideal cut-off point of each curve, according to the Youden's index.[21]

NGAL and CysC have been used as markers of AKI in observational studies of adult ICU patients, with AUCs ranging from 0.67 to 0.92[4,5,7–9] for pNGAL, 0.64 to 0.86 for uNGAL,[4,6,8–11] and 0.72 to 0.88 for pCysC,[13,15,16] respectively. The vast majority of the above studies did not use indices of reclassification improvement (NRI and/or IDI), and their predictions were mainly based on AUCs. De Geus et al.[4] reported no significant NRIs for pNGAL and uNGAL, and Siew et al.[6] reported no significant NRI for uNGAL. However, both studies categorized the patients into three groups for NRI estimation and, therefore, their results cannot be compared with ours, which are based on category-free NRI estimation. Moreover, none of the above studies estimated IDI.

Most studies so far have focused on the performance of only one of these biomarkers to detect AKI in critically ill patients, before an sCr rise. However, none of them has determined the predictive ability of their combinations in the adult ICU setting, where the etiology of AKI is multifactorial and not well understood. Because many insults are involved, it is speculated that a single biomarker will be insufficiently sensitive and specific across the full spectrum of AKI, and combinations (panels) of biomarkers with different characteristics may prove more accurate. The utility of biomarker combinations in AKI prediction has been assessed by a number of studies in cardiac surgery patients, who reported improved predictive performance for the various combinations they used.[26,27,33–35] Nevertheless, which are the optimal

Table 4. Predictive characteristics of admission biomarkers and their combinations for AKI development within 7 days post admission.

Variable	Cut-off[a]	Sn	(95% CI)	Sp	(95% CI)	(+) LR	(95% CI)	(−) LR	(95% CI)	PPV	(95% CI)	NPV	95% CI
pNGAL	62 ng/mL	72.2	(54.8–85.8)	78.1	(66.0–87.5)	3.3	(2.6–4.2)	0.36	(0.2–0.7)	65.0	(48.3–79.4)	83.3	71.5–91.7
pCysC	1.04 mg/L	61.1	(43.5–76.9)	81.2	(69.5–89.9)	3.2	(2.4–4.3)	0.48	(0.2–0.9)	64.7	(46.5–80.3)	78.8	67.0–87.9
uNGAL	58.5 ng/mL	77.8	(60.8–89.9)	71.8	(59.2–82.4)	2.7	(2.2–3.5)	0.31	(0.1–0.6)	60.9	(45.4–74.9)	85.2	72.9–93.4
sCr	0.86 mg/dL	72.2	(54.8–85.8)	79.7	(67.8–88.7)	3.5	(2.8–4.5)	0.35	(0.2–0.7)	66.7	(49.8–80.9)	83.6	71.9–91.8
pNGAL, +sCr	0.52[b]	63.9	(46.2–79.2)	96.9	(89.2–99.6)	20.4	(15.9–26.2)	0.37	(0.09–1.6)	92.0	(74.0–99.0)	82.7	72.2–90.4
pNGAL, +uNGAL, +sCr	0.42[b]	69.4	(51.9–83.7)	89.0	(78.8–95.5)	6.3	(5.0–8.0)	0.34	(0.1–0.8)	78.1	(59.7–90.9)	83.8	72.9–91.6

Notes: AKI, acute kidney injury; pNGAL, plasma neutrophil gelatinase-associated lipocalin; pCysC, plasma cystatin C; uNGAL, urine neutrophil gelatinase-associated lipocalin; sCr, serum creatinine; AUC, area under curve; CI, confidence interval; Sn, sensitivity; Sp, specificity; (+) LR, positive likelihood ratio; (−) LR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.
[a]Ideal cut-off value according to Youden's index.
[b]Predictive probability of the multivariate logistic regression model.

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Table 5. Category-free NRI and IDI of including biomarkers and their combinations in the risk factor model to predict AKI development within 7 days post-admission.

Reference clinical model	Added marker or combination	*p*-Value in the model	AUC	ΔAUC	*p*-Value vs. clinical model[a]	NRI (SE), *p*-value (total)	NRI (SE), *p*-value (in AKI patients)	NRI (SE), *p*-value (in no AKI patients)	IDI (SE), *p*-value
SAPS III + INR AUC: 0.756 95% CI: 0.66–0.83	pNGAL	0.0002	0.852	0.097	0.03	0.78 (0.208), 0.0002	0.16 (0.167), 0.31	0.62 (0.125), 0.0001	0.16 (0.045), 0.0004
	pCysC	0.01	0.786	0.03	0.40	0.45 (0.208), 0.031	0.11 (0.167), 0.51	0.34 (0.125), 0.006	0.056 (0.028), 0.049
	uNGAL	0.058	0.781	0.025	0.37	0.38 (0.208), 0.068	0.16 (0.167), 0.31	0.22 (0.125), 0.08	0.032 (0.018), 0.09
	sCr	0.002	0.816	0.06	0.13	0.28 (0.208), 0.18	0.0 (0.167), 1.0	0.28 (0.125), 0.025	0.10 (0.034), 0.003
	pNGAL +sCr	0.0008	0.858	0.102	0.04	0.78 (0.208), 0.0002	0.22 (0.167), 0.18	0.56 (0.125), 0.0001	0.23 (0.051), 0.0001
		0.01							
	pNGAL +uNGAL +sCr	0.002	0.858	0.102	0.04	0.78 (0.208), 0.0002	0.22 (0.167), 0.18	0.56 (0.125), 0.0001	0.23 (0.051), 0.0001
		0.98							
		0.01							

Notes: NRI, net reclassification improvement; IDI, integrated discrimination improvement; SE, standard error; AKI, acute kidney injury; AUC, area under curve; CI, confidence interval; ΔAUC, difference in AUCs between the model including the added marker or combination and the reference clinical model; SAPS III, simplified acute physiology score III; INR, international normalized ratio; pNGAL, plasma neutrophil gelatinase-associated lipocalin; uNGAL, urine neutrophil gelatinase-associated lipocalin; pCysC, plasma cystatin C; sCr, serum creatinine

[a]According to the method of DeLong et al.[20] for AUCs comparison.

combinations of biomarkers remain unclear. We found that the combinations of novel biomarkers with a conventional one (sCr) are promising in the heterogeneous population of a general adult ICU. Larger studies that will identify the most efficient and cost-effective biomarker combinations and will develop and validate AKI scoring systems are required.

The strengths of our study are the following: first, we used modern indices of risk stratification improvement and did not rely solely on AUC comparisons. Second, all novel biomarkers were determined by methods that are, or shortly will be, widely available to clinicians. We acknowledge that our study has certain limitations. First of all, it is a single-center study and its findings are not as generalizable as those of a multicenter study. Second, the relatively small sample size could reduce the power of the study. Third, the enrolment of only 100 patients from a total of 281 patients who were initially screened could have led to selection bias, and because the main reason of exclusion was an expected ICU stay less than 48 h, the more severely ill patients might have been selected. Fourth, the extent to which AKI per se contributes to pNGAL levels could be confounded by the release of NGAL into the bloodstream in patients with SIRS or sepsis.

We analyzed our data by using both absolute uNGAL and normalized to urine creatinine (uNGAL/uCr) values, and found that their AUCs did not significantly differ. More specifically, the AUC of uNGAL/uCr for AKI prediction was 0.69 (CI 95%: 0.58–0.80, $p < 0.15$ vs. absolute uNGAL). However, when it was used in the various multivariate models instead of absolute uNGAL, the performances of its combinations were inferior to those of absolute uNGAL. Therefore, we decided to select absolute uNGAL for all further statistical analyses. Throughout the literature, the authors use either the absolute uNGAL or uNGAL/uCr value in a spot urine sample. To date, there are no strong arguments in favor of one of them. However, there are some pitfalls when reporting absolute (raw) concentrations of a urinary biomarker; for example, oliguria will cause an increase, and polyuria a decrease, in its absolute concentration, the production and excretion rates of which are constant. Therefore, normalizing a urinary biomarker concentration to uCr takes into account the differences in urinary flow rate. On the other hand, when normalizing, one must also take into consideration the assumption that uCr excretion is constant. However, Waikar et al.[36] by using computer simulations of creatinine kinetics under nonsteady-state conditions, such as AKI, found that the uCr excretion rate changes over time. Consequently, normalization by uCr may result in an underestimation or overestimation of the biomarker excretion rate depending on the clinical context. The previous study found that the most accurate method to quantify urine biomarkers requires the collection of timed urine specimens to estimate the actual excretion rate.[36] However, this method has got some practical difficulties; for

example, many patients in the ICU are anuric, and therefore, urine collection would be impossible. Moreover, it is time-consuming compared to the spot urine sample, in which case the clinician can have the results quite shortly.

In conclusion, when we evaluated the predictive ability for AKI occurrence only by means of AUC, none of the three novel biomarkers was superior to each other or to sCr, while the two combinations were better than sCr. However, when we assessed the predictive performance with modern indices of reclassification improvement, namely the category-free NRI and the IDI, the two combinations were superior not only to sCr but also to the other three novel biomarkers. Regarding each biomarker individually, pNGAL was superior to the other three. Early AKI detection by a combination of renal biomarkers could lead to timely interventions (e.g., avoidance of nephrotoxic drugs, early RRT initiation) to prevent the detrimental effects of AKI in critically ill patients.

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