Test Characteristics of Urinary Biomarkers Depend on Quantitation Method in Acute Kidney Injury

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ABSTRACT

The concentration of urine influences the concentration of urinary biomarkers of AKI. Whether normalization to urinary creatinine concentration, as commonly performed to quantitate albuminuria, is the best method to account for variations in urinary biomarker concentration among patients in the intensive care unit is unknown. Here, we compared the diagnostic and prognostic performance of three methods of biomarker quantitation: absolute concentration, biomarker normalized to urinary creatinine concentration, and biomarker excretion rate. We measured urinary concentrations of alkaline phosphatase, \(\gamma\)-glutamyl transpeptidase, cystatin C, neutrophil gelatinase–associated lipocalin, kidney injury molecule–1, and IL-18 in 528 patients on admission and after 12 and 24 hours. Absolute concentration best diagnosed AKI on admission, but normalized concentrations best predicted death, dialysis, or subsequent development of AKI. Excretion rate on admission did not diagnose or predict outcomes better than either absolute or normalized concentration. Estimated 24-hour biomarker excretion associated with AKI severity, and for neutrophil gelatinase–associated lipocalin and cystatin C, with poorer survival. In summary, normalization to urinary creatinine concentration improves the prediction of incipient AKI and outcome but provides no advantage in diagnosing established AKI. The ideal method for quantitating biomarkers of urinary AKI depends on the outcome of interest.


The concentration of urinary biomarkers of AKI is influenced by variation in urinary concentration within and between individuals. In diabetes mellitus, the urinary albumin:creatinine ratio is an accepted method of accounting for variation in albumin concentration arising from variations in urine flow rate due to hydration, diuresis, or concentration changes induced by antidiuretic hormone or tubular injury. Consequently, many studies investigating urinary biomarker utility for the detection of AKI have normalized biomarkers to creatinine. The accuracy of this method is compromised by tubular secretion of creatinine and variations in urine creatinine excretion in non-steady state when the GFR changes. Creatinine excretion may also vary because of diurnal creatinine production, physical activity, emotional stress, diet, muscle mass (hence age, sex, and body weight variation), and disease state. A recent theoretical analysis showed that normalizing to creatinine may briefly amplify the biomarker signal soon after a reduction in GFR. To date, relatively few studies have evaluated biomarker performance by both absolute and normalized concentration, and there is no consensus on how data should be
reported. This hinders the comparison of biomarkers between trials and adds uncertainty regarding how biomarkers should be utilized in clinical practice. Alternatives to normalizing biomarker concentration to creatinine include using the absolute concentration or quantifying the excretion rate. Intuitively, the excretion rate may also account for variation in water re-absorption and urine flow rate. In addition, total biomarker excretion in AKI might more accurately reflect the mass of injured tubular cells, a function of both severity and duration, parameters associated with long-term mortality.10–12 We compared the three biomarker quantitation methods, the absolute and normalized concentrations, and the excretion rate in the diagnosis of AKI, prediction of AKI, death, and the need for renal replacement therapy (RRT) in patients admitted to two general intensive care units (ICUs). In addition, we assessed the association of estimated total biomarker excretion over 24 hours to AKI severity and mortality.

RESULTS

Demographic Profile

Of the 528 recruited patients, 484 had 4-hour creatinine clearance measurements on ICU admission from which urine output volumes could be obtained for the calculation of biomarker excretion rates. These patients comprised the analyzed cohort (Table 1). Of patients with missing clearances, 30 were a subcohort of cardiothoracic surgical patients in whom no clearance on admission to ICU postoperatively was planned, five had collection errors, and nine missed samples. Patients with AKI at anytime within 48 hours of admission were older and had higher baseline plasma creatinine, severity scores, and lengths of stay in the ICU. AKI was more common in those with abdominal aortic aneurysm rupture and repair (P<0.0001) and in sepsis (P=0.002), and was less common in those with neurologic events (P=0.001). Of the 484 patients, we measured the following on admission: γ-glutamyl transpeptidase (GGT) and alkaline phosphatase (AP) in 484, IL-18 and kidney injury molecule-1 (KIM-1) in 481, cystatin C (CysC) in 480, and neutrophil gelatinase-associated lipocalin (NGAL) in 449 patients. Compared with those with NGAL measurement, patients with missing NGAL data had more AKI on admission (P<0.05) but no differences in dialysis, subsequent AKI postadmission (AKIN48 or RIFLE24), or mortality. In this study, AKIN48 refers to the Acute Kidney Injury Network (AKIN) definition at any time within 48 hours. RIFLE24 refers to sustained AKI as defined by RIFLE (Risk, Injury, Failure, Loss, ESRD) criteria that occur for >24 hours at any time within 7 days.

Because there were no differences in biomarker performance with or without the inclusion of the erythropoietin (EPO) cohort, patients triaged to receive EPO were included to increase power.13 Table 2 presents data from all patients for the absolute and normalized biomarker concentration, excretion rate on admission to the ICU, and estimated total excretion over 24 hours (24-hour excretion) of all biomarkers. There were significant differences between the absolute and normalized concentrations and the excretion of CysC, NGAL, IL-18, and KIM-1 between those with and without AKI on admission.

Biomarker Quantitation Method Comparison

Figure 1 depicts the difference between the area under the receiver-operator characteristic (ROC) curve for the normalized concentration (referent value) and the area under the curve (AUC) for the absolute concentration and the excretion rate for each outcome measure. For a diagnosis of AKI on admission, absolute concentration performed better than the normalized concentration or excretion rate (Figure 1 and Table 3). The AUCs for absolute concentration were greater than for both normalized concentration and excretion rate for all biomarkers (P=0.003) except KIM-1. For prediction of 7-day and 365-day mortality, normalizing to creatinine produced the highest AUCs. Normalized concentrations of AP, GGT, and NGAL better predicted RRT than their excretion rates (P=0.04). Medians (interquartile range) of urine creatinine concentrations were greater in those who had AKI on admission compared with those without AKI (7.4 [3.5–10.8] versus 4.9 [2.2–8.7] mmol/l, P<0.0001). Patients with AKI also had lower urine flow rates compared with those without AKI (0.99 [0.54–1.8] versus 1.44 [0.80–2.8] ml/min, P<0.0001).

AP and GGT were not diagnostic of AKI or prognostic of outcome (Table 3). Nevertheless, the pattern of the AUC differences between the absolute and normalized concentrations and the excretion rate of AP and GGT were consistent with the other biomarkers. The time courses of biomarker excretion rates from putative time of insult were similar to those of normalized concentrations as previously published (Supplemental Figure 1).14

In the cohort of patients without AKI on admission (n=339), there were no differences in performance between absolute and normalized concentrations in the prediction of AKI within 48 hours (AKIN48) or sustained AKI within 7 days (RIFLE24). However, the normalized concentrations had higher AUCs than the excretion rates (P=0.01) (Figure 1). The creatinine excretion rate on admission was lower in those who developed AKI within 48 hours compared with those who did not (6.2±4.0 versus 8.1±4.6 μmol/min, P=0.001). The excretion rate increased in this cohort over the first 48 hours (repeated measures ANOVA, P=0.003) (Figure 2). Forty-eight hours later, urine creatinine excretion rates were similar in both groups (8.0±5.3 versus 9.1±5.4 μmol/min, P=0.16).

Estimated Total Biomarker Excretion over 24 Hours

The 24-hour excretion of CysC, NGAL, IL-18, and KIM-1 increased with the increasing severity of injury stage (maximum AKIN) reached within 48 hours (one-way ANOVA, P<0.0001) (Figure 3). Differences between successive AKIN stages of increasing severity were demonstrated for NGAL.
Table 1. Demographic profile

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cohort (N=484)</th>
<th>No AKIN48 (n=266)</th>
<th>AKIN48 (n=218)</th>
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<tr>
<td>Age (yr)</td>
<td>60±17</td>
<td>57±17</td>
<td>63±17</td>
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<td>Male sex</td>
<td>294 (60.7)</td>
<td>158 (59.4)</td>
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<td>Weight (kg)</td>
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<td>77±18</td>
<td>82±20</td>
<td>0.01</td>
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<td>APACHE II score</td>
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<td>17±6</td>
<td>19±6</td>
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<td>SOFA score</td>
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<td>5.5±2.5</td>
<td>7.3±2.9</td>
<td>&lt;0.0001</td>
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<tr>
<td>Baseline plasma creatinine (mU/L)</td>
<td>72 (60–90)</td>
<td>70 (60–86)</td>
<td>80 (60–98)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD, n (%), or median (lower quartile–upper quartile).

*Baseline plasma creatinine was determined from a chart review as previously described.13 Creatinine measurements before ICU admission were used if available (n=229). When the measurements were not available, the lowest creatinine at follow-up (n=38), last creatinine in the ICU (n=138), or creatinine on admission to the ICU (n=79) was used.

Patient survival was assessed by the extent of biomarker excretion over 24 hours ranked by tertiles (Figure 4). Only NGAL demonstrated a significant association between 24-hour excretion and survival over 365 days (log-rank test, P=0.04). We adjusted for age, sex, sepsis, EPO therapy, and Acute Physiology, Age, Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA) severity scores. After adjustment, patients with greater NGAL excretion (higher tertile, 24-hour excretion >184 μg) had a higher mortality at 365 days compared with those with lower NGAL excretion (lower tertile, 24-hour excretion of <40 μg) (hazard ratio [HR], 2.10; 95% confidence interval [95% CI], 1.21–3.65; P=0.009) (Table 4).

The 24-hour excretion of NGAL predicted 30-day and 365-day mortality, whereas CysC predicted 30-day mortality only (Table 4). To determine the added contribution of the excretion data to known clinical predictors of mortality, NGAL and CysC were added separately to a multivariable logistic regression that included age, sex, sepsis, APACHE II and SOFA scores, EPO therapy, and AKIN48. NGAL remained independently predictive of 365-day mortality (odds ratio, 1.43; 95% CI, 1.03–1.99), along with age and APACHE II score (Table 5). However, the inclusion of NGAL only marginally improved the AUC of the base model from 0.66 (0.61–0.72) to 0.68 (0.62–0.74) (P=0.31). For prediction of 30-day mortality, the addition of NGAL increased the AUC from 0.61 (0.53–0.69) to 0.69 (0.62–0.74) (P=0.035), and the addition of CysC increased the AUC from 0.60 (0.52–0.68) to 0.64 (0.57–0.72) (P=0.042).

**DISCUSSION**

Individual variation in water handling by surviving nephrons may compromise the utility of urinary biomarker concentration in the detection and prediction of AKI and mortality. Normalizing to urine creatinine and measuring the excretion rate can be used to account for variations in water reabsorption. Waikar et al.1 recently suggested that the excretion rate may provide a better measure of biomarker performance. This is the first study to address this hypothesis directly. For all injury biomarkers, absolute
concentration performed best in the diagnosis of AKI, whereas the normalized concentration performed best for the prediction of death, dialysis, and subsequent development of AKI. The excretion rate on admission was not a better diagnostic or prognostic biomarker than the absolute or normalized concentration.

**Biomarker Quantitation Method Comparison**

For all biomarkers, normalizing to creatinine or measuring the excretion rate diagnosed AKI on ICU admission more poorly than the absolute concentration, which may be explained as follows. In evolving AKI, the creatinine excretion rate decreases because the GFR decreases. As plasma creatinine increases, creatinine excretion rates increase, asymptoting toward the original rate before the GFR decreases (Figure 5). The data support this explanation. Excluding patients with obvious AKI on admission, creatinine excretion on admission was lower in those who developed AKI within 48 hours compared with those who did not. Creatinine excretion then increased over the first 48 hours to the level at admission, similarly to those who had not developed AKI at all (Figure 2). In theory, an acute loss of GFR will result in an immediate decrease in filtered creatinine but should have no effect on induced (e.g., IL-18 and KIM-1) or preformed (AP and GGT) urinary biomarker excretion. However, in the case of NGAL, final urinary concentration will be the complex sum of both distal tubular marker excretion and induced filtration and reabsorption. Consequently, normalization to urine creatinine will amplify urinary biomarker signal impossibly.

### Table 2. Urinary biomarkers on ICU admission

<table>
<thead>
<tr>
<th>Urinary Biomarker</th>
<th>All Patients</th>
<th>AKI on Admission</th>
<th>No AKI on Admission</th>
<th>P</th>
</tr>
</thead>
</table>
| AP
  normalized (U/mmol Cr) | 0.87 (0.47–1.7) | 0.71 (0.35–1.8) | 0.94 (0.51–1.7) | 0.06 |
  concentration (U/L) | 4.1 (2.0–9.0) | 5.0 (2.1–11) | 4.0 (2.0–8.3) | 0.22 |
  ER (×10^{-3} U/min) | 6.1 (3.1–11) | 4.8 (2.1–12) | 6.3 (3.7–11) | 0.08 |
  24-h excretion (U) | 8.4 (5.0–14) | 6.9 (4.1–13) | 8.7 (5.4–14) | 0.01 |
| GGT
  normalized (U/mmol Cr) | 13 (7.1–26) | 17 (7.6–44) | 11 (7.0–22) | 0.001 |
  concentration (U/L) | 74 (28–160) | 115 (51–290) | 58 (53–130) | <0.0001 |
  ER (×10^{-3} U/min) | 85 (44–180) | 110 (48–240) | 78 (42–150) | 0.01 |
  24-h excretion (U) | 120 (75–210) | 130 (72–260) | 120 (78–200) | 0.38 |
| CysC
  normalized (mg/mmol Cr) | 0.02 (0.01–0.19) | 0.06 (0.01–0.61) | 0.02 (0.01–0.10) | <0.0001 |
  concentration (mg/L) | 0.12 (0.05–1.0) | 0.44 (0.09–2.6) | 0.09 (0.04–0.37) | <0.0001 |
  ER (µg/min) | 0.16 (0.06–1.3) | 0.42 (0.09–3.6) | 0.12 (0.06–0.59) | <0.0001 |
  24-h excretion (mg) | 0.23 (0.10–2.0) | 0.88 (0.19–5.9) | 0.17 (0.08–0.72) | <0.0001 |
| NGAL
  normalized (µg/mmol Cr) | 7.7 (2.3–41) | 20 (5.1–99) | 5.6 (1.8–24) | <0.0001 |
  concentration (ng/ml) | 47 (11–260) | 149 (40–500) | 26 (6.7–140) | <0.0001 |
  ER (ng/min) | 56 (15–300) | 170 (33–760) | 38 (13–150) | <0.0001 |
  24-h excretion (µg) | 78 (2–310) | 220 (69–740) | 56 (21–190) | <0.0001 |
| IL-18
  normalized (ng/mmol Cr) | 0.54 (0.001–35) | 9.7 (0.001–84) | 0.001 (0.001–23) | <0.0001 |
  concentration (pg/ml) | 4.4 (0.05–240) | 110 (0.05–600) | 0.05 (0.05–156) | <0.0001 |
  ER (pg/min) | 1.2 (0.1–260) | 84 (0.1–600) | 0.1 (0.1–160) | <0.0001 |
  24-h excretion (ng) | 68 (0.1–300) | 115 (2.3–620) | 36 (0.14–240) | <0.0001 |
| KIM-1
  normalized (ng/mmol Cr) | 86 (38–220) | 140 (59–380) | 74 (31–180) | <0.0001 |
  concentration (pg/ml) | 500 (130–1300) | 870 (350–3500) | 360 (100–1000) | <0.0001 |
  ER (pg/min) | 580 (240–1600) | 953 (375–2994) | 470 (200–1100) | <0.0001 |
  24-h excretion (ng) | 1800 (710–3800) | 2700 (1200–4900) | 1500 (560–3200) | <0.0001 |
| Urine creatinine (mmol/L) | 5.5 (2.6–9.5) | 7.4 (3.5–10.8) | 4.9 (2.2–8.7) | <0.0001 |

Data are expressed as median (lower quartile–upper quartile). Cr, creatinine; concentration, absolute biomarker concentration; normalized, normalized biomarker concentration; ER, biomarker excretion rate; 24-h excretion, estimated total biomarker excretion over 24 hours.

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The urine creatinine concentration was higher in those who had AKI on admission compared with those without AKI. Consequently, when biomarker concentrations are normalized, the relative differences between the non-AKI and AKI cohorts are reduced, resulting in fewer true positives and a reduced AUC (Figure 6). A higher urine creatinine concentration could be due to increased tubular secretion of creatinine or, more likely, to increased reabsorption of water (decreased free water clearance) because of an increased antidiuretic hormone activity. Whereas the biomarker concentration was higher in the AKI than the non-AKI cohort, the urinary flow rate was lower, consistent with the second alternative. Therefore, the ratios of the AKI to non-AKI biomarker excretion rate and of the normalized concentration were lower than the ratio of the absolute concentration; this combination reduced the AUCs.

Normalized concentration also best predicted hard outcomes such as mortality and the need for dialysis. Without the bias introduced by the presence of AKI on one side of the ROC equation (e.g., among survivors compared with nonsurvivors, in which the ratio of AKI to non-AKI patients was similar), there was improvement in predictive performance with respect to subsequent AKI, mortality, and need for RRT. This most likely resulted from the normalization accounting for the kidney concentrating capacity in the denominator (urine creatinine) and capturing cellular injury in the numerator (biomarker concentration).

In the prediction of the development of subsequent AKI (AKIN48 or RIFLE24), the AUC for all biomarkers was greater for the normalized concentration compared with the absolute concentration or excretion rate, irrespective of whether the AUC was significantly different. This may be due to decreased excretion of urine creatinine as a result of reduced GFR, with resulting amplification of the signal of these biomarkers. Normalization to urine creatinine amplifies the biomarker signal when there is a change of GFR, irrespective of whether the biomarker is itself increased by injury. Although it may potentially be clinically useful as an amplified signal, using normalized values alone (i.e., without knowing the absolute concentration) conceals the mechanism of biomarker signal increase. We suggest reporting both absolute and normalized concentrations.
normalized concentrations to facilitate understanding of the biomarker data.

Biomarker prediction of RIFLE24 was generally better than for AKIN48. RIFLE24 represents, on average, more severe AKI than AKIN48. This is because in the majority of patients, the change in GFR is greater and more sustained because an increase of 50% requires a larger change in GFR than an increase of 0.3 mg/dl if baseline plasma creatinine is >0.6 mg/dl (87.4% of patients in this cohort). In addition, AKIN diagnosis requires only a single time point, whereas RIFLE24 requires at least two time points 24 hours apart.

The pattern of relative performance of absolute concentration and excretion rate to normalized concentration was similar for all biomarkers, even when the biomarkers were not significantly diagnostic or prognostic. The individual performance of these biomarkers in this cohort was discussed in detail in our recent publication. The generally poor performance of these markers remains an important issue that must be addressed. This poor performance compared with earlier studies can be explained by the heterogeneity of the cohort with respect to time, cause of injury, and baseline renal function. Normalizing to urine creatinine or measuring excretion rate does not turn a poor biomarker into a good one, or a good biomarker into a better one. Given the small differences observed with each approach, the issue of whether to normalize seems academic, at least with these markers. Assuming that an ideal biomarker is available, the question arises as to what properties or temporal patterns of expression such a biomarker needs, given the pattern of creatinine excretion in injury. There is no easy answer to this question. The more we understand about biomarker performance in heterogeneous groups, the harder it is to provide a simple solution, such as normalization. An ideal biomarker is probably one that changes measurably in real time with the degree of injury. However, if we use new high-sensitivity troponins as an analogous example, the absence of a baseline in most patients means that serial measurements remain essential to exclude moderate myocardial injury.

We speculate that the same conclusion will be reached with biomarkers of injury in AKI. Nevertheless, this study demonstrates that the optimal method of assessing urinary AKI

### Table 3. Biomarker performance (AUC) in patients with and without AKI on admission

<table>
<thead>
<tr>
<th>Urinary Biomarker</th>
<th>AKI on ICU Admission</th>
<th>AKIN48*</th>
<th>RIFLE24*</th>
<th>RRT</th>
<th>Mortality at 7 d</th>
<th>Mortality at 365 d</th>
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<tbody>
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<td>AP</td>
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<td></td>
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<tr>
<td>n</td>
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<td>73</td>
<td>22</td>
<td>12</td>
<td>49</td>
<td>119</td>
</tr>
<tr>
<td>normalized</td>
<td>0.45 (0.39–0.50)</td>
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<td>0.60 (0.42–0.77)</td>
<td>0.62 (0.54–0.71)</td>
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<td>0.57 (0.51–0.63)</td>
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<td>12</td>
<td>49</td>
<td>119</td>
</tr>
<tr>
<td>normalized</td>
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<td>ER</td>
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<td>10</td>
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<td>0.69 (0.57–0.80)</td>
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<tr>
<td>concentration</td>
<td>0.71 (0.66–0.77)</td>
<td>0.51 (0.43–0.60)</td>
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<td>0.82 (0.65–0.98)</td>
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<tr>
<td>ER</td>
<td>0.67 (0.61–0.72)</td>
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<td>0.60 (0.48–0.73)</td>
<td>0.80 (0.63–0.96)</td>
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<td>normalized</td>
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<td>0.55 (0.47–0.63)</td>
<td>0.75 (0.63–0.87)</td>
<td>0.80 (0.65–0.95)</td>
<td>0.67 (0.58–0.75)</td>
<td>0.56 (0.50–0.62)</td>
</tr>
<tr>
<td>concentration</td>
<td>0.63 (0.57–0.69)</td>
<td>0.52 (0.44–0.59)</td>
<td>0.70 (0.57–0.82)</td>
<td>0.80 (0.65–0.95)</td>
<td>0.64 (0.55–0.73)</td>
<td>0.55 (0.49–0.62)</td>
</tr>
<tr>
<td>ER</td>
<td>0.60 (0.54–0.65)</td>
<td>0.51 (0.43–0.58)</td>
<td>0.68 (0.55–0.81)</td>
<td>0.81 (0.66–0.96)</td>
<td>0.65 (0.56–0.74)</td>
<td>0.55 (0.49–0.61)</td>
</tr>
<tr>
<td>KIM-1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>n</td>
<td>145</td>
<td>73</td>
<td>22</td>
<td>12</td>
<td>49</td>
<td>119</td>
</tr>
<tr>
<td>normalized</td>
<td>0.66 (0.60–0.71)</td>
<td>0.54 (0.46–0.61)</td>
<td>0.61 (0.48–0.74)</td>
<td>0.69 (0.52–0.86)</td>
<td>0.56 (0.47–0.64)</td>
<td>0.58 (0.52–0.64)</td>
</tr>
<tr>
<td>concentration</td>
<td>0.68 (0.63–0.73)</td>
<td>0.51 (0.44–0.59)</td>
<td>0.56 (0.43–0.68)</td>
<td>0.65 (0.47–0.82)</td>
<td>0.50 (0.42–0.59)</td>
<td>0.56 (0.50–0.62)</td>
</tr>
<tr>
<td>ER</td>
<td>0.65 (0.59–0.70)</td>
<td>0.47 (0.39–0.54)</td>
<td>0.46 (0.34–0.58)</td>
<td>0.61 (0.44–0.78)</td>
<td>0.48 (0.40–0.57)</td>
<td>0.54 (0.48–0.60)</td>
</tr>
</tbody>
</table>

Data represent AUC of the ROC curve with 95% confidence intervals. AKI on ICU admission; AKIN definition on ICU admission; AKIN48, AKIN definition at any time within 48 hours, RIFLE24, sustained AKI as defined by RIFLE criteria that occur for >24 hours at any time within 7 days; normalized, normalized concentration; concentration, absolute concentration; ER, excretion rate.

*In the cohort without AKI on admission.
biomarkers depends on the outcome being assessed. For diagnosis of AKI on admission to ICU, absolute concentration was the best method. However, for prediction of AKI, or for mortality, normalization to urine creatinine was best. Although the choice whether to normalize to urinary creatinine may be made according to whether a clinician is attempting to diagnose existing AKI or prognose future AKI, it is likely that both measurements would be made for many patients in whom the timing of injury is unknown (e.g., sepsis patients in the ICU).

Estimated Total Biomarker Excretion over 24 hours
The 24-hour excretion of CysC, NGAL, IL-18, and KIM-1 was positively associated with AKI severity. Differences between successive AKIN stages of increasing severity were demonstrated only for NGAL. Presumably, total excretion of NGAL is a reflection of the mass of injured tubular cells in AKI. Increasing concentration of urinary NGAL has been previously associated with severity of AKI in critically ill children and cardiac surgery patients.17–19 The added value of calculating 24-hour excretion is that it integrates change over time, which captures the severity and duration of injury.

In addition to severity of injury, inclusion of duration of injury increased prediction of long-term mortality in diabetes and postcardiac surgery patients.10–12 Because it captures both severity and duration of injury, we postulated that 24-hour excretion was predictive of long-term mortality. Of the six biomarkers, only 24-hour excretion of NGAL was associated with long-term mortality. Patients with a higher 24-hour excretion of NGAL (above a threshold of >184 μg) were 2.1 times more likely to die within 365 days compared with those with a lower excretion. NGAL also remained independently

Figure 2. Creatinine excretion rate over time from ICU admission. After exclusion of patients with AKI on admission, creatinine excretion rate on admission was lower in those who developed AKI within 48 hours compared with those who did not (*P=0.001). The excretion rate later increased over the first 48 hours (repeated measures ANOVA, P=0.003). Forty-eight hours later, the excretion rates were similar in both groups (P=0.16).

Figure 3. Estimated total biomarker excretion over 24 hours compared with maximum AKIN severity stage within 48 hours. The boxes show the median and interquartile ranges, whereas the whiskers show the 10th–90th percentile in each stage. One-way ANOVA significance levels: (A) AP, P=0.04; (B) GGT, P=0.048; (C) NGAL, P<0.0001; (D) CysC, P<0.0001; (E) IL-18, P<0.0001; and (F) KIM-1, P<0.0001. *Different from previous stage with post hoc Fisher’s least squares difference analysis (P<0.05).
predictive of long-term mortality. To our knowledge, this is the first large study to examine AKI biomarkers predicting long-term outcomes at 365 days. For a more proximate mortality, both NGAL and CysC were associated with poorer survival at 30 days. Furthermore, the addition of NGAL and CysC to other mortality risk factors improved the predictive performance. If these results are supported in larger multicenter studies and across a broad demographic base, then 24-hour excretion of CysC and NGAL may be useful as outcome measures in AKI intervention trials. This is analogous to the use of the relative average value of creatinine (RAVC) metric of functional change in randomized trials in preference to categorical metrics such as AKIN or RIFLE.\(^20\) The RAVC captures both extent and duration of functional change in a single variable. A mild correlation between 24-hour excretion with RAVC at 24 hours was observed, which further supports a relationship between the extent of injury and functional loss (Supplemental Table 1). The analogy is that biomarker excretion can integrate duration and severity of injury, whereas RAVC integrates both duration and severity of functional change.

Sepsis is the leading contributing factor to AKI in the ICU.\(^21,22\) Because sepsis seems to influence urinary CysC,\(^23\) IL-18,\(^24,25\) and NGAL,\(^26,27\) we investigated the association between severity of AKI in sepsis and nonsepsis. The 24-hour excretion of CysC,
were not available in 7% of patients. Because there was a higher

This study has several limitations. First, NGAL measurements


diseases such as sepsis and in

nonsepsis cohort, and these biomarkers remained associated

with AKI and AKI severity in the sepsis cohort. In the presence

of sepsis and inflammation, plasma concentrations of IL-18

and NGAL increased due to release from activated macrophages,

Kupffer cells, activated neutrophils, and injured epithelia. It is

possible that increased plasma concentrations of IL-18 and NGAL

lead to increased urinary concentrations. We previously showed

that increased urinary CysC concentrations are independently

associated with sepsis in this cohort. Furthermore, increased

urinary NGAL and CysC in sepsis could result from reduced

reabsorption due to competition with albumin for megalin transport

at the proximal tubule.16,31,32

Study Limitations
This study has several limitations. First, NGAL measurements

were not available in 7% of patients. Because there was a higher

proportion of patients with AKI on admission in this 7% than

in the remaining 93% of patients, some caution is required

in comparing the performance of NGAL in the diagnosis of

AKI with the other biomarkers. Second, the urinary flow rate

measurements are potentially inaccurate. Urine flows over 4

hours were recorded at three separate time points. Averaging

urine flow over a longer period would limit the observation of

rapid changes in biomarker excretion rate, whereas collection

over a shorter time period might enhance the effect of fluid

loading or diuresis on urine flow rate and hence confound the

interpretation of the biomarker excretion rate. Third, because

the excretion rate is calculated from the urine flow rate, it is

already a “corrected” term and accounts for variation in water

reabsorption and urine flow rate. This form of normalization

is perhaps best visualized as the time taken to acquire sufficient

volume to measure flow (4 hours in our case) so it is not a

“snapshot in time” like the absolute concentration or the

normalized concentration. Fourth, serum creatinine–based

definitions of AKI were used in this study despite known

limitations of creatinine. The association of 24-hour excretion

with AKI severity might be better demonstrated by histology

(extent of structural injury) or by inulin clearance (extent of

functional loss). Fifth, the choice of baseline plasma creatinine

is known to affect determination of AKI outcomes. In a sub-

cohort of patients with known baselines (available from pre-

ICU admission data), the relative differences in biomarker

AUCs between normalized, absolute, and excretion rate
generally follow the same patterns as for the entire cohort

(Supplemental Table 2). Sixth, in 79 patients with no pre-

ICU baseline creatinine, the lowest creatinine was on admis-

tion to the ICU and was used as a baseline, which required

these patients to be classified as non-AKI on admission. It is

possible that some of these patients were misclassified. Finally,

we only measured the biomarkers within the first 24 hours.

Estimating the total excretion over longer periods of time (e.g.,

over 48–72 hours and with more frequent sampling) may

provide a clearer association with long-term outcome.

Normalizing to urinary creatinine improved the performance

of urinary AKI biomarkers in predicting the development

of AKI, death, and need for dialysis. The normalized

urinary biomarker concentration thus remains the method of

choice for early detection of incipient AKI. However, when

AKI had already been established long enough to increase

plasma creatinine, absolute concentration alone was a better

marker of injury than the normalized concentration. The 4-hour

excretion rate did not improve performance in the diagnosis

of AKI; however, estimated 24-hour biomarker excretion was

strongly associated with AKI severity and, for NGAL and CysC,

with survival. These associations with severity and survival sug-

gest that the 24-hour excretion may be a useful surrogate out-

come measurement in clinical trials of AKI. The ideal method

for standardizing urinary AKI biomarkers depends on the out-

come being assessed. However, observational studies should

present results on the basis of both absolute and normalized

concentrations and, where possible, excretion rates.

Figure 5. Schematic of the effect of an abrupt decrease in function
in a 70-kg male patient with a production rate of creatinine of 1
mg/min and an initial GFR of 100 ml/min. (A) Plasma creatinine
increases slowly. (B) GFR decreases suddenly. (C) Urine creatinine
excretion rate initially decreases and then recovers to the same rate
as before when plasma creatinine increases. This is because the
creatinine excretion rate is the product of plasma creatinine and
creatinine clearance (approximately by GFR). Based in part on
Figure 2 in Moran and Myers.15 Adapted by permission from
Macmillan Publishers Ltd: [KIDNEY INTERNATIONAL] (Ref. 15),
copyright 1985.
This was a retrospective analysis of urinary biomarker data from the two-center Early Intervention in Acute Renal Failure randomized, controlled trial of high-dose EPO for AKI prevention in the ICU. This study was approved by the multiregional ethics committee of New Zealand (MEC/050020029) and registered under the Australian and New Zealand Clinical Trials Registry (ACTRN01260600032550; http://www.actr.org.au).

Urinary concentrations of AP, GGT, CysC, NGAL, IL-18, KIM-1, and creatinine were measured on admission to the ICU (time 0) and at 12 and 24 hours postadmission (Figure 7). Plasma creatinine was measured daily for up to 7 days. AP, GGT, CysC, and creatinine concentrations were assayed immediately as described previously. NGAL, KIM-1, and IL-18 samples were stored at −80°C until batch analysis using a NGAL ELISA Kit 036 (AntibodyShop, Grusbakken, Denmark), microsphere-based xMAP technology (Luminex, Austin, TX, USA) with polyclonal antibodies raised against the human KIM-1 ectodomain, and a human IL-18 ELISA kit (Medical and Biologic Laboratories, Nagoya, Japan), respectively.

The normalized biomarker concentrations were derived by dividing the biomarker concentration by the urinary creatinine concentration (millimoles per liter). The 4-hour urine creatinine collections were collected from 0–4 hours, 12–16 hours, and 24–28 hours (Figure 7). An average urine hourly flow rate for each interval was calculated. Urinary biomarker excretion rates were derived by multiplication of urinary biomarker concentrations at times 0, 12, and 24 hours by the average urinary flow rate over each subsequent 4-hour period. The excretion rate between these periods was linearly interpolated. The total biomarker excretion over 24 hours (24-hour excretion) for each biomarker (i.e., integration of excretion rate with respect to time) was calculated using the trapezoidal rule (the sum of shaded areas A and B, Figure 7).

A measured baseline plasma creatinine was determined from a chart review as previously described. Briefly, creatinine measurements before ICU admission were used if available (n=229). When the measurements were not available, the lowest creatinine at follow-up (n=38), last creatinine in the ICU (n=138), or creatinine on admission to the ICU (n=79) was used. AKI status and maximum severity stage reached were determined according to the AKIN definition ($\geq 0.3$ mg/dl or $\geq 50\%$ increase in plasma creatinine above baseline) on admission to the ICU (AKI on ICU admission) and any time within 48 hours (AKIN48). Sustained AKI was defined by the RIFLE criteria ($\geq 50\%$ increase in plasma creatinine for $>24$ hours at any time within 7 days).
(RIFLE24). Other outcomes were as follows: the need for RRT within 7 days and death within 7, 30, and 365 days.

The performance of absolute and normalized concentrations and excretion rate on admission to the ICU in the diagnosis or prediction of outcome was assessed by comparison of the AUC of ROC for each parameter using the DeLong method. For the prediction of AKI outcomes (AKIN48 and RIFLE24), the cohort was restricted to patients without AKI on admission (n=339).

The association between the estimated total biomarker excretion over the first 24 hours with severity of injury and 365-day mortality was also determined. Severity of injury was defined by both a categorical variable (maximum AKIN stage within 48 hours of ICU admission inclusive of those with AKI on admission) and a continuous variable (relative average value of creatinine or RAVC). A sensitivity analysis of patients stratified according to the presence of sepsis was conducted. Sepsis was defined by the ICU physicians and required two or more systemic inflammatory response syndrome criteria, as well as suspected or confirmed bacterial or viral infection.

Statistical Analyses
Statistical analysis was performed using PASW software (version 18.0; IBM, Somers, NY) and PRISM 5.0 software (GraphPad, La Jolla, CA). All of the CIs presented are 95%. The 24-hour excretion was log-transformed before conducting ANOVA for association with AKIN severity stages. Post hoc Fisher’s least significant difference analyses were performed for all significant associations with ANOVA (P<0.05). Associations of 24-hour excretion with RAVC were analyzed by Spearman’s correlation. Tertiles of 24-hour excretion for each biomarker were determined for Kaplan–Meier and Cox regression survival analyses. HRs for each tertile were calculated relative to each biomarker were determined for Kaplan–Meier survival analyses. HRs for each tertile were calculated relative to each biomarker.

Figure 7. Time course for urine spot sampling and 4-hour collection. The average excretion rate was calculated from these three time points. Estimated total excretion over 24 hours was calculated using the trapezoidal rule (the sum of shaded areas A and B).

ACKNOWLEDGMENTS
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DISCLOSURES
P.D. is a co-inventor on patents involving NGAL as a biomarker of chronic and acute kidney disease, C.L.E. is a co-inventor on patents involving IL-18, and J.V.B. is a co-inventor on patents involving KIM-1.

REFERENCES


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