Blood Kidney Injury Molecule-1 Is a Biomarker of Acute and Chronic Kidney Injury and Predicts Progression to ESRD in Type I Diabetes

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ABSTRACT

Currently, no blood biomarker that specifically indicates injury to the proximal tubule of the kidney has been identified. Kidney injury molecule-1 (KIM-1) is highly upregulated in proximal tubular cells following kidney injury. The ectodomain of KIM-1 is shed into the lumen, and serves as a urinary biomarker of kidney injury. We report that shed KIM-1 also serves as a blood biomarker of kidney injury. Sensitive assays to measure plasma and serum KIM-1 in mice, rats, and humans were developed and validated in the current study. Plasma KIM-1 levels increased with increasing periods of ischemia (10, 20, or 30 minutes) in mice, as early as 3 hours after reperfusion; after unilateral ureteral obstruction (day 7) in mice; and after gentamicin treatment (50 or 200 mg/kg for 10 days) in rats. In humans, plasma KIM-1 levels were higher in patients with AKI than in healthy controls or post-cardiac surgery patients without AKI (area under the curve, 0.96). In patients undergoing cardiopulmonary bypass, plasma KIM-1 levels increased within 2 days after surgery only in patients who developed AKI (P < 0.01). Blood KIM-1 levels were also elevated in patients with CKD of various etiologies. In a cohort of patients with type 1 diabetes and proteinuria, serum KIM-1 level at baseline strongly predicted rate of eGFR loss and risk of ESRD during 5–15 years of follow-up, after adjustment for baseline urinary albumin-to-creatinine ratio, eGFR, and Hb1Ac. These results identify KIM-1 as a blood biomarker that specifically reflects acute and chronic kidney injury.
pathophysiology of kidney injury. The actin cytoskeleton architecture is disrupted in renal microvascular endothelial cells, with loss of cell-cell and cell-matrix adhesion junctions, and endothelial cells are detached from the basement membrane; this facilitates KIM-1 movement into the circulation. In the present study, in both rodent and human AKI and mouse and human CKD, we show that increased levels of KIM-1 can be detected in the blood and serve as a biomarker of kidney injury.

First, we evaluated whether KIM-1 was elevated in the mouse model of ischemic kidney injury. KIM-1 was quantitated in plasma and urine specimens collected from mice challenged with incremental periods (10, 20, and 30 minutes) of bilateral ischemia, followed by 24 hours of reperfusion. The extent of kidney damage was assessed by histologic analysis and changes in serum creatinine. At 24 hours, after 10 minutes of ischemia, kidney tissues showed focal tubular injury with apoptosis and necrosis, along with brush border loss in the S3 segment of the proximal tubule. These features were more prominent in mice that were subjected to longer periods of ischemia (20 or 30 minutes), where damage included tubular necrosis, intratubular casts, and brush border loss (Figure 1A). Immunohistochemical analysis revealed an increase in KIM-1 protein expression in injured tubular cells, which correlated with increased bilateral ischemic time (Figure 1B). Plasma creatinine was significantly increased by more than 10-fold in mice subjected to 30 minutes of ischemia/reperfusion but did not significantly change in mice subjected to 10 or 20 minutes ischemia/reperfusion (Figure 1C). Urinary KIM-1 levels were significantly elevated after 10, 20, or 30 minutes of ischemia by >16-fold (8.5 ± 3.3 ng/mg urinary creatinine), >48-fold (25.1 ± 10.7 ng/mg urinary creatinine), and >60-fold (31.4 ± 9.5 ng/mg urinary creatinine), respectively, compared with sham-operated mice (0.52 ± 3.3 ng/mg urinary creatinine), consistent with a high sensitivity of urinary KIM-1 levels to detect renal injury in mice (Figure 1C).

To measure KIM-1 in serum and plasma specimens, we first established an appropriate assay for rodents and humans. Accurate measurement of blood biomarkers poses several technical challenges because blood contains a high content of potentially interfering proteins and different dynamic ranges of biomarker levels compared with urine. To this end, we have rigorously re-evaluated and validated both rodent and human KIM-1 assays that we previously developed for the quantitation of serum and plasma KIM-1 levels by characterizing reproducibility, assay range, spike recovery, interference, and linearity of dilution (Supplemental Table 1). As shown in Figure 1C, compared with plasma KIM-1 levels in sham-operated mice (15 ± 2.1 pg/ml), KIM-1 levels were significantly elevated by >7-fold (112 ± 18 pg/ml), >33-fold (502 ± 17 pg/ml), and >65-fold (987 ± 15 pg/ml) 24 hours after reperfusion and after 10, 20, or 30 minutes of ischemia, respectively.

We next evaluated the sensitivity of plasma KIM-1 in detecting injury at earlier time periods. Mice were challenged with 30 minutes of bilateral ischemia and plasma and urine samples collected 3, 6, 12, 24, 48, 72, 96, 120, and 144 hours after reperfusion. Plasma creatinine levels were increased within 6 hours compared with their preoperative levels or sham-operated mice (Figure 1D). Urinary KIM-1 and plasma KIM-1 levels were significantly elevated within 3 hours compared with their preoperative levels or 3-hour values in sham-operated mice, and the levels remained elevated at 12, 24, 48, and 96 hours after reperfusion (Figure 1D).

Further, we have evaluated the utility of plasma KIM-1 levels in detecting kidney injury in a mouse model of chronic kidney injury. Mice were subjected to unilateral ureteral obstruction and plasma and urine samples were collected on day 7. Plasma and urinary KIM-1 levels increased in these mice but plasma creatinine did not change (Figure 1E).

To evaluate the specificity of plasma KIM-1 to renal injury, mice were treated with carbon tetrachloride (CCL4), a known hepatotoxicant. A single dose of CCL4 (10% CCL4, 0.5 ml/kg) resulted in liver necrosis, whereas no liver damage was observed in vehicle-treated mice (Figure 1F, upper panels). No histopathologic changes in kidney morphology (Figure 1F, lower panels) and no significant alterations in plasma creatinine, urinary KIM-1, or plasma KIM-1 occurred in CCL4-treated mice (Figure 1G). Thus, plasma KIM-1 was not affected by liver injury.

We further investigated whether plasma KIM-1 levels can serve as a biomarker in gentamicin-induced kidney injury in a different rodent species, rats. Rats were injected with gentamicin, 50 or 200 mg/kg per day, for 10 continuous days. Plasma, urine, and kidney tissues were collected on day 11. The extent of kidney damage was assessed by histopathologic analysis and changes in plasma creatinine. After 10 days of treatment with 50 mg/kg gentamicin per day, kidney tissues showed brush border loss, focal tubular necrosis, and interstitial inflammation (Figure 1H). In rats treated with daily doses of 200 mg/kg gentamicin, there was extensive diffuse cell necrosis (Figure 1, H and I). The lumens of the tubules were filled with casts and dead cells. Plasma creatinine was significantly elevated in rats treated with 200 mg/kg but not those treated with 50 mg/kg gentamicin (Figure 1I). Consistent with the histopathologic changes, both urinary (Figure 1K) and plasma (Figure 1L) KIM-1 levels were significantly increased after 10 daily doses of 50 or 200 mg/kg gentamicin in rats. Thus, plasma KIM-1 is a sensitive marker of ischemia and toxin-induced injury to the proximal tubule in mice and rats.

We extended our observations from animal models to humans to evaluate whether plasma KIM-1 is elevated in patients with AKI. Plasma and urine samples were obtained from 48 healthy volunteers, 16 post-cardiac surgery (CS) patients without AKI who were admitted to the intensive care unit (ICU), and 28 patients who developed AKI following cardiac surgery (n = 22) or after admission to the ICU due to other causes (n = 6). AKI samples were chosen to be close in time to peak serum creatinine values. AKI was defined using Kidney

Figure 1. Increase in plasma KIM-1 levels in experimental models of kidney injury in mice and rats. (A) Male BALB/c mice were subjected to 0 (sham), 10, 20, or 30 minutes of bilateral ischemia by clamping the renal pedicles for the time indicated. Urine, blood, and tissue were collected 24 hours after reperfusion. Periodic acid-Schiff staining of kidney sections indicated no injury in sham-operated mice, whereas loss of brush border, necrosis, and sloughing of cells into the tubular lumen were found in postischemic mice. (B) Immunohistochemical staining.
Disease Improving Global Outcomes (KDIGO) criteria as a ≥50% increase in plasma creatinine over baseline within 7 days or an increase in serum creatinine by 0.3 mg/dl within 2 days. An increase in serum creatinine concentration is currently used for the diagnosis of functional AKI, although it has limited sensitivity and specificity.4,14

Demographic characteristics, clinical descriptions, serum creatinine, urine albumin, and urinary and plasma KIM-1 values are shown in Table 1 and Supplemental Table 2. Plasma KIM-1 levels were significantly higher in patients with AKI than healthy volunteers (P<0.001) and patients who had cardiac surgery but did not develop AKI by creatinine criteria (Figure 2A). There was little overlap in plasma KIM-1 levels between patients with AKI and healthy controls. The slightly higher levels in some of the cardiac surgery patients without AKI may reflect subclinical kidney injury not identified as AKI using creatinine criteria or may reflect underlying subclinical CKD. Ongoing proximal tubule injury might be expected in some of these patients because 52% of cardiac surgery/ICU patients without AKI had CKD stage 3 or higher with a mean eGFR of 52 ml/min per 1.73 m². The area under the receiver-operating characteristic curve (AUC-ROC) of plasma KIM-1 for identifying AKI from all these populations, including both healthy volunteers and CS/ICU patients without AKI, was 0.96 (95% confidence interval [95% CI], 0.92 to 1.02; P<0.001), while the AUC-ROCs were 0.98 (95% CI, 0.97 to 1.00; P<0.001) (Figure 2C) for normalized urinary KIM-1 and 0.91 (95% CI, 0.85 to 0.97; P<0.001) for non-normalized urinary KIM-1 (Supplemental Figure 1B). The difference between the AUC-ROC of plasma and urinary KIM-1 was not statistically significant (P=0.31). Plasma KIM-1 was positively correlated with normalized urinary KIM-1 (r=0.43; P<0.001) (Figure 2D) and non-normalized urinary KIM-1 (r=0.24; P=0.02) (Supplemental Figure 1C). Urinary albumin-to-creatinine ratios were significantly higher (P<0.001) in CS/ICU patients without AKI than in non-hospitalized normal volunteers. Both plasma and urinary KIM-1 were positively correlated with normalized and non-normalized urinary albumin concentration (r=0.33 [P=0.001] for pKIM-1; r=0.35 [P<0.001] for uKIM-1), respectively (Figure 2, E and F). Plasma KIM-1 levels were also correlated with plasma creatinine (r=0.58; P<0.001) (Figure 2G).

To obtain information on the time course of plasma KIM-1 elevation in humans with AKI, we collected plasma and urine samples from patients (Table 2) before cardiopulmonary bypass (CPB) surgery; at the end of CPB; and then at 4 hours, 12 hours (urine only), and daily for 5 days after CPB. KIM-1 and albumin were measured in samples from nine patients with and nine patients without AKI. The time required for diagnosis of AKI (KDIGO criteria, stage 1) was a median of 3 days (range, 2–6 days). Plasma KIM-1 levels at day 2 were significantly elevated versus baseline levels in patients with AKI (P<0.01) compared with patients who did not develop AKI at this time. The AUC-ROC was 0.74 (95% CI, 0.48 to 0.91) (Figure 2H). The AUC-ROC for plasma, urinary KIM-1, and urinary albumin at various time points after CPB are provided in Supplemental Table 3. Normalized urinary KIM-1 levels were elevated on day 1 and significantly elevated at day 2 in patients who developed AKI compared with baseline levels (P=0.003) and levels in patients without AKI (P<0.02) (Figure 2I). Urinary albumin levels, normalized to urinary creatinine, fell and then rose slightly in patients with and without AKI, but did not differ statistically compared with baseline levels at any time point in patients with AKI (Figure 2J).

Blood KIM-1 levels were also evaluated in two groups of patients with various stages of CKD. The first group included clinic patients with CKD due to various causes (Supplemental Tables 4 and 5), and the second group included a cohort of 124 patients with type 1 diabetes and proteinuria (>500 mg albumin/24 hours) with longitudinal follow-up. Characteristics of the groups are provided in Supplemental Table 4. In both groups, blood KIM-1 levels increased with increasing CKD stage (Figure 3, A–C). Whereas the first group did not have follow-up, the group of patients with type 1 diabetes and proteinuria were followed for 5–15 years to ascertain the rate of eGFR loss using serial measurements of serum creatinine and the occurrence of ESRD. Figure 3D of KIM-1 on kidney tissues obtained from sham-operated mice and mice that underwent 10, 20, and 30 minutes of ischemia/reperfusion. (C) Plasma creatinine and urinary plasma KIM-1 in mice 24 hours after challenge with different durations of bilateral ischemia (n=6 per group). (D) Plasma creatinine and urinary plasma KIM-1 levels were assessed in male BALB/c mice at different times after sham surgery or after reperfusion following 30 minutes of bilateral ischemia (n=6 per group). (E) Male BALB/c mice were subjected to UUO by obstructing the ureter. Urine and blood were collected on day 7 after UUO. Plasma creatinine, urinary KIM-1, and plasma KIM-1 were measured (n=4 per group) *P<0.001. (F) Male BALB/c mice were administered one dose of 10% CCl₄ (0.5 ml/kg). Mice were euthanized 48 hours after CCl₄ administration and evaluated for liver (upper two panels) or kidney (lower two panels) toxicity by histopathology after periodic acid-Schiff staining (n=3 per group). (G) Plasma creatinine, normalized urinary KIM-1, or plasma KIM-1 concentration in vehicle (Veh) and CCl₄-treated mice (n=3 per group) *P<0.001. (H) Male Sprague-Dawley rats were administered 0.9% saline or 50 or 200 mg/kg gentamicin daily for 10 days and euthanized on day 11. Hematoxylin and eosin staining of kidney sections revealed no injury in vehicle-treated rats, whereas there was loss of brush border, necrosis, and sloughing of cells into the tubular lumen in gentamicin-treated rats. Tubular necrosis score (I), plasma creatinine (J), urinary KIM-1 normalized to urine creatinine (K), and plasma KIM-1 (L) in rats administrated gentamicin at 0, 50, or 200 mg/kg per day for 10 days. *P<0.001, #P<0.05; n=5). Scale bars, 50 μm. Error bars reflect SEM.
shows the strong relationship of baseline serum KIM-1 levels with rate of eGFR decline during the 5–15 year follow-up period with a median follow-up of 10 years, in 107 diabetic patients with stages 1–3 CKD at baseline; Spearman correlation coefficient=0.52 (P<0.001). The association of baseline serum KIM-1 levels with rate of eGFR loss was the strongest and highly statistically significant (P<0.001) in multiple regression analysis when other baseline characteristics, such as eGFR, urinary albumin-to-creatinine ratio, and hemoglobin A1c were considered. During the follow-up period, 24 of the 107 patients who had stage 1–3 CKD at baseline developed ESRD.15

To further examine the association of baseline serum KIM-1 with risk of ESRD, Kaplan–Meier survival analysis was performed. As shown in Figure 3E, after 12.5 years of follow-up, only 20% of patients with serum levels of KIM-1 below the median (97 pg/ml) progressed to ESRD compared with 63% of patients with baseline KIM-1 above the median (P<0.01). This finding was confirmed in multivariate Cox regression analysis. The effect of baseline serum level of KIM-1 on hazard ratio of ESRD was significant (P<0.01) when other baseline covariates, such as eGFR, urinary albumin-to-creatinine ratio, and hemoglobin A1c, were included. The size of the KIM-1 fragment in plasma and urine in patients with AKI and CKD was similar (molecular mass, approximately 90 kD). This is the predicted size of the cleaved ectodomain, and both plasma and urine KIM-1 is recognized by the same antibody (Figure 3F).

Currently, no other blood biomarker specifically reflects kidney proximal tubule injury. Blood KIM-1 reflects the integrated result of kidney proximal tubule injury over a period of time and nicely complements urinary levels in patients to evaluate acute or chronic kidney injury. Plasma KIM-1 may be particularly suitable for detecting chronic ongoing injury. Although spot urinary KIM-1 concentration, normalized to urinary creatinine concentration, is very attractive as a biomarker given the stability of KIM-1 and the easy accessibility of urine specimens, urinary excretion can vary widely over time in patients with AKI, such that a spot collection may not reflect these variations.16 A method that reflects a time-averaged production of a kidney injury biomarker would be a welcome complement to the urine measurement.

In both mice and rats, the increase in plasma KIM-1 levels directly reflected the degree of histologic and functional kidney injury. Both plasma and urinary KIM-1 levels were significantly elevated within 3 hours after injury in mice, indicating that both are early and sensitive biomarkers of kidney injury. Plasma KIM-1 levels are elevated in the unilateral ureteral obstruction model of fibrosis, while plasma creatinine is normal, indicating that plasma KIM-1 can also serve as a marker of chronic kidney injury. While healthy kidney tissue expresses very low or undetectable levels of KIM-1, the mRNA of one KIM-1 variant has been reported to be expressed by the liver.17 Our studies using CCl4, a known hepatotoxicant, indicate that plasma KIM-1 is not changed with liver toxicity.

In humans, plasma KIM-1 was significantly elevated in patients with established AKI compared with healthy volunteers and hospitalized individuals without AKI after cardiac surgery. Because the definition of AKI relies on changes in plasma creatinine, an insensitive and nonspecific indicator of acute kidney proximal tubule injury, the diagnostically performance of any biomarker compared with plasma creatinine as a gold standard has to be interpreted in that light.14 Tubular injury may not be associated with an increase in plasma creatinine concentration, and an increase in creatinine does not necessarily reflect injury.14 The AUC-ROC of plasma KIM-1 was 0.98 when patients with established AKI were compared with only healthy volunteers in the analysis, excluding the cardiac surgery non-AKI group, some of whom may have had subclinical injury and of whom 52% had CKD stage 3 or higher. The AUC-ROC of plasma and urinary KIM-1 did not significantly differ, although our sample size was limited. Compared with urinary KIM-1 normalized to creatinine (AUC-ROC, 0.98), use of absolute KIM-1 levels resulted in a lower AUC-ROC (0.91) and weaker correlation with plasma KIM-1 (r=0.23)

### Table 1. Characteristics of participants with and without AKI in cross-sectional study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Volunteers (n=48)</th>
<th>CS Patients without AKI (n=16)</th>
<th>CS/ICU Patients with AKI (n=28)</th>
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</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>34±1</td>
<td>74±2</td>
<td>74±2</td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>24 (50)</td>
<td>10 (63)</td>
<td>18 (65)</td>
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<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>White</td>
<td>20</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>African American</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Asian and others</td>
<td>13</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Baseline plasma creatinine (mg/dl)</td>
<td>0.88±0.02</td>
<td>1.32±0.1</td>
<td>1.23±0.1</td>
</tr>
<tr>
<td>Albumin-to-creatinine ratio (mg/g urinary creatinine)</td>
<td>5.2 (2.09 to 7.5)</td>
<td>71.7 (12.8 to 130.6)</td>
<td>193.7 (118.4 to 269)</td>
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<tr>
<td>Baseline eGFR (ml/min per 1.73 m²)²</td>
<td>93.8±2.6</td>
<td>51.7±3.8</td>
<td>60.1±4.4</td>
</tr>
<tr>
<td>Plasma KIM-1 (pg/ml)</td>
<td>64.4 (51 to 77.7)</td>
<td>205.7 (62.15 to 349.3)</td>
<td>1458 (274.8 to 2641)</td>
</tr>
<tr>
<td>Urinary KIM-1 (ng/mg urinary creatinine)</td>
<td>0.29 (0.22 to 0.35)</td>
<td>0.77 (0.59 to 0.96)</td>
<td>5.9 (3.21 to 8.70)</td>
</tr>
</tbody>
</table>

Values for continuous variables given as mean±SEM or mean (95% CI). CS, cardiac surgery.

*Baseline eGFR was calculated using the Modification of Diet in Renal Disease equation.

Cl in a lower AUC-ROC (0.91) and weaker correlation with plasma KIM-1 (r=0.23).
Figure 2. Plasma KIM-1 is a marker of renal injury in human AKI. Plasma and urine were collected from healthy volunteers and post–cardiac surgery (CS) patients with or without AKI and ICU patients with AKI from other causes. Dot plots indicate plasma KIM-1 (A) and urinary KIM-1 normalized to urinary creatinine (B) for each patient. *P<0.001; #P<0.05. (C) ROC curve analysis comparing performance of normalized urinary KIM-1 (dashed red line, AUC 0.98) and plasma KIM-1 (solid black line, AUC 0.96) levels. (D) Scatter plot demonstrating a positive correlation between plasma and urinary KIM-1 levels in all participants, including healthy volunteers (n=48) and patients with (n=28) or without AKI (n=16). (r=0.43; P<0.001). (E) Scatterplot demonstrating a correlation between plasma KIM-1 levels and urinary albumin-to-creatinine ratios (r=0.33; P=0.001). (F) Scatter plot demonstrating a correlation between urinary KIM-1 levels and urinary albumin levels (r=0.35; P<0.001 for urinary KIM-1). (G) Scatter plot demonstrating positive correlation between plasma KIM-1 and plasma creatinine in patients with or without AKI (r=0.58; P<0.001). (H) Plasma and urine were collected at various times before and after CPB from nine patients who developed stage 1 AKI and nine who did not develop AKI. Mean plasma creatinine, plasma KIM-1 (I), normalized urinary KIM-1 (J), and urinary albumin (J) concentrations were determined. #P<0.05 for difference from baseline; *P<0.05 for difference between AKI and non-AKI groups. n=9 for both AKI and no-AKI groups. Error bars represents SDs.
in patients with AKI. Normalization to urine creatinine accounts for variation in water reabsorption along the nephron. According to Waikar et al., “Lower creatinine excretion in the setting of acute kidney injury may amplify a tubular injury biomarker signal, thereby increasing its clinical utility.” The AUC-ROC of plasma and urinary KIM-1 to differentiate patients with AKI from those without AKI was high because of the cross-sectional nature of these studies, where the diagnosis of AKI was well established clinically (Figure 2C, compatible with prior studies. In prospective studies in which all nine patients developed stage 1 AKI, the AUC-ROC was lower (Supplemental Table 3). In addition to the integrated effects of kidney KIM-1 production and release into the blood, plasma KIM-1 levels will be influenced by the volume of KIM-1 distribution and the renal or extrarenal metabolism and clearance of KIM-1. The correlation coefficient of 0.43 reflects a statistically significant association between urinary and plasma KIM-1. The strong correlation between plasma KIM-1 and plasma creatinine concentration in humans and rodents in the setting of acute injury is not unexpected given that renal tubular injury will increase both these markers.

In patients with CKD of various causes, blood KIM-1 levels were correlated with increasingly advanced stages of disease. Baseline serum KIM-1 performed very well as a predictive biomarker for progressive kidney disease in a type 1 diabetic cohort even after other common covariates, including urinary albumin-to-creatinine ratio, hemoglobin A1c, and eGFR, were taken into account.

Further studies will be required to fully assess the patterns of elevation in blood KIM-1 to evaluate the potential utility of blood KIM-1 levels to risk-stratify patients, predict outcome (including progression of CKD), and serve as an efficacy biomarker in therapeutic trials. Blood KIM-1 levels may have an integrating function reflecting the extent of ongoing injury to the kidney over time. A limitation of our studies is the small sample sizes. How blood KIM-1 compares with other blood and urinary biomarkers of kidney injury and whether the addition of other kidney injury biomarkers would increase the performance of blood KIM-1 to detect kidney injury, risk-stratify patients, or predict outcome require further investigation in large cohorts.

In conclusion, we have identified blood KIM-1 as a marker of kidney injury in mice, rats, and humans. In humans, blood KIM-1 levels are significantly elevated in the setting of AKI and CKD and predict progression of renal disease in a type 1 diabetic cohort. This biomarker may have potential utility as a sensitive and specific diagnostic and prognostic marker for kidney injury. This is first blood biomarker that specifically reflects injury to the proximal tubule of the kidney, the primary site of injury for ischemia and most nephrotoxins.

**CONCISE METHODS**

**Bilateral Ischemia Reperfusion Injury in Mice**

Ischemia was induced in male BALB/c mice using a retroperitoneal approach by clamping both renal pedicles for 10, 20, or 30 minutes and then releasing the clamps according to published techniques from our laboratory. Sham operations were also performed, manipulating the pedicles without induction of ischemia. Twenty-four hours after reperfusion, mice were euthanized, and urine, plasma, and tissue samples were collected and analyzed. In another set of animals, ischemia was induced for 30 minutes; urine and plasma specimens were collected before the surgery and 3, 6, 12, 24, 48, 72, 96, 120, and 144 hours after the reperfusion. The institutional animal care committee approved all animal protocols.

**Unilateral ureteral obstruction in mice**

Unilateral ureteral obstruction was induced in male BALB/C mice, age 8–10 weeks, as described previously. Mice were anesthetized and the ureter of the left kidney was ligated with 6-0 silk at two points proximal to the kidney. In sham animals, flank incisions were made and the kidney exposed, but the ureter was not tied.

**Liver Injury in Mice**

As controls, male BALB/c mice were injected intraperitoneally once with 0.5 ml/kg of 10% CCl4 in vegetable oil (n=5) or vegetable oil alone (vehicle, n=5), as previously described. Forty-eight hours after CCl4 administration, mice were euthanized and urine, plasma, and tissue samples were collected and analyzed.

**Gentamicin-Induced Nephrotoxicity**

Male Sprague-Dawley rats weighing approximately 230–260 g were administered 0.9% saline, 50 mg/kg gentamicin, or 200 mg/kg gentamicin intraperitoneally daily for 10 days. Rats were provided with free access to food and water and subjected to a 12-hour light and dark cycle. Rats were euthanized on day 11, and urine, plasma, and tissue samples were collected and analyzed. Tissue samples were fixed in 10% neutral buffered formalin or snap frozen. For histologic assessment, 3- to 5-μm paraffin sections were prepared and stained with hematoxylin and eosin. The rat studies were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee.
Selection of Patient Study Groups

Single spot urine samples and corresponding plasma samples were obtained from healthy individuals, patients undergoing cardiac surgery, and patients admitted to the surgical intensive care unit. Healthy volunteers (n=48) who are self-reported to be free of chronic inflammatory diseases, chronic infectious diseases, and metabolic disease were participants in the Brigham and Women's Hospital PhenoGenetic Project, a large-scale tissue bank that provides a sample archive and longitudinal biosampling from its cohort. Cardiac surgery and ICU patients were participants in prospective AKI biomarker studies conducted at Brigham and Women's Hospital. Urine and plasma samples were obtained in 16 patients post cardiac surgery to serve as a complementary non-AKI cohort and in 28 patients with AKI (15 patients met KDIGO stage 1 criteria and 13 met stage 2 criteria) 22 patients following cardiac surgery and 6 non-cardiac surgery ICU patients. For prospective studies to determine the time course of biomarker changes, we collected plasma and urine samples before surgery; at the end of CPB; and then at 4 hours, 12 hours (urine only), and then daily for 5 days after CPB. The primary outcome variable was development of AKI, defined as a $50\%$ increase in plasma creatinine from baseline within 7 days or a $0.3 \text{mg/dl}$ increase within 2 days according to the KDIGO criteria.22

CKD urine and plasma samples were collected from outpatients attending a general nephrology clinic at Brigham and Women's Hospital. Urine and plasma samples were collected at approximately the same time. Plasma samples from patients with CKD were also obtained at University of Liverpool. Diagnoses included glomerular diseases (39.1%), diabetic nephropathy in type 2 diabetes (17.4%), and other causes of CKD (43.4%).

To examine the potential value of plasma concentration of KIM-1 as a predictor of progressive renal decline, we studied a subgroup of patients with type 1 diabetes and proteinuria who were previously included in the Joslin Proteinuria Cohort.15 Of 423 patients participating in the cohort, a random subgroup of 124 patients with a sufficient amount of baseline serum was selected for the current study. More clinical information of these patients is provided in Supplemental Table 4 and can be found in Rosolowsky et al.15

(F) Western blot depicting 90-kD band of urinary KIM-1 in a patient with AKI (lane 2) and plasma KIM-1 in patients with AKI (lanes 4 and 5), and CKD (lane 6). Urine (lane 1) and plasma (lane 3) from healthy volunteers were also included for comparison.
followed for 5–15 years (median follow-up, 10 years). During follow-up they had serial serum creatinine measurements to estimate the rate of renal decline rate of eGFR loss (eGFR slopes) according to methods previously described. All human studies were approved by institutional review boards.

**KIM-1 Measurement**

Microbead-based assays for rodent and human plasma and serum KIM-1 were developed, and extensive validation of the assays were performed using previously described approaches. Urinary KIM-1 in rodents and humans was measured using microbead based assays as described previously. Capture antibodies (MAB 1817 for mouse, AF1750 for human [R&D Systems]), MARKE antibody for rat (developed at Brigham and Women's Hospital) were conjugated with COOH polystyrene beads (Bio-Rad) with an amine coupling kit (Bio-Rad) using N-hydroxysuccinimide-1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chemistry according to the manufacturer’s protocol. Approximately 6000 beads in 50 μl of sample diluent buffer (0.1M HEPES, 0.1M NaCl, 0.1% Tween-20, and 1% BSA; pH, 7.4; filter sterilized) were incubated with 30 μl of sample or recombinant KIM-1 protein (1817-TM-050-CF for mice, 1750-TM for humans [R&D systems]), and KIM-FC for rats [developed at Brigham and Women’s Hospital]) for 45 minutes. After incubation, beads were washed three times with PBS with Tween and incubated with biotinylated anti-KIM-1 detection antibody (BAF 1817 for mice, BAF 1750 for humans [R&D Systems]) and MARKE-2 for rats [developed at Brigham and Women’s Hospital]) for 30 minutes. Beads were washed again with PBS with Tween and incubated for 15 minutes with the streptavidin–phycoerythrin solution (Invitrogen). The signal from the fluorochrome, which is directly proportional to the amount of antigen bound at the microbead surface, is captured using the Bio-Plex system (Bio-Rad). Data were interpreted using a five parametric logistic regression analysis.

**Statistical Analyses**

Scatterplots were used to graphically display log-transformed normalized biomarker levels in the clinical samples. Continuous variables were compared using the Wilcoxon rank-sum test and the Spearman correlation coefficient. Diagnostic performance (i.e., the ability of a biomarker to identify AKI) was assessed by using the ROC curve. The area under the ROC curve (AUC) and 95% CIs were calculated using the nonparametric method of DeLong. The eGFR was calculated using the Modification of Diet in Renal Disease equation. *P* values <0.05 were considered to represent statistically significant differences. Statistical analyses were performed using MedCalc for Windows, version 12.1.4.0 (MedCalc Software, Mariakerke, Belgium). For animal studies, all results are expressed as mean ± SEM. One-way ANOVA and t test were performed on control samples and treated samples to evaluate the difference in these groups. The level of significance was set at *P*<0.05 in all cases. The statistical methods used to analyze the follow-up data from the Joslin proteinuria cohort have been described previously.

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**REFERENCES**


**DISCLOSURES**

J.V.B. is coinventor on KIM-1 patents, which have been licensed by Partners Healthcare to several companies. He has received royalty income from Partners Healthcare and grant funding from Novo Nordisk. V.S. received funding from Novo Nordisk. J.V.B. or his family have received income for consulting from companies interested in biomarkers: Sekisui, Millennium, Johnson & Johnson, and Novartis.

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