

Urinary Netrin-1 Is an Early Predictive Biomarker of Acute Kidney Injury after Cardiac Surgery

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Background and objectives: Netrin-1, a laminin-related axon guidance molecule, is highly induced and excreted in the urine after acute kidney injury (AKI) in animals. Here, we determined the utility of urinary netrin-1 levels to predict AKI in humans undergoing cardiopulmonary bypass (CPB).

Design, setting, participants, & measurements: Serial urine samples were analyzed by enzyme-linked immunosorbent assay for netrin-1 in 26 patients who developed AKI (defined as a 50% or greater increase in serum creatinine after CPB) and 34 controls (patients who did not develop AKI after CPB).

Results: Using serum creatinine, AKI was detected on average only 48 hours after CPB. In contrast, urine netrin-1 increased at 2 hours after CPB, peaked at 6 hours (2462 ± 370 pg/mg creatinine), and remained elevated up to 48 hours after CPB. The predictive power of netrin-1 as demonstrated by area under the receiver-operating characteristics curve for diagnosis of AKI at 2, 6, and 12 hours after CPB was 0.74, 0.86, and 0.89, respectively. The 6-hour urine netrin-1 measurement strongly correlated with duration and severity of AKI, as well as length of hospital stay (all $P < 0.05$). Adjusting for CPB time, the 6-hour netrin-1 remained a powerful independent predictor of AKI, with an odds ratio of 1.20 (95% confidence interval: 1.08 to 1.41; $P = 0.006$).

Conclusion: Our results suggest that netrin-1 is an early, predictive biomarker of AKI after CPB and may allow for the reliable early diagnosis and prognosis of AKI after CPB, before the rise in serum creatinine.

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The incidence of acute kidney injury (AKI) is increasing worldwide, affecting about 6% of all hospitalized patients in whom it is an independent predictor of mortality and morbidity (1–3). In the critical care setting, the prevalence of AKI requiring dialysis is about 6%, with a mortality rate exceeding 60% (4). Once established, the treatment is largely supportive, at an annual cost surpassing \$10 billion in the United States alone (5). The diagnosis currently depends on detection of reduced kidney function by the rise in serum creatinine concentration, which is a delayed and unreliable measure in the acute setting (5). Ironically, experimental studies have identified interventions that may prevent or treat AKI if instituted early in the disease process, well before the serum creatinine rises (6). The lack of early predictive biomarkers has impaired our ability to translate these promising findings to human AKI.

Cardiopulmonary bypass (CPB) surgery is the most frequent

major surgical procedure performed in hospitals worldwide, with well over a million operations undertaken each year. AKI is a frequent and serious complication encountered in 30% to 40% of adults and children after CPB (7–14). AKI requiring dialysis occurs in up to 5% of these patients, in whom the mortality rate approaches 80%, and is indeed the strongest independent risk factor for death (15). However, even a minor degree of postoperative AKI as manifested by only a 0.2 to 0.3 mg/dl rise in serum creatinine from baseline is also associated with a significant increase in mortality (16,17). Additionally, AKI after cardiac surgery is associated with adverse outcomes, such as prolonged intensive care and hospital stay, dialysis dependency, and increased long-term mortality (18). Infants and children with congenital heart diseases may be especially vulnerable to developing AKI, because many require multiple surgeries for step-by-step repair of complex congenital anomalies (8–14). These patients comprise an important population for the initial validation of AKI biomarkers because they do not exhibit common comorbid variables that complicate similar studies in adults, such as diabetes, hypertension, atherosclerosis, and nephrotoxin use (19).

Experimental studies aimed at a better understanding of the early adaptive response of the stressed kidney have recently yielded several candidate genes and proteins that are serendipitously emerging as noninvasive candidate biomarkers of

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AKI (20,21). One example of such a protein is netrin. The netrins were discovered more than a decade ago as neuronal guidance cues (22). Netrins are molecules with a distinctive domain organization that belong to the laminin-related family of axon-guidance molecules (23). Recent studies indicate various other roles for netrins beyond axonal guidance, including development of mammary gland, lung, pancreas, and blood vessels; inhibition of leukocyte migration during sepsis; mitogenesis; and chemoattraction of endothelial cells (23,24). The kidney has one of the highest levels of netrin-1 expression, and administration of recombinant netrin-1 before ischemia reperfusion reduces kidney injury and inflammation (25). Preclinical studies also indicate that netrin-1 protein is markedly induced in kidney tubule cells and appears in the urine early (within 1 to 3 hours) after murine renal ischemic injury as well as other forms of AKI (26). Therefore, the objective of this study was to determine whether urinary netrin-1 levels predict the development of AKI in pediatric patients undergoing CPB.

Materials and Methods

Patient Population

Urine samples were prospectively obtained from consenting patients who underwent cardiac surgery using CPB at Cincinnati Children's Hospital for the correction or palliation of congenital cardiac defects during the period of July 2006 to June 2007. Exclusion criteria included preexisting renal insufficiency, diabetes mellitus, concomitant nephrotoxic drug use, and incomplete urine collections. Subjects were enrolled only if their preoperative kidney function was normal, on the basis of an estimated creatinine clearance of >100 ml/min per 1.73 m² as calculated using the Schwartz formula as described previously (27). To minimize postoperative volume depletion and prerenal azotemia, all subjects received at least 80% of their maintenance fluid requirements during the first 24 hours after surgery and 100% maintenance subsequently. AKI was defined as a 50% increase in serum creatinine from baseline, which occurred, on average, 48 hours after surgery. In addition, each patient with AKI was classified according to the recently described pRIFLE criteria, which is a modification of the RIFLE criteria for use in children (28). For each patient, six urine samples were obtained that corresponded to times 0, 2, 6, 12, 24, and 48 hours after initiation of CPB. Urine samples were centrifuged at $2000 \times g$ for 5 minutes, and the supernatants stored in aliquots at -80°C . The research protocol for collection and analysis of these samples was approved by the Cincinnati Children's Hospital Institutional Review Board and Pennsylvania State University College of Medicine.

Netrin-1 quantitation by Enzyme-Linked Immunosorbent Assay

Deidentified samples were blinded for netrin-1 analysis. Fifty microliters of urine was used for the netrin-1 assay. The assay was done using an enzyme-linked immunosorbent assay kit (catalogue no. E1827h; USCN Life Science, Wuhan, China). Briefly, netrin-1 standard and samples were added to antibody-coated 96-well plates and incubated for 2 hours at room temperature, followed by addition of biotin-conjugated polyclonal antibody specific for netrin-1 and incubation for an additional 1 hour. Plates were then washed and incubated with avidin conjugated to horseradish peroxidase for 1 hour. Color was developed using tetramethylbenzidine substrate, and reaction was arrested by adding sulfuric acid. The color change was measured using a plate reader (Labsystems) at a wavelength of 450 nm. The concentration of netrin-1 in the samples was determined by comparing the OD of the samples to the standard curve, with a minimal

limit of detection of 7.8 pg/ml. All measurements were made in duplicate. Urinary netrin-1 concentration was expressed as picograms per milligram of urine creatinine. The interassay coefficient of variation for urinary netrin was 5.3%.

Statistical Analyses

SAS version 9.1.3 was used for analyses (SAS Institute, Cary, NC), and a significance level of 0.05 was used for all analyses, controlling for multiple comparisons. Demographics and clinical outcomes were compared between patients who developed AKI and patients who did not. Continuous variables were compared using two-sample *t* test, and categorical variables were compared using χ^2 test or Fisher exact test, as indicated. Estimates of mean values of serum creatinine and urinary netrin-1 by group at various time points were calculated using repeated-measures ANOVA, which accounts for correlations of measurements from the same individuals across time. Least square (LS) means and their standard errors (SEMs) are reported.

Spearman correlation coefficients were used to show the correlation between urinary netrin-1 concentrations at various time points (baseline and at 2, 6, 12, 24, and 48 hours after surgery) and the following clinical outcomes: percent change in serum creatinine, CPB time, hospital length of stay after surgery, and days of AKI.

To measure the sensitivity and specificity for urinary netrin-1, a conventional receiver-operating characteristic (ROC) curve was generated for urinary netrin-1 at 2, 6, and 12 hours after the initiation of CPB. We calculated the area under the curve (AUC) to ascertain the utility of netrin-1 as a biomarker. An area of 0.5 is expected by chance, whereas a value of 1.0 signifies a perfect biomarker. The optimal urinary netrin-1 time point was selected to maximize prediction at the earliest time possible, thus weighing the AUC, timing of measurement, and *P* value from the predictive logistic model. We then identified the values of urinary netrin-1 that provided 95% sensitivity, 95% specificity, and optimal sensitivity and specificity using the ROC curve at the best time point.

Univariable and multivariable logistic regression analyses were then undertaken to assess predictors of AKI. Potential independent predictor variables included urinary netrin-1 concentration at the best time point, age, sex, race, CPB time, previous heart surgery, and hospital length of stay. Variables were retained in the final model if *P* < 0.05.

Results

Patient Characteristics and Renal Function Changes

During the period of enrollment, 120 subjects underwent CPB at our institution. Of these, 60 subjects met the inclusion criteria for this study. The most common reason for excluding a subject was incomplete urine collections. AKI occurred in 26 children (43%) within a 3-day period. No significant differences were noted between the two groups with respect to age, race, need for dialysis, or mortality (Table 1). Children who developed AKI had significantly longer CPB times compared with those who did not develop AKI (*P* < 0.0001), and also experienced significantly longer hospital stays (*P* = 0.0006). Figure 1 shows the changes of serum creatinine concentrations after CPB for children who developed AKI and those who did not. During the first 24 hours after CPB, serum creatinine did not differ significantly between the two groups. Significant differences between groups were seen by 48 hours after surgery and were maintained until 5 days after surgery.

Table 1. Descriptive statistics of patient characteristics

Parameter	AKI	No AKI	P
n	26	34	
Age, yr	4.3 ± 4.5	4.0 ± 4.6	0.81 ^a
Male, %	35	65	0.04 ^b
White, %	81	91	0.28 ^c
Prior surgery, %	38	35	1.0 ^b
Bypass time, min	188.4 ± 62.6	91.4 ± 47.8	<0.0001 ^a
Creatinine change, %	171.3 ± 133.9	11.6 ± 12.2	<0.0001 ^a
Duration of AKI, d	4.8 ± 4.5	–	–
Hospital stay, d	13.8 ± 11.6	4.8 ± 2.9	0.0006 ^a
Dialysis, %	8	0	0.18 ^c
Death, %	4	0	0.43 ^c

Means ± standard deviation (SD) are reported for continuous measures, proportions are reported for categorical variables.

^aWelch modified two-sample *t* test.

^bχ² test with Yates correction.

^cFisher exact test.

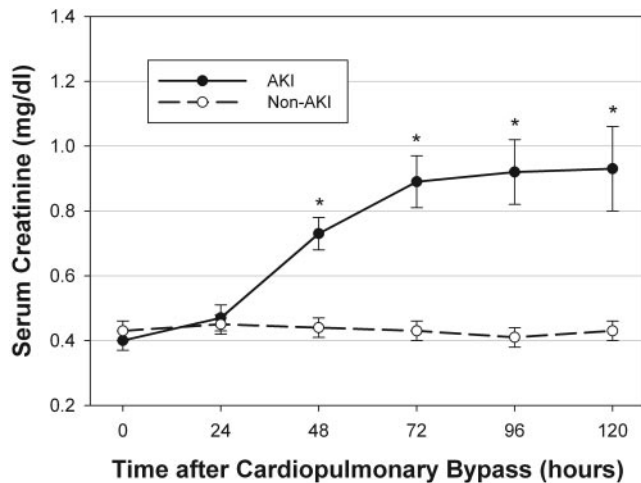


Figure 1. Changes in serum creatinine (LS mean ± SEM) at various time points after cardiac surgery in the non-AKI and AKI groups. **P* ≤ 0.0002 for differences between groups by repeated-measures ANOVA.

Associations of Netrin-1 with Patient Characteristics

Netrin-1 was not associated with patient age at any time point (all *P* > 0.14; Table 2). Netrin-1 before surgery was not

predictive of percent change in serum creatinine postsurgery, CPB time, hospital length of stay, or duration of AKI (all *P* > 0.2). Higher netrin-1 levels at all time points between 2 and 48 hours were significantly associated with greater percent change in serum creatinine, longer hospital length of stay, and longer duration of AKI. Furthermore, higher netrin-1 levels at 6, 12, and 24 hours after surgery were associated with longer CPB time.

Urinary Netrin-1 Predicts AKI after Cardiac Surgery

Urinary netrin-1 increased significantly in patients who developed AKI by 2 hours after the initiation of CPB, peaked at 6 hours after surgery, and remained significantly elevated until 48 hours after surgery (Figure 2). By contrast, patients who did not develop AKI experienced a much smaller increase shortly after surgery that resolved to baseline by 24 hours after surgery.

Among the 26 subjects who developed AKI, eight were classified as being in the risk (R) category, 12 in the injury (I) category, and six in the failure (F) category, on the basis of pRIFLE criteria. Analysis of netrin-1 concentrations by pRIFLE classification revealed that the failure group differed significantly from the group without AKI at 6, 24, and 48 hours, whereas the risk group differed from no AKI at 12 and 24

Table 2. Spearman correlation coefficients of netrin-1 with clinical characteristics

	Age	Percent Change in Serum Creatinine	CPB Time	Hospital Length of Stay	Days AKI
Baseline	−0.18	0.11	0.12	−0.07	0.07
2 h	−0.21	0.33 ^a	0.19	0.37 ^b	0.34 ^a
6 h	−0.07	0.48 ^b	0.43 ^b	0.52 ^b	0.58 ^b
12 h	−0.13	0.62 ^b	0.59 ^b	0.62 ^b	0.64 ^b
24 h	−0.10	0.36 ^a	0.43 ^b	0.47 ^b	0.41 ^a
48 h	0.007	0.36 ^a	0.31	0.36 ^a	0.34 ^a

^a*P* ≤ 0.05.

^b*P* ≤ 0.007.

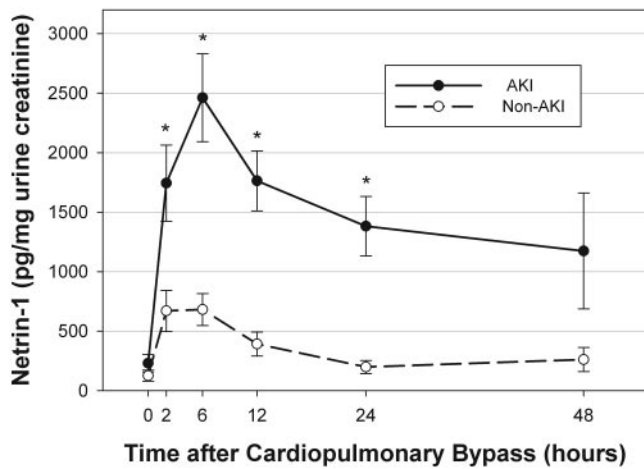


Figure 2. Changes in urinary netrin-1 concentrations at various time points after CPB surgery in non-AKI and AKI patients. Error bars are LS mean ± SEM. * $P \leq 0.003$ for differences between groups (non-AKI and AKI) by repeated-measures ANOVA.

hours, and the injury group differed from no AKI only at 12 hours (all $P < 0.003$; Figure 3).

Conventional ROC curves for AKI versus no AKI were generated for urinary netrin-1 at 2, 6, and 12 hours after surgery. The AUCs of the three ROC curves are 0.737 ($P = 0.02$), 0.858 ($P = 0.0006$), and 0.888 ($P = 0.002$), respectively. After weighing the AUC, timing of measurement, and P value from the predictive logistic model, the optimal urinary netrin-1 time point was selected at 6 hours after surgery. Figure 4 displays the unadjusted ROC curve for urinary netrin-1 at 6 hours after cardiac surgery. The sensitivities and specificities for three netrin-1 concentrations obtained at the 6-hour time point are listed in Table 3, corresponding to 95% sensitivity, optimal

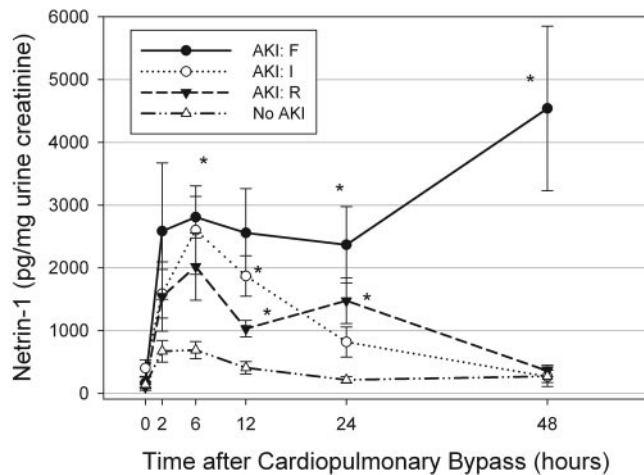


Figure 3. Changes in urinary netrin-1 concentrations at various time points after CPB surgery in non-AKI and AKI patients, stratified by pRIFLE categories. Error bars are LS mean ± SEM. * $P \leq 0.001$ for differences between groups (non-AKI and each of the pRIFLE categories) by repeated-measures ANOVA.

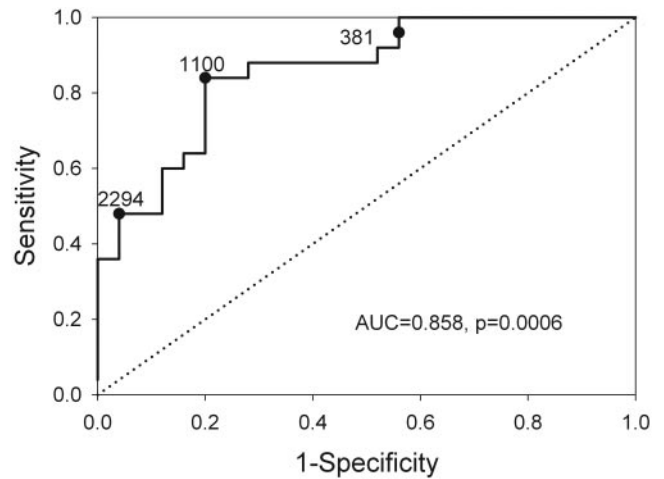


Figure 4. ROC curve analysis for urinary netrin-1 at 6 hours after cardiac surgery. The values 381.4, 1099.8, and 2294.1 are urinary netrin-1 concentrations (in picograms per milligrams urine creatinine) at 6 hours after CPB, which correspond to 96% sensitivity, optimal sensitivity and specificity, and 96% specificity, respectively.

sensitivity and specificity, and 95% specificity. A cutoff value of 1100 pg/mg creatinine at 6 hours after cardiac surgery yields the optimal combination of sensitivity (84%) and specificity (80%).

Univariable logistic regression identified longer CPB time ($P < 0.0001$), female sex ($P = 0.02$), longer hospital length of stay ($P = 0.0007$), and higher netrin-1 concentration at 2 hours ($P = 0.02$), 6 hours ($P = 0.0006$), 12 hours ($P = 0.002$), and 24 hours ($P = 0.008$) as significantly associated with higher odds of AKI. A stepwise logistic regression analysis was used to determine the most parsimonious model given a set of potential variables for predicting AKI. Potential variables for this model included age, sex, CPB time, previous cardiac surgery, hospital length of stay, and netrin-1 at the selected optimal time point (i.e., 6 hours after surgery). The final model revealed that CPB time and netrin-1 concentration at 6 hours after surgery were the only significant independent predictors of AKI in our cohort. The estimated odds ratio for every 100 pg/mg urinary creatinine increase of netrin-1 at 6 hours after surgery was 1.20 (95% confidence interval: 1.08 to 1.41; $P = 0.006$). The estimated OR for every 10-min increase of CPB time was 1.39 (95% CI: 1.16 to 1.79; $P = 0.002$).

Discussion

This is the first study to demonstrate that urinary excretion of netrin-1 is an early predictive biomarker of human AKI. In pediatric patients undergoing CPB, subjects that developed AKI displayed significantly increased urinary netrin-1 levels within the first 2 hours of the initiation of CPB, preceding the rise in serum creatinine by 48 to 72 hours.

In preclinical studies of mouse models, netrin-1 is not expressed in normal tubular epithelial cells and is undetectable in normal urine (26). However, netrin-1 protein expression is highly induced within a few hours of ischemia-reperfusion

Table 3. Urinary netrin-1 test characteristics at different cutoff values

Cutoff Value for netrin-1, pg/mg Urine Creatinine	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
381	0.96	0.44	0.63	0.92
1100	0.84	0.80	0.81	0.83
2294	0.48	0.96	0.92	0.35

The cutoff values are urinary netrin-1 concentrations at 6 h after cardiopulmonary bypass, which correspond to 95% sensitivity, optimal sensitivity and specificity, and 95% specificity, respectively.

injury and is easily detected in the urine. Similarly, urinary netrin-1 levels significantly increase 1 to 3 hours after lipopolysaccharide injection, folic acid administration, and cisplatin administration (26). These observations in animal models of AKI suggest that netrin-1 is a potential tubular injury marker. The role of netrin-1 in kidney pathophysiology is not fully understood. However, our previous studies have shown that netrin-1 suppressed ischemia-reperfusion-induced apoptosis when it was overexpressed in tubular epithelial cells (29). Moreover, netrin-1 overexpression increased tubular epithelial cell proliferation, suggesting that netrin-1 may be involved in regeneration and recovery of tubules after injury. This notion was further supported by a recent *in vitro* study, where addition of netrin-1 to proximal tubular epithelial culture increased cell proliferation and migration (30). These findings lend biologic plausibility for the phenomenon of netrin-1 overexpression in AKI, and they support the general concept of developing netrin-1 as a biologically meaningful biomarker (31).

Our results indicate that netrin-1 is an early biomarker of AKI that precedes the increase in serum creatinine by 1 to 3 days. The magnitude of rise and its correlation with AKI severity on the basis of the pRIFLE classification support the notion that netrin-1 is a highly discriminatory biomarker with a wide dynamic range and cutoff values that allow for risk stratification. Indeed, we found that other variables, such as patient demographics and previous cardiac surgery, were not predictive of AKI and could not be used for risk assessment in our cohort. However, early urinary netrin-1 levels were associated with important clinical outcomes, such as severity of AKI, duration of AKI, and length of hospital stay.

We have not measured serum netrin-1 levels in this cohort of patients to determine whether netrin-1 level in serum also has a predictive value for AKI. However, our earlier studies have shown that netrin-1 is undetectable in serum of normal humans or after kidney transplantation (Ramesh *et al.*, unpublished data). Netrin-1 is a large (72 kD) protein. Due to its size and absence in the systemic circulation, urinary netrin-1 is unlikely to be derived from glomerular filtration. These considerations suggest that the early increase in urine netrin-1 in subjects destined for AKI is a direct reflection of kidney tubule injury.

Our study has strengths. First, we prospectively recruited a relatively homogeneous cohort of pediatric subjects in whom the only obvious etiology for AKI would be the result of cardiac surgery. Second, all subjects started with normal kidney function and low levels of netrin-1 in the urine. The study design allowed for the temporal definition of altered netrin-1 concen-

trations after cardiac surgery and a direct comparison with changes in serum creatinine, the current gold standard for the definition of AKI. Third, we adjusted for urine concentration by correcting urinary netrin-1 levels with urinary creatinine.

The current study also has limitations. First, it is a single-center pilot study of pediatric subjects with congenital heart defects undergoing elective cardiac surgery. Thus, these results will need to be validated in a larger population, including adults with the usual confounding variables and comorbid conditions that normally accumulate with increasing age. Second, ours was a cohort with normal kidney function, and it will be important to confirm our findings in documented high-risk settings, such as preexisting kidney dysfunction, diabetes mellitus, volume depletion, concomitant nephrotoxic drug use, and the hemodynamically compromised patient. Third, in addition to netrin-1, simultaneous examination of other urinary biomarkers as potential predictors of AKI may provide additional information (32). Recent studies have uncovered other AKI biomarkers, such as neutrophil gelatinase-associated lipocalin (8–12), IL-18 (9), liver-type fatty acid-binding protein (11), and kidney injury molecule-1 (33), in clinical cohorts similar to that employed in this study. However, none of these are currently available for standardized clinical application, and all biomarkers have individual strengths and weaknesses. Given the multifactorial etiologies of AKI (6,34), it appears unlikely that any single biomarker will suffice. Indeed, even in the cardiac surgical population, measurements of single urinary biomarkers, such as neutrophil gelatinase-associated lipocalin, have yielded a wide range of AUCs for the prediction of AKI (35).

It is anticipated that a collection of strategically selected candidates, including netrin-1 as reported here, may prove to be of value for early and rapid diagnosis of AKI and its clinical outcomes. This approach has been recently proposed to improve the early diagnosis and risk stratification in cardiovascular diseases (36,37). It is also likely that emerging AKI biomarker panels will enable the timely initiation of interventions, such as atrial natriuretic peptide and IGF, that have been successful in smaller, phase II-level efficacy studies but not in larger phase III trials. Possibly, these interventions will be successful if they are initiated at the onset of AKI (as determined by predictive biomarkers) rather than waiting several days for serum creatinine to rise. In addition, animal studies have identified, and continue to reveal, novel therapies, such as growth factors, stem cell therapies, antiapoptotic, anti-inflammatory, and antioxidant approaches, that are effective in early AKI, far before the rise in serum creatinine (6). It is also likely

that emerging biomarker panels will enable these safe and promising agents to be investigated in humans with AKI, especially in temporally defined clinical situations, such as subjects undergoing CPB.

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Disclosures

Dr. Ramesh and Pennsylvania State University College of Medicine have submitted a provisional patent application for the use of urine netrin-1 for the early diagnosis of AKI. Dr. Devarajan is a coinventor on patents related to NGAL as a biomarker of AKI and is a consultant to Abbott Diagnostics and Biosite Inc.

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