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Marking Renal Injury: Can We Move Beyond Serum Creatinine?

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Abstract

Acute kidney injury (AKI) is a prevalent and devastating condition associated with significant morbidity and mortality. Despite marked improvements in clinical care, the outcomes for subjects with AKI have shown limited improvement in the past 50 years. A major factor inhibiting clinical progress in this field has been inability to accurately predict and diagnose early kidney dysfunction. The current gold-standard clinical and biochemical criteria for diagnosis of AKI, Risk Injury Failure Loss End-stage renal disease (RIFLE), and its modification, Acute Kidney Injury Network (AKIN) criteria, rely on urine output and serum creatinine (SCr) which are insensitive, non-specific, and late markers of disease. The recent development of a variety of analytical mass spectrometry (MS) based platforms have enabled separation, characterization, detection, and quantification of proteins (proteomics) and metabolites (metabolomics). These high-throughput platforms have raised hopes of identifying novel protein and metabolite markers, and recent efforts have led to several promising novel markers of AKI. However, substantial challenges remain including need to systematically evaluate incremental performance of these markers over and beyond current clinical and biochemical criteria for AKI. Here, we discuss the basic issues surrounding AKI biomarker development, highlight the most promising markers currently under development, and discuss the barriers towards wide-spread clinical implementation of these markers.

Keywords

Acute Kidney Injury; Biomarkers; Mass Spectrometry; Proteomics; Metabolomics

1. Acute Kidney Injury: A Diagnostic Dilemma

Acute kidney injury (AKI) is a prevalent and devastating condition associated with significant morbidity and mortality. 30–50% of critically ill patients suffer from AKI, and those requiring the initiation of renal replacement therapy carry a mortality that exceeds 50%.¹ Currently, there are no effective therapies shown to improve outcomes in established AKI, and therefore, management has remained largely supportive in nature. One of the

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major limitations hindering clinical progress in this field has been the inability to accurately predict and identify early AKI. While ultrasound and other imaging techniques can often provide prognostic information in chronic kidney disease and identify cause in obstructive renal disease, currently there is limited imaging based diagnostic capability in detecting medical kidney injury in the acute setting.

Over the past decade, there has been a shift in nomenclature from “acute renal failure” to AKI, in recognition of the fact that injury may have significant clinical consequences without overt failure of kidney function. In spite of this conceptual change, current classification of AKI still relies on indirect measures of glomerular filtration rate (GFR). Both the Risk Injury Failure Loss End-stage renal disease (RIFLE) criteria,² and its modification, the Acute Kidney Injury Network (AKIN) criteria,³ utilize serum creatinine (SCr) and urine output to define AKI leaving SCr the gold standard determiner of kidney injury for close to a century.⁴

The use of SCr as a marker of AKI has significant limitations (Table 1). Subjects with AKI (particularly those with baseline impairment in renal function) often have a significant decline in GFR prior to any observable change in SCr.⁵ Furthermore, SCr is a late marker of disease, and alterations are often not apparent until 48–72 hours post-injury leading to missed early therapeutic opportunities when treatments may be most effective.⁶ SCr is a non-specific marker both for the diagnosis of AKI and for specific disease etiology. Age, gender, muscle mass, and nutritional status all effect SCr levels. Additionally, as a non-discriminating marker of glomerular filtration, SCr can increase with volume depletion, non-steroidal anti-inflammatory drug use, or many other etiologies of decreased renal perfusion without kidney injury.⁷ Even in healthy stable subjects, SCr shows dramatic alterations with repeated measures bringing to further question its ability to accurately predict acute renal injury. It is for these well-recognized limitations of SCr and for the devastating clinical implications surrounding AKI that a search for new non-invasive markers of renal injury has been pushed to the forefront of active research.

2. The Ideal AKI Super-Marker

While the perfect marker of AKI is unlikely to exist, there are several ideal characteristics to consider in renal biomarker development as outlined in Table 2.^{8,9} First, a marker of AKI must be a sensitive and early predictor of disease. Ideally, basal levels of a marker would allow for risk stratification prior to insult, and following insult, would display a rapid and dramatic alteration spanning a large range proportional to degree of injury (e.g. cardiac troponin-I and T for acute myocardial infarction). Not only should levels increase rapidly with onset of injury, but they should remain elevated to allow a large diagnostic window.

A useful AKI biomarker should be specific for renal injury and provide insight into etiology and location of injury, rather than just a non-specific marker of glomerular filtration. Unlike SCr, the marker should be relatively unaffected by patient characteristics or clinical status, and should play a direct mechanistic role in renal cell injury or recovery to allow prognostic utility and ability to monitor therapeutic response. Further, levels should predict hard clinical outcomes, such as need for renal replacement therapy and mortality, beyond capabilities of current clinical indicators like demographic data.

In addition to overall sensitivity and specificity in the prediction and diagnosis of AKI, a useful biomarker must also be appropriate for wide spread clinical use. Samples should be obtained non-invasively and be readily available making serum, plasma, or urine sampling ideal. Urinary analysis has particular benefits, in the absence of anuric renal failure, as it may provide a more direct marker of renal injury with less alteration by systemic processes than circulating plasma.⁸ Finally, level monitoring should be cost-effective with rapid

results, utilizing clinically available enzyme-linked immunosorbent assays (ELISA) or similar techniques.

3. Challenges to Renal Biomarker Development

Despite these seemingly straight-forward criteria, AKI biomarker development faces numerous challenges. First, validation of novel biomarkers requires existence of a gold standard for diagnostic comparison. Highly accurate markers of GFR, such as inulin or radiolabeled markers, are too laborious for large scale clinical trials, thus SCr often serves this role. As previously discussed, the significant limitations of SCr in defining AKI are then extrapolated to new developing biomarkers. Specifically, studies often use correlation with SCr as a diagnostic necessity for further investigation. This leads to inclusion of markers with dependence on glomerular filtration and other similar flaws to SCr measurement, while excluding novel and perhaps more mechanism-based markers. Further, even with standardized AKI criteria,^{2, 3} a wide assortment of SCr based definitions are utilized in clinical research making cross-study comparisons difficult if not impossible. In addition to difficulties in defining AKI, the heterogeneous number of disease etiologies makes it difficult to distinguish kidney injury specific markers from those that arise from the underlying cause itself.

Identification of novel biomarkers has increasingly relied on high throughput technology. In the past two decades, a new field of science called systems biology has emerged. The goal of systems biology is to characterize an individual on a gene (genomics), transcript (transcriptomics), protein (proteomics), or metabolite (metabolomics) level. Initial large scale transcriptional based studies are more recently being followed by mass spectrometry (MS) based proteomic and metabolomic analyses as they have the advantage of utilizing readily available and non-invasively collected biofluids (plasma and urine). As such, the MS-based platforms are more amenable to biomarker discovery and translation into clinical practice. While these are powerful tools, they are limited by high cost, need for specialized equipment and expertise, and additional technique specific difficulties which restrict their wide scale application to general clinical medicine.¹⁰ In urinary proteomic analysis, albumin and other highly abundant proteins predominate in the sample.¹¹ Because of the prevalence of such proteins, low abundant proteins, perhaps those most important to early disease pathogenesis, are often left undetected.¹²

The majority of current AKI biomarker investigation is further limited by sample availability. Clinical studies are for the most part small, single center, phase 1 or phase 2 trials, with very homogenous populations. Control groups often consist of healthy volunteers making it difficult to determine if altered biomarker levels are due to renal injury or are secondary markers of systemic disease. Despite these obstacles, significant advances have been made in AKI biomarker development. Here, we will briefly review several of the most promising markers and discuss future development strategies and needs.

4. Protein Biomarkers of AKI

There is intense interest in several plasma and urinary proteins as markers of AKI, as summarized in Table 3. The schematized temporal rise of these markers following renal injury compared to an idealized biomarker and to SCr is depicted in Fig 1.

4.1 Neutrophil Gelatinase-Associated Lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25kDa protein originally isolated from neutrophil secondary granules,¹³ and is now known as a prevalent epithelial cell protein with wide distribution throughout the body. Siderophores, small iron-binding

molecules, are the major ligands of NGAL, and chelation of bacterial siderophores leads to iron deficiency and bacteriostatic effects.^{14, 15} Through transcriptional regulation by NF- κ B, NGAL can also mediate controlled eukaryotic iron delivery and stimulate cell proliferation and preservation.¹⁶ These characteristics underscore NGAL's role in both innate immunity and cellular recovery.¹⁷ NGAL is freely filtered at the glomerulus and almost completely reabsorbed in the proximal tubule. Thus, increased urinary excretion suggests proximal tubular damage with impaired reabsorption or increased primary synthesis and excretion by distal nephron segments.

4.1.1 Preclinical Investigation—A role for NGAL as a marker of AKI was first suggested by high through-put transcriptomic and proteomic studies in animal models. Supavekin and colleagues were the first to show increased NGAL gene expression in a mouse model of renal ischemia reperfusion injury.¹⁸ Mishra and colleagues not only demonstrated NGAL as one of seven highly up-regulated genes following ischemic AKI in mice, but also showed an early increase in detectable urinary NGAL excretion marking it as a potential biomarker of renal injury.¹⁶ These findings were later validated in both ischemic and nephrotoxic animal models of AKI prompting further clinical evaluation in humans.^{19–21}

4.1.2 Clinical Investigation—Clinical studies examining the utility of NGAL as a biomarker of AKI are numerous and have been extensively reviewed.^{22, 23} The predictive and diagnostic utility of NGAL in AKI has been most widely examined in the post-cardiac surgery population as there is an easily identifiable time of insult and a high incidence of renal injury.²⁴ Mishra and colleagues, in follow-up to their animal work, first demonstrated the predictive value of NGAL in human AKI through examination of 71 pediatric patients undergoing cardiopulmonary bypass (CPB).²⁵ 20 children developed AKI, defined as a 50% increase in SCr from baseline, and urinary NGAL (uNGAL), 2 hours post-bypass, was the strongest independent predictor of AKI in multivariate analysis. The area under the curve of the receiver operating characteristic (AUC-ROC) curve was 0.99 with a 2 hour uNGAL cut-off of 50ug/L.

In adult populations, multiple studies have confirmed increases in NGAL post cardiac surgery, but the role in predicting AKI has been less conclusive. In a study examining 81 adult patients before and after cardiac surgery, 16 patients developed AKI defined as a 50% increase in SCr.²⁶ uNGAL concentrations increased in all patients post-operatively, but the 16 patients with AKI had significantly higher 1 hour post-operative values compared to the patients without AKI (4,195 $\pm$ 6,520 vs. 1,068 $\pm$ 2,129 ng/ml; $P < 0.01$). Further, in the AKI group, levels remained significantly elevated at 18 hours post-operatively, while NGAL levels in the non-AKI group returned to baseline. Similar studies have also documented the ability of uNGAL to predict AKI in the immediate post-operative setting.^{27–29} However, in a larger study by Wagener and colleagues,³⁰ the AUC-ROC for uNGAL was only 0.60, and uNGAL levels directly correlated with bypass and aortic cross-clamp time suggesting a limited accuracy of NGAL in predicting AKI. Most recently, Parikh and colleagues have investigated the ability of NGAL to predict AKI in post-cardiac surgery patients utilizing two large parallel adult and pediatric cohorts.^{31, 32} Overall, plasma NGAL (pNGAL) had an AUC-ROC of 0.70 and 0.56 while uNGAL had an AUC-ROC of 0.67 and 0.71 for adult and pediatric cohorts respectively. After adjustment for demographics and intra-operative risk factors, adults with the highest quintile of pNGAL levels had a 5-fold increased risk of developing AKI, with no increased risk associated with uNGAL levels.³² Pediatric patients with the highest uNGAL levels had a 4-fold risk of AKI, with no increased risk associated with plasma levels.³¹ This suggests differences in adult and pediatric patients perhaps related to age and comorbidities.

To further examine the effects of medical comorbidities on NGAL levels, the ability of NGAL to predict AKI has been examined in various critically ill populations. Zappitelli and colleagues first reported that uNGAL had an AUC-ROC of 0.78 for predicting AKI in a heterogeneous population of critically ill children.³³ Wheeler and colleagues showed pNGAL to be a sensitive but non-specific marker of AKI in critically ill children, with levels the highest in patients with co-existent septic shock.³⁴ In a population of adult multi-trauma patients, NGAL levels on admission to the intensive care unit (ICU) were strongly predictive of AKI development (AUC-ROC = 0.98),³⁵ however in more heterogeneous populations of adult critically ill patients, AUC-ROCs range from 0.64 to 0.78 suggesting a more moderate ability to predict subsequent AKI.^{36, 37} Constantin and colleagues showed that pNGAL on ICU admission was a strong predictor for the development of AKI using a cut-off of 155nmol/L, and NGAL increased 48 hours prior to diagnosis of AKI by RIFLE criteria.³⁸ However, recent investigation continues to demonstrate a significant difference in NGAL levels in septic versus non-septic AKI further supporting the influence of comorbidities in the prognostic and diagnostic ability of NGAL.³⁹

NGAL has also been found to predict delayed graft function (DGF) following renal transplantation,^{40–42} and to predict contrast induced nephropathy (CIN) in both children and adult populations.^{43–46} Additionally, Nickolas and colleagues have shown that a single uNGAL level on admission strongly predicted AKI with an AUC-ROC of 0.95, and levels correlated with important clinical outcomes including nephrology consultation, dialysis, and admission to the ICU.⁴⁷ Finally, a recent meta-analysis by Haase and colleagues examined 2,538 patients in 19 studies.⁴⁸ The overall standardized AUC-ROC of NGAL to predict AKI was 0.83, with improved predictive ability in children versus adults (AUC-ROC 0.93 vs. 0.78). Predictive value was the best for contrast induced nephropathy (AUC-ROC=0.89), followed by post-cardiac surgery patients (AUC-ROC=0.78), and only modestly predictive in critically ill patients (AUC-ROC=0.73).

Due to the strong clinical evidence supporting NGAL as a marker of AKI, it is now being utilized as a clinical endpoint of renal injury in many clinical trials.^{49, 50} This use is likely to further increase with the recent development of rapid point of care testing methods for both plasma (Biosite Incorporated, San Diego, CA, USA) and urinary levels (Abbott Diagnostics, Illinois, USA). Still, some concern remains as NGAL appears to most accurately predict AKI in patients with normal baseline renal function and limited comorbidities, perhaps explaining the increased predictive values in pediatric populations.⁵¹ As with the other biomarkers currently in development, further clinical trials in heterogeneous populations are required to confirm the validity and clinical utility of NGAL as a biomarker of AKI.

4.2 Interleukin-18 (IL-18)

Interleukin-18 (IL-18) is a pro-inflammatory cytokine that is induced in proximal tubular epithelial cells in response to injury. Following renal injury, IL-18 is secreted in the urine prior to a significant decrease in renal function. Therefore, urinary IL-18 (uIL-18) has been identified as a potential early marker of AKI.

4.2.1 Preclinical Investigation—The rationale for examining the role of IL-18 in AKI arose from the observation that caspases play a key role in mediating renal damage.⁵² Caspase-1, previously known as IL-1 β converting enzyme, cleaves the inactive pro-form of IL-18 into the mature active form with high specificity. Early studies of caspase-1 deficient mice found that IL-18 production was defective in response to lipopolysaccharide, and that the mice were resistant to the effects of endotoxemia.⁵³ A follow-up study found that these mice also experienced less severe renal failure, defined by rise in BUN or Cr and histologic changes, than wild type mice in response to ischemic renal insults. Furthermore, the

administration of IL-18-neutralizing rabbit anti-sera to wild type mice was protective of ischemic AKI.⁵⁴ Later, a study of IL-18 binding protein transgenic mice, where IL-18 is neutralized, found that these mice were also protected against ischemic renal injury.⁵⁵ Together, these studies have established that IL-18 is not only a marker of AKI, but also plays a pathogenic role in ischemic renal injury.⁵⁶

Early animal studies measured uIL-18 with an electrochemiluminescence assay that detected both the pro and active forms of IL-18.⁵⁷ U-IL-18 levels are also expressed as a ratio to urinary Cr levels in order to account for differences in urinary concentration. Using this method in a mouse model, uIL-18 was significantly elevated in animals exposed to ischemic AKI compared to virtually undetectable levels in controls.⁵⁴ Thus a potential diagnostic role for uIL-18 was suggested.

4.2.2 Clinical Investigation—One of the first clinical studies of the diagnostic potential of uIL-18 examined 72 patients with a variety of renal disorders.⁵⁸ In patients with a clinical diagnosis of acute tubular necrosis (ATN), uIL-18 was significantly elevated compared to normal controls (mean uIL-18 814+151 vs. 23+9 pg/mg Cr, $p<.0001$). Importantly, uIL-18 levels also distinguished patients with ATN from patients with pre-renal azotemia, chronic kidney disease (CKD), urinary tract infection, and nephrotic syndrome. In a subgroup of transplant patients, uIL-18 levels measured in the first 24 hours post-transplant were significantly higher in patients receiving a deceased donor transplant who subsequently met criteria for delayed graft function (DGF, defined as requiring dialysis within the first week post-transplant) compared to transplant patients with prompt kidney function. In this study, the AUC-ROC for the diagnosis of ATN and DGF was 0.95.

Parikh and colleagues evaluated the diagnostic role of uIL-18 in a cohort of 138 critically ill patients enrolled in the Acute Respiratory Distress Syndrome Network trial and specifically examined the timing of uIL-18 levels relative to standard AKI definitions. Urinary IL-18 levels were significantly greater in AKI compared to non-AKI patients at 24 and 48hrs before Cr-based criteria for AKI were met. The AUC-ROC of uIL-18 for predicting AKI in the next 24hrs was 0.73. An additional important finding was that uIL-18 level at the time of study enrollment was an independent predictor of subsequent mortality.⁵⁹ A subsequent study further characterized the time-course of uIL-18 by demonstrating that levels begin rising as early as 4 to 6 hours following an ischemic insult, peak at 12 hours, and remain elevated for up to 48 hours.⁶⁰

Several additional studies have demonstrated the potential utility of uIL-18 as an early biomarker for AKI in a variety of clinical settings, including CIN,⁴⁶ critically ill pediatric patients,⁶¹ and renal transplant patients.⁴¹ Recently, Siew and colleagues measured uIL-18 in a general cohort of 451 ICU patients, 86 of whom developed AKI. Interestingly, in this study uIL-18 was not predictive of AKI development within 24 hours (AUC-ROC 0.62), although levels were associated with 28 day mortality.⁶² A possible explanation is that IL-18 is also produced in lymphocytes, macrophages, fibroblasts and intestinal epithelial cells, raising the possibility that levels may be influenced by generalized inflammatory states.⁶³

While this initial clinical data supports uIL-18 as a useful biomarker of human AKI, several limitations remain. In contrast to early testing in animal models, measurement of uIL-18 in humans is done by ELISA. While testing kits are commercially available, they are labor intensive, have long turnaround times, and thus are useful for research purposes only. Further, while studies of discrete patient populations have suggested AUC values of greater than 0.90 for the diagnosis of AKI, recent studies suggest that the diagnostic characteristics of uIL-18 may be more modest in heterogeneous populations. In particular, future studies are needed to clarify the potential confounding impact of generalized inflammation on

uIL-18 levels. In addition, no consensus thresholds have been identified for risk stratification, with suggested cutoffs ranging from 100 to 500 pg/mg Cr.⁵⁹

4.3 Kidney Injury Molecule-1 (KIM-1)

Kidney injury molecule 1 (KIM-1) is a membrane glycoprotein that is expressed in proximal renal tubular epithelial cells in response to cellular injury. Interest in KIM-1 first arose from genomic screening approaches, in which KIM-1 was observed to be the most up-regulated gene in a rat model of ischemic AKI.⁶⁴ KIM-1 directs phagocytosis of apoptotic cells in the tubular lumen by epithelial cells,⁶⁵ and does not appear to be expressed in normal kidneys.⁶⁶

4.3.1 Preclinical Investigation—Since the identification of KIM-1 up-regulation in the rat model of renal ischemia, additional studies have confirmed this finding and examined the diagnostic role of KIM-1 in other models of AKI. An important observation was that the extracellular domain of KIM-1 is cleaved and excreted,⁶⁷ spurring investigation of urinary KIM-1 (uKIM-1) as a biomarker of AKI.

Ichimura and colleagues examined tissue and uKIM-1 expression in a rat model of toxic AKI. Following cisplatin administration, uKIM-1 was qualitatively detectable by immunoblot assay on day 1 while serum Cr levels did not rise until day 3.⁶⁸ A further advance was the development of a sensitive ELISA test to quantitatively measure uKIM-1 in rodents.⁶⁹ Subsequent studies demonstrated a similar pattern of KIM-1 elevation in response to a variety of other nephrotoxic insults.^{70, 71} As a result, the Food and Drug Administration has recognized uKIM-1 as an appropriate biomarker for renal injury in preclinical studies of pharmacologic agents.^{72, 73}

4.3.2 Clinical Investigation—Han and colleagues conducted the first biomarker evaluations of uKIM-1 in humans.⁷⁴ In 6 patients with biopsy-proven ischemic ATN, uKIM-1 levels, normalized to urinary creatinine, were significantly higher than in controls without renal failure, where uKIM-1 was nearly undetectable. Patients with a variety of other renal conditions, including CIN, other AKI, and CKD, were also found to have elevated uKIM-1 levels compared to controls. Interestingly, these conditions had significantly lower KIM-1 levels compared to ischemic ATN, suggesting that KIM-1 may be useful in differentiating between various etiologies of AKI.

In a study of pediatric patients undergoing CPB surgery, urinary KIM-1 levels were prospectively monitored following surgery. Patients that developed AKI, defined as a 50% increase in serum creatinine, had a significant rise in normalized KIM-1 by 12 hours (but not at 2 or 6 hours) post-op when compared to patients not developing AKI, and KIM-1 levels remained elevated for 48 hours. In this study, the 12 hour KIM-1 level yielded an AUC-ROC of 0.83 for the diagnosis of AKI.⁷⁵ In contrast, a study of 103 adult patients undergoing CPB found that normalized KIM-1 levels were significantly elevated by 2 hours in AKI versus non-AKI patients, with an AUC-ROC of 0.78. Based on test characteristics, an optimal diagnostic threshold value of KIM-1 was determined to be 0.42 ng/mg Cr.⁷⁶ Another study of adult cardiac surgery patients confirmed that normalized uKIM-1 levels were significantly elevated in the early, immediately and at 3 hours, post-operative period in patients who developed AKI within the first 24 hours post-op compared to non-AKI patients.⁷⁷

A recent study of 201 patients with established AKI found that higher normalized uKIM-1 levels were independently associated with a greater risk for the composite outcome of death or need for dialysis.⁷⁸ Therefore, KIM-1 may hold important prognostic value in addition to diagnostic utility.

Recently, a rapid testing method for KIM-1 has been described, yielding semi-quantitative results in just 15 minutes.⁷² This assay provides the potential for rapid diagnosis of AKI in advance of traditional markers such as SCr. Commercial kits using this assay have become available. However, as with uIL-18, all tests remain labeled for research purposes only and further clinical evaluation is required.

While initial studies suggested that KIM-1 elevations may be delayed relative to other novel biomarkers of AKI, more recent studies suggest that KIM-1 elevations do occur within hours of renal injury. Potential advantages of this biomarker include the apparent specificity for ischemic renal injury and the availability of a rapid assay. However, there have also been inconsistent results between studies⁷⁹ and further research is needed to clarify the value of KIM-1 as a diagnostic or prognostic biomarker in AKI.

4.4 Liver FABP-1 (L-FABP)

Fatty acid binding proteins (FABPs) are a family of small cytosolic proteins which facilitate β -oxidation through binding and transportation of long chain fatty acids.⁸⁰ Additionally, selective binding to lipid peroxidation products limits subsequent cellular toxicity,⁸¹ and this protective role has triggered interest in FABPs as potential markers of cellular injury. There are currently nine tissue-specific FABPs identified. Liver-type (L)-FABP (or FABP-1) is a 14-kDa hepatically synthesized protein localized to the liver, the intestinal tract, and the renal proximal tubule epithelium, a cell type reliant on primary fatty acid energy metabolism.

4.4.1 Pre-clinical Investigation—An antioxidant role of L-FABP was displayed by Wang and colleagues by subjecting liver cells to oxidative stress *in vitro*.⁸² Transfected cells, with increased L-FABP expression, displayed a significant decrease in the generation of reactive oxidant species. To allow examination of L-FABP *in vivo*, a humanized transgenic mouse model was generated, Tg-hL-FABP, which has significant L-FABP expression compared to minimal expression in wild type mice.⁸³ Expression of L-FABP was found to be protective against renal tubulointerstitial damage and prevented accumulation of lipid peroxidation products following ureteral obstruction.⁸⁴ Later, Yamamoto and colleagues demonstrated L-FABP transitions from the cytoplasm to the proximal tubular lumen in Tg-hL-FABP mice following ischemia-reperfusion injury, further supporting the acute response of L-FABP to oxidative stress and promoting urinary L-FABP (uL-FABP) as an early marker of kidney injury.⁸⁵

Negishi and colleagues showed that cisplatin-mediated nephrotoxicity was associated with increased uL-FABP excretion in the Tg-hL-FABP mice 1 day post exposure while elevations in BUN were not detected until 3 days later.^{86, 87} In a follow-up study, the same group showed that uL-FABP could be used as a marker of differing degrees of renal histological injury following cisplatin or ischemia-reperfusion induced AKI.⁸⁸ Here, uL-FABP levels showed a better correlation with renal histological injury scores than either BUN or N-acetyl-d-glucosaminidase, and urinary levels of L-FABP increased as early as 1–2 hours post injury. The correlation of uL-FABP excretion with degree of histologic renal injury has been confirmed in subsequent studies.⁸⁹ Collectively, this evidence suggests a protective role for L-FABP against oxidant stress and cellular toxicity, with urinary excretion acting as a rapid mechanistic marker of AKI.

4.4.2 Clinical Investigation—The role of L-FABP in human AKI has been investigated in several recent studies. Nakamura and colleagues measured uL-FABP in 66 patients, with baseline SCr of 1.2–2.5mg/dL, before and after elective coronary catheterization.⁹⁰ CIN, defined as an increase in SCr of greater than 0.5 mg/dL or a relative increase of more than

25% at 2 to 5 days post procedure, occurred in 13 patients. Baseline values of uL-FABP were significantly elevated in the 13 CIN patients compared to the remaining 53 patients (18.5 ± 12.8 vs. $7.4 \pm 4.4 \mu\text{g/gCr}$; $p < 0.01$). Levels significantly increased post procedure only in the CIN group (1 day post procedure $46.8 \pm 30.5 \mu\text{g/gCr}$; $p < 0.01$) and remained elevated at 14 days despite return to baseline of SCr, supporting both a predictive and diagnostic role of uL-FABP in CIN. More recently, Yamamoto and colleagues measured uL-FABP in 12 patients receiving living-related kidney transplants immediately after organ reperfusion was achieved.⁸⁵ They found a significant inverse correlation of uL-FABP levels with peritubular capillary flow, measured with intravital video analysis, and a significant direct correlation with transplant ischemia time (both $p < 0.0001$). This supported the role of uL-FABP as a marker of ischemic renal injury.

Portilla and colleagues measured uL-FABP before and after CPB in 40 pediatric patients.⁶ AKI, defined as a 50% increase in SCr, occurred in 21 patients with increases in uL-FABP, $20 \pm 4 \text{ ng/mgCr}$ to $1885 \pm 500 \text{ ng/mgCr}$ at 4hrs to $905 \pm 320 \text{ ng/mgCr}$ at 12hrs, while increases in SCr were not seen until 24–72hrs post-surgery. Both bypass time and 4hr uL-FABP were independent risk factors for AKI, and 4hr L-FABP had an AUC-ROC of 0.810 for predicting AKI. Additionally, in patients with AKI, there were no detectable changes in serum L-FABP until 12 hours post bypass suggesting that uL-FABP is a specific marker for AKI, and not merely a marker of high plasma levels from a non-renal source. Interestingly, in a study evaluating patients undergoing cardiac catheterization, Fukuda and colleagues concluded that uL-FABP was a marker of acute coronary syndrome, sensitivity 0.80 and specificity 0.96, and not a marker of AKI, although none of the studied patients developed CIN.⁹¹

Most recently, the diagnostic ability of uL-FABP has been examined in septic shock. Nakamura and colleagues retrospectively examined L-FABP levels in 40 patients with septic shock, 20 patients with sepsis without shock, and 20 hospitalized patients with AKI with mean SCr 2.8 mg/dL and baseline SCr $< 1.2 \text{ mg/dL}$.⁹² The 40 patients with septic shock had the highest uL-FABP levels even compared to those with AKI (1860 ± 1260 vs. $120 \pm 84 \mu\text{g/gCr}$; $p < 0.0001$). uL-FABP did not correlate with SCr or with need for dialysis. Ferguson and colleagues performed a cross-sectional study of 92 hospitalized patients with AKI compared to 68 primarily ambulatory controls.⁹³ uL-FABP was a significant independent predictor of need for renal replacement therapy. When comparing AKI versus hospitalized controls, the AUC-ROC of uL-FABP for identification of AKI was 0.93 using a cutoff of 47.1 ng/mgCr . Even comparing AKI to intensive care unit controls without AKI, the AUC-ROC remained at 0.82 for the diagnosis of AKI. No adjustment for severity of illness or liver dysfunction was made in this analysis.

Collectively, these studies demonstrate the utility of uL-FABP as an early indicator of AKI, however, further investigation is needed. The clinical trials have been small and largely cross-sectional; large prospective trials including multiple etiologies of renal disease are needed to truly assess the diagnostic and prognostic ability of L-FABP levels. Further investigation is also needed to clarify systemic illness, particularly liver injury, on the role of increased uL-FABP excretion. Finally, while ELISA testing is possible, no standardized assays for L-FABP exist to allow widespread clinical use.

4.5 Cystatin C (CysC)

Cystatin C (CysC) is a cysteine proteinase inhibitor that is produced at a constant rate by all nucleated cells.⁹⁴ This middle molecular weight molecule, 13-kDa, which does not exhibit significant protein binding, is freely filtered by the glomerulus but not secreted or reabsorbed into plasma, and therefore serum cystatin C (sCysC) levels have been extensively evaluated as a measure of GFR in CKD patients.^{95–98} Following filtration, CysC

is fully catabolized by proximal tubular epithelial cells⁹⁹ and is usually undetectable in the urine of patients with normal renal function. However, tubular injury can result in measurable urinary levels, suggesting CysC as a potential biomarker for AKI.

4.5.1 Preclinical Investigation—Because of the extensive background research establishing the role of CysC as a measure of GFR, there has been a relative paucity of pre-clinical studies of its use in AKI models. Instead, most studies have been focused on humans in various clinical settings as reviewed below.

4.5.2 Clinical Investigation—In contrast to some other novel biomarkers, a rise in sCysC levels reflects a decrement in GFR and therefore there is an inherent delay between renal injury and a detectable increase in levels. However, studies have shown that sCysC levels rise prior to creatinine levels and therefore may provide an earlier diagnosis of AKI. Herget-Rosenthal and colleagues found that a 50% rise in sCysC preceded a similar rise in serum creatinine (i.e. RIFLE stage R AKI) by 2 days. In their study of 85 ICU patients at risk for developing AKI, sCysC performed well as a diagnostic test for AKI with an AUC-ROC of 0.82. Cystatin C measurements were also predictive of the need for renal replacement therapy.¹⁰⁰ A more recent study by Royakkers and colleagues also found that sCysC levels of ICU patients developing AKI were elevated 2 days before meeting RIFLE criteria, providing an AUC of 0.72. Interestingly, however, sCysC levels no longer differed 24 hours preceding AKI diagnosis.¹⁰¹ In a study of patients receiving radiocontrast, Rickli and colleagues reported that sCysC levels were not elevated at 5 hours after contrast administration but subsequently peaked at 24 hours, while serum creatinine was still rising at 48 hours.¹⁰² Other studies have provided more mixed results regarding the utility of sCysC rise compared to serum creatinine.^{27, 103, 104} In addition, there have been reports of confounding clinical conditions that can lead to sCysC elevations, including thyroid disease and/or generalized inflammation.¹⁰⁵

Because uCysC should only be detectable when proximal renal tubular function is impaired, this marker may provide earlier diagnosis of renal injury and be more specific than sCysC. Koyner and colleagues evaluated a series of biomarkers following cardiac surgery and found that uCysC levels measured 6 hours post-operatively were predictive of AKI with an AUC of 0.72, outperforming sCysC and NGAL measured at the same time point.²⁷ More recent studies have reported less favorable results using uCysC, and a recent meta-analysis (which included the above study) concluded that uCysC has only modest diagnostic value with a pooled AUC-ROC of 0.64.¹⁰⁶

Unlike other novel biomarkers for AKI, CysC measurement is readily available for clinical measurement using automated and standardized assays that provide rapid turnaround. However, there is wide variation in the reporting of both serum and urinary CysC levels, and no consensus has emerged regarding threshold levels of CysC by which to define AKI diagnosis or risk.¹⁰⁶ Additionally, as a marker of GFR, sCysC shares many of the same limitations as serum creatinine for the diagnosis of AKI, including lack of specificity in differentiating acute versus chronic kidney disease and a delay of approximately 24 hours before rising after renal injury. As such, the primary use of sCysC measurements will likely remain in the evaluation of CKD. Conversely, uCysC theoretically holds more promise for AKI diagnosis. However, clinical studies to date have reported largely mixed results, and several studies suggest fairly poor diagnostic test characteristics compared to other novel biomarkers. Additional large studies will be needed to clarify any potential role of CysC in the diagnosis of AKI.

4.6 Other Protein Biomarkers of AKI

In addition to these widely investigated potential biomarkers, multiple studies have now used various MS platforms to examine the wide spectrum of urinary proteins in AKI. These studies have identified several novel urinary proteins with biomarker potential and have been recently reviewed by Bennett.¹¹ Nguyen and colleagues examined the urine proteome of 60 pediatric CPB patients. Aprotinin, alpha-1 microglobulin, alpha-1 acid glycoprotein, and albumin were significantly increased in patients that subsequently developed AKI, defined as a 50% increase in SCr.^{107–109} Similar studies in adult CPB populations have been performed. Ho and colleagues originally identified hepcidin-25 to be significantly decreased in patients with AKI following CPB.¹¹⁰ This finding has now been validated in a recent nested case control analysis using ELISA based measurement techniques, suggesting a role of iron homeostasis in the pathogenesis of AKI.¹¹¹ Aregger and colleagues examined the urinary proteome of 36 adults undergoing CPB.¹¹² In the 6 patients that developed AKI, defined by RIFLE criteria, urinary albumin was increased, and zinc-alpha-2-glycoprotein and a fragment of adrenomedullin-binding protein were decreased. Most recently, Heller and colleagues have shown that urinary calprotectin, an activator of the innate immune system, reliably differentiates pre-renal azotemia from intrinsic AKI in a group of hospitalized patients, supporting the predictive role of the urinary proteome even in heterogeneous patient populations.¹¹³ While these initial exploratory urinary proteome studies are intriguing and will undoubtedly lead to improved mechanistic understanding and diagnostic capabilities surrounding AKI, larger clinical trials are needed for validation.

5. Metabolite Biomarkers of AKI

In addition to proteomics, recent biomarker development has utilized the growing field of metabolomics. Metabolomics attempts to identify and quantitate small molecules from biological samples and includes the analysis of amino acids, lipids, nucleic acids, and organic acids to understand the overall disease phenotype. Beger and colleagues examined the urine of 40 pediatric patients following cardiac surgery and found a significant elevation of homovanillic acid sulfate in those that developed AKI.¹¹⁴ The level drawn at 4 hours post CPB had an AUC-ROC of 0.78 in predicting AKI. Metabolomic techniques have also been utilized in kidney transplant recipients, with the hopes of identifying an early biomarker of rejection and avoidance of high risk allograft biopsy. Foxall and others have consistently found elevations in urinary trimethylamine-N-oxide, an organic amine, in patients with early allograft dysfunction.^{115, 116} Metabolic profiles have also been widely utilized in animal models of drug induced kidney injury, which has recently been reviewed elsewhere.¹¹⁷ Continued advances in bioinformatics and analytical strategies will likely increase the widespread investigation of metabolomics in human AKI.

6. Summary and Perspective

Despite the significant progress in AKI biomarker discovery, further investigation is needed to validate clinical utility. Currently, the majority of investigation has involved small single center trials. Larger trials, with more heterogeneous populations, will be required to better understand the applicability to routine clinical practice. Additionally, future studies are needed to compare various biomarkers to each other. As each individual marker has independent biological properties and kinetics, it is likely that a panel, combining several biomarkers, will have greater diagnostic and predictive capabilities (Figure 1). Several studies have in fact demonstrated the benefits of combination panels in clinical trials.^{75, 118} For example, Metzger and colleagues identified a panel of urinary proteins which predict AKI in a heterogeneous ICU population with an AUC-ROC of 0.91.¹¹⁹ Most recently, Hall and colleagues have shown that a panel combining novel biomarker levels with other clinical variables leads to a significant increase in ability to predict worsening renal failure

and in-hospital mortality.¹²⁰ In the future, the biomarkers, either alone or as a panel, will require validation within interventional trials to determine their ability to predict important clinical outcomes, particularly related to therapeutic response. Finally, in order to fully validate a given biomarker, more accurate gold standard measures of AKI, without reliance on SCr, will have to be utilized. As of now, none of the novel biomarkers or panel has demonstrated a clear superiority to current diagnostic gold-standards. While a single super-marker is unlikely to exist, future investigation in this needed field will undoubtedly lead to greater mechanistic understanding of AKI. Until then careful clinical evaluation and measurement of SCr will continue to guide clinical decision making process.

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Abbreviations

AKI	Acute Kidney Injury
AKIN	Acute Kidney Injury Network
AUC-ROC	area under the curve of the receiver operating characteristic curve
ATN	acute tubular necrosis
BUN	blood urea nitrogen
CKD	chronic kidney disease
CIN	contrast induced nephropathy
CPB	cardiopulmonary bypass
CysC	Cystatin C
DGF	delayed graft function
ELISA	enzyme-linked immunosorbent assays
GFR	glomerular filtration rate
ICU	intensive care unit
IL-18	Interleukin-18
KIM-1	Kidney injury molecule-1
L-FABP	Liver-type fatty acid binding protein-1
MS	mass spectrometry
NGAL	neutrophil gelatinase-associated lipocalin
RIFLE	Risk Injury Failure Loss End-stage renal disease
SCr	serum creatinine
UTI	urinary tract infection

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Figure 1. Schematic diagram of biomarker temporal rise following acute kidney injury
 Values are extrapolated from available clinical trials and show the variability in biomarker elevations compared to an idealized biomarker. Neutrophil gelatinase-associated lipocalin (NGAL), Interleukin-18 (IL-18), Kidney injury molecule-1 (KIM-1), Liver-type fatty acid binding protein-1 (L-FABP), Serum creatinine (SCr).

Table 1

Limitations of serum creatinine in diagnosing acute kidney injury

Limitations of Serum Creatinine
Requires steady state GFR
Insensitive to small changes in GFR
Late marker of altered GFR
Non-specific to disease process
Altered by clinical characteristics (age, weight, gender, volume status, medication use)

GFR = glomerular filtration rate

Table 2

Ideal characteristics of a biomarker for acute kidney injury.

Ideal Biomarker Characteristics
Basal levels allow risk stratification
Sensitive and specific for kidney injury
Early marker of kidney injury
Wide spectrum of values correlating to degree of injury
Remains elevated for large diagnostic window
Levels can be monitored for therapeutic response
Unaffected by demographics and clinical factors
Samples readily available and non-invasive (urine, plasma)
Cost effective
Rapid results

Table 3

Advantages and disadvantages of novel clinical biomarkers of acute kidney injury

Biomarker	Source	Characteristics	Advantages	Disadvantages
NGAL	Urine Plasma	Ubiquitous epithelial protein Freely filtered by glomerulus, reabsorption at proximal tubule	Increased levels 1–2 hours post-insult ^{25, 26} Widely investigated in diverse populations ^{22, 23} Rapid point of care testing available	Decreased specificity in critically ill populations ⁴⁸
IL-18	Urine	Pro-inflammatory cytokine Injury induced production in proximal tubular epithelial cells	Increased levels 4–6 hours post-insult ⁶⁰ Widely investigated in diverse populations ^{41, 46, 58, 59, 61}	Increased in general inflammatory conditions ^{62, 63} Research based testing only
KIM-1	Urine	Membrane glycoprotein expressed in injured proximal tubular epithelial cells Directs phagocytosis of apoptotic cells	Increased specificity for ischemic renal injury ⁷⁴ Rapid point of care testing reported ⁷²	Delayed increase up to 12 hours post-insult in certain populations ⁷⁵ Lacking data in the majority of etiologies of acute kidney injury
L-FABP	Urine	Hepatically synthesized cytosolic protein in proximal tubule epithelial cells Limits cell toxicity via sequestration of lipid peroxidation products	Increased levels 4 hours post-insult ⁶ Baseline levels may prognosticate acute kidney injury ⁹⁰	May be a non-specific marker of ischemia ⁹¹ May be altered in sepsis and liver dysfunction ⁹² Research based testing only
Cys-C	Urine Plasma	Ubiquitously produced cysteine proteinase inhibitor Freely filtered at glomerulus without secretion or reabsorption Fully catabolized by prox tubule cells, undetectable in normal urine	Increased specificity for proximal tubule dysfunction compared to SCr Rapid clinical testing available	Non-specific marker of GFR suffering similar limitations to SCr Delayed increase up to 8–12 hours post-insult, but more rapid than SCr ^{100–102} Levels altered by thyroid disease and systemic inflammation ¹⁰⁵