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Proteomic analysis of acute kidney injury: biomarkers to mechanisms

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Abstract

Acute Kidney Injury is a devastating clinical condition, both in terms of mortality and costs, and is occurring with increasing incidence. Despite better clinical care, the outcomes of AKI have changed little in the last 50 years. This lack of progress is due in part to a lack of early diagnostic biomarkers and a poor understanding of the disease mechanisms. This review will focus on the rapid progress being made in both the understanding of AKI and the promising panel of early biomarkers for AKI that have come out of both direct proteomic analysis of body fluids of AKI patients and more targeted proteomic approaches using clues from other methods such as transcriptomics. This review concludes with a discussion of the future of proteomics and personalized medicine in AKI and the challenges presented in translating these exciting proteomic results to the clinic.

Keywords

Acute Kidney Injury; Biomarkers; Proteomics

Introduction

Acute kidney injury (AKI) is a devastating clinical condition that lacks satisfactory therapeutic options, represents a huge financial burden to society and is increasing in incidence at an alarming rate. Conservative estimates have placed the annual health care expenditures attributable to hospital-acquired AKI at greater than 10 billion dollars in the United States alone.[1,2] Although many new insights into the mechanisms of AKI have been advanced in recent years and novel interventions in animal models have shown promise, translational efforts in humans have been disappointing. There are many plausible reasons for this lack of success. Among them is the scarcity of early diagnostic markers of AKI leading to delayed initiation of therapy and incomplete pathophysiologic understanding of the disease process.[3] Another major hindrance to the successful implementation of new therapies is the lack of a consensus definition of AKI (which has supplanted the term acute renal failure, or ARF). The Acute Dialysis Quality Initiative (ADQI) workgroup found that over 30 definitions for ARF were used in the literature. The definitions varied from a 25%

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increase over baseline serum creatinine to the need for dialysis[4]. The term AKI is of relatively recent origin and was proposed to better account for the diverse spectrum of molecular, biochemical and structural processes that characterize AKI [5]. In order to better classify AKI, the RIFLE classification system (Table 1) was developed (Risk-Injury-Failure-Loss-End Stage Renal Disease)[6]. The first three classes represent degrees of injury and the last two are outcome measures. This system has shown to correlate well with mortality rates [7]. In order to further refine the definition of AKI, the Acute Kidney Injury Network (AKIN) proposed a modified version of the RIFLE classification, known as the AKIN criteria. The AKIN criteria define AKI as an abrupt (within 48 hours) reduction in kidney function as measured by an absolute increase in serum creatinine ≥ 0.3 mg/dL, a percentage increase in serum creatinine $\geq 50\%$, or documented oliguria (<0.5 mL/kg/hr) for more than 6 hours [8]. Minor modifications of the RIFLE criteria include broadening the “risk” category of RIFLE to include an increase in serum creatinine of at least 0.3 mg/dL in order to increase the sensitivity of RIFLE for detecting AKI at an earlier time point. In addition, the AKIN criteria sets a window on first documentation of any criteria to 48 hours and categorizes patients in the “failure” category of RIFLE if they are treated with renal replacement therapy, regardless of either changes in creatinine or urine output. Finally, AKIN replaces the three levels of severity R, I, and F with stage 1, 2, and 3 [9].

AKI has been reported to complicate up to 7% of all hospital admissions.[10,11] AKI defined by RIFLE criteria has been found in anywhere from 25 % [12] to 36% of all ICU (intensive care unit) admissions.[13,14] Despite significant improvements in therapeutics, the mortality and morbidity associated with AKI remain high. Over the past 50 years the prognosis of AKI has remained quite poor with a mortality rate of 40–80% in the intensive care setting [15,16]. Basic research has made major advances in illuminating the pathogenesis of AKI and has led to the development of successful therapeutic approaches in animal models.[15] The primary reason these therapies, such as insulin-like growth factor 1 and anaritide,[17,18], have failed in clinical trials is the lack of early biomarkers for AKI, analogous to the troponins in acute myocardial injury, to allow for early detection and rapid initiation of therapies.[19,20]

Currently serum creatinine is the sole FDA approved diagnostic marker of human AKI. However, serum creatinine is an unsatisfactory biomarker for renal disease, especially in cases of acute kidney injury (AKI), primarily due to a lack of specificity and slow response to alterations in disease severity or treatment. Serum creatinine levels change with factors unrelated to renal disease, such as age, gender, diet, muscle mass, muscle metabolism, race, strenuous exercise and hydration status. Creatinine levels are also influenced by certain drugs.[21,22] Furthermore, in AKI, serum creatinine is not a real time indicator of kidney function, because the patients are not in steady state; so rises in serum creatinine occur long after the renal injury is sustained. In fact, serum creatinine concentrations may not change until approximately 50% of kidney function has been lost. This makes serum creatinine a poor diagnostic marker for AKI, since treatments need to be administered soon after injury to be effective. Because so many variables affect creatinine levels, it also lacks precision in assessing disease progression or risk stratification. The problems with creatinine have been evident for over thirty years [23], yet until recently little progress had been made in the search for replacement markers that will aid in earlier, more accurate and specific diagnosis of renal disease.

Identification of novel AKI biomarkers has been designated a top priority by the American Society of Nephrology and the concept of developing a new collection of tools for earlier diagnosis of disease states is a prominent feature in the National Institutes of Health road map for biomedical research.[5,24] Conventional urinary biomarkers such as casts and fractional excretion of sodium are insensitive and nonspecific for early recognition of AKI.

[25] Other traditional urinary biomarkers such as filtered high molecular-weight proteins and tubular proteins or enzymes have also suffered from lack of specificity and few standardized assays.[26] Fortunately, the application of innovative technologies such as functional genomics and proteomics to human and animal models of kidney disease has uncovered several novel candidates that are emerging as biomarkers and therapeutic targets. [3,27–29] This review will update the reader on the current status of novel AKI biomarkers that have been identified by unbiased as well as targeted proteomic approaches.

Desirable Properties of AKI Biomarkers

Besides establishing the early diagnosis, biomarkers are needed for several other purposes in AKI (summarized in Table 2). Thus, biomarkers are needed for (a) pinpointing the location of primary injury (proximal tubule, distal tubule, interstitium, or vasculature); (b) determining the duration of kidney failure (AKI, chronic kidney disease, or “acute-on-chronic” kidney disease); (c) discerning AKI subtypes (prerenal, intrinsic renal, or postrenal); (d) identifying AKI etiologies (ischemia, toxins, sepsis, or a combination); (e) differentiating AKI from other forms of acute kidney disease (urinary tract infection, glomerulonephritis, or interstitial nephritis); (f) risk stratification and prognostication (duration and severity of AKI, need for renal replacement therapy, length of hospital stay, and mortality); (g) defining the course of AKI; and (i) monitoring the response to AKI interventions [3]. Biomarkers are also needed for use as surrogate endpoints in clinical trials evaluating potential therapeutics for AKI. Surrogate markers are precise measurements that can accurately correlate with a clinical endpoint [30]. Surrogate endpoints can expedite clinical trials evaluating the safety and efficacy of new drug applications. If the intervention has the desired effect on the surrogate endpoint, then further evaluations are warranted to directly address the effect of the intervention on the appropriate clinical endpoint. This linking of the surrogate endpoint to the clinical endpoint is referred to as validation and is an essential step in the biomarker discovery process.

With respect to the desirable characteristics of AKI biomarkers, the most important remain those that are clinically applicable and can lead to early diagnosis and treatment of AKI. Other important properties of clinically relevant biomarkers of AKI should include (a) measurements from non-invasive sources, such as blood or urine; (b) easy to perform either at bedside or in a standard clinical laboratory; (c) measurements should be reliable and have a rapid turnaround time; (d) they should be sensitive for early detection and have a wide dynamic range of values with cut offs to allow for risk stratification; (e) they should be highly specific, and ideally allow for AKI subtype classification; and (f) they should be inexpensive to allow for broad global use.

Proteomic Analysis in renal disease

The application of proteomics to renal disease is still in the early phases compared to proteomics in oncology and neurology.[27] However, due to the ready availability of a non-invasive sample source (urine) and continued optimization of methods, the study of renal proteomics is growing rapidly. In fact, tremendous progress has been made in the last 10 years, in terms of biomarker discovery and understanding of pathologic mechanisms of renal injury [3,15,27,28,31–35]

Proteomics for renal disease can be accomplished in urine, plasma and serum. Of these body fluids, urine is seen as the most attractive for 3 main reasons: 1) urine can be obtained in large quantities in a non-invasive manner, 2) due to a relative lack of proteolytic activity in urine, proteins and peptides are quite stable, and 3) the protein concentration and composition in urine directly reflects changes in kidney and/or urogenital tract function.[36] Urine, however, is not without its limitations. Urine has a low protein to salt ratio, which can

interfere with certain proteomic techniques.[27] Urine also has widely variable protein concentrations, which can be especially difficult to overcome in trying to compare groups with varying grades of proteinuria. Techniques to overcome this range of protein content include normalizing to urine creatinine [37] or total protein. Problems are evident with both methods of normalization. It has long been known that the excretion of creatinine is not constant and varies during a 24 hour cycle[38] and with factors such as muscle mass, age, sex and ethnicity.[39] In fact, Waikar et al. [40] recently studied the variability in urine creatinine excretion using both computer simulations of creatinine kinetics and serial urine collections from 12 kidney transplant patients and 11 ICU patients. The authors found that urine creatinine varied greatly between individuals (from 238–2327 mg / 24 hour in the transplant patients, and 139 mg/ 24 hour in a post lung transplant cystic fibrosis patient to 7198 mg / 24 hour in a sepsis patient recovering from AKI). Even within individuals, timed readings varied up to ~40% in some patients. Normalizing to protein content can be difficult when comparing samples with vastly different concentrations of protein. Identifying increases in specific proteins, which is one of the goals of proteomic analysis, can be impaired when adding increased volumes of low protein samples due to the increase in the noise level of background proteins. Nevertheless, significant advances have been made in spite of these difficulties.

Direct Proteomic Analysis in AKI

Multiple direct proteomic approaches have been applied to analysis of urine in AKI, including 2DE, MALDI TOF MS, SELDI TOF MS and LC/MS. These methods span the spectrum of larger proteins (2DE, MALDI), to smaller proteins and peptides (SELDI, LC/MS).

SELDI

Nguyen et al [41] used SELDI to identify a urinary proteomic signature to predict AKI in 30 pediatric patients undergoing cardiopulmonary bypass surgery (CPB). Peaks at 6.4, 28, 43, and 66 kDa, were strongly enhanced within the AKI group at 2 hours post CPB. It is important to note that serum creatinine did not increase significantly in these patients until 48 to 72 hours post surgery. All four markers displayed an area under the receiver operating characteristic curve (AUROC, or AUC) of 0.90 to 0.98 for the prediction of AKI, which indicates excellent sensitivity and specificity. The 6.4 kDa protein was identified by MS/MS as aprotinin, an antifibrinolytic agent previously used therapeutically in patients undergoing CPB to decrease blood loss and the need for transfusions.[42] The remaining three AKI peaks were identified as α -1 microglobulin (A1M, 28 kDa), α -1 acid glycoprotein (AAG, 43 kDa) and albumin (66 kDa) (Table 2).[43] A functional assay for aprotinin was used to determine levels of the protein in urine from 106 patients undergoing CPB. Urinary aprotinin levels 2 hours post initiation of CPB were significantly elevated in patients destined to get AKI (AUC 0.98) and correlated well with serum creatinine, duration of AKI, and length of hospital stay. The other 3 markers were validated using nephelometry in a large CPB cohort (n=365).[43] While all showed significant increases as early as 2 hours post CPB, the strongest independent predictor of AKI was AAG > 4 mg/dL at 6 hours post CPB. Increasing levels of all 3 biomarkers were correlated with worsening severity for clinical outcomes, such as increase in serum creatinine level, length of hospital stay, and duration of AKI. All three proteins exhibit biological plausibility as potential biomarkers of AKI. A1M is an acute phase glycoprotein produced in the liver, freely filtered by the glomerulus and efficiently reabsorbed by the healthy proximal tubule. AAG is also an acute phase reactant synthesized in the liver and handled by the kidney similar to A1M. It has two major biological functions – transport of endogenous substances and an immunomodulatory role, both of which are likely to be pertinent to the early pathophysiology of AKI. Albuminuria is a well known consequence of many acute renal conditions, resulting from

changes in capillary permeability and inflammatory insults. Further validating these results, a recent study of adults presenting to the emergency department, a single measurement of urinary A1M predicted the subsequent development of AKI with an AUC of 0.89 [44]. In the same cohort, urinary AAG predicted AKI with an AUC of 0.83. Both biomarkers were also useful in predicting dialysis requirement, ICU admission, and mortality. It should be noted that later studies linking aprotinin to dialysis requirement and mortality after cardiac surgery in adults led to aprotinin being pulled from the market in 2007.[45] The true safety of aprotinin in pediatric populations, however, remains a matter of investigation.[46,47] Thus, markers identified by unbiased proteomic approaches are emerging as potential clinical tools for the early prediction of AKI and its clinical consequences.

Ho et al.[48] utilized SELDI and known measures of tubular stress to analyze urine in 44 CPB patients, half of whom developed AKI. In all patients, an 11.7 kDa peak consistent with β -2 microglobulin was found upon admission to the ICU, but resolved to baseline in both groups before multiple peaks consistent with proteolytic cleavage of β -2 microglobulin developed 3–5 days post CPB in the AKI group. A peak at 2.78 kDa, later identified as hepcidin-25, was found preferentially in urine from patients without AKI at day 1 post CPB (Table 2). Hepcidin-25 is the active form of hepcidin, an important regulator of iron homeostasis. The presence of hepcidin-25 in patients without AKI may point toward a role for iron modulation in AKI.[43] Hepcidin excretion was also found to be altered through proteomic analysis of urine by MALDI in a model of nephrotoxic nephritis.[49] The increase in hepcidin was postulated to be due to local production and renal retention. These findings continue to point to a role for iron modulation in kidney injury, but the findings have not yet been validated and further study is warranted to elucidate the exact mechanism by which this occurs.

Bennett et al.[50] used SELDI to investigate proteomic differences in the urine of pediatric patients who developed contrast-induced nephropathy (CIN) after contrast administration for cardiac imaging. Of ninety patients who enrolled in the study, 10 patients developed AKI as a result of CIN by 24 hours post administration of ioversol, a low osmolar nonionic contrast agent. Seven patients who did not develop AKI served as age and sex matched controls. At time=0 peaks at 4361 Da and 4480 Da were found to be upregulated in the patients who developed AKI and those who did not, respectively. The 4480 Da peak was identified by an on-chip immunoassay as a 41 amino acid variant of human β -defensin 1 (HBD-1). This protein was found on three chromatographic surfaces to be upregulated 4–12 fold with an AUC ranging from 0.89–0.99 in patients who did not develop CIN. A reciprocal marker at 4361 Da (hypothesized to be the 40 amino acid variant of HBD-1) was upregulated 4 fold with an AUC of 0.84 for the prediction of CIN (Table 2). HBD-1 has several active forms ranging from 36 to 47 amino acids formed through amino truncations, and represents a group of peptides with antimicrobial properties that act as part of the host defense system of the urinary tract. Evidence also suggests that HBD-1 may be involved as a regulator of cytotoxic and immune responses in the kidney.[51,52] It could be speculated that this regulatory role could confer some protection against kidney injury from contrast agents. The presence of the 4361 Da peak in patients at higher risk for developing CIN could be useful as a biomarker for the early prediction of CIN. There are limitations of this study. First, this study was conducted in a single population with congenital heart defects and needs to be validated in a prospective trial of a larger population by a standard quantitative test. This is a difficult limitation to overcome since traditional methods such as ELISA and western blot cannot be used to detect differences in concentration of proteins which only differ by one or two amino acids.

2DE

Aregger, et al.[53] used 2DE to examine the urinary proteome before and after CPB. Thirty six adults undergoing elective CPB were enrolled in the study. Using RIFLE criteria, 6 patients developed AKI. Six additional sex and age matched patients were selected as controls. Only three proteins were identified as different between AKI and control groups. Both zinc-alpha-2-glycoprotein (ZAG) and adrenomedullin-binding protein (AMBP) were down-regulated in AKI patients and only serum albumin was found to be up-regulated. Only ZAG was pursued for validation in a larger cohort (68 patients, 22 with AKI and 46 without AKI) by western blot and ELISA (Table 2). Median ZAG excretion was found to be lower in subjects with AKI, but was only weak predictor of AKI with an AUC of 0.68. Additional validation of the other markers needs to be undertaken. Limitations of this study include not excluding all comorbid conditions (other than drug use and preexisting renal disease) and small sample size, which could have both influenced the lack of major differences in AKI patients than may have been discovered in a larger, more homogeneous population.

Zhou et al.[54] used 2DE followed by MALDI or LC/MS to investigate urinary exosomal proteins in rats given the nephrotoxic drug cisplatin. Exosomes contain apical membranes and intracellular fluid and are normally secreted into the urine from all nephron segments, and contain protein markers of structural and functional renal damage. Twenty-eight differentially regulated proteins were identified by MALDI or LC/MS, but only 2 were verified to be differentially expressed by western blot. Annexin V was confirmed to be decreased in response to cisplatin. Fetuin-A was identified by immunoelectron microscopy and was found to be increased over 30 fold in the early phase of ischemia reperfusion injury (IRI) by western blot (Table 2). Additionally, 3 patients in the ICU with AKI had increased levels of Fetuin-A by western blot when compared to non-AKI patients. An advantage of exosomes as a source for biomarkers is that they lack many of the most abundant urinary proteins, such as albumin, uromodulin (Tamm-Horsfall mucoprotein) and globulin, which often interfere with biomarker discovery in urine.[3,55,56] The low rate of identification and validation of differences in this study highlights a limitation with this approach to biomarker discovery. Despite their advantages, urinary exosomes are limited in their clinical utility because of the many steps involved in their preparation. Nonetheless, clinically translatable assays, such as commercially available ELISA kits have recently become available which may lead to more widespread clinical testing of Fetuin-A as a marker for human AKI.

Puigmule et al. [57] took a different approach directed more toward *discovering functional pathways* involved in cyclosporine A (CsA) nephrotoxicity. CsA is a potent immunosuppressant that has limited use in organ transplant patients due to its potent nephrotoxicity. The authors examined the direct effects of CsA on the proteome of proximal tubule cell lines derived from both mice and humans. Three proximal tubule cell lines, as well as non-renal HeLa cells (an epithelial cell line derived from human cervical adenocarcinoma) for controls, were exposed to increasing doses of CsA for up to 72 hours. 2DE of cell lysates showed 38 differentially expressed proteins in the murine proximal tubule cell lines at the minimal toxic dose of 10 μ M CsA. The main functional classes of proteins altered (in descending order) were protein metabolism, response to damage, cytoskeletal, energy metabolism, cell cycle and nucleic acid metabolism. Results from selected proteins (α -B crystalline, RACK-1, n-actylcysteine amide (NACA) and Cyclophilin A (CypA)) were investigated by western blot in kidney lysates from male mice treated with CsA or vehicle. Decrease in expression of NACA, α -B crystalline, and CypA were all confirmed by 1D and 2D western blot. Only 2 minor RACK-1 isoforms decreased after treatment, but were not reflected in overall protein levels of RACK-1 (Table 2). This result illustrates a problem with the clinical translatability of certain proteomic findings. Often, fragments or specific isoforms of proteins are differentially found in disease groups, but these are difficult to discern in clinical assays because they can be indistinguishable from

other isoforms, or full length proteins in methods such as ELISA or immunonephelometry. However, studies like this are valuable to distinguish pathways involved in nephrotoxicity and kidney injury and could potentially lead to a better understanding of the disease, and thus more targets for therapeutics. Also, since current methods do not accurately reflect the full extent of CsA toxicity, immunohistochemical evaluation of these markers in biopsy tissue could be undertaken to evaluate and adjust doses of CsA in transplant patients to non-toxic ranges.

LC MS/MS

Sigdel et al. [35] used “shotgun proteomics” to identify proteins specific to AKI resulting from acute transplant rejection (AR). Shotgun proteomics, a form of “bottom up” proteomic analysis, refers to digesting protein samples into peptides and sequencing based on MS/MS and database searching. The authors analyzed urine from 40 patients: 10 renal transplant patients with stable grafts, 10 renal transplant patients with AR, 10 controls with non-specific proteinuria, and 10 age matched healthy controls. Two-hundred eighty four proteins were found to be differentially expressed in AR. The authors measured uromodulin (Tamm Horsfall mucoprotein), Pigment epithelium-derived factor precursor (PEDF) and CD44. ELISA confirmed decreased levels of uromodulin and CD44, and increased levels of PEDF in the AR patients compared to those with stable graft function, as well as disease and healthy controls (Table 2). The AUC for AR classification was 0.97 for CD44, 0.93 for PEDF and 0.85 for uromodulin. Uromodulin is an abundant tubule protein and its mutation or altered levels have been linked to various familial nephropathies, delayed graft function, and renal failure in type-1 diabetes.[58–60] PEDF is a serine protease inhibitor, is a potent inhibitor of angiogenesis, and has been found to be increased in the serum of patients with diabetic nephropathy.[61,62]. The authors suggest PEDF levels could be used to monitor health status in renal transplant patients.[35] The exact function of CD44 in the kidney has not been determined. While the authors were able to validate their LC/MS/MS data on uromodulin, CD44 and PEDF, this study has some important limitations. First, the groups only consisted of 10 patients each, so the data must be validated in a larger sample set. Second, LC/MS/MS, like SELDI, is semi-quantitative. It is difficult to determine the accuracy of the approach based on only 3 of 284 proteins found to be differentially expressed in the initial phase of the study. Nevertheless, the finding of PEDF having an AUC of 0.93 for the classification of AKI due to AR warrants further investigation.

Metabonomics

An extension of current methods of proteomic analysis is the emerging field of metabolomics, or metabonomics. Though often used interchangeably, the goals of metabolomics are to catalog and quantify the collection of small molecules found in biological fluids under different conditions, while metabonomics is the study of how the metabolic profile of a complex biological system changes in response to stresses like disease, toxic exposure, or dietary change.[63–65] Metabolomics or metabonomics are often nuclear magnetic resonance (NMR) or MS based, and are another avenue for discovering novel serum or urinary biomarkers of AKI.

Beger et al. [66] used LC/MS based metabonomics to investigate metabolic changes in urine from 40 pediatric CPB patients (21 developed AKI). Principal component analysis (PCA) scores were able to cluster AKI patients from non-AKI patients at 4 and 12 hours separately, most of the metabolites responsible for the clustering were unknown. The most significant metabolite distinguishing AKI from non-AKI patients was identified by MS/MS as homovanillic acid sulfate (Table 2), a metabolite of dopamine. This metabolite was found to be upregulated in AKI patients as early as 4 hours post surgery (AUC 0.78), but was most useful for prediction of AKI at 12 hours post CPB, with an AUC of 0.95 (Table 2). If

validated in a much larger sample set this marker could serve alongside other early biomarkers for AKI, such as neutrophil-gelatinase associated lipocalin (NGAL, which we will discuss later), to serve as a screening panel for AKI after CPB. Additionally, the increased presence of HVA- SO₄ in the urine of patients with AKI might yield insight into the early changes in the kidney after injury.

In addition to the more well known sympathetic nervous and adrenomedullary hormonal systems involving dopamine, a peripheral catecholamine system operates in the kidney, where dopamine functions as a natriuretic factor.[67,68,70] Dopamine excreted in the urine is believed to be of local origin, produced in the epithelium of the proximal tubules, and not simply filtration of circulating dopamine[66] In opposition to the increase in HVA in AKI, patients with chronic kidney disease have been found to have a reduction of urinary dopamine and its metabolites related to the concomitant reduction in renal function.[69]

Targeted Proteomics in AKI

Additional promising AKI biomarkers have recently been discovered by transcriptomic profiling techniques, followed by proteomic strategies for their translation from the bench to the bedside, an approach best exemplified by NGAL and kidney injury molecule-1 (KIM-1). Preclinical cDNA microarray studies in animal models of AKI identified *Ngal* (also known as lipocalin 2 or *lcn2*) to be one of the earliest and most upregulated genes in the kidney. Downstream proteomic analyses also revealed NGAL to be one of the most highly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models.[71] The serendipitous finding that NGAL protein was easily detected in the urine soon after AKI in animal studies [72] has inspired a number of translational studies to evaluate NGAL as a non-invasive biomarker in human AKI. NGAL has now emerged as an excellent stand-alone troponin-like biomarker in the urine and plasma, for the prediction of AKI, for monitoring clinical trials in AKI, and for the prognosis of AKI in several common clinical scenarios. [73] These include settings of cardiac surgery,[74] contrast-induced nephropathy,[75] kidney transplantation,[76] critical care,[77] and the emergency department,[44] to name a few [recently reviewed in Devarajan P[78]]. The deployment of standardized clinical platforms for the rapid and accurate measurement of NGAL in urine [79] and plasma [80] will facilitate the widespread use and validation of NGAL as an AKI biomarker.

A subtractive hybridization screening identified kidney injury molecule 1 (Kim-1) as a gene that was markedly up-regulated in ischemic rat kidneys.[81] Downstream proteomic studies have also shown KIM-1 to be one of the most highly induced proteins in the kidney after AKI in animal models, and a proteolytically processed domain of KIM-1 is easily detected in the urine soon after AKI.[82] Human studies have indicated that urine KIM-1 measurements can predict AKI and its clinical outcomes.[83] The recent availability of a rapid urine dipstick test for KIM-1 will facilitate its further evaluation in future preclinical and clinical studies.[84]

Challenges of Translating Proteomic Results to the AKI Clinic

This review highlights some of the successful applications of clinical proteomics and biofluid profiling in the field of AKI. However, a number of challenges remain to be addressed for this relatively new technology to be increasingly utilized in the clinical arena. First, clinical proteomics remains largely a discovery tool that potentially yields a voluminous dataset of altered protein expression profiles, which require laborious data mining and statistical analyses that have yet to be fully defined. In the AKI literature, processes for choosing the right targets for downstream confirmation and clinical translation appear to have been somewhat arbitrary. Second, standardized procedures for initial biofluid

handling and processing are still lacking, which renders it difficult to compare and contrast the results from published studies, and adds a number of technical confounding variables. Third, urinary proteomic profiling in AKI has often yielded several fragments of albumin and other common serum proteins, the biological significance of which is unclear and the translation to clinically applicable assays technically challenging. Fourth, the road from unbiased identification and initial testing, to systematic validation of AKI biomarkers, as elucidated by recently established phases of the diagnostic test development process,[85] remains long, winding, and challenging.

In addition, there are important limitations that exist in the published AKI biomarker literature that must be acknowledged. First, the majority of studies reported have been from single centers that enrolled small numbers of subjects. Validation of the published results in large multicenter studies will be essential. Second, most studies reported to date do not include patients with chronic kidney disease. This is problematic, not only because it excludes a large proportion of subjects who frequently develop AKI in clinical practice, but also because chronic kidney disease in itself can confound AKI biomarker values. Third, many studies report only statistical associations (odds ratio or relative risk), but do not report sensitivity, specificity, and AUCs for the diagnosis of AKI, which are essential to determine the accuracy of the biomarker. Fourth, only a few studies with relatively small number of cases have investigated biomarkers for the prediction of AKI severity, morbidity, and mortality – results of biomarker testing as predictors of hard clinical outcomes in large multicenter studies are anxiously awaited. Finally, the definition of AKI in the published studies varied widely, but was based largely on elevations in serum creatinine, which raises the conundrum of using a flawed outcome variable to analyze the performance of a novel assay. The studies of biomarkers for the diagnosis of AKI may have yielded different results had there been a true “gold standard” for AKI. Instead, using AKI as defined by a change in serum creatinine sets up the biomarker assays for lack of accuracy due to either false positives (true tubular injury but no significant change in serum creatinine) or false negatives (absence of true tubular injury, but elevations in serum creatinine due to pre-renal causes or any of a number of confounding variables that haunt this measurement). It will be crucial in future studies to understand the clinical outcomes of subjects who may be prone to AKI and are “biomarker-positive” but “creatinine-negative”, since this will determine whether the biomarker is overtly sensitive. It is vital that large enough future studies demonstrate (a) the association between biomarkers and hard outcomes such as dialysis, cardiovascular events, and death, and (b) that randomization to a treatment for AKI based on high biomarker levels results in an improvement in kidney function and reduction of clinical outcomes.

Despite all these challenges, it is hoped that this review provides encouraging information regarding the discovery and translation of novel AKI biomarkers. Proteomic approaches have now identified several promising biomarkers, perhaps best exemplified by NGAL, KIM-1, A1M and AAG, that have successfully passed through the pre-clinical, assay development, and initial clinical testing stages of the biomarker development process. They have now entered the prospective screening stage, facilitated by the development of standardized clinical platforms for their measurement on large populations across different laboratories. The availability of a panel of AKI biomarkers could further revolutionize and personalize renal and critical care. However, such idealistic thinking must be tempered with the enormous technical and fiscal issues surrounding the identification, validation, commercial development and acceptance of multi-marker panels. Deriving from the recent cardiology literature, a clinically useful biomarker should (a) be easily measurable at a reasonable cost with short turnaround times; (b) provide information that is not already available from clinical assessment; and (c) aid in medical decision making ([86]). In this respect, troponin as a stand-alone biomarker provides excellent diagnostic and prognostic

information in acute coronary syndromes and acute decompensated heart failure [87]. If the current prospective multicenter studies of biomarkers such as NGAL and KIM-1 measured using standardized laboratory platforms provide promising results, we may have already closed in on the “renal troponins”..

Abbreviations

AKI	acute kidney injury
ARF	acute renal failure
ADQI	Acute Dialysis Quality Initiative
RIFLE	Risk-Injury-Failure-Loss-End-stage renal disease
AKIN	Acute Kidney Injury Network
ICU	intensive care unit
ROC	receiver operating characteristic
CPB	cardiopulmonary bypass
A1M	α -1 microglobulin
AAG	α -1 acid glycoprotein
IRI	ischemia reperfusion injury
POD-1	post operative day 1
ZAG	zinc α -2 glycoprotein
AMBP	adrenomedullary binding protein
GFR	glomerular filtration rate
HBD-1	human β -defensin 1
CIN	contrast induced nephropathy
CsA	cyclosporine A
AR	acute rejection
PEDF	pigment epithelium-derived factor precursor
HVA-SO₄	homovanillic acid sulfate
NGAL	neutrophil gelatinase-associated lipocalin
KIM-1	kidney injury molecule 1

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Table 1

RIFLE Criteria (Acute Dialysis Quality Initiative)

Stage	Serum Creatinine (SCr) Criteria	GFR Criteria	Urine Output criteria
R = Risk	Increase in SCr $\geq$ 1.5 x baseline	Decrease in GFR $\geq$ 25%	< 0.5 ml/kg/hr for 6 hr
I = Injury	Increase in SCr $\geq$ 2.0 x baseline	Decrease in GFR $\geq$ 50%	< 0.5 ml/kg/hr for 12 hr
F = Failure	Increase in SCr $\geq$ 3.0 x baseline OR serum creatinine $\geq$ 4.0 mg/dL in the setting of an acute rise $\geq$ 0.5 mg/dL	Decrease in GFR $\geq$ 75%	< 0.3 ml/kg/hr for 24 hr or anuria for 12 hr
L = Loss	Persistent Failure > 4 weeks		
E = End-Stage Renal Disease	Persistent Failure > 3 months		

Adapted from reference [6]

Table 2

Summary of biomarker discoveries from direct proteomic analysis in AKI

Method	Setting	Sample	Discovery (n)	Validation (n)	Significant Proteins (AUC)	Notes
SELDI	CPB [41, 42]	Urine	30	106	Aprotinin (0.98)	Drug used to control bleeding, subsequently removed from market due to increased mortality rates.
	CPB[43]	Urine	30	365	AIM (0.84), AAG (0.87), albumin(0.76)	AIM and AAG have been independently validated in separate adult studies for prediction of AKI[44]
	CPB[48]	Urine	44	N/A	β -2 microglobulin fragments, hepcidin-25 (decreased)	Results not validated. Hepcidin-25 may link iron modulation to AKI[49]
	CIN[50]	Urine	17	N/A	HBD1-41aa (0.89-0.99 – decreased), 4361Da unidentified protein (0.84)	Fragments of HBD-1 may have protective effects against CIN, but validation by ELISA or WB will be difficult for such closely related fragments
2DE	CPB[53]	Urine	12	68	ZAG (0.68 – decreased)	AMBIP and serum albumin were also found different in AKI, but not chosen for independent validation
	Cisplatin toxicity[54]	Urine exosomes (rat and human)	151 (rats)	9 (human)	Fetuin-A	Small sample size, but recent availability of commercial ELISA kits for Fetuin-A may lead to increased testing
	CsA toxicity[57]	Proximal tubule cell lines	6 gels from 3 pooled culture plates each	16 (mice)	α -B crystalline, NACA, CypA	Rack-1 had also been found decreased in CsA treated mice, but only 2 minor isoforms were validated with no difference in total Rack-1.

Method	Setting	Sample	Discovery (n)	Validation (n)	Significant Proteins (AUC)	Notes
LC MS	AR[35]	Urine	40	40	PEDF (.93), CD44 (0.97 – decreased) uromodulin (0.86 – decreased)	Small groups (10 patients each). Of 284 proteins found different in AR, only 3 were selected for validation.
Metabonomics	CPB[66]	Urine	40	N/A	HVA-SO ₄ (0.95)	Results have not been validated, but provide an interesting link to the role of dopamine in the kidney[67–69]