Proteomics for Biomarker Discovery in Acute Kidney Injury

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Summary: Acute kidney injury (AKI), previously referred to as acute renal failure, represents a common and devastating problem in clinical medicine. Despite significant improvements in therapeutics, the mortality and morbidity associated with AKI remain high. A major reason for this is the lack of early markers for AKI, and hence an unacceptable delay in initiating therapy. Fortunately, the application of innovative technologies such as functional genomics and proteomics to human and animal models of AKI has uncovered several novel biomarkers and therapeutic targets. The most promising of these are chronicled in this review. These include the identification of biomarker panels in plasma (neutrophil gelatinase–associated lipocalin and cystatin C) and urine (neutrophil gelatinase–associated lipocalin, kidney injury molecule-1, interleukin-18, cystatin C, α1-microglobulin, Fetuin-A, Gro-α, and meprin). It is likely that the AKI panels will be useful for timing the initial insult, and assessing the duration and severity of AKI. It is also probable that the AKI panels will distinguish between the various etiologies of AKI and predict clinical outcomes. It will be important in future studies to validate the sensitivity and specificity of these biomarker panels in clinical samples from large cohorts and from multiple clinical situations. Such studies will be facilitated markedly by the development of commercial tools for the reproducible measurement of biomarkers across different laboratories.

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Acute kidney injury (AKI) is a term proposed to reflect the entire spectrum of acute renal failure (ARF), a complex disorder that occurs in a wide variety of settings with clinical manifestations ranging from a minimal increase in serum creatinine level to anuric renal failure.1 AKI represents a significant but underrecognized problem in clinical medicine, with devastating immediate and long-term consequences.2-5 The incidence of AKI varies from 5% of hospitalized patients to 30% to 50% of patients in intensive care units. There is substantial indication that the incidence of AKI is increasing at an alarming rate, and the associated mortality and morbidity have remained high despite improvements in clinical care.6-8 Although the worst outcomes in AKI traditionally have been associated with dialysis requirement,9,10 there is now mounting evidence to suggest that even very small increases in serum creatinine level portend a significant amplification in mortality and morbidity rates.11-14 Although recent advances have suggested novel mechanistic insights and therapeutic approaches in animal models, translational efforts in human beings have yielded disappointing results. The reasons for this include a lack of a consensus definition of AKI,1 an incomplete understanding of the underlying pathophysiology,15 and the lack of early biomarkers for AKI, akin to troponins in acute myocardial disease, leading to an unacceptable delay in initiating therapy.16-18 In current
clinical practice, AKI typically is diagnosed by measuring the serum creatinine level. Unfortunately, creatinine is an unreliable indicator during acute changes in kidney function.19 First, serum creatinine levels can vary widely with age, sex, muscle mass, muscle metabolism, medications, and hydration status. Second, serum creatinine concentrations may not change until about 50% of kidney function already has been lost. Third, at lower rates of glomerular filtration, the amount of tubular secretion of creatinine results in an overestimation of renal function. Fourth, during acute changes in glomerular filtration, serum creatinine does not accurately depict kidney function until steady-state equilibrium has been reached, which may require several days. However, animal studies have shown that although AKI can be prevented and/or treated by several maneuvers, these must be instituted very early after the initiating insult, well before the serum creatinine level even begins to increase.15-18 Not surprisingly, the lack of early biomarkers has negatively impacted a number of landmark clinical trials investigating highly promising therapies for AKI in human beings.20,21

The quest to improve our knowledge of AKI pathogenesis and early diagnosis is an area of intense contemporary research.22-28 Conventional urinary biomarkers such as casts and fractional excretion of sodium have been insensitive and nonspecific for the early recognition of AKI. Other traditional urinary biomarkers such as filtered high-molecular-weight proteins and tubular proteins or enzymes also have suffered from a lack of specificity and a dearth of standardized assays. Identification of novel AKI biomarkers has been designated as a top priority by the American Society of Nephrology.29 The concept of developing a new toolbox for earlier diagnosis of disease states also is featured prominently in the National Institutes of Health road map for biomedical research.30 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.31-35 This review updates the reader on current advances in proteomics that hold promise primarily in ischemic AKI, the most common and serious subtype of ARF in hospitalized patients. The reader is referred to other publications that address the role of proteomics following nephrotoxins,36,37 kidney transplantation,38,39 and glomerulonephritis.40

**DESIRABLE PROPERTIES OF AKI BIOMARKERS**

In addition to aiding in early diagnosis and prediction, biomarkers may serve several other purposes in AKI. Thus, biomarkers also are needed for the following: (1) identifying the primary location of injury (proximal tubule, distal tubule, interstitium, or vasculature); (2) pinpointing the duration of kidney failure (AKI, chronic kidney disease, or acute-on-chronic); (3) discerning AKI subtypes (prerenal, intrinsic renal, or postrenal); (4) identifying AKI etiologies (ischemia, toxins, sepsis, or a combination); (5) differentiating AKI from other forms of acute kidney disease (urinary tract infection, glomerulonephritis, or interstitial nephritis); (6) risk stratification and prognostication (duration and severity of AKI, need for renal replacement therapy, length of hospital stay, and mortality) (7) defining the course of AKI; and (8) monitoring the response to AKI interventions. Furthermore, AKI biomarkers may play a critical role in expediting the drug development process. The Critical Path Initiative issued by the Food and Drug Administration in 2004 stated that, “Additional biomarkers (quantitative measures of biologic effects that provide informative links between mechanism of action and clinical effectiveness) and additional surrogate markers (quantitative measures that can predict effectiveness) are needed to guide product development.”26,29

Desirable characteristics of clinically applicable AKI biomarkers include the following: (1) they should be noninvasive and easy to perform at the bedside or in a standard clinical laboratory, using easily accessible samples such as blood or urine; (2) they should be rapidly and reliably measurable using a standardized assay platform; (3) they should be highly sensitive to facilitate early detection, and with a wide dynamic range and cut-off values that allow for risk stratification; (4) they should be highly
specific for AKI, and enable the identification of AKI subtypes and etiologies; and (5) they should show strong biomarker properties on receiver-operating characteristic (ROC) curves.

The ROC analysis has been used extensively as a fundamental evaluation tool in clinical studies pertaining to diagnostic testing.\textsuperscript{41,42} A ROC curve is a graphic plot of the sensitivity on the y-axis versus (1 - specificity) on the x-axis for a binary classifier system because its discrimination threshold is varied. For biomarker analysis, the binary classification task typically is to determine whether a subject has a certain disease (such as AKI) or not. Characteristically, ROC curves are generated for various cut-off points for the biomarker concentration under consideration. A commonly derived statistic from the ROC curve is the area under the curve (AUC). An AUC of 1.0 represents a perfect biomarker, whereas an AUC of 0.5 indicates a result that is no better than expected by random chance. An AUC of 0.75 or greater generally is considered a good biomarker, and an AUC of 0.9 or greater would represent an excellent biomarker.

THE SEARCH FOR NOVEL AKI BIOMARKERS

The biomarker development process typically has been divided into 5 phases,\textsuperscript{43} as shown in Table 1. The preclinical discovery phase requires high-quality, well-characterized tissue or body fluid samples from carefully chosen animal or human models of the disease under investigation. Typically, tissue analysis uses genomic approaches whereas body fluids are best analyzed by proteomic techniques. Identifying biomarkers in the serum or urine is most desirable because these samples are obtained easily and allow for noninvasive testing. Urine is more likely to contain biomarkers arising from the kidney, more applicable for easy patient self-testing, and more amenable to proteomic screening because of the limited number of protein species present. However, urine samples are more prone to protein degradation, and biomarker concentrations may be confounded by changes in urine flow rate. Serum samples are available readily even in anuric patients, and serum biomarkers show better stability. On the other hand, serum markers may reflect the systemic response to a disease process rather than specific organ involvement, and the presence of a large number of normally abundant proteins (such as albumin and immunoglobulins) in blood renders proteomic approaches difficult.

The widespread availability of enabling technologies such as functional genomics and proteomics has accelerated the rate of novel biomarker discovery. The advent of the microarray, or complementary DNA chip, allows investigators to search through thousands of genes simultaneously, making the process very efficient. Such gene expression profiling studies

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<td>Prospective screening</td>
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Data from Pepe et al.\textsuperscript{43}
have identified several genes whose protein products have emerged as AKI biomarkers,\textsuperscript{15,22} as detailed later. However, microarray-based methods cannot be used for the direct analysis of biological fluids, and usually require downstream confirmation by proteomic techniques before clinical use. Proteomics is the study of both the structure and function of proteins by a variety of methods, such as gel electrophoresis, immunoblotting, mass spectrometry, and enzymatic or metabolic assays. Each method is used to determine different types of information and has its own set of strengths and limitations. Advancing technologies radically have improved the speed and precision of identifying and measuring proteins in biological fluids, and proteomic approaches also are beginning to yield novel AKI biomarkers,\textsuperscript{24-28,44} as detailed later.

PROTEOMIC ANALYSIS IN AKI: CLUES FROM TRANSCRIPTOME PROFILING

Attempts at unraveling the myriad pathways activated in AKI have been facilitated by transcriptome profiling technologies. Several investigators have used molecular techniques such as complementary DNA microarrays\textsuperscript{45-48} and subtractive hybridizations\textsuperscript{49-51} combined with downstream proteomic analysis to identify novel pathways, biomarkers, and drug targets in AKI. Findings from these approaches are voluminous, and only those that potentially are pertinent to human AKI at the present time are detailed later.

Supavekin et al\textsuperscript{45} performed detailed mouse kidney microarray analyses at early time points after ischemia-reperfusion injury to identify consistent patterns of altered gene expression, including transcription factors, growth and regenerative genes, and apoptotic molecules. Prominent among the last category included FADD, DAXX, BAD, BAK, and p53, all of which were confirmed by immunohistochemistry. Mounting evidence now indicates that apoptosis is a major mechanism of early tubule cell death in contemporary clinical AKI.\textsuperscript{52-55} Several human models of AKI consistently have shown the presence of apoptotic changes in tubule cells.\textsuperscript{56-61} Importantly, proteomic studies have identified a multitude of apoptotic pathways, including the intrinsic (Bcl-2 family, cytochrome c, caspase 9), extrinsic (Fas, FADD, caspase 8), and regulatory (p53) factors that are activated in tubule cells after human AKI.\textsuperscript{59-61} As a consequence of these studies, inhibition of apoptosis has emerged as a promising approach in human AKI.\textsuperscript{62-71} Cell-permeant caspase inhibitors have provided particularly attractive targets for study. In this regard, an orally active, small-molecule, pan-caspase inhibitor (IDN-6556; Pfizer, New York, NY) has been shown to be effective in preventing injury after lung and liver transplantation in animals.\textsuperscript{70,71}

NGAL as an AKI Biomarker

Supavekin et al\textsuperscript{45} also identified neutrophil gelatinase–associated lipocalin (NGAL, also known as lcn2) as one of the most up-regulated transcripts in the early postischemic mouse kidney, a finding that now has been confirmed in several other transcriptome profiling studies. Downstream proteomic studies also have revealed NGAL to be one of the earliest and most robustly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models, and NGAL protein is detected easily in the blood and urine soon after AKI.\textsuperscript{72-75} These findings have spawned a number of translational proteomic studies to evaluate NGAL as a novel biomarker in human AKI.

In a cross-sectional study, subjects in the intensive care unit with established ARF displayed a greater than 10-fold increase in plasma NGAL and more than a 100-fold increase in urine NGAL by Western blotting when compared with normal controls.\textsuperscript{74} Both plasma and urine NGAL correlated highly with serum creatinine levels. Kidney biopsy specimens in these patients showed intense accumulation of immunoreactive NGAL in 50% of the cortical tubules. These results identified NGAL as a widespread and sensitive response to established AKI in human beings. In a prospective study of children undergoing cardiopulmonary bypass, AKI (defined as a 50% increase in serum creatinine level) occurred in 28% of the subjects, but the diagnosis using serum creatinine only was possible 1 to 3 days after surgery.\textsuperscript{76} In marked contrast, NGAL measurements by Western blotting and by enzyme-linked immunosorbent assay (ELISA) revealed a robust 10-fold or
more increase in the urine and plasma within 2 to 6 hours of the surgery in patients who subsequently developed AKI. Both urine and plasma NGAL were powerful independent predictors of AKI, with an AUC of 0.998 for the 2-hour urine NGAL and 0.91 for the 2-hour plasma NGAL measurement. The 2-hour NGAL level represented a strong independent predictor of clinical outcomes such as duration of AKI among cases. Thus, plasma and urine NGAL have emerged as sensitive, specific, and highly predictive early biomarkers of AKI after cardiac surgery in children. These findings have been confirmed in a prospective study of adults who developed AKI after cardiac surgery, in whom urinary NGAL was increased significantly by 1 to 3 hours after the surgery. AKI, defined as a 50% increase in serum creatinine level, did not occur until the third postoperative day. However, patients who did not encounter AKI also displayed a significant increase in urine NGAL in the early postoperative period, although to a much lesser degree than in those who subsequently developed AKI. The AUC reported in this study was 0.74 for the 3-hour NGAL and 0.80 for the 18-hour NGAL, which is perhaps reflective of the confounding variables typically encountered in adults.

NGAL also has been evaluated as a biomarker of AKI in kidney transplantation. Biopsy specimens of kidneys obtained 1 hour after vascular anastomosis revealed a significant correlation between NGAL staining intensity and the subsequent development of delayed graft function. In a prospective multicenter study of children and adults, urine NGAL levels in samples collected on the day of transplant clearly identified cadaveric kidney recipients who subsequently developed delayed graft function and dialysis requirement (which typically occurred 2-4 days later). The ROC curve for prediction of delayed graft function based on urine NGAL at day 0 showed an AUC of 0.9, indicative of an excellent predictive biomarker. Urine NGAL also has been shown to predict the severity of AKI and dialysis requirement in a multicenter study of children with diarrhea-associated hemolytic uremic syndrome. Preliminary results also suggest that plasma and urine NGAL measurements represent predictive biomarkers of AKI after contrast administration and in the intensive care setting.

In summary, NGAL is emerging as a center-stage player in the AKI field, as a novel predictive biomarker. However, it is acknowledged that the studies published thus far are small, in which NGAL appears to be most sensitive and specific in relatively uncomplicated patient populations with AKI. NGAL measurements may be influenced by a number of co-existing variables such as pre-existing renal disease and systemic or urinary tract infections. Large multicenter studies to further define the predictive role of plasma and urine NGAL as a member of the putative AKI panel have been initiated, robust assays for commercialization are nearly complete, and the results are awaited with optimism.

**KIM-1 as an AKI Biomarker**

Ichimura et al performed a subtractive hybridization screening to identify kidney injury molecule 1 (KIM-1) as a gene that is up-regulated markedly in ischemic rat kidneys, a finding that has been duplicated consistently in several other transcriptome profiling studies. Downstream proteomic studies also have 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 detected easily in the urine soon after AKI. In a small, human, cross-sectional study, KIM-1 was found to be induced markedly in proximal tubules in kidney biopsy specimens from patients with established AKI (primarily ischemic), and urinary KIM-1 measured by ELISA distinguished ischemic AKI from prerenal azotemia and chronic renal disease. Patients with AKI induced by contrast did not have increased urinary KIM-1.

Recent preliminary studies have expanded the potential clinical utility of KIM-1 as a predictive AKI biomarker. In a cohort of 103 adults undergoing cardiopulmonary bypass, AKI (defined as a 0.3-mg/dL increase in serum creatinine level) developed in 31%, in whom the urinary KIM-1 levels increased by about 40% at 2 hours postsurgery and by more than 100% at the 24-hour time point. In a small case-control study of 40 children undergoing cardiac surgery, 20 with AKI (defined as a 50% increase in serum creatinine level) and 20 without AKI,
urinary KIM-1 levels were enhanced markedly, with an AUC of 0.83 at the 12-hour time point. In a larger prospective cohort study of 201 hospitalized patients with established AKI, both urinary KIM-1 as well as urinary N-acetyl-\(\beta\)-(D)-glucosaminidase levels were found to be associated with adverse clinical outcomes, including dialysis requirement and death.

Thus, KIM-1 represents a promising candidate for inclusion in the urinary AKI panel. An advantage of KIM-1 over NGAL is that it appears to be more specific to ischemic or nephrotoxic AKI, and not affected significantly by prerenal azotemia, urinary tract infections, or chronic kidney disease. It is likely that NGAL and KIM-1 will emerge as tandem biomarkers of AKI, with NGAL being most sensitive at the earliest time points and KIM-1 adding significant specificity at slightly later time points.

**Other Promising AKI Biomarkers**

Gene expression studies have provided several additional clues regarding the AKI proteome, but human data are hitherto lacking. For example, Muramatsu et al\(^50\) used a subtractive hybridization approach to identify cysteine-rich protein 61 (CYR61) (also known as \(CCN1\)) as a markedly up-regulated gene in the rat kidney very early after ischemic injury. CYR61 protein was induced in the kidney within 1 hour and was detectable in the urine at 3 to 6 hours after ischemic injury, but not after volume depletion. However, this detection required a complex bioaffinity purification step with heparin-Sepharose beads, and even after such purification several cross-reacting peptides were apparent. A more convenient platform for the evaluation of CYR61 as a urinary biomarker in human beings has not been available to date. Zahedi et al\(^51\) described spermidine/spermine N\(^1\)-acyetyltransferase, the rate-limiting enzyme in polyamine catabolism, as a novel early biomarker of tubular cell damage after ischemic injury in rats. Spermidine/spermine N\(^1\)-acyetyltransferase protein appears to play a role in the initiation of oxidant-mediated injury to tubules, raising the possibility of inhibition of polyamine catabolism as a future therapeutic approach. Tarabishi et al\(^95\) showed that another maximally induced gene identified very early after ischemic injury in animal models is Zf9, a Kruppel-like transcription factor involved in the regulation of a number of downstream targets. Zf9 protein is up-regulated markedly in the posts ischemic tubule cells, along with its major transactivating factor, transforming growth factor-\(\beta1\). Gene silencing of Zf9 abrogated transforming growth factor-\(\beta1\) protein expression and mitigated the apoptotic response to ischemic injury in vitro.\(^95\) These studies thus have identified a novel pathway that may play a critical role in the early tubule cell death that accompanies ischemic renal injury. Thakar et al\(^47\) used transcriptome profiling in rat models to identify thrombospondin 1 (TSP-1), a previously known p53-dependent pro-apoptotic and anti-angiogenic molecule, as another maximally induced gene early after ischemic AKI. The TSP-1 protein product is up-regulated in the posts ischemic proximal tubule cells, where it colocalizes with activated caspase-3. TSP-1 null mice partially were protected from ischemic injury, with striking structural preservation of kidney tissue.\(^47\) These results thus have identified yet another previously unknown apoptotic protein that is activated in proximal tubule cells early after ischemic AKI in animals.

Transcripts that have been reported consistently to be either up-regulated or down-regulated in animal models of AKI are listed in Tables 2 and 3, respectively. Although many of them now have been confirmed by downstream proteomic analysis, the majority of these studies remain in the preclinical research realm, and convincing data attesting to their utility in human AKI currently are unavailable.

**DIRECT PROTEOMIC PROFILING IN ISCHEMIC AKI**

**SELDI-TOF for AKI Biomarkers**

Recent advances in the field of direct proteomic profiling have accelerated the discovery of novel protein biomarkers and therapeutic targets for AKI.\(^31-40,111-113\) Of the various methods and platforms available, the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology has emerged as one of the preferred platforms for rapid urinary protein profiling.\(^114-116\) This approach allows for rapid high-
throughput profiling of multiple urine samples, detects low-molecular-weight biomarkers that typically are missed by other platforms, and even uncovers proteins bound to albumin. The commercial availability of the ProteinChip Biomarker System and the accompanying bioinformatics software (Bio-Rad, Hercules, CA) has provided investigators with the convenient tools to obtain reproducible results and their statistical interpretation. Previously quoted problems with calibration difficulties and variability of reagents largely have been resolved by the commercial availability of All-in-1 peptide/protein calibration standards (Bio-Rad) and standardized chromatographic solutions (BioSeptra, Villeneuve, France). However, persistent disadvantages of this method include the limited ability to resolve large-molecular-weight proteins, and the difficulties with identifying the protein peaks.

| Table 2. Genes Reported To Be Up-Regulated in at Least 3 Separate Transcriptome Profiling Studies |
|-------------------------------------------------------|----------------------------------|------------------|
| **Gene Name**                                         | **Gene Symbol**                  | **References**   |
| Cyclin-dependent kinase inhibitor 1A                  | p21/Cip1/WAF                     | 96               |
| Clusterin                                             | CLU                              | 97               |
| A kinase (PRKA) anchor protein (gravin) 12             | AKAP12/SSeCK                     | None             |
| Tubulin, β                                            | TUBB                             | 98               |
| Heme oxygenase (decycling) 1                          | HMOX1                            | 99               |
| Activating transcription factor 3                     | ATF3                             | 48               |
| Metallothionein 1A                                    | MT1A                             | 100              |
| Lectin, galactoside-binding, soluble, 3 (Galectin 3)  | LGALS3                           | 101              |
| Early growth response 1                               | EGR1                             | 102              |
| Claudin 7                                             | CLDN7                            | None             |
| CD68 antigen                                          | CD68                             | 103              |
| Lipocalin 2 (NGAL)                                    | LCN2/NGAL                        | 72               |
| KIM-1                                                 | KIM-1/HAVCR1                     | 49               |
| c-Fos                                                 | cFos                             | 104              |
| Annexin A2/Calpactin 1                                | ANXA2                            | 46               |
| Heath shock protein, 70 kd                            | HSP70                            | 105              |
| Interleukin-6                                         | IL6                              | 106              |
| Chemokine (C-X-C motif) ligand 1                      | CXCL1/Gro-1                      | 104              |

NOTE. The column on the right shows references of published proteomic studies that have confirmed the induction of the corresponding gene product.

| Table 3. Genes Reported To Be Down-Regulated in at Least 3 Separate Transcriptome Profiling Studies |
|-------------------------------------------------------|----------------------------------|------------------|
| **Gene Name**                                         | **Gene Symbol**                  | **References**   |
| Epidermal growth factor                               | EGF                              | 107              |
| Afamin/α-albumin                                      | AFM                              | None             |
| Leukemia inhibitory factor receptor                   | LIFR                             | 108              |
| Solute carrier family 9, member 3                     | SLC9A3/NHE3                      | 109              |
| Solute carrier family 16, member 7                    | SLC16A7                          | None             |
| Uromodulin (Tamm-Horsfall mucoprotein)                | UMOD                             | 110              |

NOTE. The column on the right shows references of published proteomic studies that have confirmed the suppression of the corresponding gene product.
Nguyen et al. have used SELDI-TOF-MS technology to identify urinary biomarker patterns that predict AKI in patients undergoing cardiopulmonary bypass (CPB). Urine aliquots at baseline ($t_0$) and 2 hours ($t_2h$) were assigned to control ($n_{15}$) or ARF groups ($n_{15}$). ARF was defined as a 50% or greater increase in serum creatinine level. Representative samples of spectra obtained are shown in Figure 1. The SELDI-TOF-MS analysis of the ARF group at $t_0 vs t_2h$ consistently showed a marked enhancement of protein biomarkers with a mass-to-charge ratio (m/z) of 6.4 (not shown), 28, 43, and 66 kd. The same biomarkers were significantly different when comparing control versus ARF groups at $t_2h$. No differences were detected in control versus ARF patients at $t_0$. It should be noted that the serum creatinine level in these patients did not increase until days 2 to 3 after surgery. Scatter plots revealed a dramatic increase in peak intensity of all 4 novel biomarkers in the ARF group at baseline ($t_0$) versus 2 hours post-CPB, with the AUC of the ROC curve in the 0.90 to 0.98 range, indicative of excellent biomarkers. Thus, this proteomic approach has revealed a distinctive AKI fingerprint comprising at least 4 biomarkers that are enhanced markedly within 2 hours of CPB in patients who subsequently developed AKI, and has shown that the SELDI-TOF-MS method is sensitive, non-invasive (requiring only microliter quantities of urine), rapid (with no special preparation steps needed), and reproducible. An important limitation to this study is that it represents a single-center analysis involving only children and young adults with congenital heart disease. A second limitation is the exclusion of patients with pre-existing renal insufficiency, diabetes, peripheral vascular disease, and nephrotoxin use. These results therefore need to be validated in a larger population of susceptible patients. It also will be important in future studies to confirm the identity of the 4 biomarkers uncovered by this study, and to determine their individual and collective robustness for the prediction of AKI.

MALDI-TOF for AKI Biomarkers

In another direct proteomic profiling study in human beings, Lefler et al. used 2-dimensional gel electrophoresis (2DE) followed by matrix-assisted laser desorption/ionization (MALDI-TOF-MS or MALDI-TOF/TOF to characterize proteins removed by continuous renal replacement therapy for ARF. The 2DE method allows for good separation and quantitation of individual proteins, and the resolved protein spots are directly amenable to identification by peptide mass fingerprinting (MALDI-TOF-MS) and/or peptide sequencing (MALDI-TOF/TOF). However, gel-based proteomics also have limitations. They are time- and labor-intensive, and there is considerable difficulty in detecting low-abundance proteins and insoluble membrane proteins. Nevertheless, Lefler et al. identified several proteins in the effluent by peptide mass fingerprinting, including albumin, apolipoprotein A-IV, β-2-microglobulin, lithostathine, man nose-binding lectin-associated serine protease 2–associated protein, plasma retinol-binding protein, transferrin, transthyretin, vitamin D-binding protein, and Zn α-2 glycoprotein. Direct sequencing of tryptic peptides confirmed the identity of all except apolipoprotein A-IV, transferrin, transthyretin, and serine protease 2–associated protein. The potential therapeutic or detrimental implications of the identified proteins being removed by renal replacement therapy are unclear at the present time. The identified proteins are known to be present in serum. Given their multiple physiologic roles, it
is conceivable that loss of albumin, transferrin, and vitamin D–binding protein may contribute to the complex pathophysiology of ARF in dialyzed patients. Zhou et al.\textsuperscript{119} used 2D differential in gel electrophoresis (DIGE) followed by mass spectrometry (MALDI-TOF/TOF) or liquid chromatography MS/MS to examine urinary exosomes in animal models of AKI. Urinary exosomes containing apical membranes and intracellular fluid normally are secreted into the urine from all nephron segments, and contain protein markers of structural and functional renal damage. Exosomes represent a unique source for the discovery of noninvasive urinary biomarkers that can overcome much of the interference from abundant urinary proteins such as albumin, globulin, and Tamm-Horsfall mucoprotein.\textsuperscript{120,121} Zhou et al.\textsuperscript{119} initially uncovered 74 peptide spots that showed differential expression by 2D-DIGE of urinary exosomes after nephrotoxic injury with cisplatin. Fifteen of these proteins were identified by MALDI-TOF/TOF, and an additional 13 were detected by liquid chromatography–MS/MS. Of these, Western blotting was able to confirm only 2 protein expression changes, namely Fetuin-A (increased in AKI) and annexin V (decreased in AKI). The very low rate with which differentially expressed proteins were identified and confirmed in this study exemplifies many of the limitations associated with the 2D-DIGE methodology. Nevertheless, the investigators subsequently identified Fetuin-A within urinary exosomes by immunoelectron microscopy, and validated urinary exosomal Fetuin-A to be increased more than 30-fold in the early phase of ischemia-reperfusion injury by Western blotting. Urinary exosomal Fetuin-A also was noted to be increased markedly by Western blotting in 3 patients in the intensive care unit with AKI compared with patients without AKI. This proteomic approach therefore has identified Fetuin-A as a potential biomarker for human AKI. Factors that currently limit the widespread clinical testing of Fetuin-A include the complex steps required for exosome preparation, and the lack of an easily translatable assay such as an ELISA.

Other Proteomic Approaches
Molls et al.\textsuperscript{122} used commercial protein arrays (cytokine multiplex bead–based assays) to measure 18 cytokines and chemokines in mouse kidney homogenates early after ischemia-reperfusion injury. The earliest and most consistent change noted was an increase in kidney keratinocyte–derived chemokine (KC), with a 13-fold increase within 3 hours of ischemic injury. By ELISA, serum and urinary KC levels at 3 hours after ischemia also were enhanced significantly in mice that developed an increase in serum creatinine level 24 hours after the injury. Importantly, in a small cohort of patients, the human analog of KC, namely Gro-α, was up-regulated markedly in the urine of deceased donor kidney transplant recipients with delayed graft function in comparison with recipients with good graft function.\textsuperscript{122} Thus, these studies using protein arrays have identified Gro-α as another potential candidate for inclusion in the urinary AKI panel. This approach obviously is hampered by the limited number of candidates that can be detected using a given protein array.

Holly et al.\textsuperscript{123} used 2D-DIGE followed by MALDI-TOF to identify differentially expressed urinary proteins in a rat model of sepsis-induced AKI. Sepsis is one of the most common causes of human ARF, and the resultant renal dysfunction primarily is caused by ischemic injury, resulting from a potent combination of renal vasoconstriction and systemic vasodilation.\textsuperscript{124} Although initial 2D-DIGE of urine samples identified 97 differentially expressed spots in rats with sepsis-induced AKI, subsequent peptide mass fingerprinting could identify only 30 of those. The few peptides that were up-regulated included previously known candidates such as albumin, aminopeptidase, and alpha-2 microglobulin (also known as lipocalin or NGAL). The majority of the differentially expressed urinary proteins were decreased in sepsis-induced AKI, including uromodulin (Tamm-Horsfall mucoprotein), serum protease inhibitors, and the brush-border enzyme meprin-1-alpha. The investigators chose to characterize meprin-1-alpha further. By Western blotting, septic rats with ARF displayed a decrease in meprin. Furthermore, inhibition of meprin
with actinonin partially ameliorated sepsis-induced ARF. Thus, despite the limitations described, this proteomic approach has identified meprin not only as a potential urinary biomarker that is repressed in a rat model of sepsis-induced AKI, but also as a therapeutic target. Studies of meprin in human AKI have not been reported to date.

More focused proteomic approaches recently have yielded additional biomarkers for AKI. For example, interleukin (IL)-18 is a proinflammatory cytokine that is known to be induced and cleaved in the proximal tubule, and subsequently easily detected in the urine after ischemic AKI in animal models. In a cross-sectional study, urine IL-18 levels measured by ELISA were increased markedly in patients with established AKI, but not in subjects with urinary tract infection, chronic kidney disease, nephritic syndrome, or prerenal failure. Urinary IL-18 was up-regulated significantly up to 48 hours before the increase in serum creatinine level in patients with acute respiratory distress syndrome who develop AKI, with an AUC of 0.73, and represented an independent predictor of mortality in this cohort. Both urinary IL-18 and NGAL were shown recently to represent early, predictive, sequential AKI biomarkers in children undergoing cardiac surgery. In patients who developed AKI 2 to 3 days after surgery, urinary NGAL was induced within 2 hours and peaked at 6 hours, whereas urine IL-18 levels increased around 6 hours and peaked at more than 25-fold at 12 hours after surgery (AUC, 0.75). Both IL-18 and NGAL were associated independently with duration of AKI among cases. Urine NGAL and IL-18 also have emerged as predictive biomarkers for delayed graft function after kidney transplantation. In a prospective multicenter study of children and adults, both NGAL and IL-18 in urine samples collected on the day of transplant predicted delayed graft function and dialysis requirement with an AUC of 0.9. Thus, IL-18 also may represent a promising candidate for inclusion in the urinary AKI panel. IL-18 is more specific to ischemic AKI, and is not affected by nephrotoxins, chronic kidney disease, or urinary tract infections. It is likely that NGAL, IL-18, and KIM-1 will emerge as sequential urinary biomarkers of AKI.

Herget-Rosenthal et al measured urinary excretion of a number of candidate biomarker proteins (α1-microglobulin, β2-microglobulin, cystatin C, retinol-binding protein, α-glutathione S-transferase, lactate dehydrogenase, and N-acetyl-β-D-glucosaminidase) early in the course of nonoliguric ARF in human beings. In this cohort of patients with established ARF (defined as a doubling of serum creatinine level) from a variety of causes, urinary excretion of α1-microglobulin and cystatin C were found to be predictive of severe ARF requiring renal replacement therapy, with an AUC of 0.86 and 0.92, respectively. α1-microglobulin is a tubular protein that belongs to the lipocalin superfamily, similar to NGAL. Cystatin C is a cysteine protease inhibitor that is synthesized and released into the blood at a relatively constant rate by all nucleated cells. It is filtered freely by the glomerulus, reabsorbed normally by the proximal tubule, and not secreted. Both α1-microglobulin and cystatin C are stable in the urine, and can be measured easily by immunonephelometric methods in most standard clinical chemistry laboratories. The predictive role of these urinary proteins in early AKI remains to be determined.

Because blood levels of cystatin C are not affected significantly by age, sex, race, or muscle mass, it has been proposed as a better predictor of glomerular function than serum creatinine level in patients with AKI. In the intensive care setting, a 50% increase in serum cystatin C predicted AKI 1 to 2 days before the increase in serum creatinine level, with an AUC of 0.97 and 0.82, respectively. A recent prospective study compared the ability of serum cystatin C and NGAL in the prediction of AKI after cardiac surgery. Of 129 patients, 41 developed AKI (defined as a 50% increase in serum creatinine level) 1 to 3 days after cardiopulmonary bypass. In AKI cases, serum NGAL levels were increased at 2 hours postsurgery, whereas serum cystatin C levels increased only after 12 hours. Both NGAL and cystatin C levels at 12 hours were strong independent predictors of AKI, but NGAL outperformed cystatin C at earlier time points. Thus, both NGAL and cystatin
C may represent promising tandem biomarker candidates for inclusion in the blood AKI panel.

CONCLUSIONS

The tools of contemporary proteomics have provided us with promising novel biomarkers for the clinical investigation of AKI in human beings. The most promising of these are outlined in Table 4, and their current status is chronicled in this review. These include a plasma panel (NGAL and cystatin C) and a urine panel (NGAL, KIM-1, IL-18, cystatin C, α1-microglobulin, Fetuin-A, Gro-α, and meprin). Because they represent tandem biomarkers, it is likely that the AKI panels will be useful for timing the initial insult and assessing the duration and severity of AKI (analogous to the cardiac panel for evaluating chest pain). Based on the differential expression of the biomarkers, it also is likely that the AKI panels will help distinguish between the various types and etiologies of AKI, and predict clinical outcomes. However, they hitherto have been tested only in small studies and in a limited number of clinical situations. It will be important in future studies to validate the sensitivity and specificity of these biomarker panels in clinical samples from large cohorts and from multiple clinical situations. Such studies will be facilitated markedly by the availability of commercial tools for the reliable and reproducible measurement of biomarkers across different laboratories. Ongoing and future proteomic studies likely will yield additional sensitive and specific biomarkers for the investigation of AKI resulting from diverse etiologies. Such tools will be indispensable for the early diagnosis and initiation of timely therapeutic measures.

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