

Novel biomarkers for the early prediction of acute kidney injury

Review Article

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Abbreviations: Acute renal failure (ARF); glomerular filtration rate (GFR); Acute tubular necrosis (ATN); acute kidney injury (AKI); neutrophil gelatinase-associated lipocalin (NGAL); cardiopulmonary bypass (CPB); kidney injury molecule-1 (KIM-1); sodium hydrogen exchanger isoform 3 (NHE3); Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

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Summary

Acute renal failure (ARF) remains a common problem in hospitalized patients with cancer. Despite technical advances in supportive care, the associated mortality and morbidity have remained unacceptably high. Although pre-clinical studies in animals have identified successful therapeutic interventions, translational clinical trials in humans have yielded disappointing results. The reasons for this are the lack of a consensus operational definition for acute renal failure, and the paucity of predictive early biomarkers. In this review, we first propose a consensus definition for acute kidney injury that encompasses the entire spectrum of ARF, from sub-clinical injury to minimal elevation in serum creatinine to anuric renal failure. The clinical significance of ARF, especially in the setting of cancer, is reviewed, to illustrate the urgent need for identifying novel methods for the early diagnosis of acute kidney injury. The impact of modern enabling technologies such as microarrays and proteomics on the biomarker discovery process is outlined, followed by an update on emerging biomarkers. Specifically, the utility of NGAL as a novel, sensitive, specific, highly predictive early biomarkers for human AKI is examined, and the role of urinary proteomic biomarker patterns for the early diagnosis of AKI is explored.

I. Introduction

Acute renal failure (ARF) remains a common and vexing problem in hospitalized patients with cancer. Despite technical advances in supportive care, the associated mortality and morbidity have remained unacceptably high, and have not changed appreciably in the last four decades. Although basic research and pre-clinical studies in animals have identified successful therapeutic interventions, translational clinical trials in humans have yielded disappointing results. One reason for this is the lack of a consensus operational definition for acute renal failure. This has resulted in non-uniformity in the criteria for initiating therapies, and confusion in the interpretation and comparison of existing trials. A second reason is the paucity of predictive early biomarkers. This has hindered our ability to institute potentially effective preventive and therapeutic measures in a timely manner.

In this review, we will first propose a consensus definition for acute kidney injury that encompasses the entire spectrum of ARF, and illustrate the urgent need for identifying novel biomarkers for the early prediction of acute renal injury. The impact of modern enabling technologies such as microarrays and proteomics on the biomarker discovery process will then be outlined, followed by an update on emerging biomarkers.

II. A consensus definition for acute kidney injury

Acute renal failure (ARF) has traditionally been defined as an abrupt reduction in glomerular filtration rate (GFR), leading to accumulation of waste products such as BUN and creatinine. A major quandary with this definition is the primary reliance on serum creatinine measurements for the diagnosis. Over 30 different definitions have been

used in the clinical literature, ranging widely from minimal changes in serum creatinine (0.3 mg/dl or 20% increase above baseline) to severe ARF requiring dialysis (Mehta and Chertow, 2003; Bellomo et al, 2004). This lack of consensus definition, combined with the inherent shortcomings of serum creatinine measurements in ARF (see below), have seriously jeopardized the interpretation and comparison of existing clinical trials.

Acute tubular necrosis (ATN) is the most common manifestation of ischemic or nephrotoxic ARF, and the two terms have frequently been used synonymously. However, ATN remains a pathologic diagnosis, and cannot be easily quantified since biopsy specimens are seldom obtained in patients with ARF. Furthermore, ATN is a misnomer, because frank necrosis is rarely encountered in human ARF (Devarajan, 2005). Thus, the use of the term ATN is unsuitable for translational studies.

The term acute kidney injury (AKI) has recently been proposed by the American Society of Nephrology Steering Committee on Acute Renal Failure. This definition denotes a complex disorder comprising multiple etiological factors with varied clinical manifestations ranging from sub-clinical injury to minimal elevation in serum creatinine to anuric renal failure. AKI represents a paradigm shift that incorporates the entire spectrum of ARF, and appropriately encompasses even minimal degrees of injury. However, the inability to identify sub-clinical and early AKI prior to rise in serum creatinine continues to represent an unresolved challenge. The designations ARF and AKI are used interchangeably in this review.

III. Clinical significance of acute kidney injury

ARF due to ischemic and nephrotoxic injuries continues to represent a very significant and potentially devastating problem in clinical medicine (Bonventre and Weinberg, 2003; Molitoris, 2003; Rabb, 2003; Siegel and Shah, 2003; Star, 1998; Herget-Rosenthal et al, 2004; Hewitt et al, 2004; Schrier, 2004; Schrier et al, 2004; Lameire et al, 2005b). The incidence of ARF varies from 5% of all hospitalized patients to 30-50% of patients in intensive care units, and there is now substantial evidence that this incidence is rising. Despite significant technical improvements in dialytic therapy, the mortality and morbidity associated with ARF remain dismally high and have not appreciably improved during the last four decades. The mortality rate among dialyzed patients in intensive care units exceeds 80%. Even among survivors, long term consequences are frequent, with about 50% of patients being inflicted with chronic renal insufficiency and about 15% progressing inexorably to end stage renal disease within 3 years of an ARF episode.

ARF is a particularly frequent complication in cancer patients, and a major source of mortality and morbidity (Lameire et al, 2005a). ARF in patients with malignancies not only limits our ability to deliver effective therapy, but has also emerged as a major risk factor for the development of non-renal complications. The mechanisms of ARF in cancer are similar to those encountered in other

critical illnesses. In addition, ARF can represent a primary complication of the malignancy itself (such as obstruction and infiltration), or more commonly a complication of cancer therapy (such as nephrotoxicity and sepsis). Major etiologies of ARF in cancer patients are listed in **Table 1**, but it should be emphasized that ARF in this patient population is usually multi-factorial in origin.

Nephrotoxic AKI, by itself or in combination with other mechanisms, represents one of the most frequently encountered causes of ARF in malignancy. Nephrotoxicity can result from a number of agents as listed in **Table 2**. major culprit is cisplatin, one of the most widely used and effective chemotherapeutic agents for the treatment of several human malignancies (Arany and Safirstein, 2003; Hanigan and Devarajan, 2003).

Table 1. ARF in cancer patients

PRERENAL CAUSES
Vomiting, diarrhea, dehydration
Sepsis
Hypotension
Bleeding
Congestive heart failure
Hepatorenal syndrome
RENAL CAUSES
Prolonged prerenal factors
Nephrotoxic injury
Ischemic injury
Tumor lysis syndrome
Hemolytic uremic syndrome
Thrombotic thrombocytopenic purpura
Hypercalcemia
Myeloma kidney
Lymphomatous infiltration
POSTRENAL CAUSES
Bladder outlet obstruction
Bilateral upper tract obstruction

Table 2. Nephrotoxic ARF in cancer patients

ANTIBACTERIALS
Aminoglycosides
Vancomycin
Polymyxins
ANTIFUNGALS
Amphotericin
ANTIVIRALS
Foscarnet
Cidofovir
Acyclovir
ANTINEOPLASTICS
Cisplatin
Carboplatin
Nitrosoureas
Methotrexate
Cytosine arabinoside
5-Fluorouracil
Mitomycin C
Ifosfamide

anti-neoplastic efficacy. Nephrotoxicity following cisplatin treatment may manifest after a single dose with ARF or may present with a chronic syndrome of renal electrolyte wasting. Despite various hydration protocols A The efficacy of cisplatin is dose-dependent, but the significant risk of nephrotoxicity frequently hinders the use of higher doses to maximize its designed to minimize the nephrotoxicity, approximately one-third of patients who receive cisplatin develop evidence for ARF. This can have major consequences in terms of mortality and morbidity, especially in the presence of other co-morbid conditions related to the primary malignancy or its treatment.

Bone marrow transplantation is being increasingly used for the treatment of malignancies and non-malignant conditions, and ARF is a frequent complication (Hahn et al, 2003; Patzer et al, 2003). The incidence of severe ARF varies from 10-25%, but occurs to a milder degree in over 90% of hematopoietic cell transplants (Letourneau et al, 2002; Parikh et al, 2002; Schrier, 2002). ARF in this population most commonly occurs during the period of post-transplant neutropenia, and is frequently due to a combination of nephrotoxins, sepsis and other factors. Several contributory etiologies have been identified, and may be classified according to the timing of the ARF in relation to the bone marrow transplant, as shown in **Table 3**. ARF requiring dialysis after bone marrow transplantation is associated with a very poor prognosis and a mortality rate of greater than 90% (Letourneau et al, 2002; Parikh et al, 2002; Schrier, 2002).

IV. Urgent need for biomarkers of acute kidney injury

Outstanding advances in basic research have illuminated the pathogenesis of AKI and have paved the way for successful therapeutic approaches in animal models of ischemic and nephrotoxic injuries. However, translational research efforts in humans have yielded disappointing results. A major reason for this is the lack of early markers for AKI, akin to troponins in acute myocardial disease, and hence an unacceptable delay in initiating therapy (Bonventre and Weinberg, 2003; Molitoris, 2003; Rabb, 2003; Siegel and Shah, 2003; Star, 1998; Herget-Rosenthal et al, 2004; Hewitt et al, 2004;

Table 3. ARF following bone marrow transplantation

FIRST 10 DAYS
Tumor lysis syndrome
Hemoglobinuria from infusate
Sepsis
FROM 10-21 DAYS
Veno-occlusive disease
Hepatorenal syndrome
Sepsis
AFTER 21 DAYS
Cyclosporine
Amphotericin
Hemolytic uremic syndrome
Irradiation

Schrier, 2004; Schrier et al, 2004; Lameire et al, 2005b). In current clinical practice, ARF is typically diagnosed by measuring serum creatinine. Unfortunately, creatinine is an unreliable and delayed indicator during acute changes in kidney function (Bellomo et al, 2004). First, serum creatinine levels vary widely with age, gender, diet, muscle mass, medications, and hydration status. Second, serum creatinine levels are insensitive to the small changes in GFR that are characteristic of early reversible forms of AKI. Serum creatinine concentrations may not change until about 50% of kidney function has already been lost. Third, serum creatinine does not accurately depict kidney function until a steady state has been reached. Changes in serum creatinine may lag behind alterations in GFR by several days, during decline as well as recovery of renal function. However, animal studies have shown that while AKI can be prevented and/or treated by several maneuvers, these must be instituted very early after the insult, in the initiation phase of the injury. The lack of early biomarkers for AKI in humans has hitherto crippled our ability to launch potentially effective therapies in a timely manner. Indeed, several human investigations have now established that the earlier the intervention, the better the chance of ameliorating the renal dysfunction (Schrier, 2004). Conversely, the longer the duration of ARF, the greater is the mortality rate (Schrier, 2004). Thus, there clearly exists an urgent need to identify novel methods for the early diagnosis of human AKI.

V. Genomic approaches to acute kidney injury

Attempts at unraveling the molecular basis of complex biologic processes such as AKI have been markedly facilitated by recent advances in functional genomics that have yielded new tools for genome-wide analysis (Schena et al, 1995; Eisen et al, 1998; Golub et al, 1999; Lockhart and Winzeler, 2000; King and Sinha, 2001; Kurella et al, 2001; Yoshida et al, 2002a, b; Supavekin et al, 2003). The cDNA microarray methodologies provide parallel and quantitative expression profiles of thousands of genes, which when combined with stringent bioinformatic tools can identify genes in a biologic pathway, characterize the function of novel genes, and detect disease subclasses. We have utilized the cDNA microarray technology and extensive statistical analysis to define global changes in renal gene expression during the early reperfusion periods following ischemic injury in an established mouse model (Supavekin et al, 2003). We have screened for changes in expression of 9000 sequence-verified mouse genes at various early points (3, 12, and 24 hours) following ischemic AKI. We chose to examine the immediate and early responses because the protein products of these genes may represent early biomarkers that have hitherto eluded discovery. We identified several transcripts that were known to be over-expressed or repressed following ischemic injury, thereby validating this technique (Supavekin et al, 2003).

Surprisingly, several of the transcripts that were maximally induced after ischemic AKI were novel to the field. We have focused primarily on a subset of seven

genes whose expression is upregulated more than 10 fold within the first few hours following ischemic renal injury in the mouse model. One of these transcripts, cysteine rich protein 61, has recently been confirmed to be markedly upregulated following renal ischemia in animal models, and may represent a novel biomarker for AKI (Muramatsu et al, 2002). In recent studies (Devarajan et al, 2003; Mishra et al, 2003; Mishra et al, 2004a, b, 2005; Mori et al, 2005), we have further characterized one of these previously unrecognized genes, namely neutrophil gelatinase-associated lipocalin (NGAL). We confirmed the marked upregulation of NGAL mRNA by semi-quantitative RT-PCR and protein levels by Western analysis in the early post-ischemic mouse kidney (both greater than 10-fold). NGAL protein expression was detected predominantly in PCNA-positive proximal tubule cells that were undergoing proliferation and regeneration. These findings strongly implicate a role for this maximally induced gene and protein in the repair process following AKI.

Other recent studies have also suggested that NGAL enhances the epithelial phenotype. During nephrogenesis, NGAL is expressed by the penetrating ureteric bud, and triggers nephrogenesis by stimulating the conversion of mesenchymal cells into kidney epithelia (Yang et al, 2002). These findings are especially pertinent to the mature kidney, in which one of the well-documented responses to AKI is the remarkable appearance of de-differentiated epithelial cells lining the proximal tubules (Witzgall et al, 1994). An important aspect of renal regeneration and repair after injury involves the reacquisition of the epithelial phenotype, a process that recapitulates several aspects of normal development (Hammerman, 2000). This suggests that NGAL may be expressed by the damaged tubule in order to induce re-epithelialization. Support for this notion derives from the recent identification of NGAL as a regulator of epithelial morphogenesis in cultured kidney tubule cells (Gwira et al, 2005), and as an iron transporting protein that is complementary to transferrin during nephrogenesis (Yang et al, 2002). It is well known that the delivery of iron into cells is crucial for cell growth and development, and this is presumably critical to post-ischemic renal regeneration just as it is during ontogeny. Since NGAL appears to bind and transport iron, it is also likely that NGAL may serve as a sink for iron that is shed from damaged proximal tubule epithelial cells. Because NGAL can be endocytosed by the proximal tubule, the protein could potentially recycle iron into viable cells. This might stimulate growth and development, as well as remove iron, a reactive molecule, from the site of tissue injury, thereby limiting iron-mediated cytotoxicity. Indeed, our recent findings indicate that exogenously administered NGAL ameliorates AKI in animal models by tilting the balance of tubule cell fate towards proliferation and survival (Mishra et al, 2004b; Mori et al, 2005). Importantly, we have also found that NGAL is easily detected in the urine very early following AKI in both animal and human models of ARF (Muramatsu et al, 2002). In recent studies (Devarajan et al, 2003; Mishra et al, 2003; Mishra et al, 2004a, b; Mishra et al, 2005; Mori et al, 2005). These results are detailed in

the next section. Thus, NGAL has rapidly emerged from the discovery phase using cDNA microarrays, to potentially occupying center-stage in the AKI field, not only as a novel biomarker but also as an innovative therapy.

It is important to recognize that one of the limitations to using genomic approaches is the fact that alterations in gene expression are not always predictive of downstream functional and/or pathophysiologic pathways. Although this method can suggest activation of biologic pathways at the mRNA level, additional post-transcriptional and/or post-translational events may be required to fully implicate the identified factors. Thus, the cDNA microarray results provide a stepping stone, and in the case of AKI it will be important in future studies to fully characterize the biology of genes with altered expression profiles in order to better understand their role.

VI. NGAL: a novel biomarker of acute kidney injury

In follow up studies to our initial biomarker discovery experiments by gene expression profiling, we found NGAL protein to be markedly induced in kidney tubule cells after both ischemic (Mishra et al, 2003) and nephrotoxic (Mishra et al, 2004a) AKI in animal models. NGAL protein in these tubule cells occupied a punctate cytoplasmic distribution that partially co-localized with endosomal markers, suggestive of a secreted and/or endocytosed protein (**Figure 1**). Since NGAL is known to represent a small secreted polypeptide that is protease resistant, we tested the hypothesis that it may be excreted in the urine. Indeed, we have found by Western blotting that NGAL is easily detected in the urine very early following ischemic kidney injury in both mouse and rat models of AKI after ischemia (Devarajan et al, 2003; Mishra et al, 2003) and cisplatin nephrotoxicity (Mishra et al, 2004a).

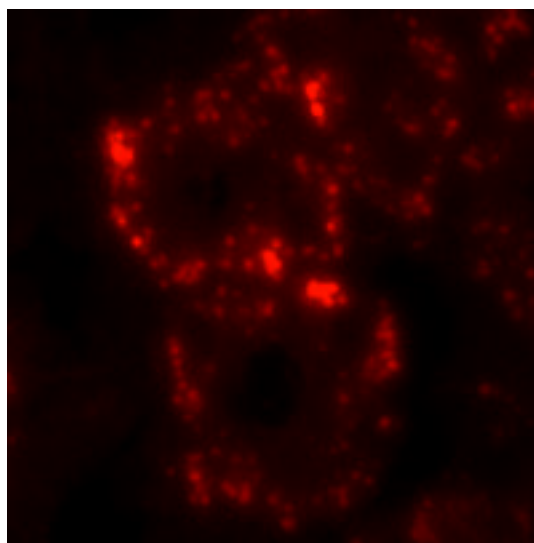


Figure 1. Mice treated with intraperitoneal cisplatin (20 mg/kg) show a rapid induction (within 3 hours) of NGAL protein in tubule cells by immunohistochemistry, in a punctate cytoplasmic distribution. NGAL staining is virtually undetectable in untreated animals (not shown). Magnification is 100X.

The appearance of NGAL in the urine is closely related to the dose and duration of renal ischemia, and precedes by far the appearance of other known urinary markers such as NAG and 2-microglobulin. Our results indicate that NGAL represents an early, sensitive, non-invasive urinary biomarker for ischemic renal injury that compares very favorably with other biomarkers that have been described in animal studies. One of the best-studied examples is KIM-1, a putative adhesion molecule involved in renal regeneration that was also first detected as a result of genomic analysis (Ichimura et al, 1998; Bailly et al, 2002). In a rat model of ischemia-reperfusion injury, KIM-1 was found to be up-regulated 24-48 hours after the initial insult, rendering it a reliable but somewhat late marker of tubular cell damage. In another recent example, *Cyr61* was found to be a secreted cysteine-rich protein that is detectable in the urine 3-6 hours after ischemic renal injury in rats (Muramatsu et al, 2002). However, this detection required a bioaffinity purification step with heparin-sepharose beads, and even after such purification several cross-reacting peptides were apparent. In contrast, our studies demonstrate that NGAL was easily and rapidly detected as a clean immunoreactive peptide in Western blots in the very first unprocessed urine output following AKI due to both ischemia and nephrotoxicity. In addition, urinary NGAL was evident even after very mild “sub-clinical” renal ischemia, in spite of normal serum creatinine levels.

These findings prompted us to test the hypothesis that NGAL represents a novel early biomarker of ischemic renal injury in a representative human population, namely

patients undergoing CPB. It is well known that over 700,000 CPB procedures are performed each year in the US alone. AKI occurs in 10-40% of patients after CPB, with 1-5% requiring dialysis in whom the mortality rate approaches 80% (Chertow et al, 1997; Fortescue et al, 2000; Tuttle et al, 2003). A variety of clinical algorithms have been proposed for prediction of dialysis-requiring ARF based on pre-operative risk factors (Chertow et al, 1997; Fortescue et al, 2000; Eriksen et al, 2003; Tuttle et al, 2003; Thakar et al, 2005), but no tools were available for the early diagnosis of lesser degrees of renal injury. We therefore prospectively studied children undergoing CPB. Exclusion criteria included pre-existing renal insufficiency, diabetes mellitus, peripheral vascular disease, and the use of nephrotoxic agents before or during the study period. Thus, we recruited a homogeneous population of patients with no confounding variables in whom the only conceivable renal insult would most likely be the result of ischemia-reperfusion injury following CPB. Serial urine and blood samples were analyzed by Western blots and a newly designed ELISA for NGAL expression. The primary outcome variable was AKI, defined as a 50% or greater increase in serum creatinine from baseline. Twenty eight percent of patients in our study cohort developed acute renal injury, but the diagnosis using serum creatinine was possible only 2-3 days after CPB. In contrast, urine NGAL in these patients rose more than 10-fold at 2 hours after CPB, as shown in **Figure 2**. Serum NGAL similarly increased 6-fold at 2

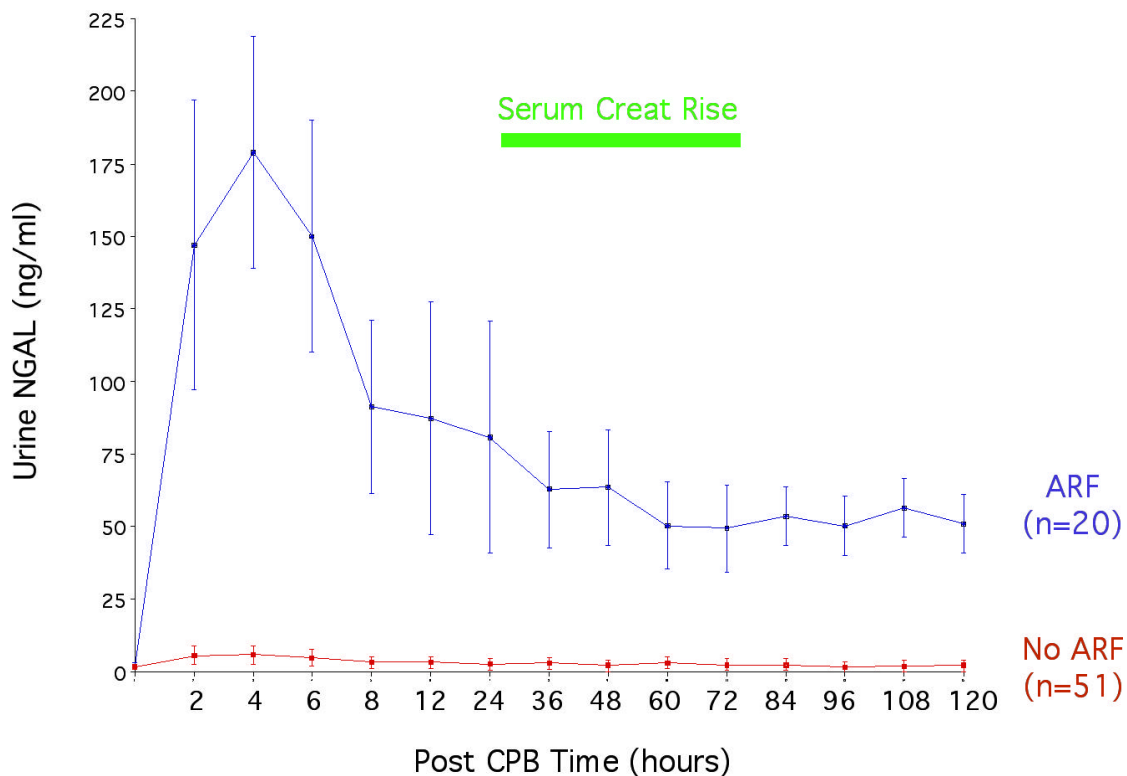


Figure 2. Panel shows urine NGAL (in ng/ml) at various times after CPB in patients who subsequently developed ARF (blue) versus those who did not (red), determined by ELISA. The green bar represents the time when the initial rise in serum creatinine was detected. At all post CPB time points examined, urine NGAL was significantly greater in subjects who developed ARF, as defined by a 50% increase in serum creatinine over baseline. Adapted from reference 38.

hours after CPB (Mishra et al, 2005). These results were similar when analyzed by either Western blotting or by ELISA. Univariate analysis showed a significant correlation between acute renal injury and the following: 2 hour urine NGAL, 2 hour serum NGAL, and CPB time. By multivariate analysis, the urine NGAL at 2 hours post CPB emerged as the most powerful independent predictor of acute renal injury. A ROC curve for the 2 hour urine NGAL revealed an area under the curve of 0.998, and a sensitivity of 1.00 and specificity of 0.98 for a cutoff value of 50 ng/ml. For the 2 hour serum NGAL, the area under the curve was 0.91, the sensitivity 0.95 and the specificity 1.00 for a cutoff value of 50 ng/ml.

Our NGAL results compare favorably with or surpass those obtained for several other biomarkers for human AKI (Rabb, 2003; Herget-Rosenthal et al, 2004; Hewitt et al, 2004). The majority of human studies reported thus far have been retrospective, have examined biomarkers in the established phase of ARF, and have been restricted to only the urine and to only one method of detection. Several tubular proteins have been measured in the urine, with conflicting and unsatisfactory results (Han et al, 2002; Westhuyzen et al, 2003; Herget-Rosenthal et al, 2004). KIM-1 is detectable by ELISA in the urine of patients with established acute tubular necrosis (Han et al, 2002). Also, the sodium hydrogen exchanger isoform 3 (NHE3) has been shown by Western blots to be increased in the membrane fractions of urine from subjects with established ARF (du Cheyron et al, 2003). However, the sensitivity and specificity of these biomarkers for the detection of renal injury have not been reported. Of the inflammatory cytokines involved in ARF, elevated levels of urinary IL-6, IL-8 and IL-18 have been demonstrated in patients with delayed graft function following cadaveric kidney transplants (Kwon et al, 2003; Parikh et al, 2003). With the exception of NGAL, none of the biomarkers have been examined prospectively for appearance in the urine during the evolution of ischemic ARF. A recent prospective study has demonstrated that an increase in serum cystatin C precedes the increase in serum creatinine in a select patient population at high risk to develop ARF (Herget-Rosenthal et al, 2004). However, the ARF in these subjects was multifactorial, due to a combination of ischemic, prerenal, nephrotoxic, and septic etiologies. Furthermore, since cystatin C is primarily a marker of GFR, it can be inferred that serum cystatin C levels will rise only after the GFR begins to fall. On the other hand, NGAL is rapidly induced in the kidney tubule cells in response to ischemic injury, and its early appearance in the urine and serum is independent of the GFR, but is highly predictive of a fall in GFR that may occur several days later.

We conclude that urine and serum NGAL represent novel, sensitive, specific, highly predictive early biomarkers for AKI following CPB (Mishra et al, 2005). A limitation to this study is that it represents a single center analysis involving children with congenital heart disease, with predominantly ischemic kidney injury. While this cohort was intentionally chosen to eliminate common confounding variables and co-morbid conditions, it is acknowledged that ARF is frequently multifactorial, and

our results will need to be validated in a larger population in whom additional mechanisms of renal injury may be invoked. Examination of urine and serum NGAL in other human conditions that predispose to AKI (including cisplatin nephrotoxicity, kidney and bone marrow transplantation, contrast nephropathy, and sepsis) is currently in progress.

VII. Proteomic approaches to acute kidney injury

Proteomics may be defined as the systematic analysis of proteins for their identity, quantity, and function (Peng and Gygi, 2001). This is a rapidly expanding field that offers several distinct advantages over microarray analysis, since it provides the technology to (a) simultaneously analyze all proteins, the primary mediators of function, within a cell or tissue of interest, (b) examine body fluids such as urine which are generally devoid of functional nucleic acids, and (c) account for post-transcriptional regulatory mechanisms that modulate protein structure and function. Recent advances in the field of clinical proteomics have greatly accelerated the discovery of novel protein biomarkers for renal diseases (Knepper, 2002; Clarke et al, 2003; Cutillas et al, 2004; Han and Bonventre, 2004; Hewitt et al, 2004; Klein and Thongboonkerd, 2004; Schaub et al, 2004a, b; Thongboonkerd, 2004; Thongboonkerd et al, 2004a, b). Of the various methods available, Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) technology has emerged as the preferred platform for urinary protein profiling (Clarke et al, 2003; Schaub et al, 2004a, b). This approach allows for rapid high throughput profiling of multiple urine samples, detects low molecular weight biomarkers that are typically missed by other platforms, and even uncovers proteins bound to albumin.

We have tested the hypothesis that urinary proteomic analysis may identify novel early biomarker patterns for AKI in a representative human population, namely CPB. Urine samples were obtained at baseline and at 2 hours post CPB, and analyzed by SELDI-TOF-MS. The primary outcome variable was ARF, defined as a 50% or greater increase in serum creatinine. We have now completed a preliminary analysis of 30 patients (15 with ARF and 15 age-matched controls without ARF). SELDI-TOF-MS analysis of urine from the ARF group at baseline versus at 2 hours post-CPB consistently showed a marked and statistically significant enhancement of protein biomarkers with m/z of 6.4, 28.5, 33, 43 and 66 kDa, as shown in **Figures 3, 4, and 5**. The same biomarkers were also enhanced when comparing control versus ARF groups at 2 hours post-CPB. The specific identity of these biomarkers is currently unknown. However, it is likely that the 28 kDa biomarker species revealed in the present study may represent NGAL, since Western blot analysis of the same urine samples with a monoclonal antibody to NGAL identified an abundant immunoreactive peptide at the 28 kDa range (not shown). Our preliminary results indicate that SELDI-TOF-MS is a novel, non-invasive, sensitive, reproducible, highly predictive, rapid (with a turnaround

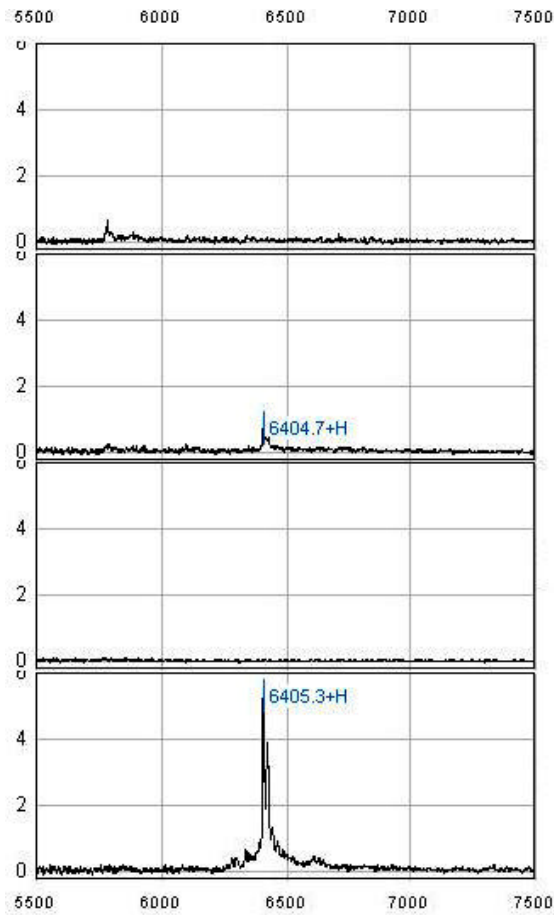


Figure 3. Representative SELDI-TOF-MS spectra of urine obtained at baseline and 2 hours post-CPB from patients in the Control or ARF group. Figure shows proteins in the 5500-7500 kDa range. Marked enhancement of a 6.4 kDa species is noted in the ARF group at 2 hours post-CPB.

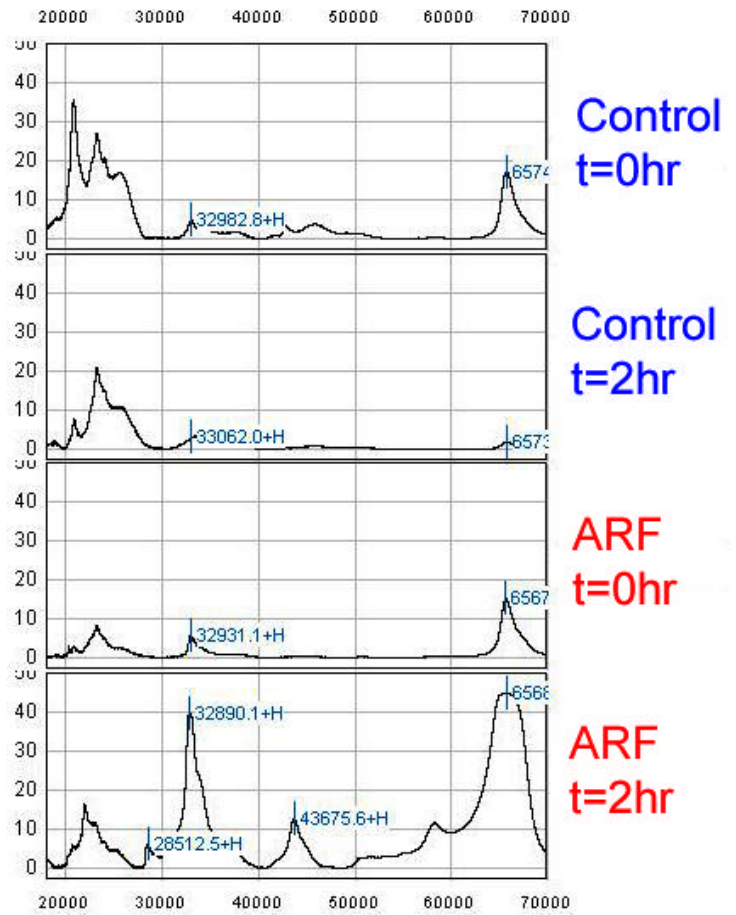


Figure 4. Representative SELDI-TOF-MS spectra of urine obtained at baseline and 2 hours post-CPB from patients in the Control or ARF group. Figure shows proteins in the 20,000-70,000 kDa range. Marked enhancement of 28.5, 33, 43, and 66 kDa species is noted in the ARF group at 2 hours post-CPB.

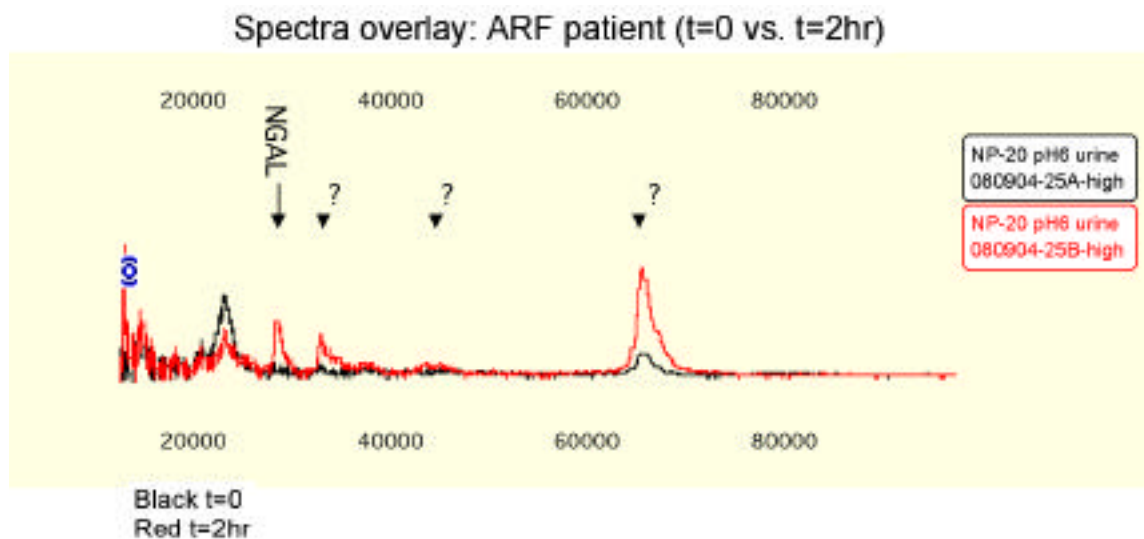


Figure 5. Overlay of representative SELDI-TOF-MS spectra of urine obtained at baseline and 2 hours post-CPB from patients in the ARF group. Marked enhancement of 28.5, 33, 43, and 66 kDa species is noted in the ARF group at 2 hours post-CPB, as highlighted by the arrows. The specific identity of these biomarkers is currently unknown. However, it is likely that the 28 kDa species may represent NGAL.

time of only 90 minutes), and non-invasive (requiring only microliter quantities of urine) method for the prediction of acute renal injury following CPB. These results will need to be validated in a larger population of susceptible patients. It will also be important in future studies to confirm the identity of the biomarkers for AKI uncovered by this study, and to determine their individual and collective robustness for the prediction of AKI.

VIII. Other emerging biomarkers of acute kidney injury

The quest for easily measured and reliable biomarkers of AKI is and has been an area of intense research interest. Many urinary proteins have been evaluated in the past as noninvasive indicators of human AKI (Han and Bonventre, 2004). Traditional urinary biomarkers include low molecular weight proteins such as retinol binding protein and 2-microglobulin, brush border proteins such as carbonic anhydrase, and a variety of urinary enzymes, as shown in **Table 4** (Taniguchi et al, 1979; Stonard et al, 1987; Tolkoff-Rubin et al, 1988; Olbricht et al, 1994; Nortier et al, 1997; Donadio et al, 1998; Bazzi et al, 2001). In general, these markers lack specificity, reproducibility, validation, and standardized assays (Wedeen et al, 1999). The utility of these biomarkers in human AKI is currently limited, although they are still commonly employed in pre-clinical studies.

Fortunately, modern enabling technologies for screening the genome and proteome have yielded promising new urinary biomarkers for human AKI (**Table 5**). In general, an ideal biomarker for AKI should be non-invasive, accurate, reproducible, measured using standardized assays, and adaptable to point-of-care testing. The potential for NGAL to satisfy all these requirements has already been alluded to. The current status of other emerging biomarkers is reviewed below.

Kidney Injury Molecule-1 (KIM-1) is an adhesion molecule that is up-regulated in tubule cells in humans and rodents after ischemic or nephrotoxic injury (Ichimura et al, 1998; Bailly et al, 2002). In a small human study, a soluble form of the cleaved protein has been detected in the urine about 12 hours after an ischemic insult (Han et al, 2002). Attractive aspects of KIM-1 as a biomarker include the fact that its expression is specific to the kidney, and that it can be measured in a standardized fashion using ELISA. However, prospective human studies, with better definition of temporal sequence of appearance, sensitivities, specificities, and predictive values are lacking.

The NHE3, the most abundant apical membrane sodium transporter, has been detected in the membrane fractions of urine from patients with ATN and post-renal ARF (du Cheyron et al, 2003). However, this detection requires isolation of membrane fractions followed by Western blotting, which are cumbersome and not easy to quantify. Additional studies with assay standardization, validation, time course and biomarker statistics are required.

Pro-inflammatory cytokines such as IL-6 (Kwon et al, 2003), IL-8 (Kwon et al, 2003) and IL-18 (Parikh et al,

Table 4. Traditional urinary biomarkers for AKI

LOW MOLECULAR WEIGHT PROTEINS
Retinol binding protein (67)
2-microglobulin (67)
1-microglobulin (68)
TUBULE BRUSH BORDER PROTEINS
Adenosine deaminase binding protein (67)
Carbonic anhydrase (69)
URINARY ENZYMES
N-acetyl- D-glucosamine (67)
Alanine aminopeptidase (67)
Neutral endopeptidase (70)
-glutamyltransferase (71)
Alkaline phosphatase (72)
Lactate dehydrogenase (72)
-glucosidase (72)
Cathepsin B (73)

Table 5. Emerging urinary biomarkers for AKI

BIOMARKER	TYPE OF INJURY	ASSAY	REF
NGAL	Ischemic, Nephrotoxic	ELISA	33-38
KIM-1	Ischemic, Nephrotoxic	ELISA	52
NHE3	Ischemic, Post-renal	Western	53
IL-6, IL-8	Delayed graft function	ELISA	54
Actin	Delayed graft function	Western	54
IL-18	Delayed graft function	ELISA	55
-GST	Proximal tubule injury	ELISA	75
-GST	Distal tubule injury	ELISA	75
Cystatin C	Acute tubular necrosis	Nephelometry	51, 76
Cyr61	Ischemic (animals)	Western	32

2003) have been shown to be up-regulated within 24 hours following kidney transplantation in the urine of patients who subsequently developed delayed graft function (Kwon et al, 2003; Parikh et al, 2003). Actin is an abundant component of tubule epithelial cells, and its urinary excretion follows a similar pattern in delayed graft function (Kwon et al, 2003). The commercial availability of standardized ELISA assays for the cytokines render them attractive AKI biomarker candidates. Additional studies are needed to validate their utility in various forms of AKI, and to further define the timing of their appearance in the urine. However, urinary actin measurement is currently dependent on Western blotting methods that are inherently difficult to quantify and standardize.

Glutathione S-transferases are cytosolic proteins that are released from proximal (-GST) or distal (-GST) tubule cells following AKI. In one human study of kidney transplant patients with dysfunction, urinary levels of -GST were elevated in acute rejection, concentrations of -GST were increased in nephrotoxic injury secondary to cyclosporine, and both isoforms were increased in ATN (Sundberg et al, 1994). However, in a more recent study of patients with ATN from various etiologies, urinary excretion of -GST was not found to be predictive of an unfavorable outcome (Herget-Rosenthal et al, 2004).

Additional studies are required to examine the utility of urinary GST measurements in various forms of AKI.

Serum cystatin C has recently emerged as an encouraging marker of GFR in ATN that precedes a rise in serum creatinine (Herget-Rosenthal et al, 2004), but the utility of urinary cystatin C is less clear. The ratio of urinary cystatin C to urinary creatinine was shown to be a sensitive measure of decreased GFR in patients with diverse chronic renal diseases (Hellerstein et al, 2004), but prospective measurements in AKI are lacking.

IX. Summary and future directions

In this review, we have redefined ARF as AKI to encompass sub-clinical injury and the initiation phase of ARF, which represents the window of opportunity for potentially effective preventive and therapeutic interventions. We have recognized the urgent need for early diagnosis of AKI prior to the rise in serum creatinine. We have reviewed the current status of promising early urinary biomarkers for AKI. It will be important in future studies to evaluate multiple potential AKI biomarkers in prospective studies of susceptible individuals. It is likely that not any one biomarker but a collection of strategically selected proteins may provide the hitherto elusive "ARF Panel" for the early and rapid diagnosis of acute renal injury. The most promising biomarkers will need to be cross-validated within a network of laboratories. We will need to partner with industry to design point-of-care kits and platforms that will enable the early diagnosis of AKI by the bedside. Such tools would be indispensable for the timely institution of potentially effective therapies in human ARF, a common clinical condition still associated with a dismal prognosis where early intervention is desperately needed.

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