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Discovery of Novel Prognostic Biomarkers of Acute Kidney Injury

By

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Molecular and Cellular Biology and Pathobiology Program

A dissertation submitted by the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies

Molecular and Cellular Biology and Pathobiology Program, 2013

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Abstract

An abstract of a dissertation on the subject of the discovery of novel biomarkers of acute kidney injury by Joseph L Alge:

Background: Acute kidney injury is a cause of significant morbidity and mortality in hospitalized patients. Prognostic biomarkers that predict at the time of diagnosis which patients will develop severe AKI and its complications would facilitate timely intervention and could lead to improved outcomes. The urinary proteome is a logical source of candidate biomarkers of kidney injury.

Methods: Urine was collected from rodents and human subjects with AKI secondary to diverse etiologies, including cardiac surgery, ischemic/hypoxic injury, and nephrotoxicity. Shotgun proteomics was used to identify candidate biomarkers in four separate discovery phase experiments. These candidates were then verified in a larger cohort and case-control studies, in which they were measured using ELISA and a multiplex, quantitative mass spectrometry assay.

Results: A total of 22 candidate prognostic biomarkers of AKI were identified by shotgun proteomic analysis of urine from rodents and humans with AKI. Of these, urinary angiotensinogen was the most promising. The prognostic predictive power of urinary angiotensinogen was verified in a cohort of post-cardiac surgery human subjects with AKI (n = 204), which found that it was a strong predictor of progression from Acute Kidney Injury Network (AKIN) stage 1 AKI to the composite endpoint AKIN stage 3 or death, having an area under the ROC curve of 0.75, 95% CI [0.65, 0.85]. In the same cohort, urinary renin concentration had

an AUC of 0.7, 95% CI [0.57, 0.83] for the outcome. A classification tree model found that the combination of these biomarkers could predict the outcome with a positive predictive value of 80.4%. The quantitative mass spectrometry assay was able to successfully measure 11 of the 22 candidate biomarkers, and using this assay, the prognostic predictive power of urinary superoxide dismutase [Cu-Zn], myoglobin was confirmed in a subset of the aforementioned cohort of post-cardiac surgery AKI patients (n =156). SOD and myoglobin predicted progression from AKIN stage 1 to AKIN stage 3 or death with an AUC of 0.76 and 0.77, respectively. Urinary angiotensinogen was also included in the assay, and it had an AUC of 0.74 for the outcome. The performance characteristics of these novel biomarkers compared favorably with urinary liver-type fatty acid binding protein (AUC = 0.69), a more established AKI biomarker which was also included in the assay.

Dedication

I would like to dedicate my dissertation to my loving wife, Mikah, and our two daughters, Eleanor and Lydia. Without your dedication and support, I never could have accomplished this task. You all inspire me. It has been my utmost joy to share this journey with you, and I eagerly look forward to taking the next steps together.

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List of Abbreviations

AKI, acute kidney injury**; Agt**, angiotensinogen; **AUC**, area under the curve**; IL-18**, interleukin-18; **KIM-1**, kidney injury molecule-1; **I/R**,ischemia-reperfusion; **L-FABP**, liver-type fatty acid binding protein; **NGAL**, neutrophil gelatinase-associated lipocalin; **NPV**, negative predictive value; **PPV**, positive predictive value; **PRM-MS**, parallel reaction monitoring mass spectrometry; **SOD**, superoxide dismutase; **ROC** curve, receiver operator characteristic curve; **RRT**, renal replacement therapy

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Chapter 1: Introduction to Acute Kidney Injury Biomarkers

Acute Kidney Injury: Definition and Classification

Acute kidney injury (AKI) is a new term for an old problem. It was recognized by ancient physicians as a decrease in urine output (oliguria) that was accompanied by the classic signs and symptoms of anorexia, nausea, and vomiting.[1] Greek and Roman physicians recognized that it could arise in a variety of settings including crush injuries, serpent bites, and poisonings, and they understood that death was imminent if a patient's condition progressed to prolonged anuria.[1] Therefore, the broadest definition of acute kidney injury, that is an abrupt reduction in renal function, has been recognized as a clinical syndrome since the time of the birth of medicine. Of course, much has changed since then regarding how we define this disease, and we now use more objective measures of renal function such as serum creatinine (sCr) and blood urea nitrogen (BUN), which are filtered at the glomerulus and accumulate in the blood when glomerular filtration rate falls, although urine output is still a useful diagnostic and prognostic indicator.[2-4]

Despite the availability of these conventional AKI biomarkers, for decades of modern medicine, there was not a consensus on the definition of AKI. In fact, there was not even an accepted nomenclature and AKI has been known by other names including acute renal failure and acute renal dysfunction. Lack of an accepted definition resulted in a broad array of diagnostic criteria being used in the literature, with the definition of AKI ranging from an increase in sCr >25% from baseline, to an increase in sCr >2.0 mg/dL,, to the need for dialysis.[5] This was recognized as a key barrier to progress in AKI research by the Acute

Dialysis Quality Initiative Group in 2004, an international consensus workgroup, which proposed the RIFLE classification system for acute renal failure. The RIFLE acronym designates the different stages of acute renal failure according to this classification system. They are: Risk of renal dysfunction, Injury to the kidney, Failure of kidney function, Loss of kidney function, and End-stage renal disease (ESRD).[2] Using RIFLE, patients are staged based upon changes in serum Creatinine (sCr) and urine output. Furthermore, the staging system includes the use of the widely accepted MDRD equation to calculate estimated glomerular filtration rate (eGFR) and changes in eGFR, when a patient's baseline sCr is unknown. A summary of the RIFLE classification scheme is shown in Table 1-1.

The RIFLE classification system was an important step toward improving the quality of AKI research and the care of patients with this disease. Two salient features of this system include the use of baseline characteristics and the inclusion of outcomes in the classification scheme. The former allows for accurate assessment of injury, including so-called "acute on chronic" kidney disease, when a patient has AKI superimposed on preexisting chronic kidney disease. The latter is helpful with regard to defining clinical end-points that can be used in clinical trials. An important limitation of this system is that it does not specify a time course for elevated sCr, although there is a recommendation for

Table 1-1. RIFLE Classification System of Acute Renal Failure[2]

Stage	sCr and GFR Criteria	Urine Output Criteria
Risk	↑ sCr of 1.5 fold (150%) or ↓eGFR >25%	UO <0.5ml/kg/h for more than 6 hr
Injury	↑ sCr of 2 fold (200%) or ↓eGFR >50%	UO <0.5ml/kg/h for more than 12 hr
Failure*	↑ sCr of >3 fold (300%) or ↓eGFR >75% or sCr ≥4mg/dl	UO <0.3ml/kg/h for 24 hr or anuria for 12 hr
Loss	Persistent ARF= complete loss of kidney function >4 weeks	
ESRD	Loss of kidney function >3 months	

Patients are staged by both GFR and urine output criteria, and are designated as the most severe stage reached by either criteria.
*Patients can be classified as Rifle-F if absolute sCr is >4.0 mg/dL with an acute increase of ≥0.5 mg/dL.
GFR, glomerular filtration rate; sCr. Serum creatinine; UO, urine output; ESRD, end stage renal disease

urine output. Despite this limitation, RIFLE was widely accepted by the nephrology community and continues to be frequently used in the literature.

In 2007, the Acute Kidney Injury Network, an international workgroup, published a new definition and classification system, which made several important changes to the RIFLE definitions (Table 1-2). First, this group proposed changing the nomenclature of acute renal failure to acute kidney injury.[3] The latter is a more inclusive term, representing the entire spectrum of acute renal failure. The newly proposed definition of AKI was

> "An abrupt (within 48 hours) reduction in kidney function currently defined as an absolute increase in serum creatinine of more than or equal to 0.3mg/dl ($\geq$26.4 μmol/L), a percentage increase in serum creatinine of more than or equal to 50% (1.5-fold from baseline), or a reduction in urine output (documented oliguria of less than 0.5ml/kg per hour for more than six hours)."[3]

The AKIN classification made several other modifications to the RIFLE definition and staging system. The specification of a brief time window allows for the clinical distinction of acute injury. Additionally, the lowering of the minimum required increase in sCr to 0.3mg/dl reflects research which had demonstrated that even small increases in sCr are associated with a higher risk of adverse outcomes.[6,7] Thus, the AKIN definition of AKI is intentionally a more sensitive diagnostic tool than the RIFLE criteria, and its increased sensitivity compared to RIFLE has been documented.[8] Using the AKIN criteria, patients are still staged according to the most severe changes in serum creatinine and urine output. However, the AKIN criteria largely omit the outcome measures included in the RIFLE classification system, although patients who receive renal replacement

Table 1-2 . Acute Kidney Injury Network (AKIN) Classification System for AKI[3]

Stage	sCr Criteria	Urine Output Criteria
1	↑ sCr of 0.3mg/dl or 1.5-2 fold (150-200%) from baseline	UO <0.5ml/kg/h for more than 6 hr
2	↑ sCr of 2-3 fold (200-300%)	UO <0.5ml/kg/h for more than 12 hr
3	↑ sCr of >3 fold (>300%) or sCr ≥4.0mg/dl with acute increase of ≥0.5mg/dl or RRT	UO <0.3ml/kg/h for 24 hr or anuria for 12 hr

sCr, serum creatinine; RRT, renal replacement therapy

therapy (RRT) are automatically considered AKIN Stage III. In summary, the AKIN definition and classification system changed the terminology of acute renal failure to acute kidney injury, added the element of time to the diagnostic criteria, and lowered the diagnostic threshold of increased sCr in order to increase sensitivity.

Finally, in 2012 the Kidney Disease Improving Global Outcomes (KDIGO) consortium issued a revised definition of AKI which unifies the RIFLE and AKIN classification systems. One of the major criticisms of the AKIN criteria has been that the specified 48 hour time window is too short, and if applied it could result in a substantial number of false negatives from patients whose sCr increases at an atypically slow rate. Therefore, KDIGO proposed the minimum threshold for AKI to be an increase in sCr ≥0.3 mg/dL over 48 hours or ≥50% from baseline over 1 week.[9] However, the KDIGO guideline was only released in 2012, and thus its proposed changes to the definition of AKI have not had time to be studied.

The Etiology of Acute Kidney Injury

AKI is one of the most common conditions seen in the hospital. Its commonness is in part a reflection of its poly-etiological nature, with possible etiologies having classically been grouped into three categories, pre-renal, intra-renal and post-renal.[10] Pre-renal AKI refers to a loss of glomerular function that is the result of decreased renal perfusion, and by definition, it is reversible following correction of renal perfusion.[10] Common causes of pre-renal AKI include hypovolemia, congestive heart failure, cardiogenic shock, and pharmacotherapy that adversely alters renal hemodynamics (such as cyclosporine or NSAIDs).[10-12] Post-renal

AKI can be caused by anything that obstructs urine flow, at any level of the urinary tract between the renal pelvis and the urethra.[10] This obstruction causes an increased pressure in Bowman's space, which opposes glomerular filtration, thereby impairing renal function. However, in a healthy person such an obstruction must be bilateral to cause AKI, since one kidney can compensate for the loss of the other. Nevertheless, post-renal AKI can occur, for example in benign prostatic hypertrophy, retroperitoneal fibrosis, or neurogenic bladder following spinal cord injury.[10]

In contrast to the relatively straightforward mechanisms underlying pre- and post-renal AKI, intra-renal, or intrinsic, AKI is a complex, often multifactorial disease process. Intrinsic AKI is the result of damage directly to the kidney parenchyma, and can be subclassified based upon the site of injury (*i.e.* glomerular, tubular, interstitial, or vascular), with the tubules being most commonly affected.[10] The most common causes of intra-renal AKI are ischemia and nephrotoxicity.[10] Because they are highly metabolically active yet exist in a hypoxic environment, renal tubular epithelial cells are exquisitely sensitive to hypoxic injury.[13] Thus, anything that impairs renal perfusion can cause tubular injury. A logical consequence is that ischemic-hypoxic tubular injury commonly occurs secondary to pre-existing pre-renal AKI. It must also be noted that AKI is a common complication of sepsis, and sepsis accounts for between 20 and 50% of AKI cases.[14,15] Sepsis drastically alters renal hemodynamics resulting in ischemic injury, and the accompanying inflammatory response further exacerbates renal injury.[16] Nephrotoxic injury to the tubules can occur as a consequence of

exposure to exogenous toxins, such as certain pharmacological agents, including the aminoglycosides and cisplatin.[10] However, the most common cause of nephrotoxic AKI is radiocontrast exposure, which accounts for approximately 10% of AKI cases in the intensive care unit.[10,15] Nephrotoxicity can also be the result of endogenous toxins, most commonly heme from hemoglobin, and myoglobin, which are released following damage to erythrocytes and skeletal myocytes, respectively. Therefore, intra-renal AKI is often seen in the settings of malarial infection and rhabdomyolysis.[17,18]

Finally, no discussion of the etiology of AKI would be complete without mentioning it as a post-operative complication, as surgical causes account for approximately one-third of AKI cases.[14] AKI is particularly common after cardiac surgery, affecting about 20% of patients who undergo cardiac procedures.[8] Of note, the cardiac surgery population is an ideal population in which to study AKI, and especially AKI biomarkers given the predictable nature of the injury and the ease of determining its timing and severity. Additionally, AKI after cardiac surgery has a complex and multifactorial pathogenesis involving ischemia-reperfusion injury, inflammation, and nephrotoxicity from lysed erythrocytes, making it likely that studies performed in this population will be generalizable to other etiologies of AKI.[19]

The Epidemiology of Acute Kidney Injury

AKI is one of the most common diseases observed in hospitalized patients, and its epidemiology has been extensively investigated.[20] Although no large,

prospective multicenter studies have been conducted to accurately assess the impact of community acquired acute kidney injury, retrospective observational studies estimate that 1.0- 9.6% of hospitalized patients are admitted with AKI, which accounts for approximately three-quarters of all AKI cases.[21-23] Community acquired AKI is usually pre-renal, secondary to volume depletion.[21,23] Consequently, it is associated with a lower mortality and a lesser risk of adverse outcomes than hospital acquired AKI.[23] In contrast, the burden of hospital-acquired AKI is well documented. Estimates from single-center retrospective studies are that 4.9 – 7.2% of patients admitted without AKI will develop AKI during their hospital stay.[24,25] Complementary of this data are the results of two large studies performed using administrative databases, which have reported that 2.1 – 3.6% of all hospital discharges are associated with ICD-9 codes related to AKI, although it should be noted that database studies are less accurate for the identification of patients with mild AKI, and likely under-report AKI diagnosis.[26-28] Nevertheless, these studies have identified trends of increasing incidence and decreasing mortality over the past 25 years.[26,27] Lastly, it should be noted that the incidence of AKI is disproportionately high in the critically ill population. Large multicenter epidemiologic studies estimate that the incidence of severe AKI is 5.7-7.7%, with an associated mortality rate in excess of 50%.[14,29,30] However, it is important to note that studies which included patients with less severe AKI, for example RIFLE risk or AKIN stage 1 as inclusion criteria, have found a much higher incidence, ranging from 16.2%-35.8 and a lower mortality rate of 24.2% -36.4%.[31-33]

AKI is associated with a number of adverse outcomes. As mentioned above, AKI increases the risk of death, both over the short and long-term, and dialysis-dependent AKI is an independent risk factor for in-hospital mortality.[6,7,22,34,35] Importantly, even mild AKI confers an increased risk of death.[6,7] In addition to mortality, AKI has been associated with an increased length of hospital stay and greater medical costs.[6] Finally, it is important to note that the risk of adverse outcomes, particularly mortality, increases proportionally with AKI severity, and patients with severe AKI bear a disproportionate burden of these outcomes.[6,32,33]

It has become increasingly apparent that AKI is causally linked with chronic kidney disease and end-stage renal disease, two conditions of tremendous importance to the health care economy in the United States.[36-38] The risk of accelerated renal function decline is greatest in those patients who already have chronic kidney disease at the onset of AKI (so-called acute on chronic kidney disease), and in these patients AKI has been called "a springboard for CKD progression".[38,39] However, it is noteworthy that an episode of AKI appears to predispose patients to ESRD, even in the absence of pre-existing CKD.[38]

Conceptual Framework of Acute Kidney Injury

The complex multifactorial, and multiphasic elements of acute kidney injury pose particularly vexing problems to researchers hoping to uncover the molecular determinants of the course of this disease. Recognizing these complexities, experts have proposed two conceptual models of AKI that serve as useful frameworks for approaching its study. The first model describes the progression of cellular and molecular pathogenesis and pathobiology of AKI. The second

model describes the interrelationships of risk factors for AKI, renal damage, and adverse outcomes. Both of these models provide important background information pertinent to the study of AKI biomarkers and will be addressed separately.

Cellular and Molecular Phases of Acute Kidney Injury

Sutton *et al.* described the progression of ischemic AKI through four distinct phases, each one characterized by a different set of cellular and molecular events (Figure 1-1).[40] The first phase is "initiation", during which the proximal insult occurs when there is a decrease in renal blood flow leading to a decrease in the glomerular filtration rate (GFR). This results in ATP depletion in the cells of renal tubular epithelium and consequent alterations in the actin cytoskeleton, loss of apical-basolateral polarity, and up-regulation of inflammatory mediators.[13,40] Cell death occurs by necrosis and apoptosis. Persistent hypoxia leads to the "extension phase", during which marked hemodynamic alterations occur due to damage to the microvascular endothelium and there is a severe reduction in perfusion to the corticomedullary junction and the outer medulla.[13,40] This phase is also accompanied by a pronounced inflammatory response and continued apoptosis and necrosis of tubular epithelial cells. During the extension phase the glomerular filtration rate continues to fall. The next phase of AKI is "maintenance", during which GFR stabilizes at its nadir, and serum creatinine plateaus.[19,40] Important cellular processes occur during this phase as there is proliferation and migration of epithelial cells to repopulate the denuded tubular lumen.[40] Strong evidence suggests a prominent role for surviving epithelial cells

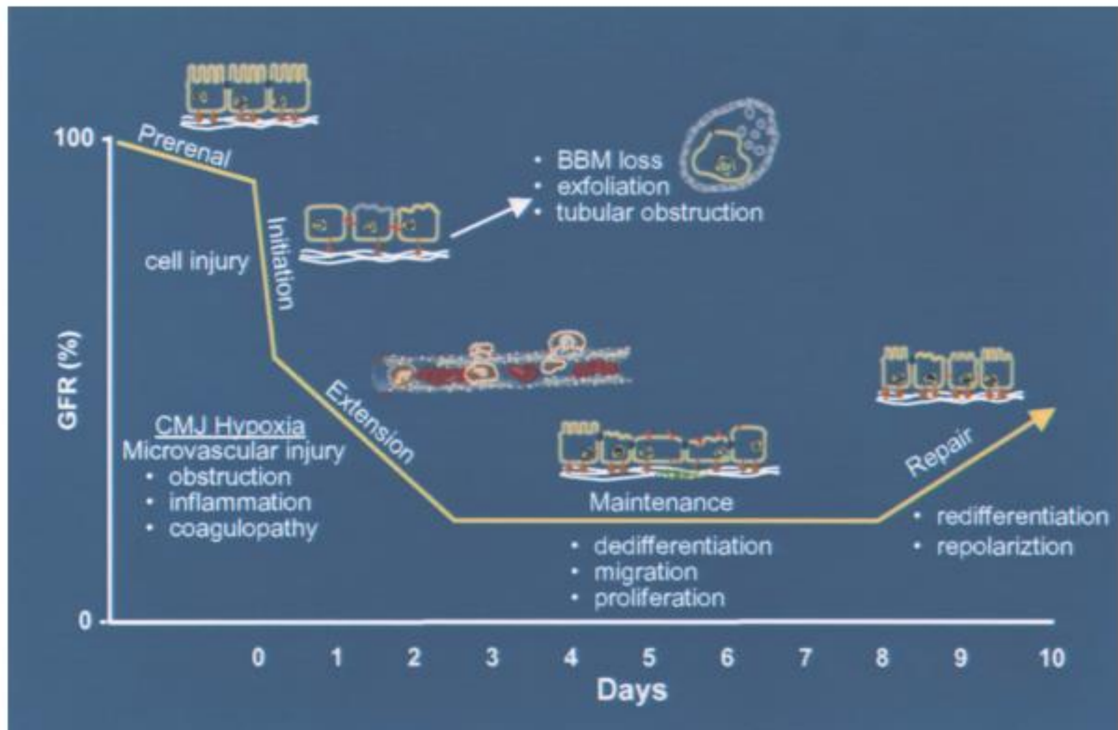

Figure 1-1. Clinical phases of acute kidney injury. This figure was taken from a review on the molecular mechanisms and phases of ishemic AKI.(1) Four distinct phase are described: initiation, extension, maintenance, and repair/recovery. GFR, glomerular filtration rate; CMJ, cortico-medullary junction; BBM, brush border membrane

in this process, although there is a small contribution from the hematopoietic stem cells, and a role for kidney specific stem cells cannot be ruled out. [13,41,42] Finally, during the “recovery” phase tubular epithelial cell polarity is reestablished, is accompanied by the return of normal cellular and renal function, and the repair process is complete.[13,40]

Conceptual Model of Acute Kidney Injury

Murray *et al.* proposed a conceptual framework for understanding AKI which begins with its antecedents (Figure 1-2).[43] Antecedents are conditions or events which put a patient at risk of developing AKI, such as existing chronic kidney disease, IV contrast exposure, or surgery. Obviously there is a connection between these risk factors, particularly exposure to known precipitants of AKI, and renal damage itself. This model points out the opportunity for scientists and physicians to intervene and mitigate the risk of AKI in these patients. If severe enough, renal damage leads to a detectable decrease in GFR, which can result in kidney failure and death. Additionally, this model recognizes the abundance of complications of acute kidney injury that can arise, such as metabolic acidosis, hyperkalemia, volume overload, all of which can result in the need for acute dialysis, which as has already been mentioned, is an independent risk factor for mortality.

These illustrative models neatly demonstrate the need for prognostic biomarkers that can quantify the severity of injury, predict the course of the disease, and assess a patient’s risk of adverse outcomes. It is clear that surrogates of GFR such as serum creatinine and urine output are inadequate for these purposes

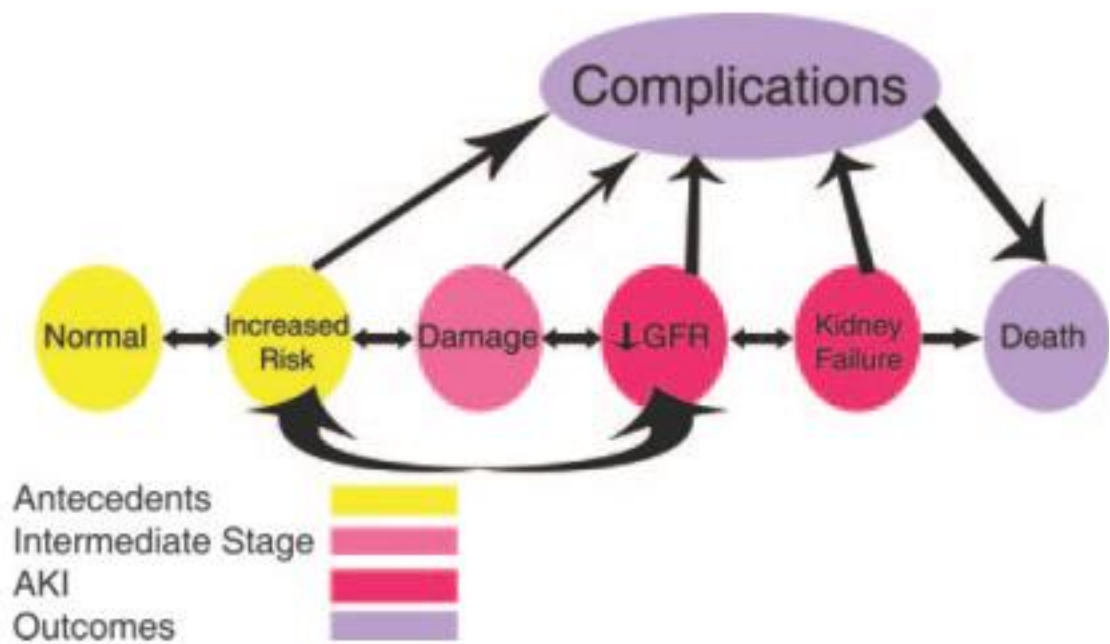

Figure 1-2. A conceptual framework of AKI. This model proposed by Murray *et al.* identifies the progression of AKI through successive risk phases during with the patient can develop adverse outcomes.[43]

because they change slowly with respect to the timing of the injury and they are mechanistically unrelated to the progression of the disease at a cellular and molecular level. Novel biomarkers of renal injury, such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), interleukin-18 (IL-18), and liver-type fatty acid binding protein (L-FABP) have been identified. These proteins have yielded valuable insight into the molecular underpinnings of AKI, and they have shown promise as early diagnostic indicators of AKI and predictors of prognosis. It is to these novel AKI biomarkers that we turn our attention to next.

Biomarkers of Acute Kidney Injury

Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a widely expressed 25 kDa protein of the lipocalin family. Several excellent reviews have been published on its role in renal disease, and the reader is referred to these for more in-depth study.[44-48] The known functions of NGAL primarily revolve around its ability to bind catechol siderophores. Toll-like receptor signaling results in significant up-regulation of NGAL gene expression and translation, and NGAL plays an important role in the innate immune response where it acts as an iron-sequestering bacteriostatic agent by binding bacterial iron-siderophore complexes, thereby preventing their uptake by the pathogen.[49,50] NGAL is also implicated in iron trafficking in the kidney epithelium. During nephrogenesis, it is secreted by the uteric bud and induces tubulogenesis in the metanephric mass, and a key component of its function is to shuttle iron into the developing kidney

epithelium.[51] It appears that this function of NGAL depends upon it being loaded with an iron-siderophore complex.[52]

Following ischemic or nephrotoxic injury, intrarenal NGAL is dramatically up-regulated at the transcript and protein levels.[53-55] Elevated NGAL protein is detectable in the urine as early as 3 hours after injury, and *in vivo* data have suggested the thick ascending limb and the collecting duct as the sites of intrarenal NGAL production, although proximal tubule cells secrete NGAL *in vitro* in response to ATP depletion.[44,54,56] Importantly, however, plasma NGAL also increases after AKI, as a result of increased hepatic production, and NGAL is filtered by the glomerulus and taken up by the proximal tubule in a megalin-dependent manner.[44,56,57] The plasma pool of NGAL protein appears to play an important role in modulating the severity of AKI, as it has been demonstrated that NGAL administered either *i.v.* or *i.p.* is delivered to the proximal tubule where it exerts a renoprotective effect.[56,58] The effect of NGAL in the injured kidney is dependent on its interaction with iron-loaded siderophore, and siderophore-free NGAL (apo-NGAL) has no effect.[56]

Both urine and plasma NGAL peak early after renal injury, within the first 6 hours, and consequently NGAL has been extensively investigated as an early predictor of AKI diagnosis (find review citation for this). The first study to evaluate the predictive accuracy of NGAL for early diagnosis of AKI was performed in a pediatric cardiac surgery cohort (n = 71) and found that urine NGAL measured at 2 hours post-operatively was a nearly perfect predictor of AKI (area under the receiver operator characteristic curve of 0.998) and had an AUC of 1.0 at the 4

hour post-operative time point.[59] Subsequent prospective studies in children that have evaluated the ability of urine and plasma NGAL to predict AKI after cardiac surgery from an early time point have confirmed these findings, and almost universally have reported high predictive power, with the calculated area under the receiver operator curve >0.90 (Table 1-3).[60-62] However, the TRIBE-AKI Consortium reported a much lower predictive power in their multicenter prospective study (n = 311).[63] In this study, urine NGAL and plasma NGAL predicted severe AKI with an area under the receiver operator characteristic curve of 0.71 and 0.56, respectively, with more disappointing results for prediction of mild AKI (defined as RIFLE-R).[63] NGAL has also been demonstrated to predict AKI after cardiac surgery in adults, although results have been less impressive in this population, where reported AUCs range from 0.61 to 0.80, and the most definitive study to date, reported disappointing results. In a prospective study of 1291 cardiac surgery patients, urine and plasma NGAL measured at the time of ICU arrival only had areas under the receiver operator characteristic curve of 0.67 and 0.70, respectively. Similar results have been reported in studies of NGAL as a predictor of contrast-induced nephropathy, and urine NGAL is currently being used to screen post-cardiac catheterization patients and identify those as high risk of contrast-induced nephropathy as a means of guiding enrollment in a clinical trial investigating early, intensive volume expansion as a prophylactic for contrast-induced nephropathy.[64,65] In the intensive care setting, both urine and plasma NGAL predict the future development of AKI, and Cruz *et al.* reported that plasma NGAL predicts the

need for renal replacement therapy with high accuracy (AUC = 0.82).[66,67] It should be noted that sepsis is a potential confounder in studies conducted on the critically ill, however, because septic AKI patients have higher urine and plasma NGAL than non-septic AKI patients, and animal models have demonstrated marked up-regulation of NGAL in response to septicemia.[50,68]

As can be noted from Table 1-3, there is a large degree of variability in the results of the various studies that have been conducted to investigate the early predictive power of NGAL for AKI. Much of this can be attributed to differences in study design such as cross-sectional versus prospective design, timing of NGAL measurement, correction for urine creatinine, and differences in the definition the primary outcome of the study. We have tried to include as much information about these differences as is reasonably possible. Nevertheless, such variability makes it difficult to interpret the sum of the results. To address these difficulties, the NGAL Meta-Analysis Group performed a systematic review and meta-analysis which collated the data from 19 studies, many of which have been mentioned.[69] They found that the predictive power of urine and plasma NGAL was similar, having areas under the summary ROC curve of 0.837 and 0.775. Importantly, it was noted that the predictive power of NGAL was highest for contrast-induced nephropathy, then for AKI after cardiac surgery, and then for AKI among the critically ill. Additionally, NGAL had a higher predictive power in children (AUC = 0.93) than adults (AUC = 0.782). These differences are thought to be attributed to the increased rates of comorbidities and heterogeneity in the adult and critically ill populations.

Table 1-3. Review of Studies Evaluating NGAL as a Diagnostic and Prognostic AKI Biomarker

First Author	Study Population	Sample Size	Primary Outcome	Timing of Measurement	Analyte Used in Prediction Analysis	Area Under the ROC Curve
Mishra[59]	Pediatric Cardiac Surgery	71 (20)	sCr > 50% from baseline	2 hr post-op	uNGAL (uncorrected)	0.998
					pNGAL	0.906
Portilla[60]	Pediatric Cardiac Surgery	40 (21)	sCr > 50% from baseline	4 hr post-op	uNGAL (corrected)	1.00
Dent[61]	Pediatric Cardiac Surgery	120 (45)	sCr > 50% from baseline	2 hr post-op	pNGAL	0.96
Bennett[62]	Pediatric Cardiac Surgery	197 (99)	sCr > 50% from baseline	6 hr post-op	uNGAL (uncorrected)	0.98
					uNGAL (corrected)	0.98
Parikh[63]	Pediatric Cardiac Surgery	311 (53)	Doubling of sCr from baseline or RRT	At ICU Arrival	uNGAL (uncorrected)	0.71
					pNGAL	0.56
			AKI (RIFLE->R)		uNGAL (uncorrected)	0.67
					pNGAL	0.53
Wagener[70]	Adult Cardiac Surgery	81 (16)	sCr > 50% from baseline	18 hr post-op	uNGAL (uncorrected)	0.80
Wagener[71]	Adult Cardiac Surgery	426 (85)	AKI (AKIN Stage 1)	18 hr post-op	uNGAL (uncorrected)	0.611
Koyner[72]	Adult Cardiac Surgery	71 (34)	sCr > 25% from baseline or RRT	Maximum value in first 6 post-op hrs	uNGAL (corrected)	0.691
					pNGAL	0.536
Haase-Fielitz[73]	Adult Cardiac Surgery	100 (50)	sCr > 50% from baseline	At ICU Arrival	pNGAL	0.8
			AKI (RIFLE- >R)			0.73
Han[74]	Adult Cardiac Surgery	90 (36)	AKI (AKIN) within 72 hr	Immediate post-op	uNGAL (corrected)	0.59
				3 hr post-op		0.65
				18 hr post-op		0.7
				24 hr post-op		0.59

Table 1-3 continued

First Author	Study Population	Sample Size	Primary Outcome	Timing of Measurement	Analyte Used in Prediction Analysis	Area Under the ROC Curve
Liangos[75]	Adult Cardiac Surgery	103 (13)	sCr >50% from baseline within 72 hr	2 hr post-op	uNGAL (corrected)	0.50*
Parikh[76]	Adult Cardiac Surgery	1219 (60)	Doubling of sCr from baseline or RRT	At ICU Arrival	uNGAL (uncorrected)	0.67
					pNGAL	0.7
			sCr > 50% from baseline or RRT		uNGAL (uncorrected)	0.61
					pNGAL	0.67
Siew[66]	Adult ICU	391 (86)	AKIN 1 within 24 hr	2nd ICU day	uNGAL (corrected)	0.71
			AKIN 1 within 48 hr		uNGAL (corrected)	0.64
Cruz[67]	Adult ICU	301 (133)	RIFLE-R within 48 hr	ICU Admission	pNGAL	0.78
Doi[77]	Adult ICU	339 (131)	Established AKI (RIFLE-R)	ICU admission	uNGAL (uncorrected)	0.8
			RIFLE-R within 1 week			0.6
Hirsch[64]	Pediatric Cardiac Catheterization	91 (11)	sCr > 50% from baseline	6 hrs post-cath	uNGAL (corrected)	0.97
					pNGAL	0.95
Vaidya[78]	Adult In-Patient*	204	Established AKI (RIFLE-R)	at renal consult	uNGAL (corrected)	0.89
Hall[79]	Adult ICU; established AKI (AKIN)	249 (72)	Progression to higher AKIN stage or death	at AKI diagnosis	uNGAL (uncorrected)	0.71
Koyner[80]	Adult Cardiac Surgery; established AKI (AKIN)	380 (45)	Progression to higher AKIN stage	at AKI diagnosis	uNGAL (uncorrected)	0.58
					pNGAL	0.74

Few studies have been conducted to specifically evaluate the prognostic predictive power of NGAL in patients with established AKI. Among patients with AKI after cardiac surgery, plasma NGAL, but not urine NGAL, appears to predict progression to a higher AKIN stage.[80] In critically-ill patients, NGAL appears to be a slightly better predictor of prognosis. Hall *et al.* reported that urine NGAL had an area under the ROC curve of 0.71 for the composite outcome progression to a higher AKIN stage or death, and it improved the predictive power of clinical model for this outcome.[79] Others have found that both urine and plasma NGAL predict AKI progression and the need for renal replacement therapy, although the predictive power of plasma NGAL is better.[68]

Kidney Injury Molecule-1 (KIM-1)

Kidney injury molecule-1 (KIM-1) is a 38.7 kDa transmembrane that contains extracellular mucin and immunoglobulin domains.[81] Basal expression of KIM-1 is low in the normal kidney. However, it is upregulated following ischemia reperfusion injury, and KIM-1 protein can be localized to proliferating epithelial cells of the proximal tubule 48 hours after injury.[81] Expression of KIM-1 has been demonstrated to confer a phagocytic phenotype on cultured primary kidney cells via its function as a phosphatidylserine receptor, which allows it to promote the phagocytosis of apoptotic bodies and necrotic debris.[82] Therefore, it has been proposed that KIM-1 could play a role in renal recovery and tubular regeneration following acute kidney injury. Importantly, the extracellular component of KIM-1 is constitutively shed from the membrane in a matrix metalloproteinase dependent

manner.[83] While the functional importance of KIM-1 shedding is unclear, it is ostensibly the mechanism underlying the increase in urinary KIM-1 observed following renal ischemic or toxic injury.[84,85] The first observation that urinary KIM-1 could be useful as an AKI biomarker was made by Han *et al.* who reported that urinary KIM-1 was significantly increased in patients with established ATN compared to patients with other types of renal failure, chronic kidney disease, and healthy controls.[84]

KIM-1 has been extensively investigated as a diagnostic biomarker of AKI, and a lateral flow immunochromatographic assay has been developed for rapid, point of care detection of urinary KIM-1 (Table 1-4).[86] Two early studies reported that KIM-1 identified established AKI in the hospitalized population with high accuracy (AUC = 0.93).[78,87] However, these studies compared patients with severe AKI to control groups that included healthy subjects, which likely resulted inflated the predictive power of KIM-1. Accordingly, a large study of ICU patients by Endre *et al.* found that KIM-1 measured at ICU admission had an AUC of 0.66 for diagnosis of AKI on entry to the ICU.[88] Studies of its usefulness as an early AKI biomarker have produced mixed results with AUCs ranging from 0.64 to 0.83.[74,87,89,90] While most of these studies have been small, single center studies, the data from the TRIBE-AKI Consortium are more conclusive. They recently reported that KIM-1 measured at the time of ICU arrival predicted severe AKI in both children and adults undergoing cardiac surgery with an AUC of 0.64

and 0.71, respectively.[90] However, the investigators noted that these results were not significant after adjustment for NGAL and IL-18.

Thus, urinary KIM-1 appears to have limited utility as an early diagnostic marker of AKI, which is plausibly explained by the temporal relationship between renal injury and KIM-1 elevation. In the TRIBE-AKI study KIM-1 did not peak until post-operative day 3 after cardiac surgery in adults and day 2 in children, which is in agreement with data from other studies, including animal models.[74,85,90] This would seem to implicate it during the later phases of AKI, and would suggest that it may be a better prognostic biomarker. However, few properly controlled, prospective studies have studies have evaluated KIM-1 and those that have been conducted have reported modest prognostic predictive power.[79,91] Finally, the ability of KIM-1 to discriminate between ATN and other renal diseases suggests that it could be used to differentiate patients with transient, pre-renal AKI from those with more severe, true AKI. This hypothesis is corroborated by a recent study by Nejat *et al.,* which found that urinary KIM-1 was elevated in patients with sustained AKI compared to pre-renal AKI.[92]

<u>Interleukin-18</u>

Interleukin-18 (IL-18) is a 22 kDa pro-inflammatory cytokine that has been implicated in many different disease processes. It is translated as a procytokine and must be subsequently cleaved by caspase-1 in order to be activated. More specifically, caspase-1 functions as part of a multiprotein complex called the "inflammasome".[93] A central component of the inflammasome is the pyrin

Table 1-4. Review of Studies Evaluating KIM-1 as a Diagnostic and Prognostic AKI Biomarker

First Author	Study Population	Sample Size	Primary Outcome	Timing of Measurement	Analyte Used in Prediction Analysis	Area Under the ROC Curve
Han[87]	Adult In-patient*	84 (29)	Established AKI	at consult	uKIM-1 (corrected)	0.93
	Pediatric Cardiac Surgery	40 (20)	sCr > 50% from baseline	12 hr post-op	uKIM-1 (corrected)	0.83
Vaidya[78]	Adult In-Patient*	204	Established AKI (RIFLE-R)	at renal consult	uKIM-1 (corrected)	0.93
Liangos[89]	Adult Cardiac Surgery	103 (13)	sCr >50% from baseline within 72 hr	2 hr post-op	uKIM-1 (corrected)	0.78
Han[74]	Adult Cardiac Surgery	90 (36)	AKI (AKIN) within 72 hr	immediately post-op	uKIM-1 (corrected)	0.68
Liangos[91]	Adult In-Patient; established AKI	201	RRT or death	at consult	uKIM-1 (corrected)	0.61
Hall[79]	Adult ICU; established AKI (AKIN)	249 (72)	Progression to higher AKIN stage or death	at AKI diagnosis	uKIM-1 (uncorrected)	0.64
Parikh[90]	Pediatric Cardiac Surgery	311 (53)	Doubling of sCr from baseline or RRT	at ICU arrival	uKIM-1 (uncorrected)	0.64
	Adult Cardiac Surgery	1219 (60)	Doubling of sCr from baseline or RRT	at ICU arrival	uKIM-1 (uncorrected)	0.71
Endre[88]	Adult ICU	381 (82)	AKI (AKIN) withing 48 hr	at ICU admission	uKIM-1 (corrected)	0.55#
		381 (27)	AKI (RIFLE) within 7 days			0.64

*ROC curve analysis included comparison to healthy controls.

#Not statistically significant because 95% CI of the ROC curve overlapped 0.5. ROC, receiver operator characteristic

domain containing members of the NOD-like receptor (NLR) family of proteins, of which are there are 14 different types.[93] The NLRs, as their name implies, recognize ligands which induce an inflammatory response. Such ligands are numerous and quite diverse, but they can be broadly grouped into two classes: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The binding of these molecules to a NLR results in the recruitment of scaffold proteins and proteolytic enzymes, and ultimately caspase-1 activation, allowing caspase-1 to proteolytically activate IL-1β and IL-18 prior to their secretion.[93] Mature IL-18 induces NF-κB signaling through the heterodimeric IL-18 receptor, and its proinflammatory effects are negatively regulated by an endogenous inhibitor, IL-18 binding protein.[94-96] The preponderance of data suggests that IL-18 contributes to renal injury during AKI. The amount of mature IL-18 increases in the kidney following ischemia-reperfusion injury, glycerol injection, and cisplatin-induced renal injury in a caspase-1 dependent manner.[97,98] Disruption of the IL-18 signaling axis by 1) knockout of NLRP3, a NOD-like receptor expressed in macrophages, 2) caspase-1 knockout 3) pharmacologic inhibition of caspase-1, and 4) pretreatment with an IL-18 neutralizing antibody have been demonstrated to attenuate the severity of AKI.[97-100] Of note, a study by *Edelstein et al.* found that mouse proximal tubules express caspase-1 and secrete IL-18 in response to hypoxia, although an immune cell source of IL-18 production during AKI cannot be definitively ruled out.[101] The precise signals that drive activation of the inflammasome and IL-18 production have not been fully characterized. However,

possible stimulants include reactive oxygen species, ATP (released from necrotic cells), and uric acid crystals (released from necrotic cells).[99,102,103]

Studies which have reported on the performance of IL-18 as an AKI biomarker are shown in Table 1-5. A cross-sectional study by Parikh *et al.* was the first demonstrate the potential of IL-18 as an AKI biomarker.[104] This study found that IL-18 was an excellent discriminator between patients with established acute tubular necrosis (n = 14) and a diverse control group including healthy controls (n = 11), prerenal azotemia (n = 8), urinary tract infection (n = 5), chronic kidney disease (n =12), and post-renal transplant patients (n = 22), with an impressive area under the receiver operator characteristic curve of 0.95.[104] A more recent, larger study confirmed the ability of IL-18 to accurately identify established AKI.[78] Urinary IL-18 has been investigated as an early predictor of AKI diagnosis in a variety of settings. In children undergoing cardiac surgery, it appears to be a moderate predictor of AKI at an early time point, with stronger predictive power for more severe AKI.[105,106] In comparison, two studies in adults undergoing cardiac surgery have reported that IL-18 is not predictive of post-operative AKI.[89,107] However, a large prospective study by the TRIBE-AKI Consortium has reported that IL-18 concentration at the time of ICU arrival (0-6 hours after the initiation of bypass) is a modest predictor of mild AKI and has stronger predictive power for severe AKI.[76] Important discrepancies between these studies exist, which could explain the contradictory results. These include sample size (studies which have reported negative results were much smaller), the use of creatinine corrected values versus uncorrected IL-18 concentration, and the timing of

Table 1-5. Review of Studies Evaluating IL-18 as a Diagnostic and Prognostic AKI Biomarker

First Author	Study Population	Sample Size	Primary Outcome	Timing of Measurement	Analyte Used in Prediction Analysis	Area Under the ROC Curve
Parikh[119]	Adult In-Patient*	72 (14)	Established ATN	at renal consult	uIL-18 (corrected)	0.95
Parikh[108]	Adult ICU	138 (52)	sCr >50% from baseline	24 hr before AKI	uIL-18 (uncorrected)	0.73
Parikh[120]	Pediatric Cardiac Surgery	71 (20)	sCr >50% from baseline	12 hr post-op	uIL-18 (uncorrected)	0.75
Haase[121]	Adult Cardiac Surgery	100 (20)	sCr >50% from baseline within 48 hr	ICU arrival 24 hr post-op	uIL-18 (corrected)	0.53[#] 0.55[#]
Siew[109]	Adult ICU	451 (86)	AKI (AKIN stage 1)	24 hr before AKI 48 hr before AKI	uIL-18 (corrected)	0.63 0.60
Parikh[106]	Pediatric Cardiac Surgery	311 (53)	Doubling of sCr from baseline or RRT AKI (RIFLE-R)	at ICU arrival	uIL-18 (uncorrected)	0.72 0.64
Parikh[76]	Adult Cardiac Surgery	1219 (60)	Doubling of sCr from baseline or RRT AKI (RIFLE-R)	at ICU arrival	uIL-18 (uncorrected)	0.74 0.65
Doi[77]	Adult ICU	339 (131)	Established AKI (RIFLE-R) New AKI (RIFLE-R)	ICU admission	uIL-18 (uncorrected)	0.78 0.59
Washburn[110]	Pediatric ICU	137 (103)	pRIFLE	24 hr before AKI	uIL-18 (uncorrected)	0.54[#]
Liangos[75]	Adult Cardiac Surgery	103 (13)	sCr >50% from baseline within 72 hr	2 hr post-op	uIL-18 (corrected)	0.66[#]
Vaidya[78]	Adult In-Patient*	204 (102)	Established AKI (RIFLE-R)	at renal consult	uIL-18 (corrected)	0.83
Hall[79]	Adult ICU; established AKI (AKIN)	249 (72)	Progression to higher AKIN stage or death	at AKI diagnosis	uIL-18 (uncorrected)	0.63

Table 1-5 continued

First Author	Study Population	Sample Size	Primary Outcome	Timing of Measurement	Analyte Used in Prediction Analysis	Area Under the ROC Curve
Koyner[80]	Adult Cardiac Surgery; established AKI (AKIN)	380 (45)	Progression to higher AKIN stage	at AKI diagnosis	uIL-18 (uncorrected)	0.63

*Cross-sectional study design that included healthy controls in analysis.

#Area under the curve not statistically significant because 95% CI overlaps 0.5.

sample collection. In the more heterogenous ICU population, IL-18 predicts the development of AKI within 24 hours after measurement.[77,108-110] However, it must be noted that although Washburn *et al.* reported that higher peak urine IL-18 concentration was associated with higher risk of developing AKI in the pediatric ICU, true prediction of future AKI was not demonstrated in this study.[110]

In addition to early diagnosis of AKI, IL-18 has also been investigated as a prognostic biomarker of AKI. Some studies have reported that IL-18 is associated with an increased risk of mortality among AKI patients.[108,109] However, few studies have robustly evaluated IL-18 as a predictor of adverse outcomes, and often prognosis is evaluated during secondary analysis of studies designed to test the ability of the biomarker to predict development of AKI at an early time point in the disease. In such studies, the majority of patients do not develop AKI and are therefore not at risk of developing adverse outcomes associated with AKI. If included in an analysis of prediction of adverse outcomes, these patients would skew the positive and negative predictive values (PPV and NPV) of any biomarker cut-off selected, since PPV and NPV depend upon prevalence. Therefore, estimates of the ability of IL-18 to predict adverse outcomes are likely unreliable. In order to properly estimate prediction of adverse outcomes, patients with IL-18 below a predetermined cut-off (signifying diagnosis of AKI) should be excluded from the analysis. Another approach is to evaluate the prognostic predictive performance of IL-18 at the time that diagnosis of AKI is made based on serum creatinine or urine output criteria, although such a time point is after the peak of IL-18 has occurred. Such a study conducted in

an ICU cohort found that IL-18 predicted worsening of AKI (defined as progression to a higher stage) or death with an AUC of 0.63, and a similar study in cardiac surgery patients by Koyner *et al.* reported that IL-18 had an AUC of 0.63 for progression to a higher stage.[79,80]

It is clear that IL-18 predicts severe AKI at an early time point after injury, it is a much less powerful predictor of mild AKI. While it is noteworthy that even mild AKI is associated with adverse events, including an increased risk of mortality, it is well-documented that the risk of adverse outcomes associated with AKI increases with the severity of injury.[6,7,22] While the prognostic value of IL-18 appears to be diminished later in the course of the disease, IL-18 its ability to identify severe AKI at an early time point could be used to identify patients at high risk of adverse outcomes who could benefit from intervention.

Liver- type Fatty Acid Binding Protein (L-FABP)

Liver-type fatty acid binding protein (L-FABP) is expressed in the renal cortex, and the resultant 14 kDa protein can be localized predominantly in the proximal tubule.[111,112] Importantly, while L-FABP can be found in the human kidney, it is not expressed in the murine kidney. Consequently, much of what is known about the factors governing the renal production of L-FABP and its effects during acute kidney injury has been discovered using a transgenic mouse model in which the genomic DNA of the human L-FABP gene, including its promoter region, was integrated into the mouse genome.[113,114] Although studies using this transgenic mouse have demonstrated that urinary L-FABP concentration is predictive of the histologic severity of AKI induced by either renal ischemia reperfusion injury or

cisplatin,[115] L-FABP has been shown to play a renoprotective role in these animal models of AKI.[116-118] The canonical function of L-FABP is to bind long chain and very long chain fatty acids and promote their uptake and subsequent metabolism by β-oxidation.[122-125] However, in the setting of renal ischemia reperfusion injury, Yamamoto *et al.* showed a significant reduction the amount of pimonidazole immunostaining, a marker of tissue hypoxia, in the outer medulla of the kidneys of mice expressing human L-FABP compared to wild type controls, suggesting an antioxidant role for L-FABP. Additionally, L-FABP binds to a number of lipid species, and it has been postulated that it also binds to the lipid peroxidation products generated during ischemia reperfusion injury and promotes their redistributed from the cytosol to the tubular lumen when L-FABP is secreted.[111,116] L-FABP gene expression is induced by hypoxia and urinary L-FABP excretion is strongly correlated with ischemic time in transplanted kidneys.[116] This relationship is presumably driven by an HIF-1α response element in the promoter region of L-FABP.[126] PPAR-α is also a potent upregulator of L-FABP gene expression, and this has been used in animal models of AKI to attenuate renal injury.[117,118] Therefore, PPAR-α agonists such as fibrates represent an attractive therapeutic target in the prevention and treatment of acute kidney injury, although two recent observational studies found an association between fibrate use and acute kidney injury.[127-129]

Similar to KIM-1, L-FABP is a highly accurate classifier of established AKI, even in the heterogenouse ICU population, among whom it had an AUC of 0.80 (Table 1-6).[77,130] It has also shown promise as an early biomarker of AKI and has been

evaluated in numerous studies in the cardiac surgery population. In the first such study, Portilla *et al.* found that urinary L-FABP concentration at the 4 hour post-operative time point predicted AKI (area under the receiver operator characteristic curve of 0.81) in a cohort of 40 pediatric cardiac surgery patients.[60] Three other studies of adult cardiac surgery patients seemed to confirm the predictive power of L-FABP, with none reporting an AUC <0.76.[131-133] The results of these and other studies were included in a meta-analysis by Susantitaphong *et al.,* which found that L-FABP was a sensitive and specific predictor for the early diagnosis of AKI (sensitivity and specificity of 74.5% and 77.6%, respectively).[134] However, this study only included a total of 7 prospective cohort studies and noted that, while L-FABP is a promising AKI biomarker, there is a paucity of high quality literature regarding the predictive performance of urinary L-FABP. In light of this, the recent publication by the TRIBE-AKI Consortium, which was not complete at the time of the aforementioned meta-analysis, is disappointing. This study, which is a much larger, multicenter trial than what had been previous published, found that L-FABP had an AUC of 0.71 and 0.66 in children and adults, respectively. In relation to NGAL, IL-18, and KIM-1, all of which have been evaluated in the same cohort, L-FABP did not appear to be useful in adult patients, although it was one of the better predictors in children. In fact, the combination of IL-18 concentration at ICU arrival and L-FABP concentration on day 2 had an AUC of 0.78 for predicting severe AKI in the pediatric cohort.[135]

Table 1-6. Review of Studies Evaluating L-FABP as a Diagnostic and Prognostic AKI Biomarker

First Author	Study Population	Sample Size	Primary Outcome	Timing of Measurement	Analyte Used in Prediction Analysis	Area Under the ROC Curve
Ferguson[130]	In-patient	160 (92)	Established AKI	at consult	uL-FABP (corrected)	0.93
Doi[77]	Adult ICU	339 (131)	Established AKI (RIFLE-R) RIFLE-R within 1 week	ICU admission	uL-FABP (uncorrected)	0.80 0.70
Portilla[60]	Pediatric Cardiac Surgery	40 (21)	sCr >50% from baseline	4 hr post-op	uL-FABP (corrected)	0.81
Krawczeski[131]	Cardiac Surgery	220 (60)	sCr >50% rom baseline within 48 hr	12 hr post-op	uL-FABP (uncorrected)	0.78
Katagiri[132]	Cardiac Surgery	77 (28)	AKI (AKIN Stage 1)	12 hr post-op	uL-FABP (uncorrected)	0.76
Matsui[133]	Cardiac Surgery	85 (48)	AKI (AKIN Stage 1)	immediately post-op	uL-FABP (corrected)	0.86
Parikh[90]	Pediatric Cardiac Surgery	311 (53)	Doubling of sCr from baseline or RRT	6-12 hr post-op	uL-FABP (uncorrected)	0.71
	Adult Cardiac Surgery	1219 (60)	Doubling of sCr from baseline or RRT	6-12 hr post-op	uL-FABP (uncorrected)	0.66
Doi[137]	Adult ICU; sepsis-associated AKI	145 (77)	In-hospital mortality	at ICU admission	uL-FABP (corrected)	0.99

ROC, receiver operator characteristic; sCr, serum creatinine

Few studies have been done investigating L-FABP in other settings. Nakamura *et al.* reported that it was diagnostic of contrast-induced nephropathy at 1 day post-exposure.[136] This same study also found that patients who developed CIN had elevated baseline urinary L-FABP levels, and pre-procedure L-FABP concentration was an independent predictor of CIN. A prospective study in ICU patients found that urinary L-FABP concentration at ICU admission predicted the development of AKI within one week (AUC = 0.70).[77] Finally, in a different study, the same investigators found that L-FABP concentration at ICU admission was a nearly perfect predictor of in-hospital mortality in patients with sepsis-associated AKI.[137] While these results are promising, the prognostic significance of L-FABP in patients with established AKI has not been adequately addressed and should be the focus of future research.

Perspectives on AKI Biomarker Research

Clearly novel AKI biomarkers have provided mechanistic insights into the molecular processes underlying AKI. However, early enthusiasm regarding the predictive power of these biomarkers has been dampened by the results of larger prospective studies, particularly by the results of the TRIBE-AKI Consortium study. Consequently, none of these biomarkers has supplanted serum creatinine in clinical use, and it remains unclear if any will attain widespread clinical use in the near future. Additionally, there is a significant gap in the amount and quality of research that has been conducted on early markers of AKI and prognostic prediction, yet the identification of high risk groups is clearly important, both before and after AKI diagnosis is made based on conventional serum creatinine

criteria. The latter seems especially important given the increasing awareness of community-acquired AKI and the significant numbers of patients who present with AKI on hospital admission. Therefore, novel renal injury biomarkers are needed, particularly biomarkers with a strong prognostic significance.

Chapter 2: Discovery Phase Proteomics Experiments

Introduction

Acute kidney injury (AKI) is associated with a number of adverse outcomes. Epidemiologic studies have reported that the risk of adverse outcomes is proportional to the severity of AKI. [6, 138-140] Accurate identification of high risk patients with severe renal injury early in the disease could augment the efficacy of available interventions and improve patient outcomes. However, it is not possible to estimate the severity of AKI at an early time point, because AKI staging is based upon the magnitude of changes in serum creatinine and urine output, surrogates of glomerular filtration rate that do not change until after renal injury has occurred and only reach their peak or nadir late in the course of the disease.[2,3,43] Therefore, the recent KDIGO clinical guideline for AKI highlighted the need for improved risk assessment for patients with established AKI.[9]

Biomarkers of AKI could be used to evaluate the severity of AKI at an early time point in the disease as a guide for clinical decision-making. They could also play a role in clinical trial design because biomarkers could be used to selectively enrich the study population with patients who have severe renal injury and are more likely to benefit from an experimental therapy, increasing the effect size of the intervention and the statistical power of the study.[141,142] Many biomarkers have been proposed as early markers of AKI, to detect of AKI prior to increases in sCr. These include NGAL, KIM-1, IL-18, Cystatin-C, L-FABP.[54, 60, 84, 104, 143, 144] However, few studies have been performed to evaluate the prognostic value of these biomarkers once AKI has been established by traditional criteria. The results of two recent studies that evaluated NGAL, KIM-1 and IL-18 have

demonstrated the suboptimal prognostic performance of these biomarkers.[79-80] The objective of the following work is to discover novel candidate prognostic biomarkers of AKI.

Materials and Methods

Human Studies

Urine samples were obtained from a bank of samples collected by investigators in the Southern Acute Kidney Injury Network (SAKINet). The SAKINet was formed in 2007 under the direction of the candidate's mentor, John Arthur, MD, PhD. It includes investigators at the Medical University of South Carolina, Duke University, George Washington University, University of Tennessee College of Medicine in Chattanooga, and Vanderbilt University. Urine was collected post-operatively from patients who had undergone cardiac surgery. Samples were collected as early as possible after AKIN serum creatinine criteria were met, and all were collected within the first 72 hours after surgery. Inclusion criteria were surgery of the heart or ascending aorta and development of AKI within 3 days of surgery. Subjects with baseline serum creatinine > 3.0 mg/dL were excluded. Prior to urine collection informed consent was obtained in accordance with the IRB-approved protocol at each institution. Samples were collected and stored using a rigorous standard operating procedure. Most patients were catheterized and urine was collected preferentially from the Foley tube or the urometer and processed immediately. Samples were centrifuged at 1,000 x *g* for 10 min and the supernatant was collected. A reversible serine and cysteine protease inhibitor cocktail tablet (Roche, Complete-mini, EDTA-free) was added to the

samples at a concentration of 1 tablet for 50 mL of urine, and samples were stored at -80°C in polypropylene tubes that had been pre-washed with 100% acetonitrile to minimize contamination of the samples with plastic polymer. For analysis urine samples were thawed in a 37°C water bath and kept on ice afterward.

Animal Studies

AKI was experimentally induced in rats (n = 6) and mice (n = 10), by glycerol injection and renal ischemia reperfusion injury, respectively. 7.5 ml/kg of 50% glycerol (in saline) was injected *i.m.* and rats were housed in metabolic cages for 24 hour urine collection. Renal ischemia reperfusion injury was performed in mice by bilateral renal pedicle clamping using a non-traumatic vascular clamp (85 g pressure; Roboz Surgical Instruments). Briefly, mice were anesthetized with 3% isofluorane and given buprenorphine *s.c.* (0.05 – 0.1 mg/kg). Kidneys were exposed using a ventral surgical approach and the clamps were applied. Mice were kept on a heating pad and under a heat lamp to maintain body temperature throughout the procedure. Ischemic time was 16 minutes and the reperfusion of the kidney was visually documented. Afterward, mice were housed in metabolic cages and urine was collected for 16 hours.

Proteomic Analysis

A 100 µL aliquot of urine obtained from each human and animal subject was used for proteomic analysis. 100 µL of 0.2% Rapigest SF surfactant (Waters) in 100 mM ammonium bicarbonate was added to the samples to improve digestion efficiency. Urine proteins were reduced by the addition of dithiothreitol (DDT;

final concentration) and heated to 60°C for 30 min. After cooling to room temperature, proteins in the samples were alkylated with iodoacetamide (final concentration of) and incubated at room temperature in the dark for 30 min. Samples were digested with trypsin overnight (10 µg added to each sample) at 37°C. The digestion was stopped by sample acidification via the addition of 750 µL of 0.1% formic acid to the samples (approximately 3 volumes). Each digested sample was pre-fractionated by offline reversed phase solid phase extraction (SPE) using Strata-X (Phenomenex; 30 mg/mL) SPE cartridges. The SPE cartridge was activated and equilibrated with 1 mL of methanol and 0.1% formic acid, respectively. The sample was then loaded onto the SPE cartridge and serial elutions using increasing concentrations of acetonitrile in 0.1% formic acid were performed. After elution, sample fractions were dried in a centrifugal vacuum concentrator. For liquid chromatography tandem mass spectrometry, samples were reconstituted in mobile phase A (98% water, 2% acetonitrile, 0.1% formic acid), and each sample fraction was then individually analyzed using LC-MS/MS using an Eksigent 2D+ nanoHPLC in-line with an AB SCIEX Triple ToF 5600 mass spectrometer. Samples were loaded onto a 1 or 2 cm Acclaim PepMap 100 nanotrap column (Thermo Scientific; 100 µm ID x 1 or 2 cm, C18, 5 µm, 100 Å). Sample fractions were then eluted from the nanotrap column using a gradient of increasing percentage of mobile phase B (95% acetonitrile, 5% water, 0.1% formic acid) and separated using an Acclaim PepMap 100 analytical column (75 µm ID x 15 cm, C18, 3 µm, 100 Å). Tandem mass spectrometry was performed in iInformation dependent acquisition using the following parameters:

250 ms TOF MS accumulation time; 50 ms MS/MS accumulation time; 20 ions monitored per cycle; total cycle time 1.3 s; 4 s dynamic exclusion time after one occurrence; rolling collision energy. The scanning windows for the TOF-MS and MS/MS were 300 – 1250 m/z and 55 – 2000 m/z, respectively.

The above proteomic methods describe the general framework used in the four described experiments. However, there were minor differences between experiments because the proteomic protocol was still in development at the time that these experiments were performed. For example, the concentrations of acetonitrile used in the Stata-X SPE elution series differed from experiment to experiment. A typical elution series included eluents of 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, and 60% acetonitrile in 0.1% formic acid. Additionally, in some cases a 1 cm nanotrap was used for in-line sample separation, in other cases a 2 cm nanotrap was used. Finally, the HPLC gradient used for in-lin sample separation differed between experiments, but generally was a two-step, continuous gradient increasing from 5% mobile phase B to 80% mobile phase B over a period of 40 to 60 minutes. It is important to note that while these are differences between the four proteomics experiments, all samples within an experiment were treated in the same way.

Protein Identification and Quantification.

Acquired spectra (.wiff files) were converted to the MGF format using the AB SCIEX converter (version 1.1 beta). MGF files from all the fractions of each sample were merged and searched against the appropriate database using the Mascot search engine with trypsin as the specified enzyme. For human studies

this was the 2011_6 release of the Human UniProtKB/Swiss-Prot database (20,127 entries) with addition of the common contaminants (112 entries). Carbamidomethyl (C) was selected as a fixed modification, and oxidation (M) and deamidation (NQ) were selected as variable modifications. Monoisotopic masses were used, and the error tolerances were 10 ppm and 0.5 Da for peptides and MS/MS fragments, respectively. Mascot search results were loaded into Scaffold (Proteome Software, Inc), which used the Peptide Prophet and Protein Prophet algorithms to validate peptide and protein identifications. The relative abundance of identified proteins was determined using Scaffold quantitative values (a type of normalized spectral count) of identified proteins.

Statistical Analysis

Within each experiment, differentially abundant proteins were identified using the Wilcoxon Rank-Sum test, since it has been reported as a robust statistical test for biomarker discovery studies with small sample sizes.[145] Correction for multiple comparisons was not used. Mean fold change between the two experimental groups was calculated, and the MFC was plotted against the $-\log_{10}$(p-value), in order to enhance selection of candidate biomarkers.

Results

Early AKI Study

In order to describe changes in urine protein abundance that occur early in the course of AKI, the urinary proteome of patients who developed severe AKI (defined as AKIN stage 3) after cardiac surgery (n=4) was compared to that of patients who did not develop AKI of any grade (n=4). The average time of urine

sample collection in was 9.6 hrs post-operatively, and at the time of sample collection, two of the AKI patients did not have elevated serum creatinine (sCr) meeting AKIN criteria for diagnosis of AKI. There were no statistically significant differences between the two groups with respect to demographic variables, type of surgery, use of intraoperative cardiopulmonary bypass, bypass time, pre-operative/baseline sCr, sCr at the time of sample collection, and the time of sample collection. A complete description of patient characteristics is shown in Table 2-1. A total of 230 proteins were identified with no false identification (calculated protein false discovery rate <1%). Of these 109 proteins were unique to the AKI group (*i.e.* the protein was identified in ≥1 sample in this group, but not identified in any sample from the other group); 27 proteins were unique to the No AKI group (Figure 2.1a). However, no proteins were consistently observed in one group but not detected in the other group (defined as identification of the protein in ≥3 samples of one group, but 0 samples in the other group). Because of its significance to experiments that will be described later, it is noteworthy that angiotensinogen was detected in the urine of 2 of the 4 AKI patients, but none of the patients without AKI. The two patients who did have detectable angiotensinogen had already met diagnostic criteria for AKI based on the magnitude of increased sCr that had already occurred at the time of sample collection. Four proteins were identified which had $P < 0.05$: uromodulin, CD59 glycoprotein, kinninogen-1, and vesicular integral-membrane protein VIP36. The distribution of mean fold change and p-value for the identified proteins is presenting in Figure 2.1b. Because candidate biomarkers were selected using

Table 2-1 Characteristics of patients used in the discovery phase proteomics studies comparing severe AKI to No AKI

	No AKI	**AKI**	***P***
n	4	4	
Demographic Variables			
Female[a]	25% (1)	0% (0)	1
Caucasian[a]	100% (4)	75% (3)	1
Age (yrs)[b]	63.8 ± 3.9	55.3 ± 12.2	0.34
Weight (kg)[b]	100.4 ± 8.3	104.9 ± 8.3	0.67
Sample Collection Time (hrs post-op)[b]	10.2 ± 5.3	9.0 ± 4.5	0.49
Operative Variables			
CABG[a]	100% (4)	25% (1)	0.14
Valve Replacement[a]	0% (0)	0% (0)	1
CABG + Valve Replacement[a]	0% (0)	0% (0)	1
Other Surgery[a]	0% (0)	75% (3)	0.14
Bypass[a]	75% (3)	100% (4)	1
Bypass Time (min)[b]	134.3 ± 32.1	147.3 ± 76.2	0.86
Serum Creatinine (mg/dL)			
Pre-Op Value[b]	1.0 ± 0.3	1.5 ± 0.3	0.11
At Sample Collection[b]	1.2 ± 0.3	1.9 ± 0.5	0.06
Maximum Post-Op Value[b]	1.3 ± 0.3	4.8 ± 1.9	0.03
Outcomes			
Days to Max sCr (from surgery)[b]	1.0 ± 0.8	3.0 ± 1.2	0.06
RRT[a]	0% (0)	25% (1)	<0.01
Death[a]	0% (0)	25% (1)	0.06

[a]Percentage and n; [b]Mean ± SD
Groups were compared using Fisher's Exact test for categorical variables and the Mann-Whitney U test for continuous variables.

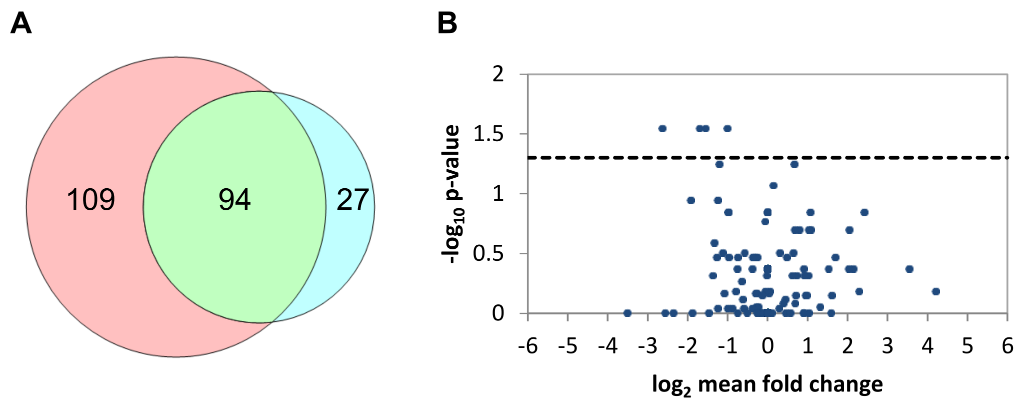

Figure 2-1. Urinary proteomic analysis of patients who either developed severe AKI after cardiac surgery or did not. Liquid chromatography tandem mass spectrometry was used to identify and quantify urinary proteomic changes during AKI. (A) Venn diagram showing the number of identified proteins by group. Red represents the group with AKI; blue represents the group without AKI. (B) Volcano plot shows the mean fold change in protein abundance between the two groups versus the statistical significance. Smaller p-values are larger due to the transformation. Data points above the dotted line have $P < 0.05$.

the combined data of this and three other proteomics experiments, the complete dataset is presented in Appendix A.

Renal Replacement Therapy

In order to identify candidate biomarkers of severe AKI, urine samples from 12 patients who had undergone cardiac surgery and developed post-operative AKI were analyzed by liquid chromatography tandem mass spectrometry. Six of these patients developed severe AKI requiring renal replacement therapy (RRT); six patients developed mild AKIN stage 1 AKI. There were no statistically significant differences between the two groups with respect to demographic variables, urine sample collection time, use of cardiopulmonary bypass, bypass time, type of surgery, preoperative/baseline serum creatinine (sCr), and sCr at the time of sample collection. A complete description of patient characteristics is shown in Table 2-2.

A total of 343 proteins were identified with a false discovery rate of 1.9%. Of these, 59 proteins were unique to the RRT group (*i.e.* they were identified in ≥1 patient in the RRT group but none of the patients in the No RRT group), and 5 proteins were unique to the No RRT group (Figure 2.2a). Twenty-six proteins were identified as being differentially abundant between the two groups ($P < 0.05$). The distribution of mean fold change and P value for the identified proteins is shown in the volcano plot (Figure 2.2b). Several candidate biomarkers can be selected using the criteria of large mean fold change and low P value. However, our objective was not to select candidate biomarkers using data from a single proteomics, but to collate data from several such studies. Therefore, the complete proteomics data from this experiment are presented in

Table 2-2 Characteristics of patients used in the discovery phase proteomics studies comparing severe AKI requiring RRT to mild AKI

	No RRT	**RRT**	***P***
n	6	6	
Demographic Variables			
Female[a]	33% (2)	33% (2)	1
Caucasian[a]	100% (6)	100% (6)	1
Age (yrs)	63.8 ± 7.9	72.5 ± 17.1	0.29
Weight (kg)	75.9 ± 40.2	85.3 ± 37.5	0.33
Sample Collection Time (hrs post-op)	29.2 ± 14.4	38.0 ± 12.0	0.3
Operative Variables			
CABG[a]	50% (3)	33% (2)	1
Valve Replacement[a]	17% (1)	17% (1)	1
CABG + Valve Replacement[a]	33% (2)	17% 1)	1
Other Surgery[a]	0% (0)	33% (2)	0.46
Bypass[a]	67% (4)	67% (4)	1
Bypass Time (min)	160.8 ± 67.0	165.0 ± 88.2	1
Serum Creatinine (mg/dL)			
Pre-Op Value	13. ± 0.4	1.4 ± 0.3	0.76
At Sample Collection	1.9 ± 0.4	2.6 ± 0.6	0.37
Maximum Post-Op Value	2.1 ± 0.5	4.2 ± 1.4	0.006
Outcomes			
Days to Max sCr (from surgery)	1.7 ± 1.0	4.8 ± 2.4	0.02
RRT[a]	0% (0)	100% (6)	<0.01
Death[a]	0% (0)	67% (4)	0.06

[a]Percentage and n; [b]Mean ± SD

Groups were compared using Fisher's Exact test for categorical variables and the Mann-Whitney U test for continuous variables.

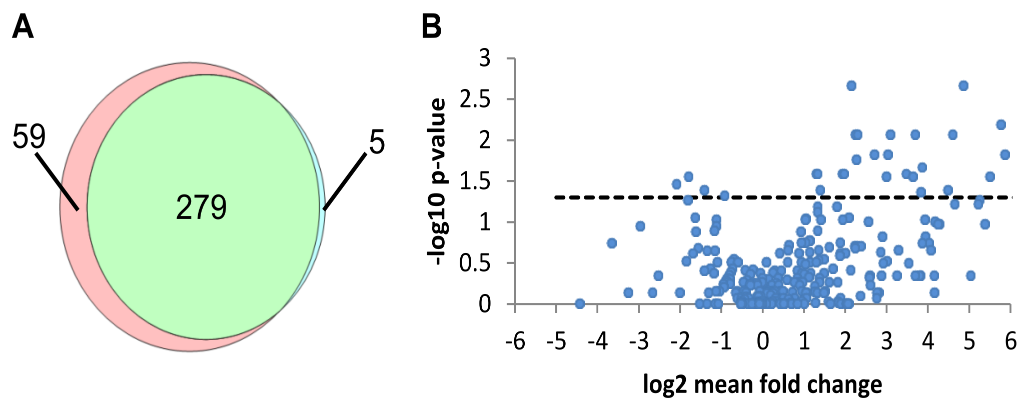

Figure 2-2. Urinary proteomic analysis of cardiac surgery patients who either developed severe AKI requiring renal replacement therapy (RRT) or mild AKI. Liquid chromatography tandem mass spectrometry was used to identify and quantify urinary proteomic changes during AKI. (A) Venn diagram showing the number of identified proteins by group. Red represents the group that required RRT; blue represents the group that only developed mild, AKIN stage 1 AKI. (B) Volcano plot shows the mean fold change in protein abundance between the two groups versus the statistical significance. Smaller p-values are larger due to the transformation. Data points above the dotted line have $P < 0.05$.

Appendix A. The combined data from this and other proteomics experiments is presented below. Nevertheless, it is worthwhile to mention the results of one candidate biomarker, urinary angiotensinogen, at this point. Of all the identified proteins in this experiment, it was the best discriminator based upon its large mean fold change (9.67-fold elevated in the RRT group) and low p-value (p = 0.002). Urinary angiotensinogen was undetectable in the 4 of the 6 patients in the No RRT group, and it discriminated with 100% accuracy between the two groups.

Rat Glycerol Induced AKI Study

In order to identify candidate biomarkers of AKI secondary to nephrotoxic causes, urine from rats (n = 3) in which AKI had been experimentally induced by glycerol injection was compared to that of vehicle injected controls (n = 3). A total of 259 proteins were identified with no false discoveries (calculated FDR <1%). Of the identified proteins, 33 were unique to the No AKI group and 48 were unique to the AKI group (Figure 2-3). However, only 37 of these proteins were consistently identified in the urine of the animals in AKI group (*i.e.* identified in all three group members but none of the other group). These proteins are shown in Table 2-3. Of note, of the 178 proteins shared between the groups, 100 had a P value = 0.1, the lowest possible p-value given the small sample size of this experiment. Of note, angiotensinogen was 10-fold elevated in the rats with AKI ($P = 0.1$), and it discriminated with 100% accuracy between the two experimental groups. The complete list of identified proteins and group comparisons is shown in Appendix A.

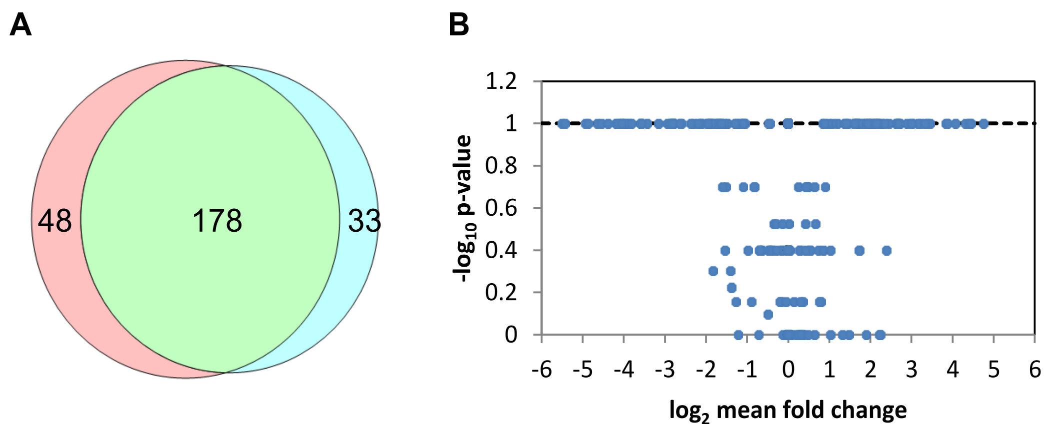

Figure 2-3. Urinary proteomic analysis of rats with glycerol injection induced AKI compared to controls. Liquid chromatography tandem mass spectrometry was used to identify and quantify urinary proteomic changes during AKI. (A) Venn diagram showing the number of identified proteins by group. Red (B) Volcano plot shows the mean fold change in protein abundance between the two groups versus the statistical significance. Smaller p-values are larger due to the transformation. The dashed line represents P= 0.1.

Table 2-3. Urinary proteins uniquely identified in rats with and without AKI

Protein Name	Uniprot Acc. Num.
Proteins Uniquely Identified in the Urine of Rats with AKI	
Fibrinogen alpha chain	P06399
Protein NOV homolog	Q9QZQ5
Creatine kinase M-type	P00564
L-lactate dehydrogenase B chain	P42123
Complement factor I	Q9WUW3
Uteroglobin	P17559
Cystatin-B	P01041
Gastrotropin	P80020
Hydroxyacid oxidase 2	Q07523
Myosin-4	Q29RW1
Sodium/potassium-transporting ATPase subunit alpha-1	P06685
Keratin, type II cytoskeletal 2 epidermal	Q6IG02
Calreticulin	P18418
Cartilage oligomeric matrix protein	P35444
Alanine--glyoxylate aminotransferase 2, mitochondrial	Q64565
Proteins Uniquely Identified in the Urine of Rats without AKI	
Phosphotriesterase-related protein	Q63530
Dipeptidyl peptidase 2	Q9EPB1
Pancreatic alpha-amylase	P00689
Neprilysin	P07861
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Q9JJ19
Meprin A subunit beta	P28826
Endothelial cell-selective adhesion molecule	Q6AYD4
Na(+)/H(+) exchange regulatory cofactor NHE-RF3	Q9JJ40
Ezrin	P31977
Retinoid-inducible serine carboxypeptidase	Q920A6
Glutamyl aminopeptidase	P50123
Calbindin	P07171
CD48 antigen	P10252
Aquaporin-1	P29975
Lysosomal alpha-glucosidase	Q6P7A9
Aflatoxin B1 aldehyde reductase member 3	P38918
Beta-microseminoprotein	P97580
Neuroplastin	P97546
Glutathione S-transferase alpha-1	P00502

Table 2-3 continued

Protein Name	Uniprot Acc. Num.
Proteins Uniquely Identified in the Urine of Rats without AKI	
Lysosomal acid phosphatase	P20611
Protein FAM151A	Q642A7
RT1 class I histocompatibility antigen, AA alpha chain	P16391

Mouse Renal Ischemia-Reperfusion Injury Induced AKI Study

To identify candidate biomarkers of AKI due to ischemic injury, urine from mice that had been subjected to renal ischemia-reperfusion injury (n = 5) was compared to that of sham operated controls (n = 5). This study identified 163 proteins (calculated FDR = 3.4%), of which 56 were differentially abundant between the two experimental groups ($P < 0.05$). Of these, only nine proteins were detected in the urine of all five mice with AKI but none of the sham operated controls. Notably, angiotensinogen was one of the proteins identified only in the urine of mice with AKI. These are displayed in Table 2-4, and the complete list of protein identifications is shown in Appendix A.

Selection of Candidate Biomarkers

Candidate biomarkers were selected based upon the combined data from the four proteomics experiments (Table 2-5). However, the experiments were not given equal weight. Since our objective was to identify prognostic biomarkers, we preferentially selected candidates based on the results of the RRT experiment, which compared patients with severe AKI requiring RRT to those with mild AKI. As mentioned above, angiotensinogen was the best discriminator in this experiment. Additionally, although it did not reach statistical significance, it was only observed in the urine of patients with AKI in the early AKI study. Similarly, angiotensinogen was observed in all 5 mice with renal ischemia-reperfusion injury, but it was not detected in any of the sham operated controls. Finally, it appeared to be a good discriminator of rats with glycerol- induced AKI,

Table 2-4. Urinary proteins uniquely identified in mice with AKI

Protein Name	Uniprot Acc. Num.
Proteins Uniquely Identified in the Urine of Mice with AKI	
Alpha-2-HS-glycoprotein	P29699
Angiotensinogen	P11859
Uteroglobin	Q06318
Vitamin D-binding protein	P21614
Plasminogen	P20918
Polyubiquitin B	P0CG49
Gelsolin	P13020
Transthyretin	P07309
Carboxylesterase 1C	P23953

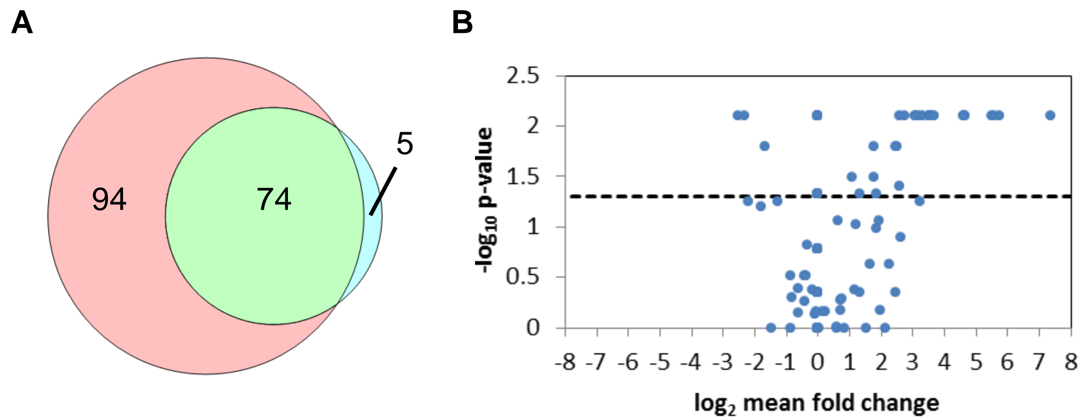

Figure 2-4. Urinary proteomic analysis of mice with renal ischemia reperfusion injury induced AKI versus sham operated controls. Liquid chromatography tandem mass spectrometry was used to identify and quantify urinary proteomic changes during AKI. (A) Venn diagram shows the number of identified proteins by group. Red represents the group with AKI; blue represents the group without AKI. (B) Volcano plot shows the mean fold change in protein abundance between the two groups versus the statistical significance. Smaller p-values are larger due to the transformation. Data points above the dotted line have $p<0.05$.

being markedly elevated in all three rats with AKI compare to controls ($P = 0.1$). Although the P value was >0.05, the lowest possible P-value for the Wilcoxon Rank-Sum test when n = 6 is 0.1, and so this difference was considered statistically significant. Figure 2-5 summarizes the angiotensinogen measurements made in the four proteomics studies. Qualitatively, angiotensinogen compared favorably with the well-established AKI biomarkers NGAL, KIM-1, and L-FABP (Table 2-5), suggesting that it could have similar or perhaps improved performance characteristics for the prediction of early AKI and AKI severity.

Discussion

A total of 22 novel candidate AKI biomarkers were identified using the combined results of four discovery phase proteomics experiments. A relative strength of our study is the heterogeneity of the study groups. The two human studies compared the urinary proteome of patients with early, severe AKI to that of patients without AKI (Early AKI Study) and the proteome of patients with early, severe AKI that eventually required renal replacement therapy (RRT) to that of patients with mild AKI (RRT study). To complement these data, two studies of different animal models of AKI were conducted, renal ischemia reperfusion injury in mice and glycerol-induced AKI in rats. The former has a similar mechanism to AKI after cardiac surgery, whereas the latter is a model of rhabdomyolysis-induced AKI. We hypothesized that we would have a greater probability of identifying translatable biomarkers by collating the data from these four experiments and by selecting proteins that discriminated between the groups of

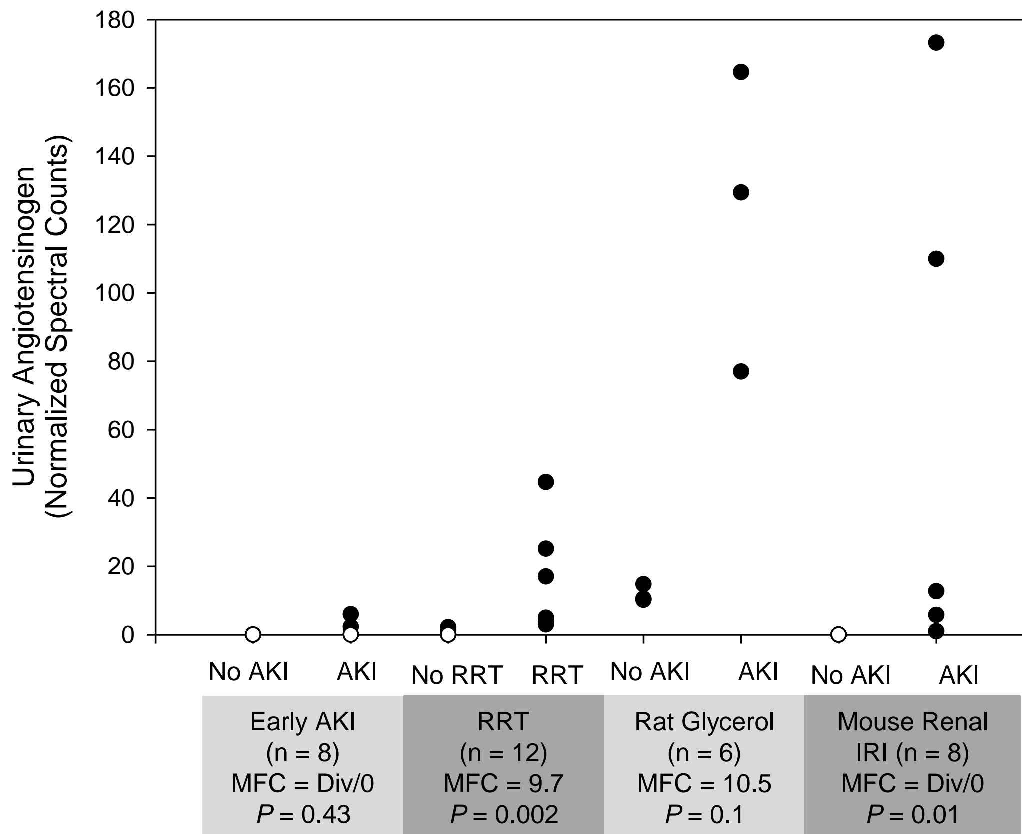

Figure 2-5. Point plots of urinary angiotensinogen abundance. The data from the four previously described proteomics studies are displayed, showing the relative abundance of angiotensinogen in each of the individual subjects of the two experimental groups of each study. For graphical representation, subjects in which angiotensinogen was not detected are represented as an open circle on the x-axis. Statistical significance was determined with the Wilcoxon Rank-Sum test. MFC, mean fold change; Div/0, angiotensinogen was not detectable in any of the subjects of the control group.

Table 2-5. Candidate Biomarkers of Acute Kidney Injury Identified by Urine Proteomic Analysis

	Study	RRT		Early		Glycerol		Renal IR/I	
	Species	Human		Human		Rat		Mouse	
	Protein	Fold change	p value/ direction	Fold change	p value/ direction	Fold change	p value/ direction	Fold change	p value/ direction
Increased	Angiotensinogen	9.7	0.002 ↑	Div/0	0.43 ↑	10.5	0.10 ↑	Div/0	0.008 ↑
	Apolipoprotein A-IV	9.1	0.007 ↑	Div/0	0.43 ↑	5.3	0.40 ↑	Div/0	0.17 ↑
	Vitamin D-binding protein	12.1	0.009 ↑	4.9	0.66 ↑	6.4	0.10 ↑	Div/0	0.008 ↑
	Complement C4-B	8.6	0.009 ↑	Not observed		4.4	0.10 ↑	Not observed	
	Superoxide dismutase [Cu-Zn]	2.4	0.009 ↑	Div/0	0.43 ↑	1.6	0.20 ↑	6.0	0.008 ↑
	Complement C3	5.7	0.009 ↑	2.0	0.49 ↑	3.2	0.10 ↑	53	0.008 ↑
	Profilin-1	4.1	0.02 ↑	Div/0	0.43 ↑	Div/0	0.40 ↑	Div/0	1.0 ↑
	Pigment epithelium-derived factor	9.1	0.03 ↑	Div/0	0.43 ↑	Not observed		Div/0	1.00 ↑
	Thymosin Beta 4	7.6	0.05 ↑	Div/0	0.43 ↑	Not observed		Not observed	
	Insulin-like growth factor-binding protein 1	Div/0	0.06 ↑	Div/0	0.43 ↑	Div/0	0.40 ↑	Not observed	
	Myoglobin	28.6	0.06 ↑	Div/0	1.0 ↑	Not observed		Not observed	
	Glutathione peroxidase 3	9.0	0.10 ↑	Div/0	1.0 ↑	8.0	0.10 ↑	Div/0	0.05 ↑
	Neutrophil defensin 1	1.8	0.20 ↑	Div/0	0.14 ↑	Not observed		Not observed	
	Lysozyme C	12.6	0.22 ↑	Div/0	0.43 ↑	1.3	1.0 ↑	Div/0	0.05 ↑
	Antithrombin-III	2.4	0.37 ↑	Div/0	1.0 ↑	Not observed		Div/0	0.05 ↑

Table 2-5 continued

	Study	RRT		Early		Glycerol		Renal IR/I	
	Species	Human		Human		Rat		Mouse	
	Protein	Fold change	p value/ direction	Fold change	p value/ direction	Fold change	p value/ direction	Fold change	
Decreased	Secreted Ly-6/uPAR-related protein 1	1.4	0.03 ↓	3.0	1.0 ↑	Not observed		Not observed	
	Non-secretory ribonuclease	2.2	0.04 ↓	2.0	0.14 ↓	Not observed		Not observed	
	Uromodulin	1.7	0.30 ↓	3.2	0.03 ↓	10.7	0.10 ↓	1.3	0.55 ↓
	CD59 glycoprotein	1.7	0.31 ↓	2.9	0.03 ↓	1.1	1.0 ↑	Not observed	
	Pro–Epidermal Growth Factor	2.0	0.38 ↓	Div/0	1.0 ↓	30.5	0.10 ↓	5.7	
	Polymeric immunoglobulin receptor	1.1	0.81 ↓	2.4	0.34 ↓	6.2	0.10 ↓	Not observed	
	Hepcidin	1.1	1.0 ↓	Div/0	1.0 ↓	16.5	0.10 ↓	Not observed	
Previously Described AKI Biomarkers	NGAL	Div/0	0.06 ↑	Div/0	1.0 ↑	9	0.10 ↑	Div/0	
	Clusterin	2.6	0.09 ↑	2.1	0.20 ↑	3.5	0.10 ↑	8.5	
	L-FABP	10.4	0.11 ↑	Not observed		Not observed		Not observed	
	KIM-1	Not observed		Not observed		Div/0	1.0 ↑	Div/0	

Data are shown as the mean fold change of protein abundance between experimental groups and the corresponding *P* value obtained from the Wilcoxon rank-sum test. Arrows indicate the direction of change where ↑ indicates higher in the case subjects and ↓ indicates higher in control subjects. Green boxes highlight proteins which increased in cases and red boxes highlight subjects that decreased in controls. Proteins are grouped based on whether they increased or decreased in cases. Data on known AKI biomarkers is also displayed. Div/0 indicates that the protein was not seen in any sample from the group with lower abundance. Not observed indicates that protein was not detected in any sample in that study. RRT-Renal replacement therapy study; I/RI- ischemia-reperfusion injury; NGAL-Neutrophil gelatinase associated lipocalin; L-FABP-L type fatty acid binding protein; KIM-1 – Kidney injury molecule-1.

each experiment. In fact, we identified NGAL using this approach, which we believe validates our data. It is important to acknowledge the limitations of discovery phase proteomics experiments. Due to the small number of samples and multiple statistical comparisons that are made, there is a high probability of type I error and false discovery of candidate biomarkers. To mitigate this probability, we used the non-parametric Wilcoxon Rank-Sum test for statistical comparison because it has been demonstrated to have a lower type I error than other tests.[19] However we did not use an adjustment for multiple comparisons such as the Bonferroni or Benjamini-Hochberg corrections, largely because the studies were underpowered for such adjustments. Instead, we selected biomarkers based on trends observed in the combined data of the four experiments. Nevertheless, it is important to recognize that discovery proteomics experiments are only the first phase of a multistep biomarker identification workflow which has been proposed by Rifai *et al* (Figure 2-6).[146] Phases which will need to be completed include qualification and verification, in which the differential abundance of the candidates is confirmed using a more targeted analytical approach, and verification, during which the biomarker is measured in populations other than the one used in the discovery phase in order to evaluate its specificity. In the final phase, validation, a clinical assay is developed and rigorously characterized. These later phases will be addressed in subsequent chapters, which describe the qualification and verification of angiotensinogen, the most promising candidate biomarker that we identified.

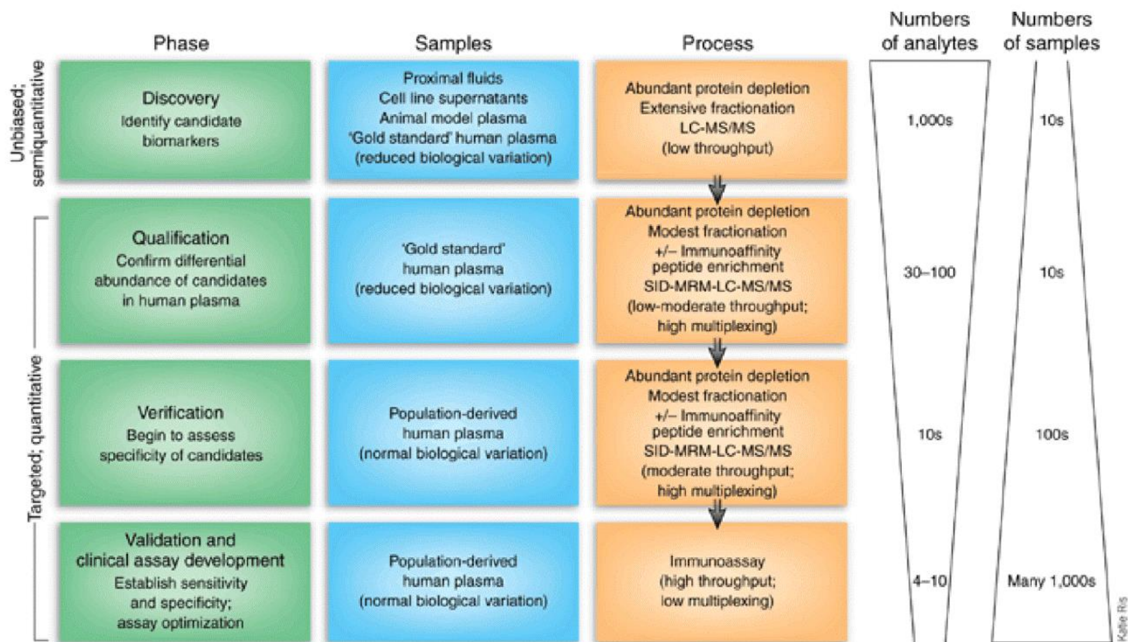

Figure 2-6. Paradigm for understanding the biomarker discovery process.[146] Four distinct phases are described: discovery, qualification, verification, and validation. In each subsequent phase, the number of proteins being measured (analytes) decreases and the number of biological samples being evaluated increases. There is also a shift from low throughput methodologies to more high throughput, targeted approaches. Rifai *et al.* Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nature Biotechnol. 2006 Aug; 24(8):971-83.

Chapter 3: Qualification of Urinary Angiotensinogen as a Prognostic Biomarker of Acute Kidney Injury After Cardiac Surgery

Introduction

Our previous study using urinary proteomic analysis identified a number of candidate prognostic biomarkers of acute kidney injury (AKI). The most promising of these was urinary angiotensinogen. Collated data from the four proteomic experiments showed that the relative abundance of urinary angiotensinogen was increased in every experimental state of each of the studies (see Table 2-4 and Figure 2-5) to a greater degree than other candidates, as determined by the mean fold change, and in 3 of the 4 experiments statistical significance was met. Furthermore, the significance of elevated urinary angiotensinogen is intriguing from a mechanistic standpoint. Angiotensinogen is the principal substrate of the renin-angiotensin system (RAS), a hormonal cascade that has pleiotropic effects in the kidney, including the regulation of hemodynamics, sodium reabsorption, aquaresis, cellular proliferation and apoptosis, fibrosis, and inflammation.[147] Urinary angiotensinogen concentration is thought to reflect the level of activation of the intrarenal RAS.[148] Thus, from a pathobiologic point of view, angiotensinogen is a logical prognostic biomarker.

Materials and Methods

Patients and Urine Samples

Urine samples were selected from those banked by the Souther Acute Kidney Injury Network (SAKINet; see Chapter 2) in order to fit the criteria described below. Patients had undergone cardiac surgery, and urine samples were collected at the time of AKI diagnosis according to AKIN serum creatinine (sCr)

criteria.[3] Inclusion criteria were consent by the patient or appropriate surrogate, surgery of the heart or ascending aorta, and development of AKI (defined by the AKIN sCr criteria) within 2 days of surgery. Only subjects who had collection of urine within 48 hours after surgery were used in order to conform to the AKIN staging criteria and to attempt to eliminate confounding effects of events that were not directly related to the cardiac surgery. The only exclusion criterion was a baseline sCr > 3 mg/dL. A total of 97 patients were included in this study. Urine samples from 10 of the patients used in this study were also used in the discovery phase proteomic analysis described in Chapter 2. Of the 97 patients enrolled, 79 were classified as AKIN stage 1 at the time of urine sample collection.

Angiotensinogen ELISA

The Human Total Angiotensinogen Assay Kit (Immuno-Biological Laboratories Co., Ltd.), a solid phase sandwich ELISA, was used according the manufacturer's protocol to measure urinary angiotensinogen. Urine samples were diluted 1:8 in enzyme immunoassay (EIA) buffer provided by the manufacturer. One hundred μL of diluted sample was added to the appropriate well and incubated for 60 min at 37°C. The plate was then washed 7 times by pipetting 250 μL of the provided wash buffer into each well using a multichannel, repeating pipet. After drying the plate, 100 μL of 30x diluted HRP-conjugated anti-angiotensinogen antibody was added to each well and incubated for 30 minutes at 37°C. The plate was washed 9 times as before and dried. 100 μL of chromogen (TMB) was added to each well, and the plate was incubated for 30

min in the dark at room temperature. One hundred µL of stop solution was added to each well, and the absorbance was measured at 450 nm using a SpectraMAX 340PC 96-well plate reader. The linear range of the assay is 0.31 to 30 ng/mL. Intra- and inter-assay variability (coefficient of variation) were calculated by measuring the standards and three selected biological samples in quadruplicate once, and in duplicate on all remaining plates. Values for intra- and interassay variability were 2.4% and 9.9%, respectively. Data were analyzed using Softmax Pro3.1.2. Samples whose values were above the upper limit of quantification for the assay were diluted 1:10 in EIA buffer and re-run on a separate plate. If the value remained above the limit of quantification, a concentration of 20 ng/mL assigned to that sample.

Urine Creatinine Determination.

Urine creatinine was used to normalize the urine angiotensinogen concentration to account for biological variability in the concentration of urine. This is accordance with the findings of Ralib *et al.* who reported that creatinine correction was appropriate and increased the prognostic predictive value of urine biomarkers of AKI.[149] Values were reported as the ratio of angiotensinogen in ng/ml to creatinine in mg/ml (uAnCR, ng/mg). Urine creatinine was measured using the Jaffe assay. Three µL of sample was combined with 100 µL of 1% picric acid (Sigma-Aldrich), 100 µL of 0.75M NaOH (Genomic Solutions), and 300 µL distilled deionized H_2O. Samples were incubated at room temperature for 15 min and absorbance at 490 nm was measured using a SpectraMAX 340PC 96-well plate reader. Data were analyzed using Softmax Pro 3.1.2.

Outcomes

The primary outcome was worsening of AKI, defined as progression to a higher AKIN stage after the time of sample collection. Secondary outcomes were progression to AKIN stage 3, the need for renal replacement therapy (RRT) within 10 days of sample collection, progression to AKIN stage 2 or 3, progression to AKIN stage 3 or death, RRT or death, and discharge >7 days from the time of sample collection or in-hospital mortality. Outcomes were tested using the entire cohort and in a subset of patients classified as AKIN stage 1 at the time of sample collection.

Statistical Analysis

Count data were analyzed using the χ^2 or Fisher's exact test as appropriate. Continuous variables were analyzed using the Student's *t test* or Mann Whitney U test when comparing two groups. ANOVA or Kruskal-Wallis ANOVA on Ranks test and the *post- hoc* Dunn's test for pairwise comparison were used to evaluate continuous variables when more than two groups were compared. Odds ratios (OR) were used to test the association of uAnCR with selected outcomes. Patients were stratified by uAnCR into quartiles, the effect of uAnCR on the risk of developing an outcome was tested by calculating the OR of the upper and lower quartiles and estimating the 95% confidence interval of the OR. Receiver operator characteristic curves were constructed to determine the predictive power of uAnCR. The area under the ROC curve (AUC) was used as an estimate of an overall accuracy of the biomarker. An AUC of 1.0 represents 100% accuracy, whereas an AUC of 0.5 indicates 50% accuracy, which is no better

than random chance. Univariate ROC curves were considered statistically significant if the AUC differed from 0.5, as determined by the z-test. Optimal cut-offs were determined by selecting the data point that minimized the geometric distance from 100% sensitivity and 100% specificity on the ROC curve.[150] Additionally, cut-offs that maximized the positive likelihood ratio and minimized the negative likelihood ratio were reported since they could be useful in assigning high or low risk of adverse outcomes to a patient. Likelihood ratios of positive and negative predictive value were used since they are insensitive to changes in prevalence (unlike PPV and NPV) and can be used to infer post-test probability. Kaplan-Meier curves were used to visualize the relationship between uAnCR and length of stay. Patients who died were censored. The log-rank test was used to compare the curves, and the Holm-Sidak test was used for *post-hoc* pairwise comparison. Category free net reclassification improvement was used to determine if addition of uAnCR to a multivariate logistic regression model for prediction of risk increased the ability of the model to predict worsening of AKI. First, a multiple logistic regression model (reference model) was created using the variables percent change in serum creatinine from baseline and Cleveland Score, a perioperative risk score that has been demonstrated to predict AKI outcomes after cardiac surgery.[151, 152] Then, a new model was created which included uAnCR, in addition to these two variables. Each patient's probability (risk) of experiencing worsening of AKI after sample collection was calculated with both models. The category free net reclassification index was calculated as previously described, and was used to quantify the improved prognostic

predictive power gained by including uAnCR in the model.[153, 154] Statistical tests were performed in either Matlab or SigmaPlot.

Results

We measured urinary angiotensinogen by ELISA and verified its ability to predict outcomes in patients who had developed AKI after cardiac surgery (n= 97). These patients were divided into three groups by maximum AKIN stage: stage 1 (n= 59), stage 2 (n= 19), and stage 3 (n= 19). Of these, 79 patients (stage 1 n= 59, stage 2 n = 10, and stage 3 n = 10) were classified as AKIN stage 1 (*i.e.* had less than a doubling of serum creatinine) at the time of urine sample collection. There were no statistically significant differences among the groups with respect to the following potential confounders: gender, race, age, use of intraoperative bypass, bypass time, pre-operative sCr, and type of surgery (Table 3-1). Since our primary objective was to identify a prognostic biomarker among patients with mild AKI, we performed a two analyses, one using the entire cohort and a second subset analysis using only patients who had not progressed beyond AKIN stage 1 at the time of sample collection (n= 79).

Urinary Angiotensinogen Concentration and AKI Severity

Among all patients who had developed AKI of any stage at the time of urine sample collection, urinary angiotensinogen corrected for creatinine (uAnCR; ng angiotensinogen / mg creatinine) was correlated with both maximum sCr ($r=0.49$; $p< 0.001$) and maximum percent change in sCr ($r= 0.29$; $p= 0.01$), and uAnCR increased with AKI severity (as determined by maximum AKIN stage) in both the

whole cohort and the subset of patients classified as AKIN stage 1 at collection (Table 3-1; Figure 3-1). *Post-hoc* pair-wise comparison revealed a significant difference between the patients who developed AKIN stage 3 and those who reached a maximum of stage 1.

Urinary Angiotensinogen and Adverse Outcomes

We evaluated the ability of angiotensinogen to predict the primary outcome of worsening of AKI (defined as progression to a higher AKIN stage). Comparing patients in the top quartile of uAnCR to those in the bottom quartile, the odds ratio for worsening of AKI was 5.0 (95% CI 1.16-21.46) in the whole cohort and 4.64 (95% CI 1.02-21.0) in the subset of patients who were classified as AKIN stage 1 at collection. Several secondary outcomes were also evaluated, including AKIN stage 3, the need for renal replacement therapy (RRT) within 10 days, and the composite outcomes of development of AKIN stage 2 or 3, AKIN stage 3 or death, and RRT or death. Ingeneral, patients with higher uAnCR were at increased risk of these outcomes (Table 3-2). Receiver operator characteristic (ROC) curve analysis found that uAnCR was predictive of worsening AKI in both the whole cohort (AUC= 0.70) and in the subset classified as AKIN stage 1 at collection (AUC= 0.71). It also predicted the other tested outcomes, with the exception of RRT in the subset of patients classified as AKIN stage 1 at collection (Figures 3-2, 3-3, and 3-4). While the ROC curve for RRT prediction in these patients was not statistically significant (p= 0.1), it is likely that it was

Table 3-1. Characteristics of patients used to qualify urinary angiotensinogen as a prognostic AKI biomarker

	AKI of Any Stage at Time of Sample Collection (n=97)				AKIN Stage 1 at Time of Sample Collection (n=79)			
Maximum AKIN Stage Achieved	AKIN Stage 1	AKIN Stage 2	AKIN Stage 3	p-value	AKIN Stage 1	AKIN Stage 2	AKIN Stage 3	p-value
n	59	19	19		59	10	10	
uAnCR[a]	22.6 (13.1-54.0)	34.1 (11.1-50.4)	58.8 (20.4-217.1)	0.01	22.6 (13.1-54.0)	35.3 (22.6-270.3)	77.0 (30.9-329.4)	0.01
Male	71% (42)	74% (14)	68% (13)	0.94	71% (42)	60% (6)	70% (7)	0.78
Caucasian	64% (38)	63% (12)	79% (15)	0.47	64% (38)	60% (6)	80% (8)	0.58
Age (yrs)[b]	65.8 +/- 10.8	64.5 +/- 10.0	68.5 +/- 11.9	0.53	65.8 +/- 10.8	68.2 +/-10.8	69.0 +/- 14.7	0.58
Weight (kg)[b]	88.2 +/- 24.3	94.41 +/- 23.8	88.9 +/- 27.3	0.63	88.2 +/- 24.3	84.7 +/- 21.7	88.1 +/- 33.3	0.69
Sample Collection Time (post-op hrs)[b]	27.9 +/- 11.8	31.6 +/- 14.5	36.0 +/- 11.0	0.04	27.9 +/- 11.8	26.2 +/- 15.6	35.2 +/- 12.2	0.23
Operative Variables								
CABG	61% (36)	53% (10)	58% (11)	0.81	61% (36)	40% (4)	50% (5)	0.41
Valve Replacement	29% (17)	37% (7)	21% (4)	0.56	29% (17)	50% (5)	20% (2)	0.3
CABG + Valve Replacement	10% (6)	11% (2)	21% (4)	0.44	10% (6)	10% (1)	30% (3)	0.21
Bypass	76% (45)	84% (16)	74% (14)	0.71	76% (45)	80% (8)	70% (7)	0.88
Bypass Time (min)[b]	143.2 +/- 72.5	145.4 +/- 75.6	118.9 +/- 67.3	0.31	143.2 +/- 72.5	154.4 +/- 74.7	146.4 +/- 77.8	0.95
Serum Creatinine (mg/dL)								
Pre-op sCr[b]	1.2 +/- 0.3	1.2 +/- 0.4	1.2 +/- 0.5	0.19	1.2 +/- 0.3	1.2 +/- 0.4	1.4 +/- 0.5	0.74
sCr at Collection[b]	1.7 +/- 0.4	2.0 +/- 0.7	2.5 +/- 0.8	0.001	1.7 +/- 0.4	1.7 +/- 0.6	2.3 +/- 0.8	0.14
Max sCr[b]	1.9 +/- 0.4	2.7 +/- 0.8	4.0 +/- 1.9	<0.001	1.9 +/- 0.4	2.6 +/- 0.8	4.3 +/- 2.6	<0.001
Outcomes								
Days to Max sCr (post-op)[a]	2.0 (1.0-3.0)	2.0 (2.0-3.0)	4.0 (2.0-5.0)	0.001	2.0 (1.0-3.0)	3.0 (2.0-5.25)	4.5 (2.7-6.75)	<0.001
RRT 10 days	0	0	47% (9)	<0.001	0	0	80% (8)	<0.001
Death	0	11 % (2)	32 %(6)	<0.001	0	20% (2)	30% (3)	<0.001

[a]Median and interquartile range; [b]Mean and SD; Categorical data are shown as percentage and n

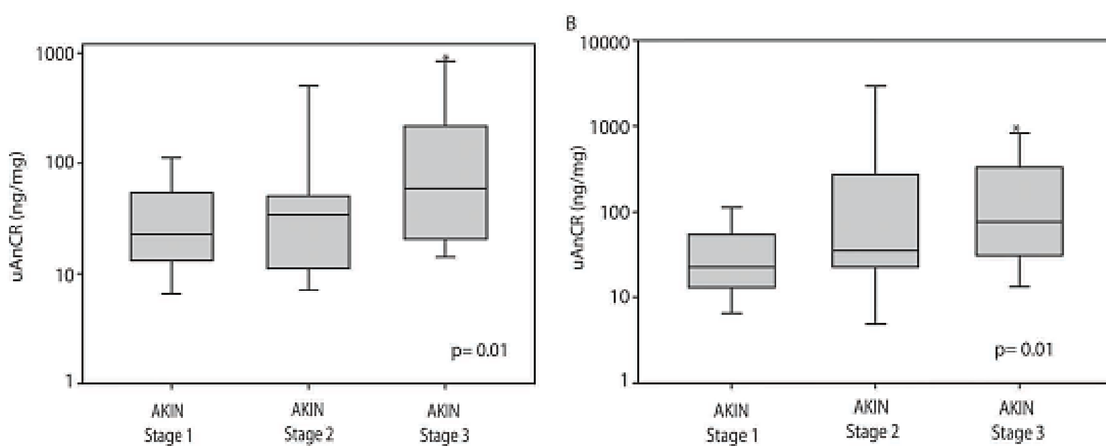

Figure 3-1. uAnCR increases with AKI severity. Box and whisker plots showing the distribution of uAnCR by group in patients who developed AKI after cardiac surgery. (A) Among patients who had AKI of any AKIN stage at the time of sample collection (n = 97), and (B) among the subset of patients who were classified as AKIN stage 1 at the time of sample collection (n = 79), uAnCr increased in a graded manner with AKI severity. Box plots show the median (solid line), 25th and 75th percentiles. Error bars represent the 5th and 95th percentiles. AKIN stage groups were compared using the Kruskal-Wallis test (p-value shown in bottom right) and *post-hoc* Dunn's test for pairwise comparison. *, $p < 0.05$ when compared to AKIN stage 1 group

Table 3-2. Association of Elevated uAnCR and Adverse Outcomes

Patients who were any stage AKI at the time of sample collection (n=97)									
	Worsening of AKI			AKIN stage 3			RRT		
	Yes	No	OR	Yes	No	OR	Yes	No	OR
1st Quartile	13% (3)	87% (21)	5.0	4% (1)	96% (21)	13.8	4% (1)	96% (23)	6.1
2nd Quartile	16% (4)	84% (21)	(1.16-21.46)	16% (4)	84% (21)	(1.58-120.37)	4% (1)	96% (24)	(0.65-56.37)
3rd Quartile	29% (7)	71% (17)		21% (5)	79% (19)		8% (2)	92% (22)	
4th Quartile	42% (10)	58% (14)		38% (9)	62% (15)		21% (5)	79% (19)	
	LOS			RRT or Death			AKIN stage 3 or Death		
	Yes	No	OR	Yes	No	OR	Yes	No	OR
1st Quartile	25% (6)	75% (18)	21.0	4% (1)	96% (23)	9.5	4% (1)	96% (23)	13.8
2nd Quartile	56% (14)	44% (11)	(4.58-96.23)	12% (3)	88% (22)	(1.06-84.37)	16% (4)	84% (21)	(1.58-120.38)
3rd Quartile	62% (15)	38% (9)		8% (2)	92% (22)		21% (5)	79% (19)	
4th Quartile	87% (21)	13% (3)		29% (7)	71% 17)		38% (9)	62% (15)	
	AKIN stage 2 or 3								
	Yes	No	OR						
1st Quartile	29% (7)	71% (17)	2.9						
2nd Quartile	28% (7)	72% (18)	(0.87-9.45)						
3rd Quartile	46% (11)	54% (13)							
4th Quartile	54% (13)	46% (11)							
Patients who were classified as AKIN stage 1 at the time of sample collection (n=79)									
	Worsening of AKI			AKIN stage 3			RRT		
	Yes	No	OR	Yes	No	OR	Yes	No	OR
1st Quartile	15% (3)	85% (17)	4.64	5% (1)	95% (19)	8.14	5% (1)	95% (19)	4.75
2nd Quartile	10% (2)	90% (18)	(1.02-21.0)	5% (1)	95% (19)	(0.88-75.48)	5% (1)	95% (19)	(0.48-46.91)
3rd Quartile	32% (6)	68% (13)		11%(2)	89% (17)		11% (2)	89% (17)	
4th Quartile	45% (9)	55% (11)		30% (6)	70% (14)		20% (4)	80% (16)	
	LOS			RRT or Death			AKIN stage 3 or Death		
	Yes	No	OR	Yes	No	OR	Yes	No	OR
1st Quartile	25% (5)	75% (15)	17.0	5% (1)	95% (19)	8.14	5% (1)	95% (19)	8.0
2nd Quartile	50% (10)	50% (10)	(3.46-83.44)	5% (1)	95% (19)	(0.88-75.48)	5% (1)	95% (19)	(0.91-70.34)
3rd Quartile	63% (12)	37% (7)		11% (2)	89% (17)		11% (2)	89 (17)	
4th Quartile	85% (17)	15% (3)		30% (6)	70% (14)		30% (8)	70% (12)	

The percentage and number of patients who met each outcome are shown by quartile of uAnCR. The odds ratio comparing the 4th and 1st quartiles and 95% CI are also reported.

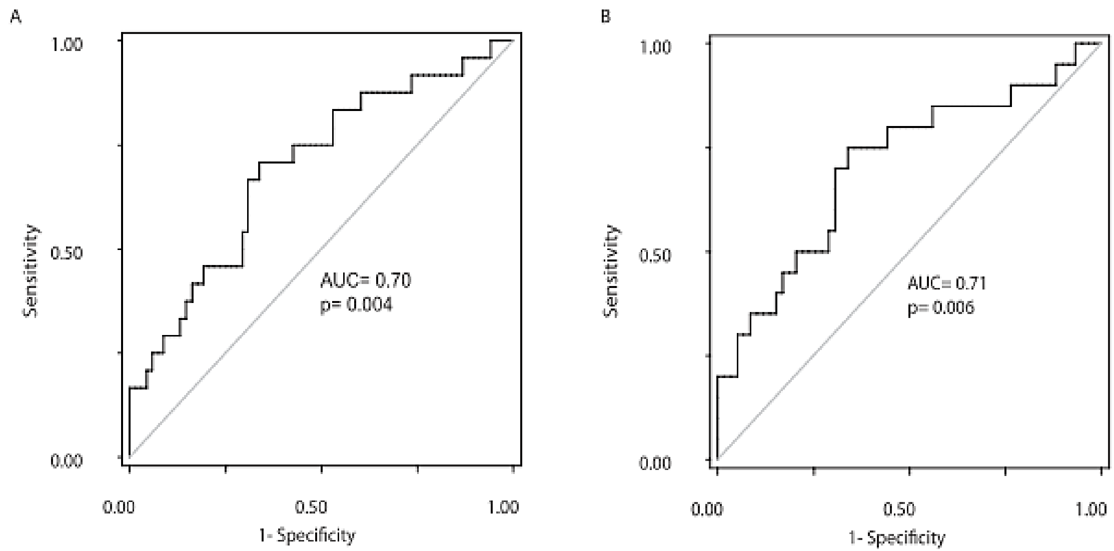

Figure 3-2. uAnCR Predicts Worsening of AKI. Receiver operator characteristic (ROC) curves demonstrate the ability of uAnCR to predict worsening of AKI after sample collection among (A) patients who were any stage AKI at the time of collection and (B) the subset of patients who were classified as AKIN stage 1 at collection. Worsening of AKI was defined as progression to a higher AKIN stage. A perfect biomarker would have an area under the ROC curve (AUC) of 1, whereas random chance has an AUC of 0.5. The ROC curve was considered statistically significant if the AUC differed from 0.5.

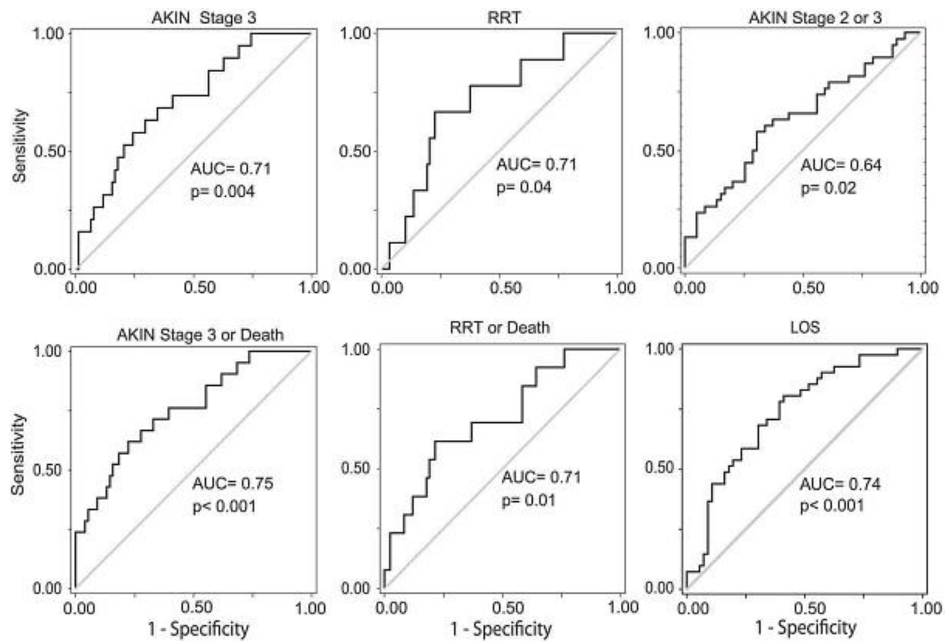

Figure 3-3. Receiver operator characteristic (ROC) curves showing the predictive power of uAnCR for multiple adverse outcomes in patients who developed AKI after cardiac surgery. The entire cohort (n = 97) was used in these analyses.

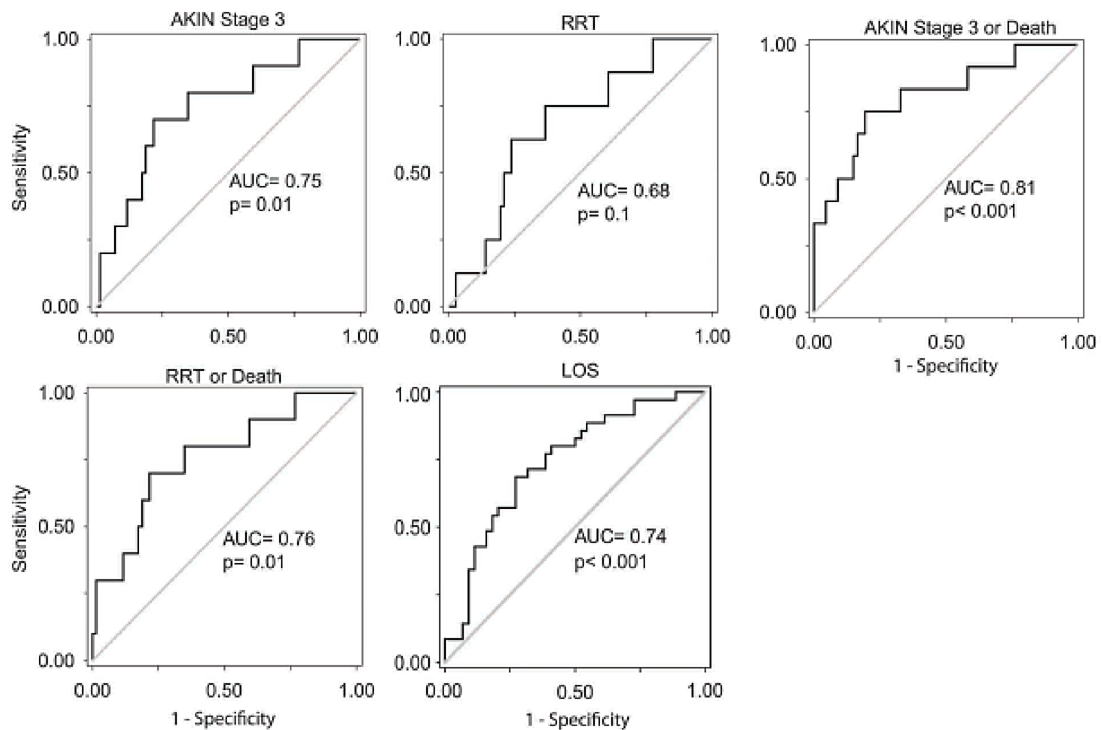

Figure 3-4. Receiver operator characteristic (ROC) curves showing the predictive power of uAnCR for multiple adverse outcomes in patients who developed AKI after cardiac surgery. Only the subset of patients who were classified as AKIN stage 1 at the time of sample collection were included in these analyses (n = 79). The outcome AKIN stage 2 or 3 is not reported here because it was previously reported as worsening of AKI in Figure 3-2.

under powered since only eight patients required RRT in this group Notably, the predictive power for most outcomes among those patients classified as AKIN stage 1 at collection appeared to be slightly augmented in comparison to the analysis including the entire cohort. In addition to the prediction of the renal and mortality outcomes, we noted a relationship between uAnCR and length of hospital stay. This relationship is visualized in survival curves plotting the time todischarge (defined as days after sample collection) of patients in the upper, middle or lower tertiles of uAnCR. Among all AKI patients and in the subset of patients classified as AKIN stage 1 at the time of collection, those patients with higher uAnCR concentrations had longer hospital stays (Figures 3-5a and 3-5b). ROC curve analysis indicated that uAnCR was predictive of longer length of stay defined as discharge >7 days from the time of sample collection or death ≤7 days from collection in both the whole cohort (Figures 3-3) and in the subset of patients who were classified as AKIN stage 1 at collection (Figure 3-4). Tables 3-3 and 3-4 summarize the performance characteristics of uAnCR as a predictor of the tested outcomes in patients who had AKIof any stage at the time of sample collection and those who had not progressed beyond AKIN stage 1 at the time of sample collection, respectively.

Net Reclassification Improvement

We determined the ability of uAnCR to improve the prediction of worsening AKI of a clinical risk model. The clinical model was a multivariate logistic regression model consisting of the percent change in sCr from baseline that had already

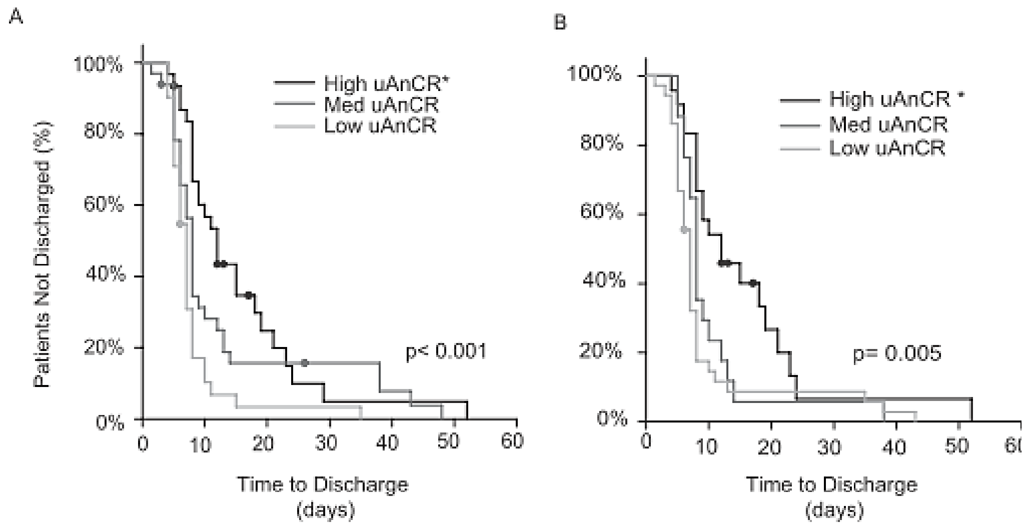

Figure 3-5. Survival curves showing the association of increased uAnCR and increased length of stay in cardiac surgery patients with post-operative AKI. Patients were stratified into tertiles by uAnCR. Kaplan-Meier survival curves show that (A) in patients with any stage AKI at the time of sample collection (n = 97), and (B) in the subset of patients classified as AKIN stage 1 at collection, patients with higher uAnCR had increased length of stay (defined as days to discharge from the time of sample collection. The log-rank test was used to determine if the survival curves differed statistically (p-value shown), and the Holm-Sidak test was used for *post-hoc* pairwise comparison. *, p< 0.05 compared to Low uAnCR group

Table 3-3. Performance characteristics of uAnCR as a prognostic biomarker of AKI among patients who were any stage AKI at the time of sample collection (n = 97)

Outcome	AUC	Cut-Off (ng/mg)		% (n)[a]	Sensitivity	Specificity	LR+	LR-	PPV	NPV
Worsening AKI	0.70	Best	>33.27	44.3% (43)	70.8%	66.2%	2.09	0.44	67.7%	69.4%
	(0.57-0.82)	Max PPV	>392.5	6.2% (6)	16.7%	98.5%	11.34	0.85	91.9%	54.2%
		Max NPV	>12.55	20.6 (20)	91.7%	26.5%	1.25	0.31	55.5%	76.1%
AKIN Stage 3 AKI	0.71	Best	>34.33	41.2% (40)	68.4%	65.4%	1.98	0.48	66.4%	67.4%
	(0.59- 0.84)	Max PPV	>572.0	4.1% (4)	15.8%	98.7%	12.34	0.85	92.5%	54.0%
		Max NPV	>14.06	25.8% (25)	94.7%	30.8%	1.37	0.17	57.8%	85.4%
RRT[b]	0.71	Best	>58.63	26.8% (26)	66.7%	77.3%	2.93	0.43	74.6%	69.9%
	(0.54-0.88)	Max PPV	>572.0	4.1% (4)	11.1%	96.6%	3.26	0.92	76.5%	52.1%
		Max NPV	>20.01	38.1% (37)	88.9%	40.9%	1.50	0.27	60.1%	78.6%
AKIN Stage 2 or 3 AKI	0.64	Best	>34.33	41.2% (40)	57.9%	69.5%	1.90	0.61	65.5%	62.3%
	(0.52-0.73)	Max PPV	>392.5	6.2% (6)	13.2%	98.3%	7.79	0.88	88.6%	53.1%
		Max NPV	>6.777	7.2% (7)	97.4%	10.2%	1.08	0.26	52.0%	79.5%
AKIN 3 or Death[c]	0.75	Best	>37.36	36.1% (35)	66.7%	72.4%	2.41	0.46	70.7%	68.5%
	(0.64-0.87)	Max PPV	>392.5	6.2% (6)	23.8%	98.7%	18.04	0.77	94.7%	56.4%
		Max NPV	>14.06	25.8% (25)	95.2%	31.6%	1.39	0.15	58.2%	86.9%
RRT or Death[c]	0.71	Best	>58.63	26.8% (26)	61.5%	78.6%	2.87	0.49	74.2%	67.1%
	(0.55-0.86)	Max PPV	>466.6	5.2% (5)	23.1%	97.6%	9.70	0.79	90.7%	55.9%
		Max NPV	>16.28	32% (31)	92.3%	35.7%	1.44	0.22	58.9%	82.3%
Length of Stay[d]	0.74	Best	>26.38	54.6% (52)	69.6%	68.3%	2.19	0.44	68.7%	69.2%
	(0.64-0.84)	Max PPV	>67.97	24.7% (24)	37.5%	92.7%	5.12	0.67	83.7%	59.7%
		Max NPV	<13.78	24.8% (24)	89.3%	43.9%	1.59	0.24	61.4%	80.3%

[a]The percentage and number of patients who were above the Best and Max PPV cut-offs or below the Max NPV cut-off; [b]RRT- renal replacement therapy initiated within 10 days of surgery; [c]Death-defined as in-hospital mortality; [d]Length of stay- outcome defined as discharge >7 days from sample collection or death before post-operative day 7

Table 3-4. Performance characteristics of uAnCR as a prognostic AKI biomarker among patients classified as AKIN stage 1 at the time of urine sample collection (n = 79)

Outcome	AUC	Cut-Off (ng/mg)		% (n)[a]	Sensitivity	Specificity	LR+	LR-	PPV	NPV
Worsening AKI	0.71 (0.57-0.85)	Best	>33.27	44.3% (35)	75.0%	66.1%	2.21	0.38	68.9%	72.6%
		Max PPV	>392.5	6.3% (5)	20.0%	98.3%	11.83	0.81	92.2%	55.1%
		Max NPV	>19.95	36.7% (29)	85.0%	44.1%	1.52	0.34	60.3%	74.6%
AKIN Stage 3 AKI	0.75 (0.58-0.92)	Best	>58.63	27.8% (22)	70.0%	78.3%	3.22	0.38	76.3%	72.3%
		Max PPV	>572.0	3.8% (3)	20.0%	98.6%	13.79	0.81	93.2%	55.2%
		Max NPV	>19.95	36.7% (29)	90.0%	40.6%	1.51	0.25	60.2%	80.2%
RRT	0.68 (0.49-0.87)	Best	>34.33	40.5% (32)	75.0%	63.4%	2.05	0.39	67.2%	71.7%
		Max PPV	>572.0	3.8% (3)	12.5%	97.2%	4.43	0.90	81.6%	52.6%
		Max NPV	>19.95	36.7% (29)	87.5%	39.4%	1.44	0.32	59.1%	75.9%
AKIN 3 or Death	0.81 (0.66-0.95)	Best	>58.63	27.8% (22)	75.0%	80.6%	3.87	0.31	79.4%	76.3%
		Max PPV	>392.5	6.3% (5)	33.3%	98.5%	22.37	0.68	95.7%	59.6%
		Max NPV	>19.95	36.7% (29)	91.7%	41.8%	1.57	0.20	61.2%	83.4%
RRT or Death	0.76 (0.59-0.93)	Best	>58.63	27.8% (22)	70.0%	78.3%	3.22	0.38	76.3%	72.3%
		Max PPV	>466.6	5.1% (4)	30.0%	98.6%	20.69	0.71	95.4%	58.5%
		Max NPV	>19.95	36.7% (29)	90.0%	40.6%	1.51	0.25	60.2%	80.2%
Length of Stay	0.74 (0.63-0.85)	Best	>26.38	54.4% (43)	72.7%	68.6%	2.31	0.40	69.8%	71.5%
		Max PPV	>109.0	16.5% (13)	27.3%	97.1%	9.5	0.75	90.5%	57.1%
		Max NPV	>13.78	74.5% (59)	89.3%	43.9%	1.59	0.24	61.4%	80.3%

[a]The percentage and number of patients who were above the Best and Max PPV cut-offs or below the Max NPV cut-off; [b]RRT- renal replacement therapy initiated within 10 days of surgery; [c]Death-defined as in-hospital mortality; [d]Length of stay- outcome defined as discharge >7 days from sample collection or death before post-operative day 7

occurred at the time of sample collection and the patient's Cleveland Clinic score, a perioperative risk score that predicts AKI severity after cardiac surgery.[8,9] The ability of uANCR to improve the prediction by the clinical model was determined in both the entire cohort and in the subset of patients classified as AKIN stage 1 at the time of urine collection. When uAnCR was added to the clinical model, we found that it predicted worsening of AKI independently of the percent change in sCr and the Cleveland Clinic score (p= 0.02). Category free net reclassification improvement (cfNRI) was used to capture the added benefit of including uAnCR in the model. cfNRI compares each patient's calculated risk for an outcome using a reference model to a new model (reference model plus uAnCR). Addition of uAnCR to the clinical model improved the ability to predict a patient's risk of experiencing worsening of AKI in both the entire cohort and the subset of patients who were classified as AKIN stage 1 (cfNRI= 0.457 and 0.428, respectively). To visualize the improvement in prediction, we constructed a risk assessment plot, as proposed by Pickering and Endre.[7] This plot compares the sensitivity and 1-specificity of the reference and new models across the spectrum of calculated risk for each model. Figure 3-6a and 3-6b show that, in both the entire cohort and in the subset, the addition of uAnCR into the model resulted in patients who met the outcome (events) having a greater calculated risk, and patients who did not meet the outcome (nonevents) had a lower calculated risk. Therefore, both sensitivity and specificity were improved by including uAnCR in the prediction model.

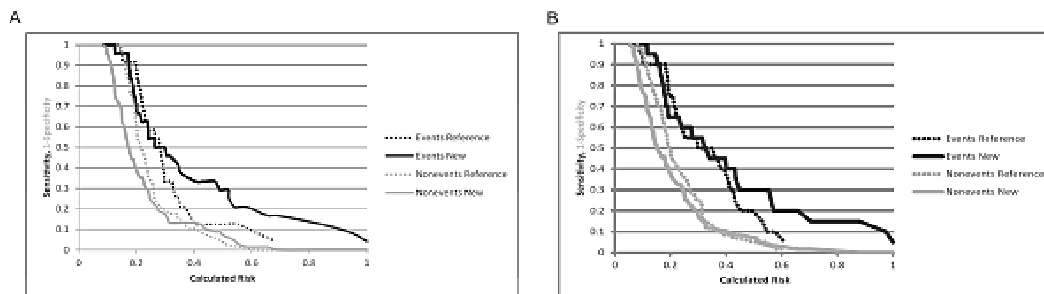

Figure 3-6. Risk assessment plots showing the improved prediction of worsening AKI when uAnCR is included in the model. Results from both (A) the entire cohort and (B) the subset of patients who were classified as AKIN stage 1 at the time of sample collection are shown. Of 97 patients in the whole cohort (A), 39 patients met the outcome worsening of AKI after sample collection, whereas in the subset analysis, 20 patients met the outcome. Two multivariate logistic regression models were created to predict risk of worsening of AKI after sample collection. The first model (reference) used percent change in sCr from baseline and Cleveland Clinic score created by Thakar *et al.* The second model included these variables plus uAnCR. Each patient's probability (*i.e.,* risk) of meeting the outcome worsening of AKI was calculated with both models. The sensitivity (proportion of events with a calculated risk equal to or above the defined threshold) and 1-specificity (proportion of nonevents with a calculated risk below the defined threshold) was calculated across all possible unique thresholds using both models. uAnCr, urine angiotensinogen/creatinine ratio

Discussion

The urinary angiotensinogen/creatinine ratio (uAnCR) was elevated in patients who developed more severe AKI. Elevated uAnCR was associated with worsening of AKI, independent of changes in sCr and Cleveland Clinic score, and it was also associated with several secondary outcomes. The prognostic predictive power of uAnCR was improved when only patients who were classified as AKIN stage 1 at the time of sample collection were used in the analysis, indicating that angiotensinogen could be used to predict adverse outcomes among patients who have not yet developed severe AKI as measured by serum creatinine. Our data suggest that angiotensinogen could be used at the time of AKI diagnosis to assess the risk of adverse outcomes. This risk assessment could lead to improved outcomes by identifying high risk patients in need of therapeutic intervention, as was highlighted in the KDIGO Clinical Practice Guideline for Acute Kidney Injury.[9] The guidelines suggest several interventions in patients with stage 2 and 3 AKI that are not recommended for patients with stage 1 AKI, including checking for drug dosing, considering renal replacement therapy, and considering ICU admission. Elevation in uAnCR could suggest a population of patients with stage 1 AKI who are likely to continue to worsen and could benefit from more intensive intervention. While we did not directly compare the prognostic predictive power of angiotensinogen to that of other biomarkers, our results compare favorably with what has been reported in the literature for previously described AKI biomarkers. In a cohort of critically ill patients with AKI, Hall *et al.* reported unadjusted AUCs of 0.71, 0.64, and 0.63 for the prediction of

the composite outcome of worsening of AKI or death for urine NGAL, KIM-1 and IL-18, respectively.[79] In patients who developed AKI after cardiac surgery, Koyner *et al.* recently reported unadjusted AUCs of 0.58, 0.63 and 0.74 for urine NGAL, urine IL-18, and plasma NGAL, respectively, for the outcome of worsening of AKI.[80] We found that uAnCR predicted this outcome with an AUC of 0.7 in a similar cohort of patients, and we confirmed that it predicted worsening of AKI in a subset of patients who were classified as AKIN stage 1 at collection (AUC = 0.71). Thus, uAnCR, alone or in combination with other biomarkers could improve risk classification models in these patients.

The results of the current study are limited by the heterogeneous etiologic nature of AKI (see the discussion on AKI etiology in Chapter 1), which was not taken into account in our study design. This study was performed in post-operative cardiac surgery patients because both the timing and the severity of renal injury are readily determined in these patients, making them an ideal population for AKI biomarker research. Additionally, the objective of this study was to confirm our findings from proteomic analysis (*i.e.* qualification of angiotensinogen). However, it is plausible that the prognostic significance of urinary angiotensinogen concentration as an AKI biomarker could vary with the underlying etiology. Therefore, an important next step is verification of angiotensinogen as an AKI biomarker, during which we will attempt to confirm its association with adverse outcomes in AKI secondary to causes other than cardiac surgery. This is addressed in Chapter 4, where a case-control study is described in which the

prognostic predictive power of urinary angiotensinogen was tested in a critically ill, non-surgical population.

Chapter 4: Qualification of Urinary Angiotensinogen as a Prognostic Biomarker of AKI Secondary to Non-Surgical Causes

Introduction

Discovery phase proteomic analysis identified urinary angiotensinogen as a promising AKI biomarker, and it was subsequently qualified in a retrospective cohort study designed to test its ability to predict adverse outcomes among post cardiac surgery patients with established AKI. This study confirmed that elevated urinary angiotensinogen is associated with more severe AKI and increased risk of adverse outcomes such as worsening of AKI, the need for renal replacement therapy, and increased length of hospital stay. However, as has been discussed in Chapter 1, AKI can be the result of a number of different precipitating factors, and it is likely that the performance characteristics of AKI biomarkers vary with the underlying etiology. For this reason, an important step in evaluating novel AKI biomarkers is verification of their predictive power in patients with AKI secondary to diverse etiologies.

The critically ill are the ideal population in which to test the effect of AKI etiology on biomarker performance, because of the heterogeneity of the causes of AKI and its associated comorbidities in these patients. Common causes of AKI in the ICU population include sepsis, cardiac disease, liver disease, prolonged and unresolved pre-renal factors such as hypovolemia and hemorrhage, cardiogenic shock, radiocontrast, and rhabdomyolysis.[14,15] The diversity of the underlying molecular mechanisms of these etiologies present a unique challenge to biomarker studies, and the heterogeneity of the ICU population tends to decrease the performance of AKI biomarkers compared to studies performed in cardiac surgery patients. However this population also offers unique insight

because biomarkers which are correlated with AKI severity in this population are more likely to be mechanistically involved in AKI pathobiology on a fundamental level.

Experimental Design

In order to verify angiotensinogen as an AKI biomarker, a retrospective case-control study was designed to evaluate the prognostic predictive power of urinary angiotensinogen in a population of critically ill, non-surgical patients with AKI. The composite outcome of the need for renal replacement therapy (RRT) or death was selected as the primary outcome since these are the most clinically relevant endpoints. Secondary outcomes that were also evaluated included: 1) worsening of AKI, which was defined as an additional increase in serum creatinine (sCr) >0.3 mg/dL from the sCr at the time of the urine sample collection 2) worsening of AKI or the initiation of RRT; and 3) increased length of hospital stay (LOS), which was defined as hospital discharge >7 days from the day of sample collection or death ≤7 days from sample collection. In comparison to the previous study on cardiac surgery patients, worsening of AKI was defined as an absolute increase in sCr after sample collection. This approach was chosen because it is notoriously difficult to determine the baseline sCr value in critically ill patients, which impedes accurate staging using the AKIN or RIFLE classification systems.

Materials and Methods

Patients and Urine Samples

All patients (n = 45) had been admitted to the intensive care unit at the Medical University of South Carolina Hospital. Patients either had AKI at ICU admission or developed AKI during their stay in the ICU. AKI was defined according to the AKIN criteria.[3] When possible, baseline sCr was defined as the most recent (within 1 month) value prior to the AKI episode. When antecedent sCr values were not available, the lowest sCr observed during the patient's hospital stay was used as the baseline. Informed consent was obtained from the patients or their next of kin prior to urine sample collection, in accordance with our Institutional Review Board approved protocol. The only exclusion criteria were initiation of renal replacement therapy prior to sample collection and non-consent.

Urine samples were collected in collaboration with the MUSC Biomedical Research Bank. Patients for this study were selected retrospectively in order to perform a case-control study of ICU patients diagnosed with AKI at to the time of urine sample collection. The primary outcome was the need for renal replacement therapy or death, and patients who had AKI at the time of sample collection but did not meet the primary outcome were selected as controls. Samples were collected at the time that the diagnosis of AKI was made. If patients had AKI on admission, samples were collected immediately after admission. Urine samples were processed according to a standard operating procedure. Tthey were treated with a protease inhibitor cocktail (Roche,

cOmplete, Mini, EDTA-free), centrifuged for 10 min at 1,000 *x* g and the supernatant was aspirated and stored at -80°C until the time of use. Clinical data was obtained by retrospective chart review.

AKI Etiology

Etiology of AKI was determined by retrospective chart review and patients were assigned to one of four categories: pre-renal, ischemic ATN, sepsis-associated AKI, and other. Pre-renal AKI was defined as an episode of AKI in the setting of hypotension or hypovolemia in which the patient's sCr decreased to <150% of baseline within 48 hours after diagnosis. Ischemic ATN was defined as severe, prolonged AKI following any event that compromises renal blood flow or oxygen delivery. The specific events observed in our cohort included ruptured abdominal aortic aneurysm, cardiogenic shock, and congestive heart failure exacerbation. Patients for whom the etiology could not be determined or was multifactorial were included in the "other" category.

Determination of Urinary Angiotensinogen-to-Creatinine Ratio

Urinary angiotensinogen was measured using the Human Total Angiotensinogen Assay Kit (Immuno-Biological Laboratories Co., Ltd., IBL-America, Minneapolis, MN), a solid phase sandwich ELISA, according to the manufacturer's protocol. Urine creatinine was measured using the Jaffe assay and used to correct the urine angiotensinogen concentration. Values were reported as the ratio of angiotensinogen in ng/ml to creatinine in mg/ml (uAnCR, ng/mg).

Statistical Analysis

The Kruskal-Wallis test and *post-hoc* Dunn's test were used to compare the

uAnCR values of patients grouped by AKI etiology. The Mann-Whitney U test was used when only two groups were compared. Other continuous variables were compared using the Student's *t-test* or Mann-Whitney U test. Categorical variables were compared using the χ^2 or Fisher's Exact tests. Logistic regression was used to determine the multiplicative odds ratio for a one standard deviation increase in uAnCR. However, because uAnCR was not normally distributed, it was first $\log_{10}$ transformed for this analysis. Receiver operator characteristic (ROC) curves were used to test the ability of uAnCR to predict outcomes. The area under the ROC curve (AUC) was used as an estimate of overall accuracy of the biomarker. ROC curves were considered statistically significant if the AUC differed from 0.5, as determined by the z-test. Optimal cut-offs were determined by selecting the data point that minimized the geometric distance from 100% sensitivity and 100% specificity on the ROC curve.[24] Additional cut-offs were determined by selecting the points on the ROC at which the positive and negative likelihood ratios were maximized and minimized, respectively. The Spearman's correlation coefficient was used to determine the correlation between uAnCR and length of hospital stay. Kaplan-Meier curves were used to visualize the relationship between uAnCR and length of hospital stay. Patients who died were censored. The log rank test was used to compare the curves. Cox regression was used to calculate the proportional hazard ratio for time to discharge comparing patients with high and low uAnCR (defined as > the median or ≤ the median of the cohort). The Cox proportional hazard model included both the patients' uAnCR and AKIN stage at collection.

Results

Patient Characteristics

Urine samples were obtained from patients with AKI in the intensive care unit (n=45). At the time of sample collection, five patients were classified as AKIN stage 3, 12 patients as AKIN stage 2, and 28 patients as AKIN stage 1. Baseline patient characteristics are described in detail in Table 4-1. In approximately one-third of patients, the etiology of AKI could not be determined or was multifactorial (n= 16). Sepsis-associated AKI was the most common established etiology (n=15), followed by pre-renal AKI (n= 8), and ischemic acute tubular necrosis (n=5). Twenty-three patients met the primary outcome, the need for renal replacement therapy (RRT) or death. Pre-renal AKI was significantly more common among the patients who did not meet this outcome ($p = 0.01$). There were no significant differences between the group of patients who required RRT or died compared to those who did not with respect to age, race, gender, the day of sample collection (defined as days after the date that AKI criteria were met), baseline serum creatinine (sCr), sCr at the time of sample collection, or the percent change in sCr from baseline at the time of sample collection. However, patients who met the primary outcome had lower rates of hypertension, diabetes mellitus, and the use of angiotensin converting enzyme inhibitors or angiotensin receptor blockers.

Angiotensinogen Predicts RRT or Death

Urinary angiotensinogen was elevated in the group of patients who met the primary outcome RRT or death (median uAnCR = 89.4 ng/mg, IQR 35.9 – 335.6

Table 4-1. Characteristics of ICU patients used to verify the prognostic predictive power of urinary angiotensinogen as an AKI biomarker

	No RRT and Survival	RRT or Death	*P*
n	22	23	
Age (yrs)[a]	62.9 ± 16.1	54.4 ± 17.6	0.1
Caucasian	64% (14)	65% (15)	0.84
Male	55% (12)	65% (15)	0.67
AKI Etiology			
Sepsis	23% (5)	43% (10)	0.25
Pre-renal	32% (7)	4% (1)	0.01
Ischemic ATN	9% (2)	13% (3)	1
Other	36% (8)	39% (9)	0.91
Serum Creatinine (mg/dL)			
Baseline sCr[b]	1.15 (0.8-1.6)	1.1 (1.0-1.5)	0.98
sCr at Collection[a]	2.1 ± 0.8	2.5 ± 0.8	0.06
% Change in sCr[b]	150% (130-189%)	200% (150-257%)	0.07
Other Variables			
MAP on day of collection[b]	74.9 (70.4-86.8)	68.6 (64.5-84.1)	0.08
History of HTN	91% (20)	48% (11)	0.005
History of Diabetes Mellitus	55% (12)	22% (5)	0.05
History of ACE Inhibitor or ARB Use	48% (12)	17% (4)	0.03

[a]Mean and SD; [b]Median and IQR; Categorical data are shown as percentage and n
P-values are shown for the χ^2 or Fisher Exact test, as appropriate.

ng/mg) compared to the group who did not (median uAnCR = 25.4 ng/mg IQR 5.8 – 120.4 ng/mg; Figure 4-1A). Elevated uAnCR was associated with an increased risk of meeting this outcome. The multiplicative odds ratio for a one standard deviation increase in a patient's uAnCR was 2.61 (95% CI 1.23 - 5.53). The receiver operator characteristic (ROC) curve for this outcome had an area under the curve (AUC) of 0.73 (Figure 4-1B; $p = 0.01$). The optimal cut-off was 34.76 ng/mg, at which the test had a sensitivity and specificity of 78.3% and 54.6%, respectively. The cut-off at which the test had the highest positive likelihood ratio ($LR^+ = 9.6$) was 230.0 ng/mg. Eleven of the 45 AKI patients had uAnCR values greater than 230.0 ng/mg, of which 10 met the outcome. At this cut-off, the sensitivity and specificity of the prediction of RRT or death were 43.5% and 95.5%, respectively. The positive predictive value of a value this high was 90.9%. Similarly, the lowest negative likelihood ratio of the test was achieved at a cut-off of 7.58 ng/mg ($LR^- = 0.14$). Eight patients had uAnCR values ≤7.58 ng/mg, of which 7 did not meet the outcome. The test had a sensitivity and specificity of 95.7% and 31.8%, respectively at this cut-off.

Worsening of AKI

Elevated uAnCR was associated with an increased risk of worsening AKI after sample collection (Figure 4-2). The ROC curve for this outcome had an AUC of 0.77. At the optimal cut-off, 34.76 ng/mg, the sensitivity and specificity were 87.0% and 63.6%, respectively. At the cut-off with the maximum LR^+, 230.0 ng/mg ($LR^+ = 4.31$), the sensitivity and specificity were 39.1% and 90.9%, respectively; at the cut-off with the lowest LR^-, 21.24 ng/mg ($LR^- = 0.07$), the

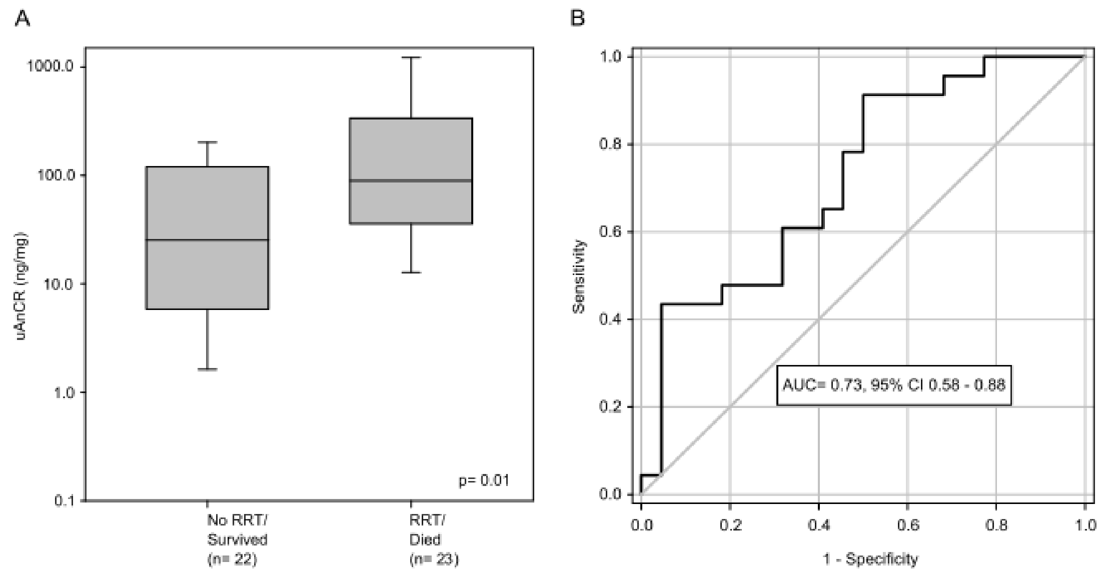

Figure 4-1. The urinary angiotensinogen-to-creatinine ratio in patients who met the outcome renal replacement therapy or death compared to patients who did not. (A) Box and whisker plots show the median and interquartile range. Error bars represent the 5th and 95th percentiles. Groups were compared with the Mann-Whitney *U* test. (B) Receiver operator characteristic curve was performed to evaluate the ability of uAnCR to predict the outcome renal replacement therapy (RRT) or death. A perfect biomarker would have an area under the ROC curver (AUC) of 1, whereas random chance has an AUC = 0.5

sensitivity and specificity were 95.7% and 59.1%. Eleven patients had uAnCR values above the cut-off of maximal LR^+, 10 of whom met the outcome worsening of AKI. Fourteen patients had uAnCR values below the threshold of minimal LR^- ,of whom only one met the outcome.

Length of Hospital Stay

Among patients who survived to discharge (n = 26), uAnCR was correlated with days to hospital discharge (r = 0.57, p = 0.002). Patients who had high uAnCR values (defined as >55.21 ng/mg, the median value) had an increased length of stay compared to patients who had low uAnCR (≤55.21 ng/mg). The median LOS (defined as days after the time of sample collection) for these groups were 22 and 7 days, respectively (Figure 4-3A; p = 0.01), and the AKIN stage adjusted hazard ratio for discharge was 0.367 (95% CI 0.17 – 0.91) for patients with high uAnCR compared to those with low uAnCR, indicating that uAnCR affects LOS independently of changes in sCr. Elevated uAnCR was strongly associated with an increased risk of the composite outcome discharge >7 days from the time of sample collection or death ≤7 days from collection. The multiplicative OR for one SD increase in uAnCR was 3.31 (95% CI 1.36 - 8.04). ROC curve analysis demonstrated that uAnCR was a strong predictor of this outcome (Figure 4-3B; AUC= 0.77). At the optimal cut-off, 59.61 ng/mg, the sensitivity and specificity of the prediction of prolonged hospital stay were 60.6% and 83.3%, respectively. The cut-off at which the test had the highest positive likelihood ratio (LR^+ = 5.5) was 123.5 ng/mg. Sixteen patients were above this cut-off, of which 15 met the outcome. At this cut-off, the sensitivity and specificity of the test were 43.5% and

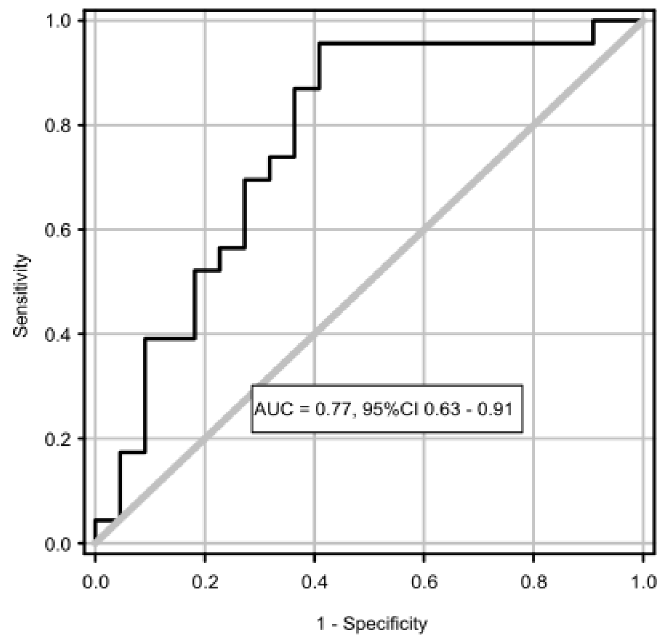

Figure 4-2. The urinary angiotensinogen-to-creatinine ratio as a predictor of the outcome worsening of AKI. ROC curve for the composite outcome worsening of AKI (defined as an increase in serum creatinine >0.3 mg/dL after the time of sample collection or RRT).

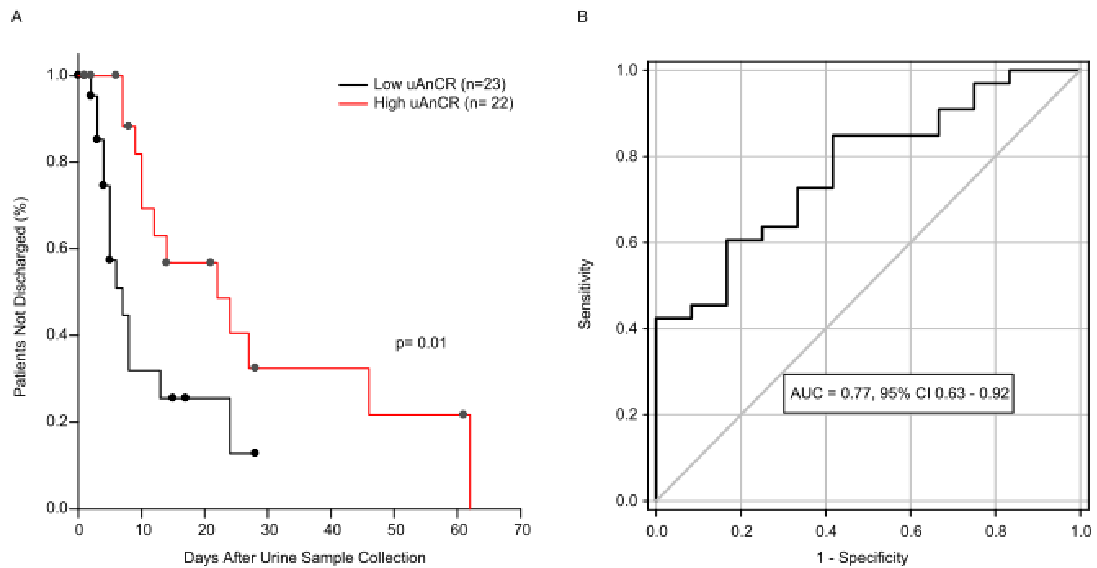

Figure 4-3. Urinary angiotensinogen-to-creatinine ratio and length of stay. (A) Patients were stratified into groups by urinary angiotensinogen-to-creatinine ratio (uAnCR). Patients with uAnCR > the median for the cohort were classified as high (red line), whereas patients with uAnCR ≤ the median were classified as low (black line). Patients who died were censored. The median times to discharge (defined as days after sample collection) were 22 and 7 days for the high and low uAnCR groups, respectively. (B) ROC curve analysis was performed to evaluate the ability of uAnCR to predict the composite outcome discharge >7 days after sample collection or death ≤7 days from sample collection.

96.5%, respectively. Similarly, the lowest negative likelihood ratio of the test was achieved at a cut-off of 3.31 ng/mg (LR^- = 0.12). Four patients had uAnCR values ≤ 3.31 ng/mg, of which three did not meet the outcome. The test had a sensitivity and specificity of 97.1% and 25.0%, respectively at this cut-off.

Urinary Angiotensinogen by AKI Etiology

The urinary angiotensinogen/creatinine ratio (uAnCR) differed statistically by the underlying etiology of AKI (Figure 4-4). Patients with AKI secondary to ischemic ATN had the highest median uAnCR (260.2 ng/mg, IQR 69.6 - 1213.2), followed by patients with AKI due to other or unknown causes, including multifactorial etiology (90.6 ng/mg, IQR 12.1 - 251.5), patients with sepsis-associated AKI (48.1 ng/mg, IQR 23.5 - 222.4), and patients with pre-renal AKI (11.3, IQR 5.2 - 61.5). *Post-hoc* pairwise comparison found a statistically significant difference between patients with ischemic ATN and patients with pre-renal AKI. Patients were categorized into the dichotomous groups of pre-renal AKI and AKI of other etiologies (Figure 4-5). The median uAnCR for patients with pre-renal AKI (n=8) was 11.3 ng/mg (IQR 5.2 - 61.5) while the median for patients with AKI not classified as pre-renal etiology (n=37) was 80.2 ng/mg (IQR 22.7 – 259.2). There was a statistically significant difference between the uAnCR values of this group compared to the group of patients with pre-renal AKI (p= 0.03).

Discussion

The prognostic predictive power of urinary angiotensinogen in the setting AKI after cardiac surgery was described in Chapter 3. However, it was unclear if its prognostic significance was generalizable to AKI secondary to causes other than

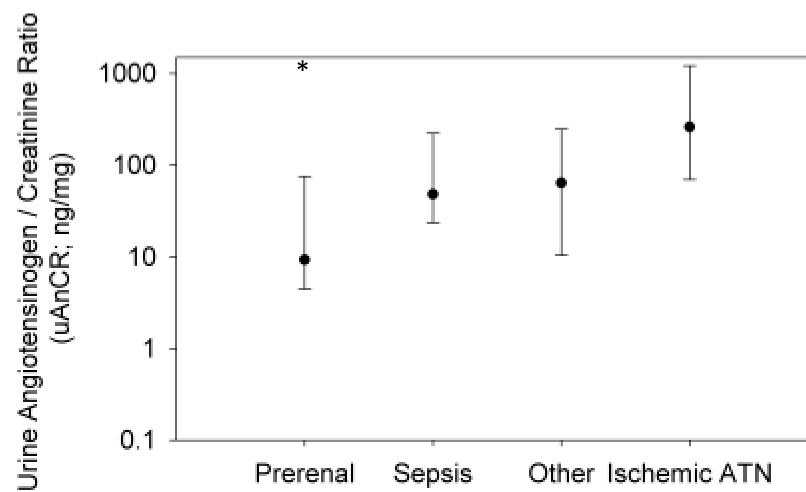

Figure 4-4. Urinary angiotensinogen/creatinine ratio (uAnCR) by AKI etiology. Patients who developed AKI in the ICU were grouped by the etiology underlying the AKI. The median (black dot) and interquartile range (error bars) are shown. *, $p < 0.05$ compared to ischemic ATN group

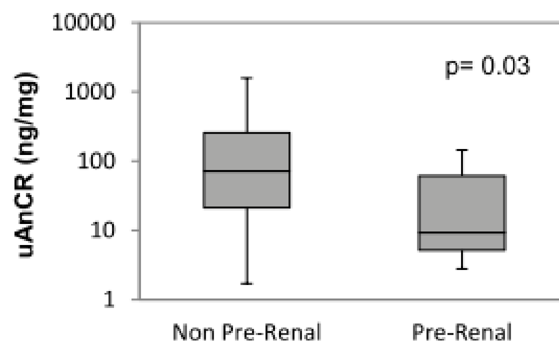

Figure 4-5. Urinary angiotensinogen-to-creatinine ratio (uAnCR) in pre-renal AKI compared to AKI of other etiologies. The box and whiskers plots show the median and interquartile range. Error bars represent the 5th and 95th percentiles. Groups were compared with the Mann-Whitney U test.

cardiac surgery. In the study described in this chapter, urinary angiotensinogen was measured in ICU patients who developed AKI secondary to diverse etiologies. We found that elevated urinary angiotensinogen was associated with an increased risk of RRT or death, longer time to hospital discharge, and worsening of AKI after the time of sample collection, and that angiotensinogen was a strong predictor of these outcomes using ROC curve analysis. A potential confounding factor of our results is that urinary angiotensinogen was statistically decreased in patients with pre-renal AKI compared to those with AKI of other etiologies, and the former was more common in the group of patients who did not die or require RRT. Therefore, it is not clear if the relationship between elevated urinary angiotensinogen and this outcome is in fact driven by its association with etiologies that tend to produce more severe AKI. Nevertheless, this would not negate the findings of this study because it is clinically important to distinguish between pre-renal AKI and AKI of other causes Pre-renal AKI typically is transient and resolves with fluid resuscitation, whereas other more severe forms of AKI do not. Pre-renal AKI is classically differentiated from AKI of other etiologies by FeNa $<1\%$ or FeUrea <35.[155-157] However, FeNa can be confounded by diuretic use and is altered in the setting of sepsis, whereas FeUrea decreases with age and a multicenter trial reported that it was not diagnostic of transient AKI.[158-160] Better biomarkers of pre-renal AKI are clearly needed. In a recent study, Cystatin C, NGAL, IL-18 and KIM-1 were found to be elevated in ICU patients with pre-renal AKI compared to those without AKI, but were lower than values for patients whose AKI did not resolve within 48 hrs.[92]

Finally, an important limitation of this verification study is that it was a relatively small retrospective biomarker qualification study, and our findings will need to be confirmed in a larger prospective study. Nevertheless, these data are encouraging and indicate that urinary angiotensinogen could have potential utility as an AKI biomarker in a variety of clinical settings.

Chapter 5: Concomitant Elevation of Urinary Angiotensinogen and Renin Predicts Severe AKI

Introduction

Epidemiologic studies have reported that the risk of adverse outcomes is proportional to the severity of acute kidney injury (AKI).[6, 138-140] Accurate identification of high risk patients with severe renal injury early in the disease could augment the efficacy of available interventions and improve patient outcomes. However, it is difficult to estimate the severity of AKI at an early time point, because AKI staging is based upon the magnitude of changes in serum creatinine and urine output, surrogates of glomerular filtration rate that do not change until after renal injury has occurred and reach their peak or lowest point later in the course of the disease.[2,3,43] The recent KDIGO clinical guideline for AKI highlighted the need for improved risk assessment for patients with established AKI.[9] Biomarkers of AKI could be used to evaluate the severity of AKI at an early time point in the disease as a guide for clinical decision-making. They could also play a role in clinical trial design because they could be used to selectively enrich the study population with patients who have severe renal injury and are more likely to benefit from an experimental therapy, increasing the statistical power of the study.[141,142] Many biomarkers have been proposed as early markers of AKI which may be useful for the detection of AKI prior to increases in serum creatinine. These include NGAL, KIM-1, IL-18, Cystatin-C, and L-FABP.[54, 60, 84, 104, 143, 144] While many studies have included an analysis of the the ability of these biomarkers to predict adverse outcomes, most have done so as a secondary analysis in cohorts designed to test early diagnostic capability. Due to the inclusion of large numbers of patients without AKI, results derived

from such analyses may not be generalizable to patients with established AKI. In support of this, the results of two recent studies that excluded patients without AKI have reported that NGAL, KIM-1 and IL-18 are substantially less accurate predictors of AKI progression and mortality than would be inferred from studies including patients without AKI, highlighting the need for other prognostic biomarkers.[79,80]

We recently identified urinary angiotensinogen as a novel prognostic biomarker of AKI.[161, 162] In the current retrospective cohort study we further evaluated the prognostic predictive power of angiotensinogen and its combination with renin and uromodulin. Renin was evaluated because we hypothesized that it would predict AKI severity because it cleaves angiotensinogen in the rate-limiting step of the renin-angiotensin-system (RAS). Since renin and angiotensinogen concentrations reflect different components within the renal RAS, combinations of the two candidate markers may improve prediction. Uromodulin was chosen on the basis of discovery-phase proteomic analysis, which showed that it decreased during AKI. This result was in agreement with recently published results of a pediatric AKI biomarker study which reported lower urinary concentration of uromodulin during AKI.[163] Uromodulin potentially plays a renoprotective, anti-inflammatory role during AKI, and we hypothesized that patients with more severe AKI would have lower concentrations of uromodulin, and that the combination of elevated urinary angiotensinogen and decreased uromodulin would be an accurate predictor of AKI severity.[164,164] Our findings show that the combination of angiotensinogen and renin is able to identify a

subset of patients with stage 1 AKI with a very high risk (80%) of progression to stage 3 AKI or death.

Materials and Methods

Patients and Urine Samples

Urine samples were obtained from 204 consecutively enrolled patients who had cardiac surgery at one of the SAKInet institutions between the dates of August 1, 2008 and June 1, 2012. This cohort included 74 samples included in our previously described study.[161] Informed consent was obtained in accordance with the IRB-approved protocol at each institution. Samples were collected and stored using a standard operating procedure which included centrifugation, addition of protease inhibitors and storage at -80°C. Urine samples were collected as early as possible after AKIN serum creatinine criteria were met, and all were collected within the first 72 hours after surgery. Inclusion criteria were surgery of the heart or ascending aorta and development of AKIN stage 1 AKI by creatinine criteria within 3 days of surgery. Subjects with ESRD, baseline serum creatinine > 3.0 mg/dL or AKI greater than AKIN stage 1 at the time of collection were excluded. Patients were followed until either time of death or hospital discharge, and were staged according to the maximum increase in serum creatinine using the AKIN classification system.[3] Urine output criteria were not used in diagnosis or staging because urine output data was not available.

Measurement of Biomarkers

Urine samples were thawed at 37°C and urinary angiotensinogen was measured using a sandwich ELISA (Immunobiologic Laboratories) according to the

manufacturer's protocol. Urinary renin was measured using a sandwich ELISA (R and D Systems) according to the manufacturer's protocol. Urine was diluted 1:2000 and uromoduin was measured by ELISA (BioVendor). All biomarker concentrations were corrected for urine creatinine (biomarker/creatinine ratio). Urine creatinine was measured using the Jaffe assay.

Statistical Analysis

Patients were grouped according to maximum AKIN stage and biomarker concentrations were compared using the Kruskal-Wallis test with *post-hoc* pairwise comparison. Univariate receiver operator characteristic (ROC) curve analysis was performed to determine if Cleveland Clinic score, percent increase in serum creatinine from baseline, the urinary angiotensinogen/creatinine ratio (uAnCR), and the urinary renin/creatinine ratio (uRenCR) predicted the composite outcome development of AKIN stage 3 or 30 day mortality (AKIN stage 3 or death). Variables were considered predictive if the area under the ROC curve differed statistically from 0.5. Cleveland Clinic score and percent increase in serum creatinine were combined in a multivariate logistic regression model (clinical model) to predict the outcome. Biomarker concentrations were $\log_{10}$ transformed and added individually to the clinical model and category free net reclassification improvement and integrated discrimination improvement were used to determine if the addition of biomarkers improved prognostic predictive performance.[153, 154] A classification tree was created using the four inputs (uAnCR, uRenCR, Cleveland Clinic score and percent change serum creatinine at collection) to determine optimal cut-offs to be used together in a clinical test to

identify patients at high risk of meeting the outcome AKIN stage 3 or death. The tree was grown using Chi-squared Automatic Interaction Detection (CHAID), specifying at least 10 cases per parent node and 5 cases per daughter node. Nodes were split using the Pearson's Chi-squared test if $P < 0.05$. Bonferroni correction was applied to the *P*-values to adjust for multiple comparisons. 25-fold cross-validation was performed. Statistical tests were performed in SPSS and SigmaPlot.

Results

Patients Characteristics

Urine samples were retrospectively analyzed from 204 cardiac surgery patients. Samples were obtained post-operatively at the time of diagnosis with AKI. All patients were classified as AKIN stage 1 AKI at the time of urine sample collection. Twenty-six patients progressed to AKIN stage 2, and 22 progressed to AKIN stage 3. Twenty-six patients met the primary outcome AKIN stage 3 or death. When patients were grouped by the primary outcome, there were no statistically significant differences in demographic variables, time of sample collection, or operative variables. However, compared to those who did not meet the outcome, patients who met the outcome had elevated pre-operative serum creatinine (median 1.1 versus 1.3 mg/dL; $P = 0.02$), serum creatinine (sCr) at collection (median 1.6 vs 1.9 mg/dL; $P < 0.001$), and percent increase in sCr from baseline that had occurred at the time of collection (median 41% versus 64%; $P = 0.003$). A description of the study population is found in Table 5-1.

Table 5-1. Characteristics of cohort of post-cardiac surgery patients enrolled in study

	AKIN Stage 1 or 2 and Survived	AKIN Stage 3 or Death	*P*
n	178	26	
Age[b]	68 (59.0 – 76.0)	65.5 (58.0 – 79.0)	0.97
Gender (female)[a]	32.6% (58)	38.5% (10)	0.71
Race (Caucasian)[a]	70.2% (125)	69.2% (18)	0.9
Operative Variables[b]			
CABG[a.c]	46.6% (83)	34.6% (9)	0.35
Valve Replacement[a]	28.7% (51)	26.9% (7)	0.96
CABG + Valve[a]	16.9% (30)	23.1% (6)	0.62
Other[a]	7.9% (14)	15.4% (4)	0.37
Bypass[a]	86.0% (153)	88.5% (23)	0.97
Bypass Time[d]	141.0 (83.0 – 192.0)	159.5 (62.0 – 203.0)	0.66
Collection Time (hr post-op)	21.8 (19.2 – 43.0)	21.6 (19.2 – 33.6)	0.69
Serum Creatinine (sCr mg/dL)			
Pre-Op sCr[d]	1.1 (0.9 – 1.3)	1.3 (1.0 – 1.8)	0.02
sCr at Collection[d]	1.6 (1.3 – 1.9)	1.9 (1.6 – 3.1)	<0.001
Percent Increase in sCr at Collection[d]	41% (30% – 56%)	64% (35% – 80%)	0.003
Outcomes			
Days to Max sCr[d,e]	2.0 (1.0 – 3.0)	5.0 (3.75 – 8.0)	<0.001
Days to Discharge or Death[d,e]	7.0 (6.0 -10.0)	14.0 (9.75 – 24.75)	<0.001
AKIN Stage 3[a]	0% (0)	84.6% (22)	<0.001
AKIN Stage 3 or Death [a]	0% (0)	100% (26)	<0.001
Death[a]	0% (0)	34.6% (9)	<0.001
RRT[a]	0% (0)	50.0% (13)	<0.001

Statistical significance was determined by the χ^2 test for categorical variables and the Mann-Whitney U test for continuous variables.

[a]Categorical variables are reported as percentage (n).

[b]Type of surgery is reported as CABG only, Valve replacement only, CABG + Valve replacement, and other procedures.

[c]CABG, coronary artery bypass graft

[d]Continuous variables are reported as median (interquartile range).

[e]Days are reported as the number of days after surgery.

Biomarker Concentrations by AKIN Stage and According to Primary Outcome

The urinary angiotensinogen/creatinine ratio (uAnCR) was correlated with both maximum serum creatinine (sCr) and the maximum percent increase in sCr (rho = 0.383 and 0.256, respectively; $P < 0.001$). Similarly, the urinary renin/creatinine ratio (uRenCR) was correlated with maximum sCr (rho = 0.392; $P < 0.001$) and the maximum percent increase in sCr (rho = 0.308; $P < 0.001$). However, urinary uromodulin/creatinine ratio was not correlated with either maximum sCr or the maximum percent increase in sCr. There was also a statistically significant correlation between uAnCR and uRenCR (rho = 0.341; $P < 0.001$). Accordingly, there was a trend for both urinary angiotensinogen and urinary renin to increase with the maximum AKIN stage that a patient achieved (Table 5-2), and there was a statistically significant difference in uAnCR and uRenCR between patients who developed AKIN stage 3 AKI compared to those who only developed AKIN stage 1 AKI. Uromodulin concentrations did not change with maximum AKIN stage, and so uromodulin was not investigated further. When patients were grouped according to the primary outcome, development of AKIN stage 3 or death, those who met the outcome had higher uAnCR compared to those who did not (median and IQR of 30.84 and 10.75 to 89.95 compared to 96.7 and 38.23 to 457.34 ng/mg; $P < 0.001$). Patients who met the primary outcome also had higher uRenCR than those who did not (median and IQR of 280.72 and 118.98 to 638.96 compared to 894.71 and 335.43 to 2894.06 pg/mg; $P < 0.001$).

Table 5-2. Distribution of urinary biomarker concentrations by maximum AKIN stage

	AKIN Stage 1	AKIN Stage 2	AKIN Stage 3/death	P^a
n	156	26	22	
uAnCR (ng/mg)	29.22 (10.72 – 82.42)	36.39 (14.56 – 163.54)	96.7[#] (38.23 – 457.34)	0.002
uUroCR (mg/mg)	4.23 (2.5 – 6.14)	4.01 (2.61 – 7.83)	5.09 (3.21 – 9.52)	0.33
uRenCR (pg/mg)	257.28 (113.88 – 564.34)	406.79 (144.06 – 922.47)	894.71[#] (335.43 – 2894.06)	0.001

Biomarker concentrations are reported as median (interquartile range)

[a]p-value according to Kruskal-Wallis test

[#], p< 0.05 in *post-hoc* pairwise comparison

Univariate Prediction of AKIN Stage 3 or Death

Selected clinical variables and putative prognostic biomarkers were tested for the ability to predict the outcome AKIN stage 3 or death. Cleveland Clinic score (Figure 5-1a) and the percent change in sCr at the time of sample collection (Figure 5-1b) both predicted the outcome, having an area under the receiver operator characteristic curve (AUC) of 0.72 (95% CI [0.62, 0.83]) and 0.68 (95% CI [0.55, 0.82]), respectively. In comparison, angiotensinogen and renin were also moderately strong predictors (Figures 5-1c and 5-1d). The AUC of the urinary angiotensinogen/creatinine ratio (uAnCR) was 0.75 (95% CI [0.65, 0.85]). The AUC of the urinary renin/creatinine ratio (uRenCR) was 0.70 (95% CI [0.57, 0.83]). Additionally, the prognostic predictive power of these variables was evaluated in the subset of the cohort (n = 81; data not shown) that were classified as RIFLE-R at the time of collection, since it has been reported that this criterion has a lower false positive rate than AKIN stage 1 for diagnosis of AKI. Compared to the entire cohort this analysis found little difference in the ability of uAnCR, uRenCR, and Cleveland Clinic score to predict AKIN stage 3 or death, whereas the predictive power of the percent increase in serum creatinine was substantially improved.

Multivariate Prediction of AKIN Stage 3 or Death

A model including relevant clinical variables and biomarkers was created to predict the outcome AKIN stage 3 AKI or death. First, Cleveland Clinic score and percent change in serum creatinine were combined into a multivariable logistic

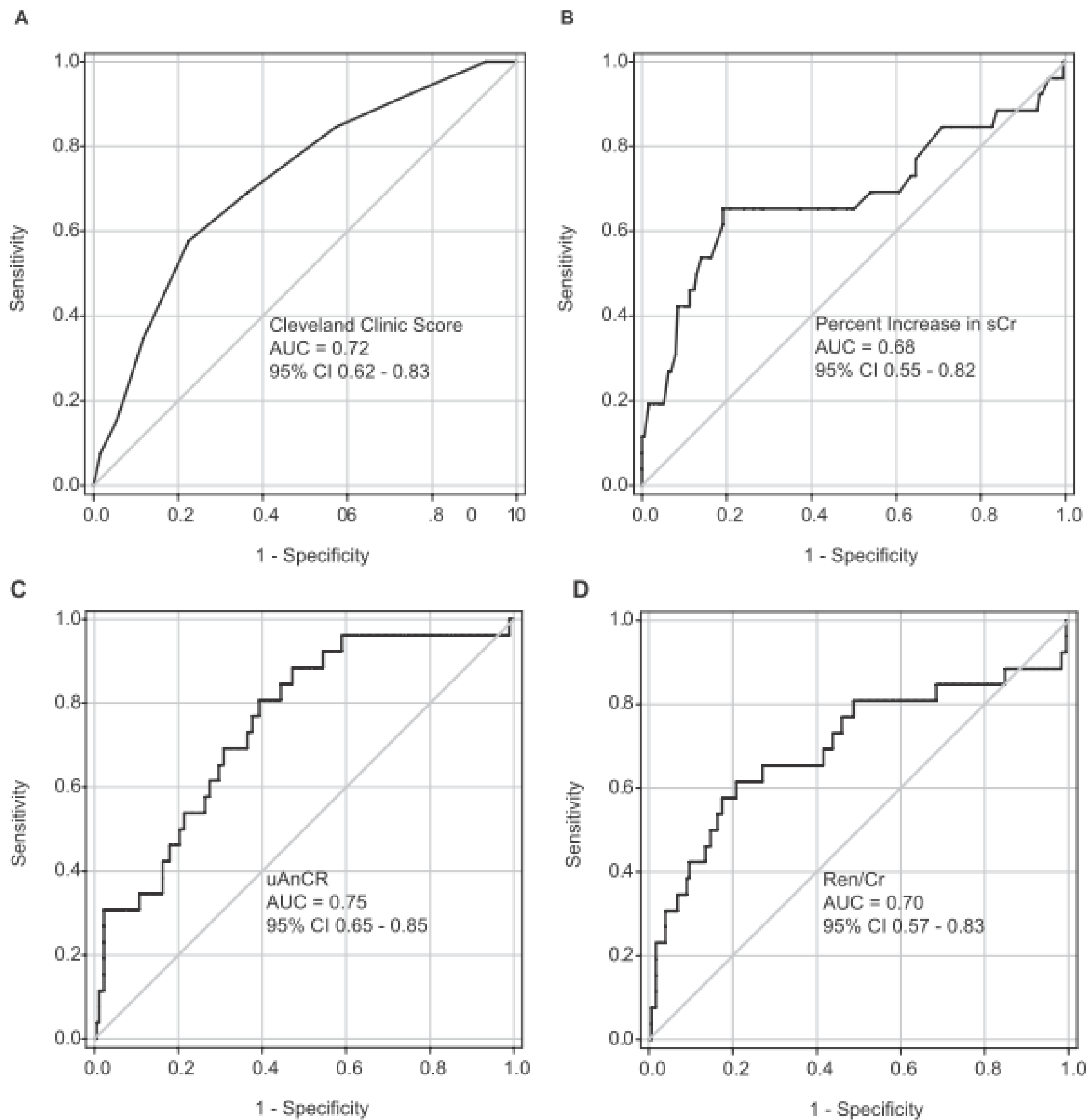

Figure 5-1. Univariate receiver operator characteristic (ROC) curves for the outcome AKIN stage 3 or death. Clinical variables Cleveland Clinic score (A) and percent increase in serum creatinine (sCr) (B) at the time of sample collection, as well as the biomarkers urinary angiotensinogen to creatinine ratio (C) and urinary renin to creatinine ratio (D) were tested for the ability to predict the outcome. The diagonal gray line shows the line of identity for between the true positive (sensitivity) and false positive (1-specificity) rates of the test, and has an area under the ROC curve (AUC) of 0.5. Variables were considered predictive if the AUC was >0.5 and the 95% confidence interval (CI) did not overlap 0.5.

regression model. This clinical model had an AUC = 0.79 (95% CI [0.69, 0.88]) for the outcome (Figure 5-2). Then uAnCR and uRenCR were added stepwise to the clinical model to determine the incremental increase in the accuracy model gained by the addition of each biomarker (Table 5-3). When uAnCR was added to the clinical model, the AUC improved to 0.85 (95% CI [0.78, 0.92]; $P = 0.01$ compared to clinical model; Figure 5-2). This was the result of augmented sensitivity and specificity (Figure 5-3a and 5-3b). The category free net reclassification improvement (cfNRI) for addition of uAnCR was 0.67 (95% CI [0.26, 1.09]; $P = 0.001$; Figure 5-4a and 5-4b), and the integrated discrimination improvement (IDI) was 0.06 ($P = 0.09$). Although the IDI did not reach statistical significance, the median calculated risk of the event group increased significantly following addition ofuAnCR to the model from 0.22 to 0.26 ($P = 0.04$). In contrast to uAnCR, the addition of uRenCR to the clinical model did not improve the area under the ROC curve (AUC = 0.79 95% CI [0.69, 0.89]; Table 5-3). Although the IDI (0.02; $P = 0.26$) was not statistically significant, there was an improvement in risk reclassification (cfNRI 0.52 95% CI [0.10, 0.93]; $P = 0.01$; Table 5-3; Figure 5-4c and 5-4d). Addition of uRenCR to a three variable model that included Cleveland Clinic Score, percent increase in sCr, and uAnCR did improve the accuracy of the model (cfNRI = 0.55 95% CI [0.14, 0.96], $P < 0.01$), although the IDI was 0.01 ($P = 0.38$) and there was no improvement in the AUC of the ROC curve (AUC = 0.85 95% CI [0.77, 0.92]; Table 5-3; Figure 5-4e and 5-4f). This marginal increase in the in the discriminative slope resulted in more events having a calculated risk between 0.2 and 0.55, as can be observed in the

Table 5-3. Incremental Improvement in Prognostic Predictive Power by Addition of Angiotensinogen and Renin to a Clinical Model

Reference Model	New Model	$cfNRI_{events}$[b]	$cfNRI_{nonevents}$[b]	cfNRI[b]	*P*	IDI[c]	*P*
Clinical Model[a]	Clinical Model[a] + uAnCR	0.28	0.39	0.67 [0.26, 1.09]	0.001	0.06	0.09
Clinical Model[a]	Clinical Model[a] + uRenCR	0.31	0.21	0.52 [0.10, 0.92]	0.01	0.02	0.26
Clinical Model[a] +uAnCR	Clinical Model[a] +uAnCR +uRenCR	0.44	0.11	0.55 [0.14, 0.96]	<0.01	0.01	0.38

[a]Clinical model is a multivariate logistic regression model including the Cleveland Clinic score and the percent increase in serum creatinine from baseline at the time of sample collection.

[b]cfNRI is a means of calculating the effect of adding a new variable to a predictive model on the overall accuracy of the model. cfNRI is the sum of $cfNRI_{events}$ and $cfNRI_{nonevents}$. $cfNRI_{events}$ and $cfNRI_{nonevents}$ are the proportion of patients who met the outcome (events) or those who did not, respectively, which are correctly reclassified by the new model minus the proportion of patients who are incorrectly reclassified. Correct reclassification is defined as a calculated risk of meeting the outcome that is higher for events and lower for nonevents when compared to the reference model. If all events and nonevents were correctly reclassified, the $cfNR_{Ievents}$ and $cfNRI_{nonevents}$ would be +1, and the cfNRI would be 2.

[c]IDI is a means of quantifying the effect of addition of a new variable to a predictive model on the magnitude of the change in the difference between the average calculated risk of patients who met the outcome compared to those who did not. The mean risk of the two groups is calculated using the reference model and the new model, and IDI is simply the difference between the discrimination slopes of the two models.

uAnCR, urinary angiotensinogen/creatinine ratio; uRenCR, urinary renin/creatinine ratio; cfNRI, category free net reclassification improvement; IDI, integrated discrimination improvement

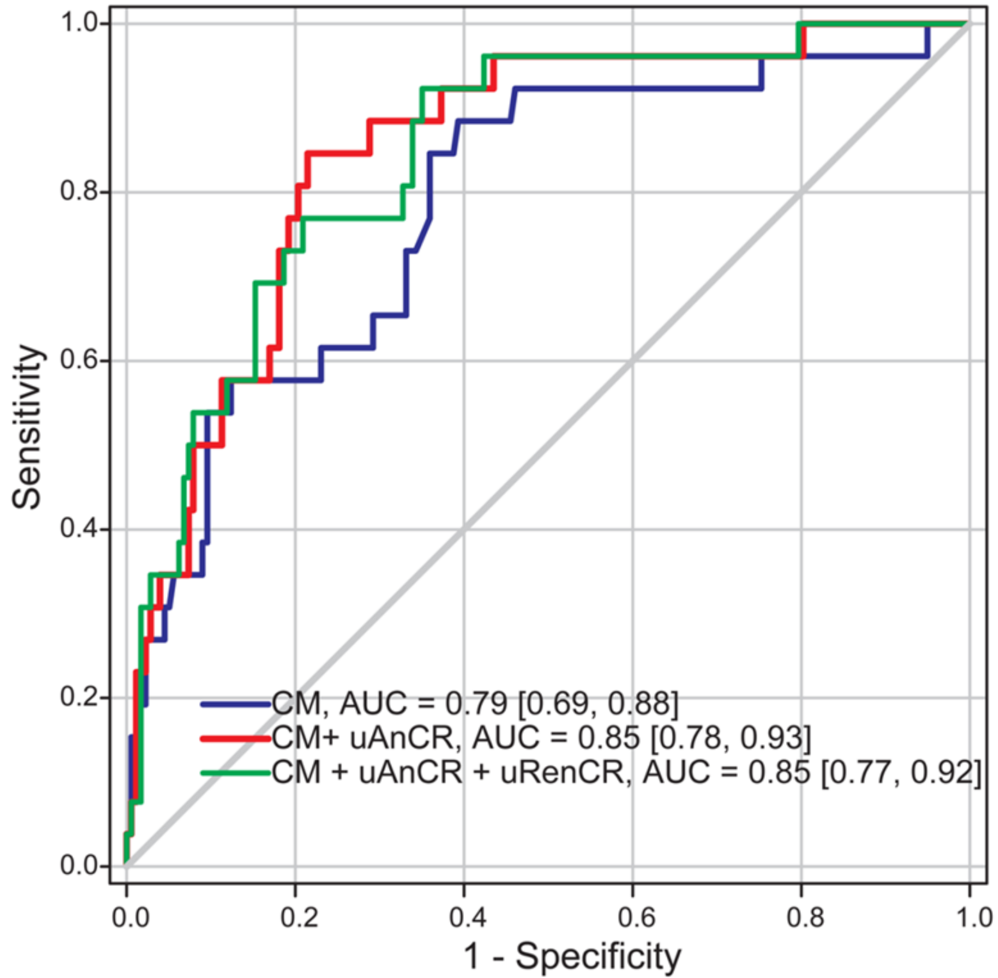

Figure 5-2. Multivariable Prediction Models for AKIN Stage 3 or Death. Receiver operator characteristic (ROC) curves are shown for the Clinical Model (CM; includes Cleveland Clinic Score and percent increase in serum creatinine from baseline), the clinical model plus creatinine-corrected urine angiotensinogen (CM + uAnCR), and the clinical model plus creatinine-corrected urinary angiotensinogen and renin (CM +uAnCR +uRenCR).

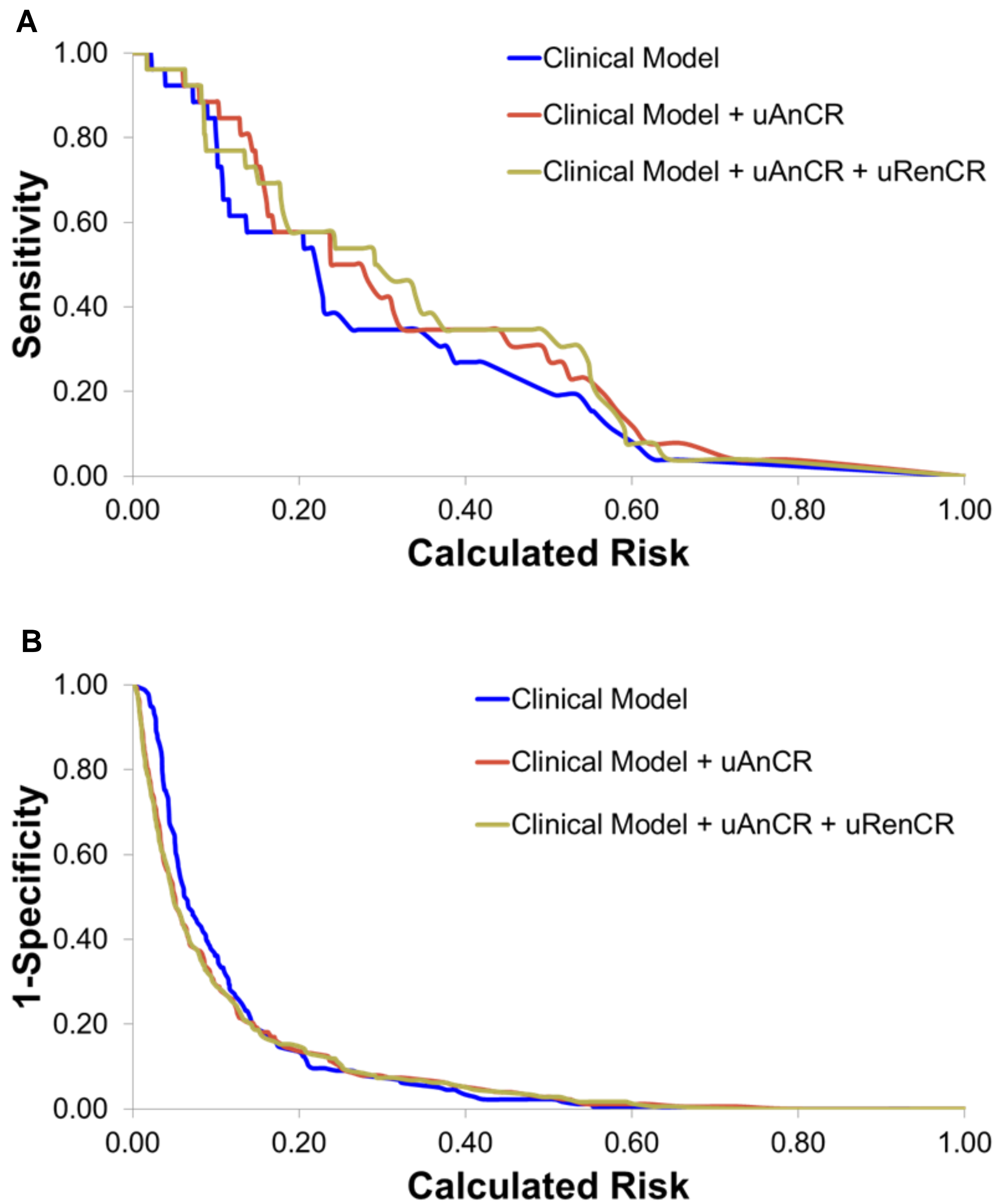

Figure 5-3. Risk assessment plots showing the incremental changes in sensitivity and specificity resulting from stepwise addition of angiotensinogen and renin to the clinical model. Addition of angiotensinogen to the clinical model improved sensitivity and specificity. Subsequent addition of renin to the model resulted in a gain in sensitivity with no effect on specificity.

increased sensitivity of the model in this range (Figure 5-3a). However, there was no improvement in specificity (Figure 5-3b). Finally, the best cut-off for the ROC curve of the final model had a calculated sensitivity of 76.9% and a specificity of 79.1% (PPV and NPV of 35.0% and 76.5%, respectively).

Classification Tree

Multivariate logistic regression is a powerful technique for evaluating the predictive power of biomarkers, but due to its complexity it is unlikely to be clinically useful in the setting of acute illness where the decision to intervene is especially time sensitive. Additional limitations include the inability to identify subsets of patients in whom biomarkers under or over perform and insensitivity to potentially important nonlinear interactions between covariates in the model, both of which could be informative from a mechanistic perspective. Therefore, we sought to create a simple algorithm that identifies patients at high risk of meeting the outcome AKIN stage 3 or death, which could be used to guide decision making. To accomplish this we chose to use Chi-squared Automatic Interaction Detection (CHAID) to grow a classification tree that assigned patients to risk groups by identifying interactions among the same variables that were previously used in multivariate logistic regression. In this analysis uAnCR, uRenCR, Cleveland Clinic score and percent change in serum creatinine were all statistically significant classifiers for the outcome (Figure 5-5). However, the model selected only urinary angiotensinogen and renin for use in prediction of the outcome. Using a cut-off of > 337.89 ng/mg for uAnCR, the model divided patients into low (n = 184) and intermediate (n = 20) risk groups, in which 9.8

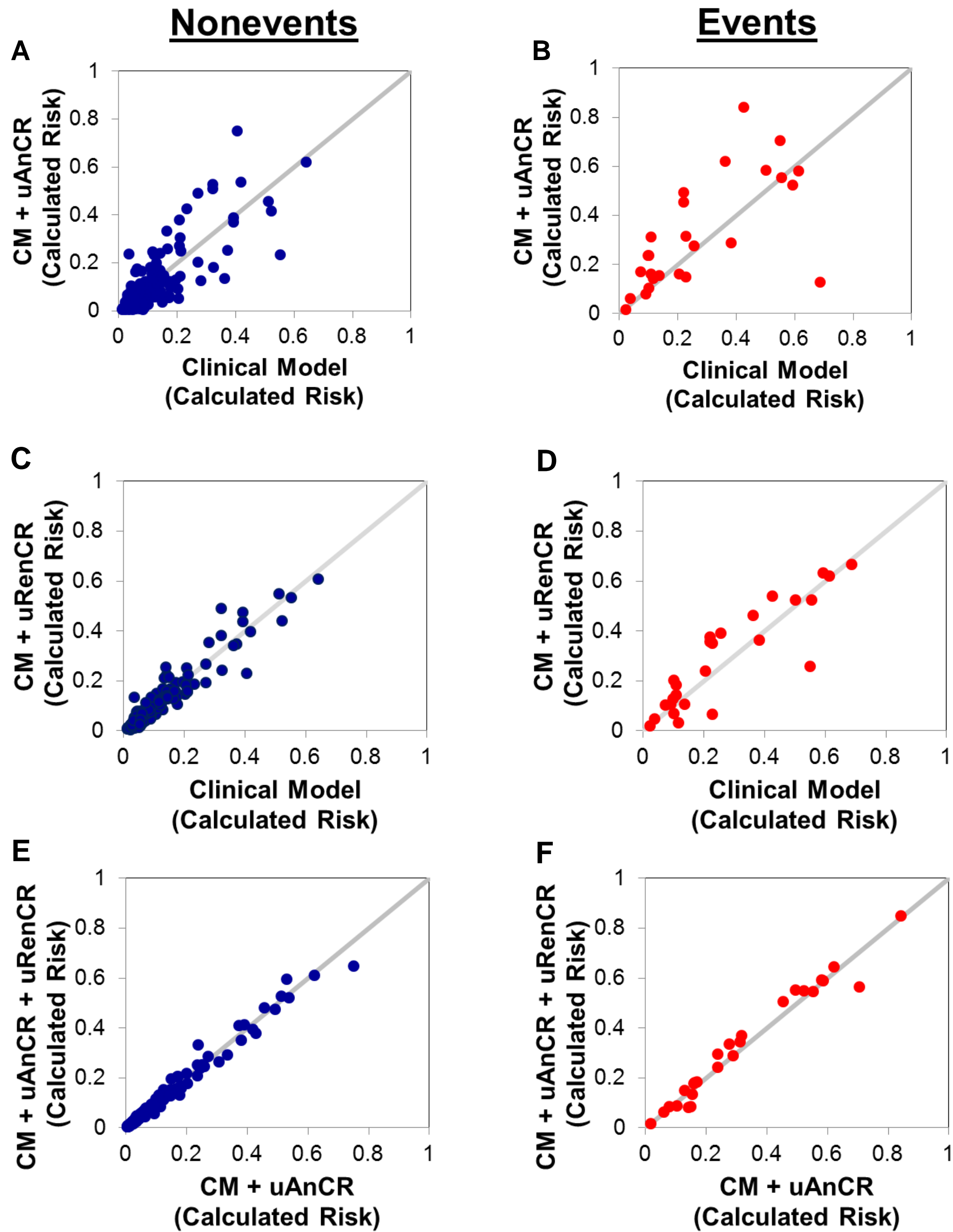

Figure 5-4. Scatterplots showing the calculated risk of meeting the outcome AKIN Stage 3 or death. The incremental changes in predictive accuracy gained by addition of angiotensinogen (A and B) and renin (C-F) was evaluated using category free net reclassification improvement (cfNRI) and integrated discrimination improvement (IDI). These scatterplots are a visual representation of cfNRI and IDI. Each data point represents the calculated risks of a single patient using a reference model (x-axis) and a model with an added biomarker (y-axis). The gray diagonal line is the line of

Figure 5-4. continued identity,indicating calculated risk that does not change with addition of a biomarker to the reference model. The proportion of nonevents (patients who did not meet the outcome) below the line and the proportion of events (patients who met the outcome) is used to calculate the category free net reclassification improvement (cfNRI). The magnitude of the changes in calculated risk, which is the vertical distance of a point from the line of identity, is included by the integrated discrimination improvement (IDI).

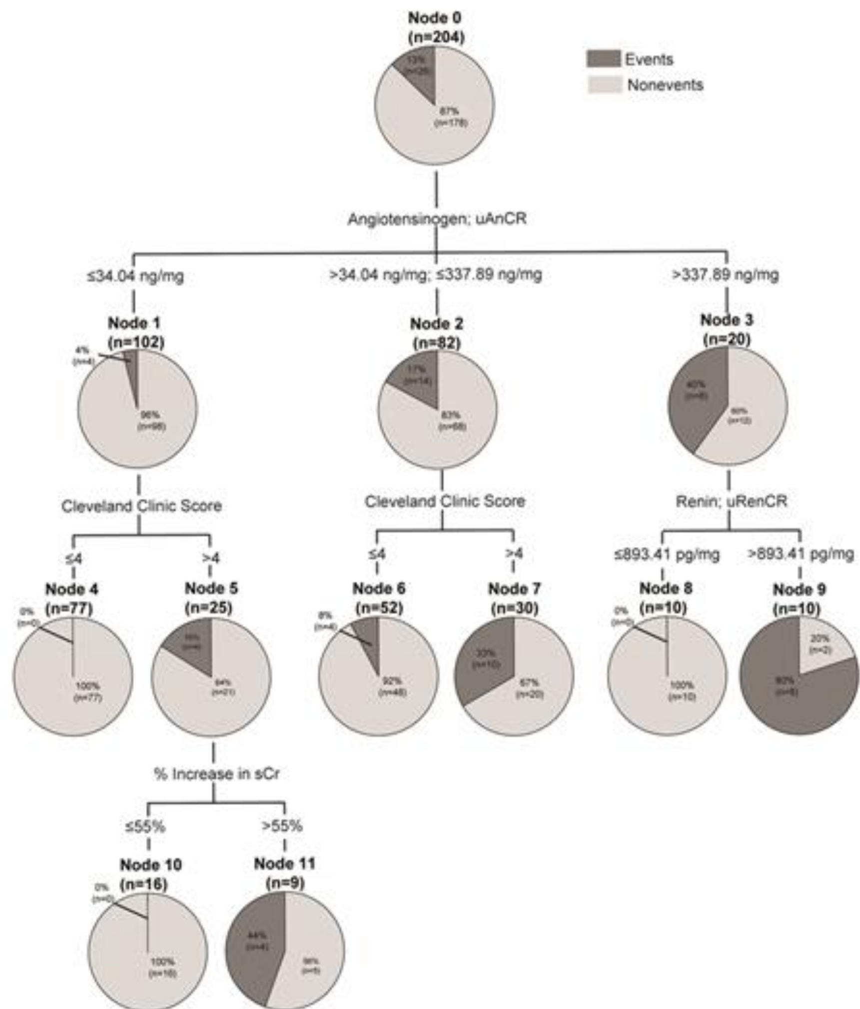

Figure 5-5. CHAID classification tree for the outcome AKIN stage 3 AKI or death. Chi-squared automatic interaction detection (CHAID) was used to grow the classification tree using the following variables: Cleveland Clinic score, percent increase in serum creatinine from baseline at the time of sample collection, urinary angiotensinogen (ng/mg creatinine; uAnCR), and urinary renin (pg/mg creatinine; uRenCR). Pie charts represent the proportion of patients who met the outcome (events) or not (nonevents) at each node of the tree. The model only used angiotensinogen and renin to predict the outcome.

% and 40.0% of the patients met the outcome, respectively. The intermediate risk group was then further subdivided into a low and high risk group using the cut-off of uRenCR >893.41 pg/mg. Applying the combination of these two cut-offs for uAnCR and uRenCR resulted in a group of 10 patients, 8 of whom met the outcome, yielding a sensitivity of 30.8% and specificity of 98.9%. The PPV and NPV of this model were 80.4% and 90.7%, respectively. Overall, the model correctly classified 90.2% of patients correctly, and the 25-fold cross-validation estimate of the risk of misclassification of events was 0.132 (SE = 0.024). ROC curve analysis found that CHAID model had an AUC of 0.91, and compared favorably with the multivariate logistic regression model (Table 5-4; Figure 5-5).

Discussion

In this retrospective cohort study, we measured the candidate AKI biomarkers angiotensinogen, uromodulin, and renin in spot urine samples that had been obtained from post cardiac surgery patients early after AKI diagnosis had been made on the basis of AKIN serum creatinine criteria. Of these patients, only 81 had an increase in sCr >50% and none had an increase ≥100% at the time of collection. Both urinary angiotensinogen and renin predicted the composite outcome AKIN stage 3 or death, whereas uromodulin did not. While our study is limited by the use of a composite outcome that relatively few (26 of 204) patients met, it was chosen because our objective was to identify patients at high risk of severe adverse outcomes. Selection bias cannot be entirely ruled out because of the retrospective design of our study. However, we used multivariate analysis to

Table 5-4. Comparison of Multivariate Logistic Regression Model and CHAID Classification Tree

Model	AUC[a]	Sensitivity	Specificity	PPV	NPV
Multivariate Logistic Regression[b]	0.85 [0.77, 0.92]	76.9%	79.1%	35.0%	76.5%
CHAID[c]	0.91 [0.87, 0.96]*	30.8%	98.9%	80.4%	90.7%

[a]AUC, area under the receiver operator characteristic curve; 95% CI estimates are shown in brackets

[b]Cut-off specific performance characteristics shown are from the point on the ROC curve closest to the point of 100% sensitivity and specificity.

[c]CHAID, Chi-squared automatic interaction detection; cut-off specific performance characteristics shown are for the node representing uAnCR >337.89 ng/mg and uRenCR >893.41 pg/mg.

*$P = 0.02$ compared to multivariate logistic regression model.

Multivariate logistic regression and CHAID models were generated using the following variables: Cleveland Clinic score, percent increase in serum creatinine at the time of sample collection, urinary angiotensinogen/creatinine ratio (uAnCR), and urinary renin/creatinine ratio (uRenCR).

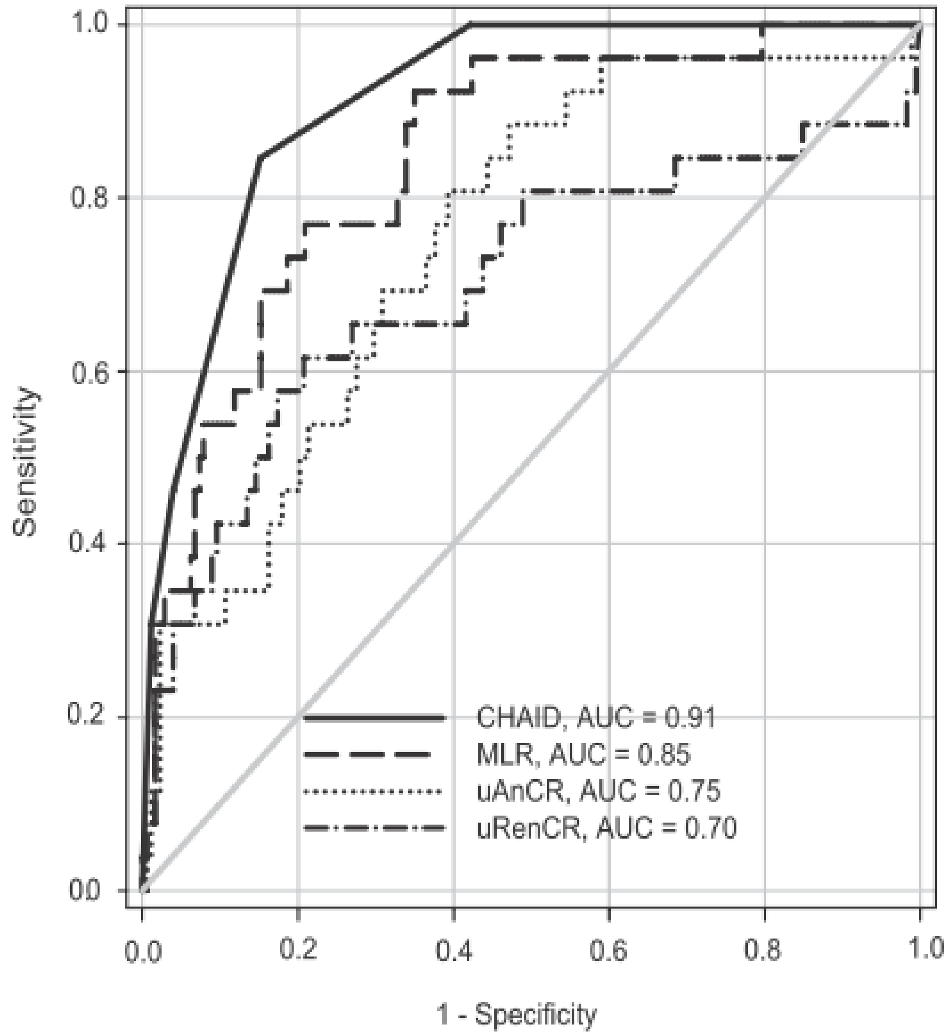

Figure 5-5. Multivariate receiver operator characteristic (ROC) curves for prediction of AKIN stage 3 AKI or death. The ROC curves of two multivariate models, a CHAID classification tree (CHAID) and a multivariable logistic regression model (MLR) are shown. Both models included four variables: Cleveland Clinic score, percent increase in serum creatinine from baseline at that time of sample collection, urinary angiotensinogen (ng/mg creatinine; uAnCR), and urinary renin (pg/mg creatinine; uRenCR). Additionally, the ROC curves for angiotensinogen (uAnCR) and renin (uRenCR) are shown for comparison, although they were previously reported in Figures 1c and 1d). The CHAID classification tree model was the most accurate predictor (*P* =0.02 compared to the multivariable logistic regression model).

adjust for known confounders (Cleveland Clinic score and percent increase in serum creatinine at collection). We demonstrated that urinary angiotensinogen improved the predictive power of a clinical model that included these variables by using the net reclassification improvement and integrated discrimination improvement. These data confirm our previous findings regarding the prognostic predictive power of urinary angiotensinogen, although it is not a true validation study because approximately one-third of the patients used in this cohort (74 patients, 10 of whom met the outcome) were included in our previous study (Chapter 3).[161] We also found that elevated urinary renin is associated with severe AKI, and further addition of renin to the multivariate model appeared to improve sensitivity, indicating that the interaction between urinary angiotensinogen and renin concentrations is an important prognostic indicator. We believe that these findings are strongly suggestive of a role for the renin-angiotensin system (RAS) in the pathobiology of AKI. This notion is in agreement with data from animal models, and will be addressed in Chapter 7.

Chi-squared automatic interaction detection was used to grow a classification tree to identify risk subgroups. Others have reported CHAID to be less accurate than multivariate logistic regression in ROC curve analysis.[166] Nevertheless, we chose to use CHAID because of its relative simplicity and the ease of graphic representation of the results. Additionally, CHAID offers some analytical advantages compared to logistic regression. Whereas logistic regression identifies independent predictors, CHAID is adept at identifying interactions among the variables, including non-linear relationships, and it can be used to

identify subgroups in which covariates exert the greatest influence in a predictive model.[166-168] Thus, CHAID can be used to generate or test hypotheses regarding the potential role of the relationship between variables and the outcome of interest, which is a particularly intriguing feature for testing combinations of biomarkers.[167] The CHAID classification tree identified patients with concomitant elevation of both angiotensinogen and renin as the group with the highest risk for the outcome AKIN stage 3 or death, and found that clinical variables drove the predictive model when biomarker concentrations were low. We interpret these results as an indication that activation of the renin-angiotensin system could modulate AKI severity. Importantly, this analysis found that angiotensinogen was a stronger predictor than renin, and renin was not a useful predictor when angiotensinogen was below 337.89 ng/mg. This is congruent with our hypothesis that renin improves the predictive accuracy of angiotensinogen based on the biological relationship between the two proteins, as angiotensinogen is the only known natural substrate for renin.[169] Therefore, the CHAID model is also an informative guide for the potential use of the combination of these biomarkers in the event of discordance between the two.

Urinary angiotensinogen and renin could be a useful AKI biomarker combination and could be used to guide clinical trial enrollment. The PPV of the combination of uAnCR > 337.89 ng/mg and uRenCR >893.41 pg/mg was 80.4%, a 6.3-fold enrichment for the endpoint of stage 3 AKI or death (compared to 12.8% incidence). This would increase the effect size of an intervention and result in an improved statistical power and a reduction in the number needed to enroll.

However, the trade-off is that a large number of patients would need to be screened. Using our cohort as an example, the ratio of enrolled to screened patients would be approximately 1:19 if the results from the CHAID model were used as inclusion criteria enrollment, since only 4.9% of patients had uAnCR and uRenCR values above these cut-offs. Therefore, the cost of screening would need to be weighed against the potential benefits of enrichment (increased power and reduced enrollment), taking into account the assumed effect size of the intervention. A more complete description of how biomarkers can be used to improve clinical trial design is presented in Chapter 7.

Chapter 6: Verification of Candidate Biomarkers by Targeted Mass Spectrometry

Introduction

Acute kidney injury (AKI) is a common and serious disease that is associated with a number of adverse outcomes. Conventional biomarkers (*i.e.* creatinine, blood urea nitrogen, and urine output) do not reliably predict the course of the disease from an early time point after renal injury, and are of limited prognostic utility. AKI biomarkers that predict adverse outcomes are needed to guide clinical decision making.[19, 47] Novel biomarkers of AKI such as neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), and liver-type fatty acid binding protein (L-FABP) have been proposed.[54, 60, 84, 104, 143, 144] The TRIBE-AKI Consortium recently conducted a large, multicenter prospective trial, which reported disappointing results regarding the early predictive power of these biomarkers, highlighting the need for new discoveries in this important area of AKI research.[63, 76, 90]

As described in the previous chapters, we have identified urinary angiotensinogen as a novel prognostic biomarker of AKI. We have verified its prognostic predictive power in cardiac surgery patients and in the nonsurgical critically ill population. However, in these studies, angiotensinogen was quantified using a sandwich ELISA, which can be negatively affected by the urine matrix, impeding accurate quantification. Additionally, these studies did not evaluate other novel AKI biomarkers such as the ones mentioned above, and we do not know how angiotensinogen compares to these. Finally, the discovery phase proteomics studies described in Chapter 2 identified other candidate AKI biomarkers which we have not yet evaluated. This chapter describes the

development of two parallel reaction monitoring mass spectrometry (PRM-MS) assays designed to quantify urinary angiotensinogen, the other candidate biomarkers, as well as the more established AKI biomarkers NGAL, KIM-1, and L-FABP.

Tandem Mass Spectrometry and Protein Quantification

Targeted tandem mass spectrometry techniques allow for highly accurate quantification of selected peptides and proteins. First described over thirty years ago, selected reaction monitoring (SRM-MS) is the most simple of this family of techniques and is performed using a triple quadrupole mass spectrometer.[13] SRM-MS makes use of this instrument's ability to select specific precursor and fragment ions for detection (Figure 6-1).[170] Quantification is performed using the extracted ion chromatogram (XIC) of the precursor-fragment ion pair (called a reaction or transition). The accuracy of quantification is improved through the use of stable isotope-labeled peptides, which have the same amino acid sequence as the target peptide, but are labeled on the C-terminus with C-13 and N-15 lysine or arginine. Peak area ratio of the native-to-SIS XIC for the quantitypic transition is used for quantification, and can be compared to an external calibration curve for absolute quantification. Additionally, as can be seen in Figure 6-1b, the SIS peptide has the same chromatographic retention time as the native peptide, which increases the specificity of the SRM-MS quantification. A major advantage of the triple quadrupole platform is its speed, requiring approximately10 ms per transition.[171] Thus this technique is highly amenable to multiplexing. Additionally, as technology has improved, it has

allowed for the monitoring of several fragment ions per precursor ion, a technique called multiple reaction monitoring (MRM-MS). MRM-MS provides improved specificity for the target peptide compared to SRM-MS, and it has become a popular method of protein biomarker quantification.[172] This technique has been used to measure up to 67 proteins in a single 30 minute analytical run.[173-175] Newer generation hybrid mass spectrometers such as quadrupole ion trap and quadrupole time of flight instruments offer further advantages for peptide quantification due to their superior resolution and mass accuracy and are capable of running in a data dependent acquisition mode similar to MRM. However, an important distinguishing characteristic is that the mass analyzer of these instruments allows for detection of the all the fragment ions generated from a given precursor ion in parallel. Peptide quantification based on all of the transitions generated during fragmentation has been termed parallel reaction monintoring (PRM-MS), and this method of quantification has been demonstrated to have improved linear range, reduced technical variability, and greater specificity for the target peptide compared to MRM-MS.[176] Therefore, this relatively new technique is likely to become the new gold standard for protein quantification and an indispensable tool in the proteomics biomarker toolbox.

Methods

Patients and Urine Samples

Urine samples were obtained from 204 consecutively enrolled patients who had undergone cardiac surgery at one of the SAKInet institutions between the dates

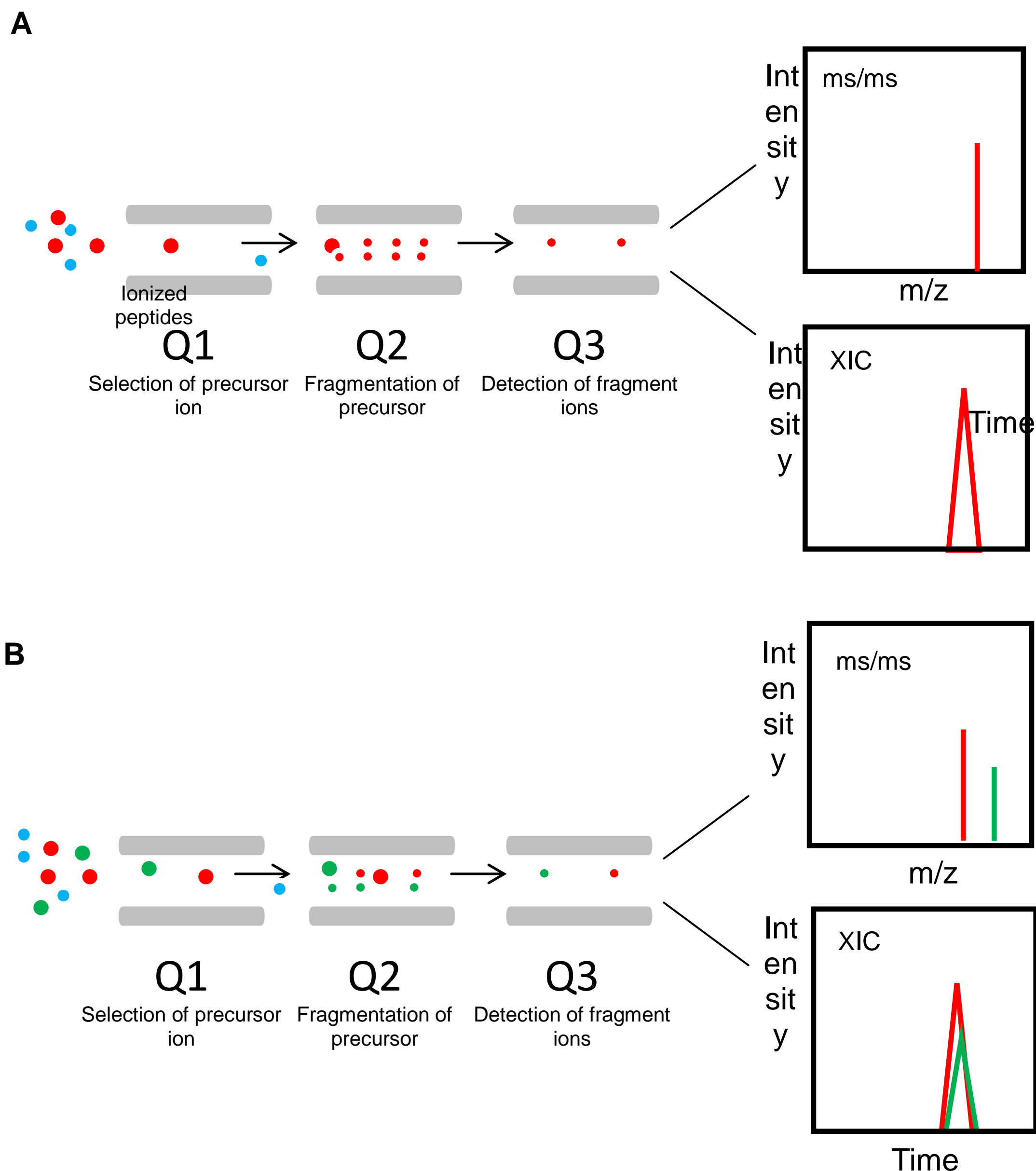

Figure 6-1. Schematic of selected reaction monitoring mass spectrometry. (A) In SRM-MS mode peptides derived from a biological sample are chromatographically separated, are ionized by electrospray ionization, and enter the first quadrupole (Q1). Only preselected precursor ions are allowed by the first quadrupole to enter the collision cell (Q2; second quadrupole). After fragmentation a single fragment ion is allowed to pass into the third quadrupole (Q3) and to the detector for quantification using the peak area from the corresponding extracted ion chromatogram (XIC). (B). Quantification with SRM-MS is accomplished using a stable isotopically –labeled peptide that is identical in sequence to native peptide, but can be differentiated by its increased mass. The ratio of the peak areas of the XICs of the two peptides is used for quantification and can be compared to an external calibration curve for absolute quantification.

of August 1, 2008 and June 1, 2012. All samples used were included in our previously described study (Chapter 3).[177] Informed consent was obtained in accordance with the IRB-approved protocol at each institution. Samples were collected and stored as previously described using a standard operating procedure which included centrifugation, addition of protease inhibitors and storage at -80°C. Urine samples were collected as early as possible after AKIN serum creatinine criteria were met, and all were collected within the first 72 hours after surgery. Inclusion criteria were surgery of the heart or ascending aorta and development of AKIN stage 1 AKI by creatinine criteria within 3 days of surgery. Subjects with ESRD, baseline serum creatinine > 3.0 mg/dL or AKI greater than AKIN stage 1 at the time of collection were excluded. Patients were followed until either time of death or hospital discharge, and were staged according to the maximum increase in serum creatinine using the AKIN classification system.[3] Urine output criteria were not used in diagnosis or staging because urine output data was not available. For PRM-MS analysis, patient samples were grouped by the maximum AKIN stage attained by the patient, and a block randomization was used to divide the cohort into four batches of 40 samples consisting of 30 patients who did not progress beyond AKIN stage 1, six patients who progressed to AKIN stage 2, four who progressed to AKIN stage 3.

Selection of Peptides for Parallel Reaction Monitoring Assay

With the exception of angiotensinogen, one tryptic peptide from each of the candidate biomarkers was selected for inclusion in the assay. Peptides identified during discovery phase proteomic analysis were preferentially selected for use in

the assay. When no suitable peptide could be identified from previous data, theoretical peptides were generated by *in silico* protein digestion (Peptide Mass) and an appropriate peptide was selected.[178] Only peptides which were identified by BLAST alignment as unique to the protein of interest were used for PRM-MS. Stable isotope-labeled versions of these peptides (SIS peptides) were synthesized (New England Peptide), and were used during assay development and as internal standards for quantification of urine biomarkers. These SIS peptides are C13- and N15-labeled on the C-terminus, and are only distinguishable from the endogenous (light) peptides by a shift in the mass of the precursor and fragment ions (y-ion series).

Peptide Characterization

To determine the predominant charge state of target peptides, an SIS peptide mixture was analyzed in information dependent acquisition mode using a Eksigent 2D+ HPLC in-line with an AB SCIEX Triple ToF 5600 mass spectrometer. Theoretical collision energy values were calculated using the formula published by Kuzyk *et al.* and the formula suggested by the manufacturer.[174] These values were used as a guide for empirical determination of optimal collision energy. The elution profile of each peptide from the Strata-X solid phase extraction (SPE) cartridge used in our workflow was determined empirically. The SIS peptide mix was spiked into a urine matrix and the SPE cartridge was washed with 5% acetonitrile 0.1% formic acid. Serial elutions were performed using eluents of 10%, 15%, 20%, 25%, and 30% acetonitrile 0.1% formic acid solutions. The peak area of the extracted ion chromatogram of the

most abundant fragment ion of each peptide precursor ion was calculated in each eluate to generate the elution profile.

External Calibration Curve

SIS peptides were brought to a concentration of 1 nmol/μL in 10% acetonitrile 0.1% formic acid. The peptide mix was then serially diluted and analyzed by liquid chromatography PRM-MS using an Eksigent 2D nano-HPLC and AB SCIEX Triple ToF 5600 mass spectrometer. PRM-MS data were analyzed in Multiquant and the sum of three most abundant fragment ions of each peptide (except for the peptides for complement C4b and LFABP, for which only 2 ions were used) was used to generate an external calibration curve (standard curve) with 1/x weighting using OLS regression.

Protein Quantification

Batches of 40 samples were analyzed by the PRM-MS method outlined below. Within each batch three analytical blanks and a standard reference material (SRM) were also analyzed. The SRM consisted of a pooled urine sample obtained from 5 patients with acute kidney injury after cardiac surgery.

Trypsin Digestion

Frozen samples were thawed in a 37°C water bath. A 50 μL aliquot of each sample was diluted 1:4 in 100 mM ammonium bicarbonate. To estimate digestion efficiency, 40 ng of stable isotope-labeled angiotensinogen protein (C13- N15-labeled lysine and arginine residues; Origene) was spiked into each sample. Proteins were denatured by the addition of 100 mM DTT and heating to 60°C for 30 min. Cysteine alkylation was performed by the addition of 0.5 M iodoacetamide followed by 30 min incubation in the dark. Trypsin (Promega)

was added at a ratio of 1:20 with total protein and samples were digested for 12 hr at 37°C.

Solid Phase Extraction

Following trypsin digestion samples were acidified by the addition of 3 volumes of 0.1% formic acid. At this time, a known concentration of SIS peptides was spiked into the samples to account for technical variability and to be used in downstream quantification. Solid phase extraction (SPE) was used for sample clean up and preparation for liquid chromatography tandem mass spectrometry. Strata-X (Phenomenex) SPE cartridges were conditioned with 1 mL acetonitrile (Honeywell) and equilibrated with 1 mL 0.1% formic acid (Sigma). Acidified sample digests were loaded on the SPE cartridge. After waiting for 5 min, samples bound to the SPE cartridges were washed with 1 mL of 5% acetonitrile 0.1% formic acid solution. Sample elution was performed using 30% acetonitrile 0.1% formic acid eluent. Sample eluate was collected in Protein LoBind polypropylene tubes (Eppendorf), frozen at -80°C and dried in a centrifugal vacuum concentrator.

Liquid Chromatography Parallel Reaction Monitoring Mass Spectrometry

Dried samples were reconstituted by the addition of 50 µL of 10% acetonitrile 0.1% formic acid followed by mild vortexing at room temperature for 20 min. Reconstituted samples were centrifuged at 15,000 *x g* 4°C for 15 min. A 5 µL aliquot of supernatant was diluted 1:15 in 10% acetonitrile 0.1% formic acid in order ensure that peptides of higher abundance proteins (uromodulin, nonsecretory ribonuclease, myoglobin, superoxide dismutase, and liver fatty acid binding protein) were below the upper limit of quantification. The remainder of

the supernatant was transferred directly into autosampler vials (Wheaton) and used to quantify lower abundance proteins. Each sample was analyzed using two separate analytical runs, one optimized for low abundance proteins and one for high abundance proteins. A 10 μL injection of undiluted sample was used for measurement of low abundance proteins, and a 5 μL injection of diluted sample was used for measurement of high abundance proteins. Liquid chromatography was performed on a 2D Eksigent HPLC. Briefly, samples were loaded onto a 2 cm Acclaim PepMap 100 C18 nanotrap column (Thermo Scientific) and washed extensively with mobile phase A (98% water, 2% acetonitrile, 0.1% formic acid). Chromatographic separation was then performed using a continuous gradient on increasing percentage of mobile phase B (95% acetonitrile, 5% water, 0.1% formic acid) on a 15 cm Acclaim PepMap 100 C18 analytical column (Thermo Scientific). Peptides were ionized using a nanospray ion source, and analyzed using an AB SCIEX Triple ToF 5600 mass spectrometer. Parallel reaction monitoring data acquisition parameters include MS accumulation time of 150 ms, fragmentation only of masses corresponding the empirically derived m/z of each peptide/SIS peptide pair selected during assay design using empirically derived collision energy, and 100 ms of MS/MS accumulation time was specified. The total cycle time for the data acquisition method was 2.15 s for low abundance proteins and 1.8 s for high abundance proteins. The extracted ion chromatrogram (XIC) of the sum of no fewer than 2 precursor ion-fragment ion transitions was used for quantification of each target protein by comparison to the

corresponding SIS peptide and external calibration curve All PRM-MS data were analyzed in Multiquant (AB SCIEX).

Assay Characterization

A standard reference material (SRM), which was a pooled urine sample obtained from 5 patients who had developed AKIN stage 1 AKI after cardiac surgery was used as a standard reference material (SRM). A 50 µL aliquot of the SRM was processed and analyzed with each batch of 40 patient samples. The coefficient of variation of the calculated protein concentrations was used to assess interassay variability. Analytical blanks were used to calculate the limit of detection (LOD) and lower limit of quantification (LLOQ) of the assay, as described by Mani *et al.* The distribution of the peak areas of the twelve blanks was determined, and outliers were identified using the upper and lower fences of a box plot (defined as 75th percentile + 1.5*IQR and 25th percentile – 1.5*IQR, respectively). Blanks which fell outside of this range were deemed unreliable estimates of assay performance. LOD and LLOQ were then calculated according the method described by Mani *et al.*[172]

Statistical Analysis

The primary outcome was the development of AKIN stage 3 AKI or death. Creatinine corrected molar concentration of target proteins was not normally distributed, and so comparison of biomarker concentrations between the group of patients who met the outcome and those who did not was performed using the Mann-Whitney U test. Receiver operator characteristic (ROC) curve analysis was used to evaluate the ability of each biomarker to predict the primary outcome. Logistic regression was used to test the association between

biomarker concentration and risk of meeting the primary outcome. Because a significant number of patients had calculated biomarker, biomarker concentrations were transformed into an ordinal scale (<LOD = 0, <LLOQ =1, 1st quartile = 2, 2nd quartile = 3, 3rd quartile = 4, 4th quartile = 5). Ordinalized biomarkers were also combined in a backwards stepwise multivariate logistic regression model to identify independent predictors of the primary outcome. Goodness of fit was assessed with the Hosmer-Lemeshow, Pearson Chi-square, and Likelihood ratio tests. Statistical Analysis was performed in SPSS (version 21 and Sigma Plot (version 11).

Results

Patient Characteristics

Urine samples collected from 157 patients who had developed AKI after undergoing cardiac surgery were analyzed using the PRM-MS assay that we developed. All patients had developed AKI within 72 hours after surgery and were classified as AKIN stage 1 by serum creatinine (sCr) criteria at the time of sample collection. Twenty-one patients met the primary outcome of development of AKIN stage 3 or death. Patients who met the outcome were well-matched to those who did not with respect to demographic and intraoperative variables. However, sCr at the time of collection and the change in sCr from baseline were higher in the group of patients who met the outcome, and there was a trend toward higher pre-operative sCr in this group. A comparison of patients who met the outcome and those who did not is shown in Table 6-1.

Table 6-1. Characteristics of post-cardiac surgery patients enrolled in study

	AKIN Stage 1 or 2 and Survived	AKIN Stage 3 or Death	*P*
n	136	21	
Age (yrs)[b]	68 (59.0 – 75.8)	65.5 (54.5 – 76.0)	0.58
Gender (female)[a]	33.8% (46)	42.9% (9)	0.57
Race (Caucasian)[a]	73.0% (99)	59.1% (13)	0.29
Collection Time (hr post-op)[b]	24.0 (19.2 – 43.2)	21.6 (16.8 – 38.4)	0.53
Operative Variables			
Bypass[a]	115 (84.5%)	18 (85.7%)	0.85
Bypass Time (min)[b]	137.0 (53.5 – 191.0)	137.0 (78.0 – 194.3)	0.9
CABG[a]	65 (47.8%)	8 (38.1 %)	0.55
Valve Replacement[a]	37 (27.2%)	4 (19.1%)	0.6
CABG + Valve[a]	23 (16.9%	6 (28.6%)	0.33
Other Surgery[a]	11 (8.1%)	3 (14.3%)	0.61
Serum Creatinine (sCr mg/dL)			
Pre-Op sCr[b]	1.1 (0.9 – 1.3)	1.2 (0.95 – 1.95)	0.07
sCr at Collection[b]	1.6 (1.3 – 1.8)	1.8 (1.6 – 3.1)	< 0.01
Percent Increase in sCr at Collection[b]	41% (30% – 56%)	6% (34% – 79%)	0.01
Max sCr[b]	1.75 (1.4 – 2.2)	4.2 (3.6 – 5.4)	< 0.001
Outcomes			
AKIN Stage 3[a]	0 (0%)	19 (90.5%)	< 0.001
RRT[a]	0 (0%)	10 (47.6%)	< 0.001
Death[a]	0 (0%)	5 (23.8%)	< 0.001

Statistical significance was determined by the χ^2 test for categorical variables and the Mann-Whitney U test for continuous variables.

[a]Categorical variables are reported as percentage (n).

[b]Continuous variables are reported as median (interquartile range).

Assay Development

A total of 22 candidate biomarker were identified by discovery phase proteomic analysis (see Chapter 2, Table 2-4). We attempted to design a PRM-MS assay using a single tryptic peptide to quantify each of the proteins, with the exception of angiotensinogen for which we selected three peptides (Table 6-2). Peptides which had been identified during discovery proteomic analyses of human urine were screened to determine their suitability for inclusion in the assay. In order to be included peptides had to have been unmodified, not contain any cysteine or methionine residues, not contain any missed cleavages, and be unique to the target protein. Unfortunately, we could not identify suitable peptides for Secreted Ly-6/uPAR-related protein, Complement C3 and Hepcidin, so these candidate biomarkers were not be included in the assay. The SIS peptides were analyzed in information dependent acquisition mode to ascertain their predominant charge state. The peptides that were selected to quantify Thymosin Beta-4 and Insulin-like growth factor binding protein 1 were not detectable during this analysis, indicating that they were either insoluble or failed to ionize well. After the optimal collision energy was empirically determined for the remaining peptides, an external calibration curve was generated. The sum of the intensities of the three most dominant fragment (y) ions was used for quantification, with the exceptions of the peptides for Complement C4-B and L-FABP, which only had two detectable y-ions. The peptides for Profilin-1, Glutathione peroxidase-3, Lysozyme C, and Polymeric immunoglobulin receptor did not have linear standard curves, and so these were also excluded from the assay. In contrast, 12 of the 16 peptides had excellent linearity ($r \geq 0.98$) with the

Table 6-2. Characteristics of peptides used in development of a multiplex parallel reaction monitoring assay for AKI biomarkers

Protein	Native/SIS Peptide Pair	m/z	CE	Fragment Ions Used for Quantification	External Calibration Curve Characteristics: Range	Linearity (r)	Slope
Angiotensinogen	ALQDQLVLVAAK	634.9	29	y8, y7, y6	0.03 - 125.0	0.995	3.19585E+04
	ALQDQLVLVAA^K^	640.9					
	FMQAVTGWK	534.3	28	y8, y6, y4	0.03 - 125.0	0.997	3.56913E+04
	FMQAVTG^WK^	539.8					
	SLDFTELDVAAEK	719.4	35	y11, y9, y4	0.03- 250.0	0.986	2.93185E+04
	SLDFTELDVAA^EK^	725.4					
Apolipoprotein A-IV	LGEVNTYAGDLQK	704.8	33	y9, y8, y7	0.03 - 250.0	0.992	3.50246E+04
	LGEVNTYAGDLQK^	708.4					
Vitamin D binding protein	THLPEVFLSK	390.9	s	excluded from assay because peptide did not ionize well			
	THLPEVFLSK^	393.6					
Superoxide Dismutase [Cu-Zn]	GDGPVQGIINFEQK	751.4	39	y9, y8, y6	1.0 - 250.0	0.970	1.00752E+03
	GDGPVQGIINFEQK^	755.4					
Complement C4-B	DHAVDLIQK	519.8	28	y7, y6	0.03 - 125.0	0.992	4.88203E+03
	DHAVDLIQK^	523.8					
Profilin-1	TFVNITPAEVGVLVGK	822.5	39	excluded from assay because peptide did not ionize well			
	TFVNITPAEVGVLVGK^	826.5					
Pigment Epithelium Derived Protein	TVQAVLTVPK	528.3	24	y8, y7, y6	0.03 - 125.0	0.997	4.24798E+04
	TVQAVLTVPK^	532.3					
Thymosin Beta-4	ETIEQEK			excluded from assay because peptide did not ionize well			
	ETIEQEK^						
Insulin-like growth factor binding protein 1	AQETSGEEISK			excluded from assay because peptide did not ionize well			
	AQETSGEEISK^						

SIS, stable isotope standard; CE, collision energy; ^ indicates the amino acid that is isotopically labeled with heavy carbon

Table 6-2 (cont)

Protein	Native/SIS Peptide Pair	m/z	CE	Fragment Ions Used for Quantification	External Calibration Curve Characteristics: Range	Linearity (r)	Slope
Myoglobin	VEADIPGHGQEVLIR VEADIPGHGQEVLIR^	545.0 548.3	33.0	y10, y9, y7	1.0 -250.0	0.96	3.81E+03
Glutathione peroxidase-3	YVRPGGGFVPNFQLFEK YVRPGGGFVPNFQLFEK^		31	excluded from assay because peptide did not ionize well			
Neutrophil defensin1	not included in assay because a signature peptide could not be identified in BLAST search						
Lysozyme C	STDYGIFQINSR STDYGIFQINSR^	700.8 705.8	34	excluded from assay because transitions did not produce an external calibration curve with appropriate linear range			
Antithrombin III	VAEGTQVLELPFK VAEGTQVLELPFK^	715.9 719.9	33.0	y10, y7, y6	0.12 - 250.0	0.98	1.98E+04
Secreted Ly-6/uPAR-related protein	not included in assay because a signature peptide could not be identified in BLAST search						
Non-secretory Ribonuclease	DPPQYPVVPVHLDR DPPQYPVVPVHLDR^	544.6 548.0	27.0	y10, y9, y6	1.0 - 250.0	0.96	2.51E+03
Uromodulin	VGGTGMFTVR VGGTGMFTVR^	512.8 517.8	27.0	y9, y8, y6	0.5 - 250.0	0.99	3.42E+03
CD59 glycoprotein	AGLQVYNK AGLQVYNK^	446.8 450.8	22.0	y7, y5. y4	25.0 - 250.0	0.99	3.01E+01
Pro-Epidermal growth factor	IESSSLQGLGR IESSSLQGLGR^	573.8 578.8	32.0	y10, y9, y8	0.03 - 62.5	1.00	5.95E+04
Polymeric immunoglobulin receptor	VYTVDLGR VYTVDLGR^	461.8 466.8	20	excluded from assay because peptide did not ionize well			
Hepcidin	not included in assay because a signature peptide could not be identified in BLAST search						

SIS, stable isotopically labeled standard; ^, isotopically labeled amino acid; CE, collision energy

Table 6-2 (cont)

Protein	Native/SIS Peptide Pair	m/z	CE	Fragment Ions Used for Quantification	External Calibration Curve Characteristics		
					Range	Linearity (r)	Slope
Neutrophil gelatinase-associated lipocalin (NGAL)	VPLQQNFQDNQFQGK	597.6		y7, y6, y4	0.12 - 125.0	0.99	2.32E+04
	VPLQQNFQDNQFQGK^	600.3					
Liver-type fatty acid binding protein (L-FABP)	AIGLPEELIQK	605.9	28.0	y9, y7	0.5 - 250.0	0.97	1.26E+04
	AIGLPEELIQK^	609.9					
Kidney injury molecule-1 (KIM-1)	ITVSLEIVPPK	598.4	28.0	y10, y9, y8	0.12 - 500.0	0.99	4.89E+02
	ITVSLEIVPPK^	602.4					

SIS, stable isotopically labeled standard; ^, isotopically labeled amino acid; CE, collision energy

linear range of the standard curve of 11 of these at attomolar concentrations. Therefore, the final assay contained 16 native/SIS peptide pairs representing 14 proteins, including 3 established AKI biomarkers: neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, and liver-type fatty acid binding protein.

We attempted to estimate the average abundance of each of the proteins in the assay because others have reported that the accuracy of quantification decreases when the SIS peptide spike differs from the endogenous peptide by greater than a factor of 10.[174] A literature search returned estimates for CD59, myoglobin, KIM-1, L-FABP, and NGAL. The concentrations of urinary angiotensinogen and uromodulin were estimated using the mode of the values obtained by ELISA in the experiment described in Chapter 5. The molar abundance of the remaining proteins was estimated using exponentially modified protein abundance index (emPAI) values obtained from discovery phase proteomic analysis in Chapter 2, indexing them to ELISA measurements that we had previously made on angiotensinogen and uromodulin. These estimates indicated that the target proteins spanned several orders of magnitude, which complicated our analysis because it was obvious that all of the proteins would not be in the linear range of the standard curve if a single sample injection were used (Table 6-3). Therefore, we decided to adapt the analytical approach such that each sample was analyzed twice, once in undiluted form (that is reconstituted with the amount of volume equal to the starting sample volume) and once as a 1:15 dilution.

Assay Performance: LOD, LLOQ and CV

Assay performance characteristics for each target peptide are reported in Table 6-4. Unfortunately, many of the samples had values that were undetectable (below the limit of detection; LOD). More samples had calculated values below the lower limit of quantification (LLOQ). In some cases the target proteins were only observed in a very small percentage of patients (CD59), and KIM-1 and apolipoprotein A-IV was not detected in any samples. Therefore, these proteins could not be evaluated. The external calibration curves for CD59, and KIM-1 were among the lowest quality included in the assay, which could explain the poor measurements made on these proteins. In contrast, the large number of patients with apolipoprotein A-IV and values below the LOD was surprising given excellent calibration curve for this peptide, suggesting that either the endogenous protein is outside of the range of the assay or that this peptide is not observed due to ion.

The coefficient of variation (CV) of the measurements made on the standard reference material (SRM) that was analyzed with each batch was used to estimate interassay variability. Unfortunately, the CV could not be calculated for many of the proteins because the native peptide was undetectable in the SRM. This likely reflects a low concentration of the target proteins in the urine samples that were pooled to make the SRM. Therefore, the technical variability of the assay could not be evaluated for several of the proteins. To overcome this limitation in future studies, the SRM should include a spike of unlabeled peptide or protein to ensure that assay variability can be assessed. Additionally, the CVs

Table 6-3. Estimated molar concentration of target proteins included in PRM-MS assay.

Protein	Estimated Concentration
Angiotensinogen	0.188 fmol/µL
Pigment epithelium derived protein	0.188 fmol/µL
Antithrombin III	0.188 fmol/µL
Complement C4-B	0.188 fmol/µL
Apolipoprotein A-IV	0.188 fmol/µL
Myoglobin	5.82 fmol/µL
Proepidermal growth factor	0.188 fmol/µL
NGAL	1.33 fmol/µL
KIM-1	0.052 fmol/µL
Uromodulin	71.7 fmol/µL
CD59	282.0 fmol/µL
L-FABP	63.3 fmol/µL
Superoxide dismutase	71.7 fmol/µL
Nonsecretory ribonuclease	71.7 fmol/µL

Table 6-4. Performance Characteristics of PRM-MS Assay

Protein	Peptide	LOD	LLOQ	<LOD	<LLOQ	CV
Angiotensinogen	ALQDQLVLVAAK	0.29	0.97	39 (24.8%)	98 (62.4%)	31.1%
LFABP	AIGLPEELIQK	139.74	465.83	21 (14.4%)	21 (13.4%)	26.9%
SOD [Cu-Zn]	GDGPVQGIINFEQK	0.85	2.84	64 (40.8%)	22 (14.0%)	25.3%
NSRN	DPPQYPVVPVHLDR	0.38	1.26	4 (2.5%)	23 (14.7%)	8.9%
PEDP	TVQAVLTVPK	11.01	36.70	73 (46.5%)	74 (47.1%)	ND
Pro-EGF	IESSSLQGLGR	2.40	7.99	63 (40.1%)	63 (40.1%)	ND
Antithrombin III	VAEGTQVLELPFK	11.98	39.92	70 (44.6%)	70 (44.6%)	22.5%
Complement C4B	DHAVDLIQK	0.04	0.13	114 (72.6%)	117 (74.5%)	ND
Uromodulin	VGGTGMFTVR	0.42	1.39	16 (10.2%)	75 (47.8%)	ND
Myoglobin	VEADIPGHGQEVLIR	134.90	449.67	30 (19.1%)	45 (28.7%)	ND
CD59	AGLQVYNK	187.62	625.41	156 (99.4%)	157 (100.0%)	ND
Apo A-IV	LGEVNTYAGDLQK	<0.1	<0.3	ND	ND	ND
KIM-1	ITVSLEIVPPK	0.11	0.37	ND	ND	ND
NGAL				ND		

The limit of detection (LOD) and lower limit of quantification (LLOQ) for each peptide included in the PRM-MS assay is shown. The number and percentage of patients (n = 157) with protein concentrations below the LOD and LLOQ is reported. The coefficient of variation (CV) was calculated using a standard reference material that was concurrently processed and analyzed with each batch of urine samples.

[a]Concentration reported as amol on column

[b]Concentration reported as fmol on column

that could be calculated were higher others have reported.[174,175] This is likely due to a difference in study design. We chose to digest and process the SRM concurrently with each batch, whereas others make an SRM batch which is digested and processed separately from the samples of each batch and is only analyzed concurrently with the samples. Thus the CV that is reported here incorporates variability in each step of the workflow from digestion to instrumental analysis, whereas the use of an SRM batch only accounts for instrumental variability.

Qualification of Candidate Biomarkers by Targeted Mass Spectrometry

Because many patients had biomarker concentrations that were below the limit of detection for the assay, we compared the proportion of patients with biomarker concentrations above the LOD who met or did not meet the AKIN stage 3 or death (Table 6-5). Patients who met the outcome were more likely to have urinary concentrations of L-FABP, superoxide dismutase [Cu-Zn], and myoglobin above the LOD of the assay, compared to patients who did not meet the outcome. Next, the association of elevated biomarker concentration (not creatinine-corrected) and risk of development of AKIN stage 3 or death was determined using an ordinalized protein concentration scale that included the following cut-offs: <LOD, >LOD but <lower limit of quantification (LLOQ), 1st, 2nd, 3rd, and 4th quartiles above the LLOQ (Table 6-5). Complement C4B, and CD59 were excluded from this analysis because only a small number of patients who had concentrations of these proteins above the LOD of the assay. Higher

Table 6-5. Association of Biomarker Concentration and Development of AKIN Stage 3 or Death

	AKIN 1 or 2 and Survived (n = 136)[a]	AKIN 3 or Death (n = 21)[a]	*P*	OR [95% CI][b]
Angiotensinogen	100 (73.5%)	18 (85.7%)	0.23	1.56 [1.19, 2.05]
Superoxide Dismutase [Cu-Zn]	73 (53.7%)	20 (95.2%)	<0.001	1.89 [1.31, 2.73]
L-FABP	115 (84.6%)	21 (100%)	0.08	1.46 [1.01, 2.11]
Myoglobin	107(78.7%)	20 (95.2%)	0.08	1.69 [1.16, 2.48]
Uromodulin	123 (90.4%)	18 (85.7%)	0.45	0.92 [0.72, 1.17]
Nonsecretory Ribonuclease	132 (97.1%)	21 (100%)	0.43	1.18 [0.89, 1.65]
Pigment Epithelium Derived Protein	71 (52.2%)	13 (61.9%)	0.49	1.08 [0.88, 1.33]
Pro-EGF	82 (60.3%)	12 (57.1%)	0.81	0.94 [0.74, 1.19]
Antithrombin III	75 (55.1%)	12 (57.1%)	1.0	0.99 [0.81, 1.22]
Complement C4B	38 (27.9%)	5 (23.8%)	0.8	ND
CD59	1 (0.7%)	0 (0%)	1	ND

[a]The number and percentage of patients who had protein concentrations above the limit of detection (LOD)of the assay is shown by protein. The Chi-squared and Fisher's Exact test were used as appropriate to compare the two groups of patients.

[b]Protein concentrations were converted to an ordinal scale and the continuous OR was calculated to evaluate the association of increasing biomarker concentration with the probability of development AKIN stage 3 or death.

L-FABP, liver-type fatty acid binding protein; Pro-EGF, pro-epidermal growth factor; ND, not determined due to the small number of patients with protein concentrations above the LOD

Table 6-6. Analysis of the Prognostic Predictive Power of Candidate Biomarkers

	AKIN 1 or 2 and Survived (n = 136)[a]	AKIN 3 or Death (n = 21)[a]	*P*	AUC[b]
Angiotensinogen[c]	73.7 (39.0 - 187.4)	312.3 (122.0 - (493.8)	<0.001	0.74 [0.63, 0.85]
SuperoxideDismutase [Cu-Zn][d]	4.78 (2.40 - 18.56)	21.96 (9.25 – 80.33)	<0.001	0.76 [0.66, 0.86]
L-FABP[d]	10.97 (5.12 – 21.24)	18.23 (14.96 – 34.47)	<0.01	0.69 [0.59, 0.80]
Myoglobin[d]	0.36 (0.18 – 0.84)	0.90 (0.66 – 3.30)	<0.001	0.77 [0.67, 0.87]
Uromodulin[d]	4.74 (2.35 – 12.89)	6.44 (4.84 – 13.26)	0.12	0.61 [0.49, 0.72]
Nonsecretory Ribonuclease[d]	8.2 (5.4 - 12.7)	11.25 (7.2 - 20.7)	0.08	0.62 [0.47, 0.76]
Pigment Epithelium Derived Protein[d]	15.3 (5.17 – 145.31)	67.18 (10.37 – 354.91)	0.13	0.60 [0.47, 0.74]
Pro-EGF[c]	71.01 (0.27 – 240.51)	40.73 (0.28 – 160.38)	0.83	0.52 [0.38, 0.65]
Antithrombin III[c]	77.12 (1.04 – 257.93)	87.4 (2.16 – 430.42)	0.25	0.58 [0.44, 0.72]
Complement C4B[d]	5.64 (2.87 - 26.62)	7.06 (4.12 - 15.56)	0.29	0.57 [0.44, 0.71]
CD59[d]	ND	384.2 (254.8 - 497.6)	0.01	0.68, 0.55, 0.80]

[a]The median and interquartile range of creatinine-corrected candidate biomarker concentrations are reported by outcome group. Groups were compared using the Mann-Whitney U test.

[b]Prediction of the outcome was assessed using receiver operator characteristic (ROC) curve analysis.

[c]Concentration is reported as pmol of protein per µg of creatinine.

[d]Concentration is reported as fmol of protein per µg of creatinine.

L-FABP, liver-type fatty acid binding protein; Pro-EGF, pro-epidermal growth factor; AUC, area under the receiver operator characteristic curve

concentrations of angiotensinogen (OR = 1.56 95% CI [1.19, 2.05]), superoxide dismutase [Cu-Zn] (OR =1.89 95% CI [1.31, 2.73]), L-FABP (OR = 1.46 95% CI [1.01, 2.11]), and myoglobin (OR = 1.69 95% CI [1.16, 2.48]) were associated with an increased risk of meeting the outcome. Finally, we evaluated the ability of creatinine-corrected biomarker concentrations to discriminate between patients who would meet the outcome and those who did not. In order to complete this part of the analysis, the LOD of each biomarker was applied as a threshold such that patients who had biomarker concentrations below the LOD were made equal to the LOD. The median concentrations of angiotensinogen, L-FABP, and superoxide dismutase were higher in patients who developed AKIN stage 3 or died Table 6-6). Receiver operator characteristic curve analysis found that all of these proteins were predictive of the primary outcome (Figure 2).

Discussion

A multiplex tandem mass spectrometric assay was developed to quantify 10 candidate prognostic biomarkers of AKI, as well as angiotensinogen, NGAL, L-FABP, and KIM-1. Peptides for an additional 6 peptides from 6 other candidate biomarkers were evaluated for inclusion in the assay but did not perform well during the development phase and were excluded. Future studies should attempt to quantify these proteins using different proteotypic peptides. This assay was similar in design to PRM-MS assays that have been described, in that it utilized an analytical platform with a high resolution and mass accuracy, and so our data benefitted from enhanced precision compared to a conventional MRM-MS approach.[176] However, our assay was not a true PRM-MS assay because only the most abundant fragment ions of the y-series were used for quantitation

(although the entire fragmentation spectrum was collected). This approach was chosen because it has been our experience with our instrument that lower abundance transitions introduce chemical noise and reduce the sensitivity of the measurement.

Urine samples from a total of 157 cardiac surgery patients were analyzed and the concentrations of the 14 proteins were evaluated for prediction of the outcome AKIN stage 3 or death. Disappointingly, many of the measurements that we made were below the lower limit of quantification (LLOQ) or the protein was undetectable. There are several possible explanations for this including: 1) matrix effects that suppress the ionization of the target peptide, 2) poor digestion efficiency resulting in lower abundance of the target peptide, 3) protein modification that either reduces digestion efficiency or shifts the mass of the target peptide, and 4) low protein abundance that is below the range of the assay. In the unique case of NGAL, the incorrect precursor ion was selected for fragmentation, and so no data could be acquired to quantify it. This is a limitation of the study, because NGAL is a well-validated AKI biomarker and novel candidate biomarkers should be compared to it. Despite these significant limitations, we attempted to draw a valid conclusion from the data by assuming that data points below the LOD were due to low levels of the endogenous protein, and so the LOD was applied as a threshold to the data and all values <LOD were made equal to the LOD.

Using this approach, we found that the candidate biomarkers angiotensinogen, superoxide dismutase [Cu-Zn], and myoglobin predicted AKIN stage 3 or death.

Although we admit that the accuracy of our measurements must be viewed with caution because the assay was not thoroughly validated, we believe that the precision is sufficient for relative quantification and that our inferences are valid. This is evidenced by three confirmatory findings present in our data. First, we found a good correlation between angiotensinogen values obtained by PRM-MS and those previously measured by ELISA, and the data obtained by PRM-MS showed that angiotensinogen predicted AKIN stage 3 or death with an AUC nearly equal to that obtained using ELISA measurements. Similarly, we confirmed that uromodulin did not predict the outcome, which we had also previously found using an ELISA for quantitation. Finally, we found that liver-type fatty acid binding protein (L-FABP), a well-established AKI biomarker, predicted this outcome that has been reported to be an excellent predictor of mortality in patients with sepsis-associated AKI.[137]

These results are the first to report superoxide dismutase [Cu-Zn] and myoglobin as AKI biomarkers. While these finding will need to be confirmed with a more well-validated assay, the prognostic significance of these proteins is congruous with our understanding of the role of oxidative stress in AKI. Specifically, myoglobin, a heme-containing protein, can generate free radicals and is a known nephrotoxin. It is plausible that higher concentrations of myoglobin in the urine would be associated with more severe renal injury.[179] Conversely, SOD [Cu-Zn] is a free radical scavenger which converts superoxide to hydrogen peroxide and has been shown to be renoprotective.[169, 180] Elevated concentrations of SOD [Cu-Zn] could represent a response to severe oxidative stress. Additionally, we

have previously found that angiotensin predicts adverse outcomes in patients with established AKI, but had not directly compared it to more well-characterized AKI biomarkers. In this analysis, we found that angiotensinogen compared favorably with L-FABP. These data solidify our previous findings and emonstrate that urinary angiotensinogen could have clinical utility as an AKI biomarker.

Chapter 7: Insights, Unanswered Questions, and Future Directions

Mechanistic Insights and Unanswered Questions: The Renin-Angiotensin System and AKI

Not only do biomarkers provide information useful for guiding clinical care, but they can also yield mechanistic insights into the molecular underpinnings of the associated disease. The studies that have been presented have convincingly shown that elevated concentrations of urinary angiotensinogen and renin are associated with more severe AKI and adverse outcomes. These two proteins are the substrate and enzyme, respectively, of the rate limiting step of the renin angiotensin system (RAS).[169] It has long been recognized that the RAS plays a central role in chronic kidney disease (CKD), and RAS inhibitors are the standard of care for patients afflicted with CKD.[181, 182] An increase in angiotensinogen is prerequisite for activation of the RAS and generation of downstream effector angiotensin peptides, and it is believed that urinary angiotensinogen is a surrogate for intrarenal activation of the renin-angiotensin system (RAS) during chronic kidney disease.[148, 183-185] Importantly, Kim *et al.* have shown that urinary angiotensinogen concentration correlates with urinary TGF-β, a profibrotic cytokine, and with the degree of the severity of the renal histopathology of patients with CKD.[186] Given its biological relationship with angiotensinogen, it is logical that urinary renin excretion could also be an important index of intrarenal RAS activation in chronic kidney disease.

In fact, it has been demonstrated that intrarenal angiotensin II increases renin expression

in the collecting duct via AT1R signaling, which results in tubular secretion of prorenin and renin.[187] In light of what is known about RAS and the importance of urinary angiotensinogen and renin in CKD, our findings require us to consider the possibility that the RAS could modulate the severity of renal injury during an episode of AKI. Certainly this would be congruent with the data from animal models of AKI (see below). Furthermore, as mentioned in the introduction in Chapter 1, AKI can precipitate CKD and accelerate its progression. Could the RAS be the mechanism which underlies this epidemiologic association? It is certainly plausible. If indeed this is the case, then it is likely that monitoring urinary angiotensinogen and renin levels during the course of AKI could predict which patients will develop CKD or progress from CKD to end stage renal disease (ESRD).

Animal models of AKI have repeatedly demonstrated that RAS activation occurs during AKI, and that it exerts a negative effect on the severity of the injury. Allred *et al.* showed that angiotensin II increases in kidney tissue following ischemia reperfusion injury in rats, whereas angiotensin I and angiotensin 1-7 increase in the urine.[188] These findings are supported by a study by da Silveira *et al.*, which showed that not only does angiotensin II increase, but angiotensin 1-7, a potential counterbalance of angiotensin II effects, decreases in renal tissue following ischemia reperfusion injury in a rat model of AKI.[189] Greater amounts of renal angiotensin II that are observed during AKI could result in increased intrarenal inflammation, since models of chronic renal injury have shown that angiotensin II contributes to renal injury through pro-inflammatory effects

mediated by the NF-κB pathway.[190-194] Indeed, inhibition of angiotensin converting enzyme and the angiotensin II type 1 receptor with captopril and losartan, respectively, reduce renal inflammation in rats subjected to renal ischemia-reperfusion injury and mitigate the severity of AKI in this model.[195, 196]

It is commonly believed that RAS inhibitors can precipitate and exacerbate AKI. Some observational studies have noted an increased risk of AKI associated with RAS blockade, which is presumably due to the inhibition of angiotensin II mediated vasoconstriction of the efferent arteriole.[197-199] This results in lower hydrostatic pressure in the glomerulus, resulting in a decreased glomerular filtration rate (GFR) and an increase in serum creatinine. Therefore, the effect of RAS inhibitors on AKI incidence could be artificial and unrelated to the effect of RAS inhibitors on renal injury, and it could be possible that RAS inhibitors attenuate renal injury while simultaneously decreasing renal function. Unfortunately, the effect of RAS inhibitors of the incidence of severe AKI and its complications has not been thoroughly investigated. Our work would seem to suggest that the incidence might be decreased, and therefore an observational study on the effect of RAS inhibitor use and AKI severity would be a logical extension of the biomarker data that has been presented. We would hypothesize that the effect of RAS inhibitors on GFR could result in an increased rate of complications from AKI such as uremic encephalopathy, hyperkalemia, metabolic acidosis, and volume overload, but that if these complications are appropriately managed, RAS inhibitors could mitigate the mortality rate associated with severe AKI and potentially reduce the rate of long term complications of AKI. While we

recognize that this is in stark contrast to conventional wisdom, it is noteworthy that a recent review proposed the continuation of RAS inhibitors during AKI.[200]

Intrarenal or Systemic: The Question of Location

Classically, the RAS has been understood as a systemic hormonal cascade in which angiotensinogen is produced by the liver and renin is released into circulation by the juxtaglomerular apparatus in the afferent arterioles of kidney. However, the existence of a local RAS in the kidney is widely recognized, and an important question that remains unanswered by our work is whether this or the systemic RAS is the source of increased angiotensinogen and renin that we have observed in the urine of patients with more severe AKI.[147,201] We believe that the tubular compartment is the likely source of these urine proteins during AKI. Early work by Ingelfinger *et al*. demonstrated localization of angiotensinogen messenger RNA in rat proximal tubules, a finding that was subsequently verified in human tissue.[202, 203] This indicates that the proximal tubule itself is capable of angiotensinogen biosynthesis. In support of this idea, a recent multiphoton imaging study reported negligible glomerular filtration of plasma angiotensinogen and concluded that urinary angiotensinogen concentration reflects intrarenal production.[204] On the contrary, elegant studies using tissue specific and conditional knockout mice have demonstrated that under normal conditions tubular angiotensinogen protein is primarily derived from the liver and depends on megalin for its uptake.[205, 206] Thus, it is possible that the urinary angiotensinogen detected in our assays was synthesized in the liver and sequestered in the proximal tubule, but was released by the proximal tubule upon

injury. However, a limitation of the knockout animal studies is that they have investigated intrarenal angiotensinogen in the uninjured state, and so their findings may not reflect what occurs during AKI. Therefore, AKI induced angiotensinogen biosynthesis by the proximal tubule cannot be ruled out, since this is a well-established phenomenon.[202, 207] Similarly, renin is classically understood as secreted by the juxtaglomerular apparatus into circulation. Renin protein can be localized in the proximal tubule, and like angiotensinogen, appears to be dependent on megalin uptake.[206] However, renin is expressed along renal tubular epithelium, and *in vivo* microscopy has shown that renin production increases in the collecting duct during diabetic nephropathy.[208] A similar mechanism could underlie the increased concentration of urinary renin that we observed in patients with more severe AKI.

Despite the evidence for intrarenal production of angiotensinogen and renin during chronic renal injury, which could be paralleled during acute injury, we must acknowledge that such deductive reasoning is inconclusive. A potential future direction of this project is to determine the site of angiotensinogen and renin production during AKI using animal models of AKI (such as renal ischemia/reperfusion injury). Increased intrarenal expression of these genes and protein abundance after renal injury suggest the kidney as the source of these urine proteins. However, kidney-specific knockouts of angiotensinogen and renin would be needed to definitively prove intrarenal production.

Implications for Clinical Trials of AKI

Despite strong evidence from animal models and numerous randomized clinical

trials, there are no effective interventions for AKI, and treatment is supportive in nature. There are at least three reasons for this translational bottleneck: 1) late initiation of therapy during the trial 2) the complex and multifactorial nature of AKI pathobiology and 3) underpowered study design. Novel biomarkers of AKI can address each of these issues. Serum creatinine, the conventional AKI biomarker, is a surrogate of the glomerular filtration rate, and as described in chapter 1, it increases as GFR declines during the early phases of AKI. However, it increases slowly and elevated sCr is often not detected until 1-3 days after the renal injury has occurred. Therefore, clinical trials which rely upon increased serum creatinine for AKI diagnosis and enrollment are unlikely to find a positive effect on acute outcomes. Recognition of this limitation of creatinine has been the primary driving force behind AKI biomarker research, leading to the discovery of several “early” AKI biomarkers. Secondly, biomarkers can function as molecular phenotyping tools that distinguish between different subtypes of disease, perhaps identifying a subpopulation in which intervention is effective. While no AKI biomarkers have been proposed for this purpose, examples of biomarker driven clinical care abound in the oncology literature, and our findings regarding angiotensinogen and renin are particularly attractive for this purpose. Given the discussion above, it seems likely that the RAS is involved mechanistically in AKI, and it could be that RAS blockade could have a benefit in patients with elevated urine concentrations of these proteins. Finally, the inclusion of patients with mild AKI in clinical trials, while commensurate with the evolving definition of this disease, results in a lower baseline prevalence of

severe adverse outcomes and diminishing the effect size of an intervention. Thus very large sample sizes are needed to demonstrate efficacy. Prognostic biomarkers such as angiotensinogen and renin, which predict progression to more severe AKI and a higher risk of adverse outcomes, could therefore be used to screen for inclusion in a trial.

Despite the strong rationale for incorporating biomarkers in AKI trial design, only one trial, the EARLYARF Trial, has used biomarker screening as a guide to enrollment.[142] In this trial, urinary alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT) values were monitored in ICU and post cardiac surgery patients and were used to screen patients for enrollment in a randomized, prospective, placebo-controlled clinical trial investigating the effect of erythropoietin on AKI incidence. Unfortunately, this trial failed to demonstrate an effect. However, it serves as an illustrative example. The underlying reason for the failure of erythropoietin is not likely to be late initiation of therapy, since increased urine concentrations of these brush border enzymes is thought to be an early marker of renal injury. Rather, there are two more plausible explanations. First, the putative renoprotective mechanism of erythropoietin is attenuation of ischemia-reperfusion injury.[209, 210] While GGT and AP do increase following ischemic tubular injury, they are not specific for ischemic injury. The heterogeneity of the study cohort itself is evidence of this, as there were a significant number of the patients who had septic AKI and AKI after cardiac surgery, both of which have complex, multifactorial pathogenesis.[211, 212] This heterogeneity could have attenuated any therapeutic benefit gained by

erythropoietin administration. Secondly, the investigators ran an observational study in parallel with the trial to determine the effectiveness of biomarker screening at enriching the study population. It seems that GGT and AP guided enrollment only marginally enriched the study population, resulting in only a small increase in statistical power of the study. For example, biomarker guided enrollment increased the incidence of AKI from 15.1% to 23.0% and 7 day mortality from 8.1% to 14.2% ($P = 0.034$). While the .investigators explicitly stated in the manuscript that AP and GGT were the only confirmed AKI biomarkers available for rapid detection at the time the study was conducted, it begs the question of how the trial would have concluded if a more accurate biomarker had been used.

The design of the EARLY ARF trial raises an important question for AKI biomarker research. Namely, how good does a biomarker need to be in order to be useful in clinical trial design? A recent NIDDK workshop on AKI clinical trial design stated,

> "Although numerous novel biomarkers have been proposed for early identification of intrinsic AKI, to date no biomarker has been shown to possess sufficient predictive ability to be used as a primary enrollment criterion and should not supplant SCr [serum creatinine] for enrollment into trials exploring the effects of agents on established AKI."[213]

However, the participants noted that potential use of biomarkers in risk

stratification in AKI trials should continue to be investigated. Specific recommendations for assessing this potential were not prescribed, and it remains largely unclear how to define the advantages gained by using a biomarker as a guide for enrollment. It is obvious, however, that the advantages and disadvantages of biomarker guided clinical trial enrollment are best understood in terms of the benefit of enrichment and the cost of screening. It is our objective to provide a logical framework for performing a cost-benefit analysis to determine if biomarker guided enrollment might be beneficial for future AKI clinical trials.

Enrichment: The Benefit of Biomarker Guided Enrollment

Enrichment strategies are commonplace in clinical trial design.[141] Their purpose is to increase the proportion of patients in the study population who might benefit from an experimental intervention, with the results being increased effect size (*i.e.* absolute risk reduction), increased statistical power, and a decrease in the sample size needed to achieve a given power. To describe the advantages of enrichment, let us consider the fenoldopam trial conducted by Tumlin *et al.*[214] This prospective, randomized, double-blind, placebo controlled clinical trial (n = 155) found that fenoldopam, a dopamine receptor alpha-1 specific agonist, failed to reduce the rate of renal replacement therapy or death within 21 days (p = 0.163). The incidence of this outcome in the placebo group (n = 75) was 38.7%, whereas in the treatment arm (n = 80) 27.5% of the patients met the outcome (relative risk reduction of 28.9%). The power to detect an effect size of 11.2% with this sample size is 0.331. In order to achieve a power of 0.8, the investigators would have needed to enroll 553 subjects. In order to understand

how biomarker guided enrollment might have altered the outcome of this trial, consider the combination of urinary angiotensinogen and renin that was described in Chapter 5. This biomarker test identified patients at high risk of progressing from AKIN stage 1 to the composite endpoint AKIN stage 3 or death (sensitivity = 30.4%; specificity = 98.9%). This outcome is quite comparable to the composite outcome renal replacement therapy or death chosen by Tumlin *et al*. Assuming the same sensitivity and specificity, this test would have a PPV of 94.6% in the population used in the fenoldopam trial. If the angiotensinogen/renin combination had been used to enrich the study population of the fenoldopam trial, the power would have been 0.996, assuming no change in sample size or the relative risk reduction conferred by fenoldopam treatment. The increase in power is due to an increase in the absolute difference in the proportions of subjects meeting the endpoint between the experimental groups from 11.2% (38.7% -27.5%) to 27.4% (94.6% - 67.2%). Importantly, however, because of the increased power, one of the most significant benefits of enrichment is that, compared to the unenriched population, fewer patients will need to be enrolled in the trial to detect a difference between the treatment group, and we calculate that the number needed to enroll trial to attain a power of 0.8 ($NNE_{Power\ 0.8}$) in an angiotensinogen/renin enriched fenoldopam AKI clinical is only 62 patients.

Mathematically, enrichment is simply defined as the ratio of PPV to prevalence. The benefit of enrichment can be appreciated by examining the changes in the number needed to enroll (NNE) and number needed to treat (NNT), which are

illustrated in Table 7.1. The number needed to enroll (NNE) is the number of patients that need to be enrolled in order to enroll one patient who will meet the outcome (one event). In an unenriched trial, NNE is simply the reciprocal of the prevalence of the outcome (Equation 7.1a); in a biomarker enriched trial, NNE is the reciprocal of the PPV of the biomarker test (Equation 7.1b). If the desired number of events is known, then the total enrollment that will be necessary is given by the product of NNE and the target number of events. Equivalently, because enrichment increases the absolute risk reduction (ARR) of an intervention, it results in a decrease in the number needed to treat (NNT), which is the reciprocal of the ARR. Importantly, the PPV/prevalence ratio is equal to the unenriched-to-enriched NNE and NNT ratios. Therefore we propose that when evaluating the potential use of a biomarker in clinical trial design that this "enrichment index" be calculated (Equation 7.2), because of the ease of interpreting this number as a measure of the degree of enrichment and its effect on the observed treatment response rate. Furthermore, the statistical significance of the enrichment index can be readily determined using the χ^2 test. However, since this test relies both on proportions and frequencies, we propose that by convention, the proportion of 100 patients who meet the outcome be calculated using the prevalence and compared to the number (out of 100) calculated using the PPV (Figure 7.1). While this is a somewhat crude approach, it immediately reveals if there is a statistically significant enrichment that could be

Table 7-1. The Relationship Between Enrichment, Number Needed to Enroll, and Number Needed to Treat

			Reference					Enriched					Enrichment Index		
Prevalence	PPV	RRR	Placebo Arm	Tx Arm	ARR_U	NNE_U	NNT_U	Placebo Arm	Tx Arm	ARR_E	NNE_E	NNT_E	PPV/ Prevalence	NNE_U/ NNE_E	NNT_U/ NNT_E
0.10	0.15	0.50	0.10	0.05	0.05	10.00	20.00	0.15	0.08	0.08	6.67	13.33	1.50	1.50	1.50
0.10	0.15	0.25	0.10	0.03	0.08	10.00	13.33	0.15	0.04	0.11	6.67	8.89	1.50	1.50	1.50
0.10	0.15	0.10	0.10	0.01	0.09	10.00	11.11	0.15	0.02	0.14	6.67	7.41	1.50	1.50	1.50
0.10	0.20	0.50	0.10	0.05	0.05	10.00	20.00	0.20	0.10	0.10	5.00	10.00	2.00	2.00	2.00
0.10	0.20	0.25	0.10	0.03	0.08	10.00	13.33	0.20	0.05	0.15	5.00	6.67	2.00	2.00	2.00
0.10	0.20	0.10	0.10	0.01	0.09	10.00	11.11	0.20	0.02	0.18	5.00	5.56	2.00	2.00	2.00
0.10	0.30	0.50	0.10	0.05	0.05	10.00	20.00	0.30	0.15	0.15	3.33	6.67	3.00	3.00	3.00
0.10	0.30	0.25	0.10	0.03	0.08	10.00	13.33	0.30	0.08	0.23	3.33	4.44	3.00	3.00	3.00
0.10	0.30	0.10	0.10	0.01	0.09	10.00	11.11	0.30	0.03	0.27	3.33	3.70	3.00	3.00	3.00
0.20	0.30	0.50	0.20	0.10	0.10	5.00	10.00	0.30	0.15	0.15	3.33	6.67	1.50	1.50	1.50
0.20	0.30	0.25	0.20	0.05	0.15	5.00	6.67	0.30	0.08	0.23	3.33	4.44	1.50	1.50	1.50
0.20	0.30	0.10	0.20	0.02	0.18	5.00	5.56	0.30	0.03	0.27	3.33	3.70	1.50	1.50	1.50
0.20	0.40	0.50	0.20	0.10	0.10	5.00	10.00	0.40	0.20	0.20	2.50	5.00	2.00	2.00	2.00
0.20	0.40	0.25	0.20	0.05	0.15	5.00	6.67	0.40	0.10	0.30	2.50	3.33	2.00	2.00	2.00
0.20	0.40	0.10	0.20	0.02	0.18	5.00	5.56	0.40	0.04	0.36	2.50	2.78	2.00	2.00	2.00
0.20	0.60	0.50	0.20	0.10	0.10	5.00	10.00	0.60	0.30	0.30	1.67	3.33	3.00	3.00	3.00
0.20	0.60	0.25	0.20	0.05	0.15	5.00	6.67	0.60	0.15	0.45	1.67	2.22	3.00	3.00	3.00
0.20	0.60	0.10	0.20	0.02	0.18	5.00	5.56	0.60	0.06	0.54	1.67	1.85	3.00	3.00	3.00

The rates of patients meeting the hypothetical primary outcome of the placebo and treatment (tx) arms of a theoretical randomized controlled trial for AKI are shown. The reference numbers (denoted by subscript U) are calculated in the unenriched population using the baseline prevalence, whereas the enriched numbers (denoted by subscript E) are calculated using the biomarker PPV. NNT is shown as a surrogate measure of statistical power.

PPV, positive predictive value; RRR, relative risk reduction- the hypothesized effect size of the intervention; ARR, absolute risk reduction; NNE, number needed to enroll- the number of patients who would need to be enrolled to accrue one subject who would meet the adverse outcome; NNT, number needed to treate- the number of patients that will need to be treated using the intervention in order to prevent one adverse outcome

Equation 7.1a $NNE_u = 1/Prevalence$

Equation 7.1b $NNE_E = 1/PPV$

Equation 7.2 $EI = PPV/Prevalence = NNE_u/NNE_E = NNT_U/NNT_E$

The subscripts U and E indicate unenriched and enriched populations, respectively. EI, enrichment index; PPV, positive predictive value of the biomarker test; NNE, number needed to enroll; NNT, number needed to treat

worth the cost of screening. Using this approach, it appears that the enrichment index of angiotensinogen/renin is statistically significant (P< 0.001).

Screening: The Cost of Biomarker Guided Enrollment

The second aspect that must be considered when evaluating the potential of a biomarker for guided enrollment is the cost of screening. This can be estimated by calculating the number of patients who will need to be screened in order to enroll one patient (number needed to screen; NNS). Because only patients with a positive test result will be enrolled, the NNS is mathematically determined by the proportion of patients who test positive (*i.e.* the sum of the true positive and false positive rates). The NNS is equal to the reciprocal of the positive rate of the test (Equation 7.3a). Assuming prevalence = 38.7%; sensitivity = 30.4%; and specificity = 98.9%, the NNS of the angiotensinogen/renin CHAID model would be 8. Therefore, in order to match the enrollment of the fenoldopam trial, 1240 patients need to be screened. From this analysis, the NNS seems to unreasonably large, and we might erroneously reject the use of the angiotensinogen/renin combination to guide enrollment. However, the NNS does not tell us how many patients will need to be screened to conduct the trial; it is merely an estimate of the rate of screening to enrollment. Furthermore, we have already demonstrated that due to benefits of enrichment, a biomarker guided trial will need to enroll fewer patients than an unenriched trial to reach a given statistical power. In order to calculate how many patients will be needed conduct the trial if a biomarker were used to enrich the study population, power and sample size calculations must first be performed. Once the sample size has

	Unenriched	Enriched
Outcome	P*100	PPV*100
No Outcome	(1-P)*100	(1-PPV)*100

n = 200

Figure 7.1. 2 x 2 Contingency table evaluating the statistical significance of enrichment. By convention, 100 subjects are considered for both the unenriched and biomarker enriched populations. Prevalence (P) and positive predictive value (PPV) are used to calculate the estimated frequencies of patients meeting the outcome or not. Statistical significance is determined with the $\chi 2$ test.

Equation 7.3a NNS = 1/[(P*Sensitivity) +[(1-P)(1-Specificity)]]

Equation 7.3b $NNS_{Power\ 0.8} = NNS * SS_{Power\ 0.8}$

Where P = prevalence; NNS = number of patients that need to be screened to enroll one patient; $SS_{Power\ 0.8}$ = the number of patients that would need to be enrolled to attain Power of 0.8; $NNS_{Power\ 0.8}$ = number patients that will need to be screened to enroll $SS_{Power\ 0.8}$ patients in the trial.

been determined, then the NNS can be used to calculate the number of patients that will need to be screened to meet that target enrollment ($NNS_{Power\ 0.8}$; Formula 7.3b). Therefore, if we use the sample size calculation performed above for a power of 0.8, then we estimate that 491 patients would need to be screened to meet the target enrollment of 62. Thus the number screened is actually less than the number of patients who would need to be enrolled to achieve a power of 0.8 if all comers were enrolled (n = 553), indicating that the cost of screening is less than the benefit of enrichment. A schematic for evaluating the costs and benefits of biomarker guided enrollment is shown in Figure 7.2.

Discussion of Cost-Benefit Analysis

It must be noted that the framework which we have provided has some obvious limitations. The suggested calculations are performed *a priori* and consequently are heavily dependent upon assumptions of prevalence, biomarker test performance characteristics, and the relative risk reduction of the intervention. Accordingly, prevalence of the primary outcome of a clinical trial should be estimated from historical data in the study population to maximize the accuracy of the estimate. Similarly, biomarker test performance should be well characterized in a prospective observational study in the same population that will be used in the clinical trial. This was the major pitfall of the EARLYARF trial, because the investigators used a cut-off that had been determined from an extremely small (n = 26) prospective study in which only 4 subjects developed AKI.[142, 215] This is also limitation of the data that we have presented since

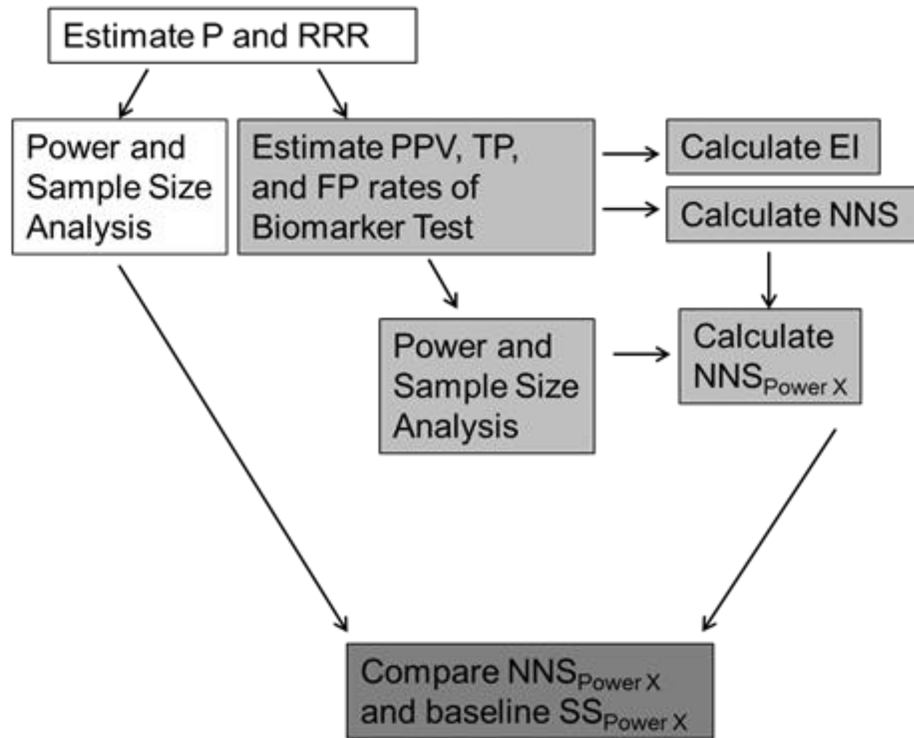

Figure 7-2. Schematic for evaluating the benefit and cost of incorporating biomarker guided enrollment into clinical trial design. The first step is to estimate the prevalence of the primary outcome (P) and the relative risk reduction (RRR) conferred by the intervention. Prevalence should then be used to calculate the positive predictive value (PPV) of the biomarker test. The enrichment index (EI) should be calculated and statistical significance determined using the $\chi 2$ test as described in the text. Power and sample size analysis should be performed for the unenriched (white box) and enriched trials (light gray boxes) using the P and PPV, respectively, for the event rates in the placebo arm, keeping RRR constant. The sample size (SS) calculated for a give power (Power X) should be determined for both studies, and the NNS should be used to calculate the total number of patients that would need to be screened to enroll that number of patients in the biomarker enriched trial ($NNS_{Power\ X}$). This should then be compared to the SS calculated for the unenriched trial at that power ($SS_{Power\ X}$).

angiotensinogen and renin were evaluated in a retrospective cohort study in a population that differed from that of the fenoldopam trial. Finally, the relative risk reduction conferred by an intervention in a biomarker enriched trial has been assumed to be identical to that of an unenriched trial. Unfortunately, this may not be true. Because biomarkers subclassify a disease according to molecular phenotype, the study populations of a biomarker enriched and unenriched trials are not identical, and thus the effect size of an intervention is unlikely to be the same. Unfortunately it is not possible to determine what the difference in the performance of an intervention might be in the enriched population. There is even the possibility that biomarker guided enrichment could abrogate the therapeutic effect of an otherwise efficacious intervention, which would lead us to falsely conclude that it is not effective. For this reason, it would be advisable to adopt the 2 stage clinical trial strategy proposed by Jones and Holmgren.[216-218] In the first stage, 2 pilot trials are conducted in which the intervention is tested in biomarker enriched population and an unenriched population. The second stage is a larger, phase II trial conducted using the population in which the intervention demonstrated efficacy in stage 1. If there is no difference in the efficacy between the enriched and unenriched groups, then the biomarker enriched population could be used to reduce the enrollment in the second stage, potentially accelerating the conclusion of the trial and decreasing the time needed to bring an intervention to the general population. An additional advantage of the 2 stage approach is that it allows us to determine if the intervention is only efficacious in the biomarker enriched population. Such a result would be expected if the

biomarker were an index of the mechanism which is targeted by an intervention, and in fact, the oncology literature is replete with examples of biomarker driven therapy. In addition to benefitting biomarker positive patients, correlating efficacy with biomarker status is an important finding from an ethical perspective because it would prevent the treatment of biomarker negative patients with an intervention from which they will not benefit. Therefore, we hypothesize that patients with elevated urinary angiotensinogen and renin could represent a subpopulation in which inhibitors of the renin-angiotensin system could attenuate the severity of AKI, whereas other patients without elevated urinary angiotensinogen and renin would not benefit from RAS blockade.

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Biography of the Author

I was born in Charleston, SC and raised there by my grandparents. It was my grandfather, a missile engineer for Lockheed Martin, who imparted to me his love for science, and as a consequence, I have always been fascinated with human biology and the biomedical sciences. Even during high school, I was sure that I wanted to become a physician. After graduating from First Baptist Church School in Charleston, I matriculated to Wofford College, where I completed a pre-medical studies program while double majoring in Biology and French. It was during the spring semester of my junior year at Wofford that I was introduced to biomedical research through my participation in an introductory research course, and I have known since that time that I wanted to pursue a career as a physician-scientist. After graduating *magna cum laude* from Wofford in 2006, I spent a year serving as an English teacher at Soai Christ Church in Yokkaichi, Japan. During this year, I applied to the Medical Scientist Training Program at the Medical University of South Carolina. Much to my dismay, I was not accepted due to my relative lack of research experience.

I did, however, gain entrance into medical school at the Medical University of South Carolina to pursue a MD, and because I was still intent on becoming a physician-scientist, I availed myself of every opportunity to obtain training in biomedical research. I worked in the laboratory of Drs. Robert Gemmill and Harry Drabkin in the Hollings Cancer Center during the summer between my first and second year of medical school. During my second year of medical school, I applied and was accepted to a Master’s of Clinical Research training program,

which I completed after my second year of medical school. I completed my thesis on the discovery of prognostic biomarkers of acute graft-versus-host-disease, and I conducted my research in the laboratory of John Arthur, a MD/PhD nephrologist, who encouraged me to reapply to the Medical Scientist Training Program and offered to support me to remain in his lab to complete my doctoral dissertation. This was a wonderful opportunity to fulfill my desire to become a physician-scientist, which was first born at Wofford in 2005.

As anyone familiar with the process of molding a scientist will know, it has not been an easy process. My scientific training has challenged me in ways that I never imagined it would, but emerging from this crucible, I am so much the better person for having gone through it. It has endowed me with the ability to skillfully wield the scientific method and apply it to any problem, and it has taught me the value in persevering through adversity. As I now embark upon the final stage of my journey to complete my medical training, I am immensely grateful for the skills that I have been imparted to me by my mentors. I have no doubt that they will serve me and my patients well in the future.