Full Review

Cell-cycle arrest and acute kidney injury: the light and the dark sides

John A. Kellum[1] and Lakhmir S. Chawla[2]

[1]Center for Critical Care Nephrology, Department of Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA, USA and [2]Department of Medicine, Division of Intensive Care Medicine and Division of Nephrology, Veterans Affairs Medical Center, Washington, DC, USA

Correspondence and offprint requests to: John A. Kellum, E-mail: kellumja@ccm.upmc.edu

ABSTRACT

Acute kidney injury (AKI) is a common consequence of systemic illness or injury and it complicates several forms of major surgery. Two major difficulties have hampered progress in AKI research and clinical management. AKI is difficult to detect early and its pathogenesis is still poorly understood. We recently reported results from multi-center studies where two urinary markers of cell-cycle arrest, tissue inhibitor of metalloproteinases-2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7) were validated for development of AKI well ahead of clinical manifestations—azotemia and oliguria. Cell-cycle arrest is known to be involved in the pathogenesis of AKI and this 'dark side' may also involve progression to chronic kidney disease. However, cell-cycle arrest has a 'light side' as well, since this mechanism can protect cells from the disastrous consequences of entering cell division with damaged DNA or insufficient bioenergetic resources during injury or stress. Whether we can use the light side to help prevent AKI remains to be seen, but there is already evidence that cell-cycle arrest biomarkers are indicators of both sides of this complex physiology.

Keywords: acute kidney injury, cell-cycle arrest, insulin-like growth factor-binding protein 7, tissue inhibitor of metalloproteinases-2

INTRODUCTION

Acute kidney injury (AKI) causes considerable harm [1, 2]. AKI is associated with short-term morbidity, a long-term risk of

chronic kidney disease (CKD) and cardiovascular events and decreases survival [3–8]. Hospitalized patients, particularly those with comorbidities and those undergoing complex procedures are at high risk for developing AKI [7, 9]. Despite increasing attention in recent years [1, 2, 8, 10], little improvement in outcomes for AKI has occurred and many authors have speculated that this is largely due to the inability to identify AKI early, in a potentially easily reversible stage [1, 2]. Common functional markers such as serum creatinine or urine output are late indicators of AKI. Indicators of kidney damage would potentially provide valuable 'lead time' to counter AKI—markers of renal 'stress' might be even better. There are numerous potential strategies that could be deployed to mitigate AKI if sufficient warning was provided [10].

In this review, we will summarize the current evidence that supports the use of cell-cycle arrest biomarkers for risk assessment of AKI, we will consider various responses to this warning and we will speculate on how this mechanism could be exploited to protect the kidney.

WHY ARE BIOMARKERS NEEDED?

Serum creatinine (sCr), the most widely used marker of kidney function, is a component of the definition of AKI, and yet as a single measurement is completely useless in differentiating AKI from CKD. While sCr is an adequate marker of glomerular filtration rate (GFR), sCr itself does not correlate with hospital survival whether measured at the time of presentation [11] or the start of dialysis [12]. What predicts short- and long-term outcomes is the *change* in renal function and herein lies the problem. Changes in sCr require several hours to days before they reach steady state following an injury to the kidney.

Thus the change in sCr is an excellent tool for defining when a change in function has occurred but not particularly good for detecting that it is occurring (or about to occur). Worse yet, sCr, like all functional markers, may be both insensitive to and non-specific for injury.

The criteria for defining AKI also includes urine output (UO). UO will often decrease before sCr increases making it a more time sensitive marker of GFR. However, UO is not nearly as specific for GFR. While in the absence of obstruction if there is no UO, there can be no GFR, not all reductions in UO signal AKI. Sustained oliguria is invariably associated with AKI but then, the timeliness of UO as an early indicator is lost.

Importantly, functional change is neither necessary nor sufficient to define AKI *as it is occurring*. Over time, a persistent renal functional change can be used to infer kidney injury in the same way that regional wall motion abnormalities or a decrement in ejection fraction can be used to infer myocardial injury. However, acute changes in function may not be due to or result in damage. Conversely, the absence of functional change cannot be used to exclude injury, especially in previously healthy individuals with normal renal reserve [13]. As such, markers that can detect actual damage or warn of impending damage are required in the same way that functional measures alone (e.g. left ventricular ejection fraction) are insufficient to manage acute coronary syndrome.

DISCOVERY AND VALIDATION OF CELL-CYCLE ARREST MARKERS

In 2013, we reported the results of a prospective, observational, international investigation (Sapphire study) of tissue inhibitor of metalloproteinases-2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7) in a heterogeneous group of critically ill (evidence of respiratory or cardiovascular failure) patients [14]. This report actually included two multi-center studies—discovery and validation. In the discovery phase we enrolled 522 adults in three distinct cohorts including patients with sepsis, shock, major surgery and trauma and examined over 300 markers in urine and blood. In the validation study we enrolled 744 adults with critical illness and without evidence of AKI at enrollment; the final analysis cohort was a heterogeneous sample of 728 critically ill patients. The primary end point was moderate-severe AKI [Kidney Disease: Improving Global Outcomes (KDIGO) stage 2–3] [10] within 12 h of sample collection which occurred in 14% of Sapphire subjects. The two top biomarkers from discovery, IGFBP7 and TIMP-2, were validated—area under the receiver operating characteristic curve (AUC) of 0.80 together (0.76 and 0.79 alone). [TIMP-2]•[IGFBP7] was significantly superior to all previously described markers of AKI (P < 0.002) including neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule (KIM)-1, none of which achieved an AUC > 0.72 [14]. However, this result might be due in part to varying kinetics of different early AKI biomarkers. [TIMP-2]•[IGFBP7] significantly improved risk stratification when added to a 9-variable clinical model when analyzed using Cox proportional hazards model, generalized estimating equation, integrated discrimination improvement or net reclassification improvement.

Finally, in sensitivity analyses [TIMP-2]•[IGFBP7] remained significant and superior to all other markers regardless of changes in reference creatinine method.

Other investigators have confirmed that [TIMP-2] and [IGFBP7] are useful in the detection of AKI. In a small study of patients undergoing cardiac surgery, Wetz *et al.* found that levels of urinary [TIMP-2]•[IGFBP7] were higher in the patients who developed AKI than those that did not [15]. Yamashita demonstrated that urinary [TIMP-2] performed well to predict severe AKI in critically ill patients with and without sepsis with a ROC AUC range of 0.81–0.84 [16].

Next, we developed and separately evaluated, two clinical cutoffs for a test using urinary [TIMP-2]•[IGFBP7] [17]. In order to do so, we first had to consider the intended use of the test. There are no specific means to prevent AKI, no equivalent to thrombolytic therapy and recommended actions are low-risk and generally applicable to most if not all critically ill patients (see Figure 1) [10]. However, these actions are difficult to implement in all patients and some are time-consuming and potentially expensive. Thus, the main purpose of the test would be to identify the majority of high-risk patients, and to ensure all such patients receive these recommended actions. As such, the test would need to have a primary cutoff that provides high sensitivity and high negative predictive value (NPV)—the proportion of true negatives over all patients testing negative. Although this would be the main use for the test, a cutoff with a high specificity and high positive predictive value (PPV) would have value as well. For example, in judging the risk-benefit to a patient for a decision to postpone some form of therapy that carries a risk of kidney damage (e.g. cardiac surgery) one might wish to have a test with a high PPV.

Thus, we derived cutoffs for urinary [TIMP-2]•[IGFBP7] based on sensitivity and specificity (as well as NPV and PPV) for prediction of AKI using data from the Sapphire study [14]. We set the high sensitivity/high NPV cutoff at 0.3 $(ng/mL)^2/1000$ and the high specificity/high PPV cutoff at 2.0 $(ng/mL)^2/1000$. Next, we verified these cutoffs in a new study (Opal) enrolling 154 critically ill adults from six sites in the United States [17]. In total, 27 subjects met the primary end point (stage 2–3 AKI within 12 h). The results of the Opal study replicated those of Sapphire (Figure 2) where the sensitivity at the 0.3 cutoff was 89%, and NPV was 97%. For 2.0, specificity was 95% and PPV was 49%. Relative risk of AKI for subjects testing in each strata is shown in Figure 2. Compared with baseline risk, subjects in the middle strata (>0.3–2) were more than 4-fold and those in the highest strata (>2) were more than 10-fold more likely to manifest moderate to severe AKI (KDIGO stage 2–3) in the next 12 h. In Opal, 46% of patients tested ≤0.3 while the upper two strata included 39% and 16% of patients. We also examined these cutoffs for urinary [TIMP-2]•[IGFBP7] in Sapphire with respect to subsequent development of major adverse kidney events at 30 days $(MAKE_{30})$, which included death, dialysis or persistence of renal dysfunction (creatinine twice baseline). Risk for $MAKE_{30}$ was lowest when [TIMP-2]•[IGFBP7] was ≤0.3 (raw risk 18%) and increased to 23% for >0.3–2.0 and 40% for >2.0 (P < 0.001).

Finally we conducted a third study, Topaz [18], and enrolled another heterogeneous cohort of 420 critically ill patients in

FIGURE 1: Stage-based management of AKI. Shading of boxes indicates priority of action—solid shading indicates actions that are equally appropriate at all stages whereas graded shading indicates increasing priority as intensity increases. AKI, acute kidney injury; ICU, intensive care unit. Source: www.KDIGO.org.

order to prospectively validate the lower (0.3) biomarker cutoff value for risk assessment of AKI. This was the first study to evaluate urinary [TIMP-2]•[IGFBP7] where the end point was determined by clinical adjudication by AKI experts blinded to the results of the test. We found that the urinary [TIMP-2]• [IGFBP7] test significantly improved risk assessment by stratifying patients into distinct risk categories, with a 7-fold increase in risk for patients with a [TIMP-2]•[IGFBP7] test value >0.3 compared with those ≤0.3. When two cutoffs are used, relative risk for >0.3–2.0 was 5, and >2.0 relative risk was 17 ($P < 0.002$). Furthermore, using a multivariate model including clinical information, urinary [TIMP-2]•[IGFBP7] remained statistically significant and a strong predictor of AKI. The clinical model alone exhibited an AUC 0.70, 95% CI 0.63–0.76 while the AUC was 0.86, 95% CI 0.80–0.90 for clinical variables plus [TIMP-2]•[IGFBP7]).

Thus, urinary [TIMP-2]•[IGFBP7] has now been shown to provide early risk stratification for imminent AKI in over 1200 critically ill patients in three multi-center studies enrolling diverse groups of patients (Table 1) with the prevalence of major exposures for AKI, such as sepsis, similar to other reports in the literature [19, 20].

CLINICAL APPLICATION OF AKI BIOMARKERS

Taken together the results of these studies of urinary [TIMP-2]• [IGFBP7] should enable early triage and risk stratification in a wide range of critically ill patients from the emergency room, acute admission areas and intensive care. Specific approaches might also be used in settings such as cardiac surgery. Early identification of these at-risk patients should improve delivery

of KDIGO-recommended interventions [10] and might also enable interventional studies for the treatment of AKI in the not-so-distant future [13].

Importantly, both TIMP-2 and IGFBP7 may increase in response to a wide variety of insults (inflammation, oxidative stress, ultraviolet radiation, drugs and toxins) [21–23]. This may help explain why they correspond to risk for AKI, a syndrome known for its multiple etiologies even in the same patient. However, these insults may not actually destroy cells and these molecules appear to be able to signal in autocrine and paracrine fashions [23, 24] thus behaving more like an 'alarm' spreading to adjacent cells (Figure 3). In terms of timing, this signal could represent the earliest point of cellular stress. Biomarkers that can detect cellular stress (or conversely cell health) may be more useful than markers of injury or cell death [13].

As urinary [TIMP-2]•[IGFBP7] can aid in the risk assessment for AKI, it is important to recognize that, like all diagnostic tests, it does not take the place of clinical judgment. Furthermore, while the test was designed to risk assess for stage 2–3 AKI, the biomarker concentrations correspond to severity of AKI across all three stages [17]. Thus, clinical applications may vary depending on the clinical question. However, the markers do not appear to persist in the urine for long after AKI has occurred and thus they may be normal in patients who have already manifest AKI by functional criteria (e.g. sCr). Finally, the cutoffs for the test are based on overall behavior of the biomarkers in the majority of patients. While this performance appears to be very consistent across various exposures and susceptibilities for AKI, specific groups of patients may require more fine-tuning. For example, Meersch et al. examined sensitivity and specificity of [TIMP-2]•[IGFBP7] for AKI (stage 1 or greater) in a high-severity group of patients undergoing cardiac
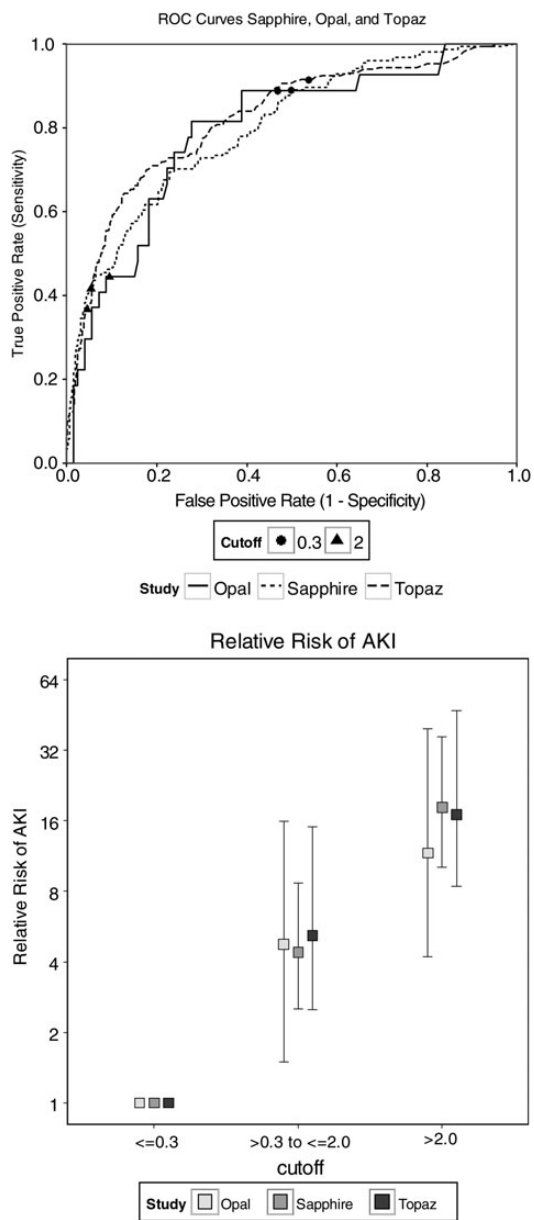
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ROC Curves Sapphire, Opal, and Topaz

Cutoff ● 0.3 ▲ 2

Study — Opal ··· Sapphire --- Topaz

Relative Risk of AKI

Study ☐ Opal ◼ Sapphire ■ Topaz

FIGURE 2: *Top:* [TIMP-2]•[IGFBP7] ROC curves for the Opal (solid), Sapphire (short dash) and Topaz (long dash) cohorts. Closed circles and triangles indicate [TIMP-2]•[IGFBP7] cutoffs of 0.3 and 2.0, respectively. Area under the ROC curve (95% CI) = 0.79 (0.69–0.88), 0.80 (0.74–0.84) and 0.82 (0.76–0.88) for Opal, Sapphire and Topaz, respectively. *Bottom:* Relative risk of AKI stage 2 or 3 within 12 h in the Opal (light gray), Sapphire (medium gray) and Topaz (dark gray) cohort.

surgery [25]. These investigators found a sensitivity of 0.92 and specificity of 0.81 for a cutoff value of 0.5 (AUC 0.90) using the maximum urinary [TIMP-2]•[IGFBP7] concentration achieved in the first 24 h following surgery (composite time point).

Taking the cardiac surgery example, we propose a set of actions based on pre-test assessment of risk and the urinary [TIMP-2]•[IGFBP7] test result (Figure 4). Low, moderate and high risk are based on a combination of (i) underlying clinical predisposition (susceptibility) which includes most of the same

Table 1. Baseline characteristics of subjects included in three large multi-center trials evaluating cell-cycle arrest markers

	Sapphire[a]	Opal[b]	Topaz[c]
All patients	728	153	408
Male	449 (62%)	87 (57%)	219 (54%)
Age, years	64 (53–73)	65 (54–77)	65 (54–76)
Race			
White	573 (79%)	119 (78%)	339 (83%)
Black	87 (12%)	13 (8%)	56 (14%)
Other/Unknown	68 (9%)	21 (14%)	13 (3%)
History of CKD	65 (9%)	13 (8%)	32 (8%)
ICU type			
Medical	225 (31%)	53 (35%)	180 (44%)
Surgical	179 (25%)	13 (8%)	70 (17%)
Combined ICU	147 (20%)	43 (28%)	62 (15%)
Cardiac surgery	61 (8%)	1 (1%)	38 (9%)
Neurologic	39 (5%)	2 (1%)	14 (3%)
Coronary care unit	30 (4%)	27 (18%)	10 (2%)
Trauma	24 (3%)	9 (6%)	27 (7%)
Other/Unknown	23 (3%)	5 (3%)	7 (2%)
Reason for ICU admission[d]			
Respiratory	310 (43%)	81 (53%)	206 (50%)
Surgery	247 (34%)	23 (15%)	128 (31%)
Cardiovascular	243 (33%)	64 (42%)	165 (40%)
Sepsis	136 (19%)	29 (19%)	97 (24%)
Neurological	70 (10%)	15 (10%)	52 (13%)
Trauma	55 (8%)	12 (8%)	44 (11%)
Other	126 (17%)	57 (37%)	120 (29%)
Enrollment sCr, mg/dL	0.9 (0.7–1.2)	1.1 (0.8–1.6)	0.9 (0.7–1.3)

Shown are N (proportion) or median (interquartile range).
[a]Results reproduced from Table 1 of [14].
[b]Results reproduced from Table S1 of [17].
[c]Results partially reproduced from Table 1 of [18].
[d]Subjects may have multiple reasons for ICU admission.

variables that determine STS predicted risk of mortality; (ii) acute evidence of AKI (oliguria or increasing sCr); (iii) clinical suspicion of AKI based on exposures and (iv) urinary [TIMP-2]• [IGFBP7]. When [TIMP-2]•[IGFBP7] is ≤0.3 (ng/mL)^2/1000, the high NPV means that patients are low-risk unless clinical suspicion is high or unless they already have clinical evidence of AKI. When [TIMP-2]•[IGFBP7] is >0.3 but <2.0 (ng/mL)^2/1000, risk is moderate but should be increased to high if any of the following exist: clinical evidence of AKI, STS predicted risk of mortality ≥4%, clinical suspicion for AKI high. Finally, given the high PPV when urinary [TIMP-2]• [IGFBP7] >2.0 (ng/mL)^2/1000, anyone regardless of clinical risk assessment should be considered to be at high risk.

TWO SIDES TO CELL-CYCLE ARREST

Each phase of the cell cycle has a specific function that is required for appropriate cell proliferation. Quiescent cells are normally in G_0. In order for cells to divide and begin the process of repair, they must enter and exit each phase of the cell cycle on schedule [26]. If the cell exits a phase too soon, or stays in a phase too long, the normal repair and recovery process can become maladaptive [26]. For instance, if epithelial cells remain arrested in G_1 or G_2, it favors a hypertrophic and fibrotic phenotype [27, 28]. Conversely, exit from cell cycle in late G_1 leads to apoptosis [29]. Cyclins and cyclin-dependent kinases, and inhibitors control each phase
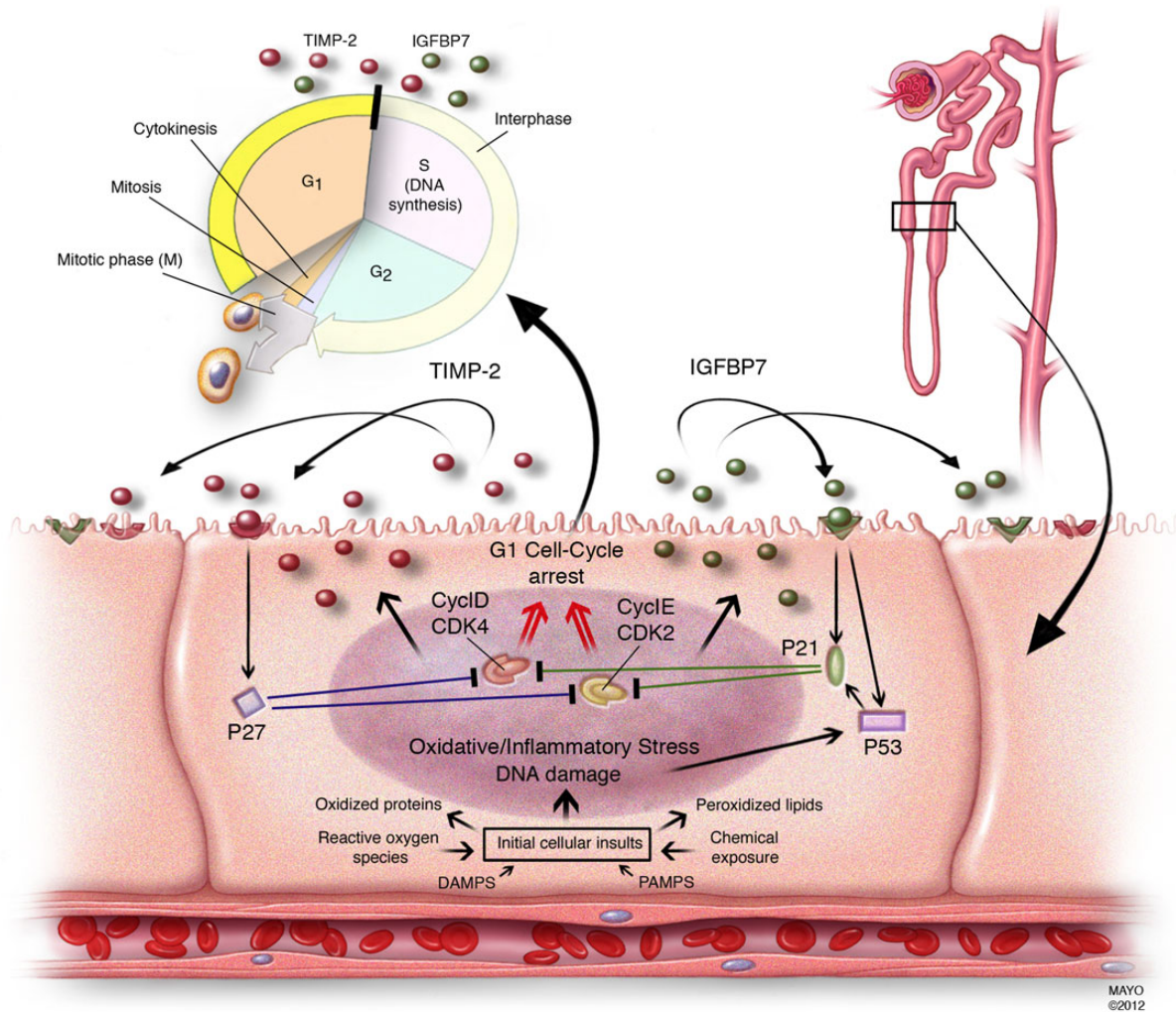
FIGURE 3: Proposed mechanistic involvement of the novel biomarkers in AKI: Initial tubular cells sustain injury by various insults. In response to DNA and possibly other forms of damage IGFBP7 and TIMP-2 are expressed in the tubular cells. IGFBP7 directly increases the expression of p53 and p21 and TIMP2 stimulates p27 expression. These effects are conducted in an autocrine and paracrine manner via IGFBP7 and TIMP-2 receptors. The p proteins in turn, block the effect of the cyclin-dependent protein kinase complexes (CyclD-CDK4 and CyclE-CDK2) on the cell-cycle promotion, thereby resulting in G1 cell-cycle arrest for short periods of time presumably to avoid cells with possible damage from dividing. Source: [14].

of the cell cycle [26]. The cell uses cell-cycle arrest as a protective mechanism to avoid cell-division when potentially damaged [30]. By initiating cell-cycle arrest, cells can thus avoid cell-division during stress and injury, which is protective. However, if the cells do not re-initiate the cell-cycle and remain arrested at G1 or G2 (or possibly other phases of cell cycle), a fibrotic phenotype can ensue.

Both TIMP2 and IGFBP7 have been implicated in the G_1 cell-cycle arrest phase noted to occur during the very early phases of cellular stress (Figure 3) [21–23]. Specifically, it has also been shown that renal tubular cells also go through this G_1 cell-cycle arrest phase following stress due to a variety of insults [31]. Induction of cell-cycle arrest is not only associated with increased risk for AKI but may also serve as a mechanistic link between AKI and CKD [28]. Sustained cell-cycle arrest will result in a senescent cell phenotype and lead to fibrosis. Interestingly, the various sub-types of TIMP proteins may play different roles in the kidney. Wang *et al.* have shown that

TIMP-3 protects the cells from damage, whereas TIMP-2 appears to promote injury through matrix metalloproteinase activation [32]. Similarly, research studies in renal transplant allografts show that matrix metalloproteinase activity is important in mediating scarring in chronic allograft nephropathy [33].

Indeed there is already evidence that urinary [TIMP-2]•[IGFBP7] is strongly associated with a composite end point of death or need for dialysis. We found that [TIMP-2]•[IGFBP7] >2.0 $(ng/mL)^2/1000$ was associated with increased risk for mortality or receipt of RRT over the next 9 months (hazard ratio [HR], 2.11; 95% confidence interval [95% CI], 1.37 to 3.23; P,0.001). Interestingly, in a multivariate analysis adjusted for the clinical model, [TIMP-2]•[IGFBP7] >0.3 $(ng/mL)^2/1000$ were associated with death or RRT only in subjects who developed AKI (P = 0.002) [34]. Additionally, Aregger *et al.* recently demonstrated that IGFBP7 levels predicted mortality, renal recovery and severity/duration of AKI in a small cohort of critically ill adults [35].

Clinical Evidence of AKI	Clinical Suspicion	STS Predicted Mortality	Urinary [TIMP-2]•[IGFBP7]	Risk Assessment
Negative	Low or Moderate	Any	≤ 0.3	LOW
Negative	Low or Moderate	<4%	>0.3 <2.0	Moderate
Any	Any	≥ 4%	>0.3 <2.0	High
Positive	Any	Any	Any	High
Any	High	Any	Any	High
Any	Any	Any	≥ 2.0	High

Action	Low Risk	Moderate Risk	High Risk
Monitor sCr	Standard Care (daily)	Every 12 hrs until decrease	Every 12hrs until decrease
Monitor Urine Output	(I/0s reviewed every 12 hours)	Strict I/0s keep Foley	Strict I/0s keep Foley
Ensure volume status	Standard Care Lasix as needed	For Oliguria, may use balanced fluid IF CVP < 8; Hold Lasix unless pulmonary edema	May use balanced fluid IF CVP < 8 AND evidence of hypovolemia (not just oliguria); hold Lasix
Avoid Nephrotoxic meds	Standard care	No NSAIDS or ACE/ARBs	No NSAIDS or ACE/ARBs Adjust doses (narcotics)*
Cardiac management	Usual care	Monitor SCVO2 if h/o abnormal LV Fx	Monitor SVO2, Echo or PA catheter if < 55% – Inotropes to keep CI >2.2
Recheck markers	NA	24 hrs	12 hrs

FIGURE 4: Proposed clinical application of risk assessment for patients immediately after cardiac surgery. STS, Society of Thoracic Surgery; I/O, input and output; sCr, serum creatinine; CVP, central venous pressure; NSAIDS, Non-steroidal anti-inflammatory drug; ACE, Angiotenson converting enzyme inhibitor; SCVO2, central verous oxygen saturation; h/o, history of; LV Fx, Left ventricular function; PA, pulmonary artery; CI, cardiac index.

As discussed above, cell-cycle arrest is nonetheless a protective mechanism to avoid the cell entering the cell-cycle when it is injured or even in an adverse environment [36]. Thus, temporary G_1 cell-cycle arrest should reduce kidney damage. Using an animal model of septic AKI secondary to cecal ligation and puncture we hypothesized that a pharmacologically induced early cell-cycle arrest would be associated with less AKI [37]. We used cyclosporine A, a known inducer of cell-cycle arrest and previously shown to attenuate kidney damage in the setting of folic acid-induced AKI [38], and found that a single dose given 18 h after cecal ligation and puncture and along with initial antibiotics was successful in reducing AKI [37]. Thus manipulation of cell-cycle may represent a new therapeutic strategy in the prevention and treatment of AKI.

These results are also important because they have implications for how we understand the pathogenesis of AKI. There is a growing appreciation for the concept of secondary injury to the kidney as danger signaling molecules (damage and pathogen-associated molecular patterns) are delivered to the renal tubule via both glomerular filtration and the blood stream [39]. These molecules are detected by pattern recognition receptors on the tubular cell surface where they initiate a cascade leading to inflammation and/or apoptosis. In essence, the available data suggest that cell-cycle arrest signaling is a protective response, but when engaged by multiple cells such that increases in markers like TIMP-2 and IGFBP7 can be detected in the urine, it is often followed by AKI. Furthermore, if cell-cycle arrest persists the result can become maladaptive and lead to a fibrosis phenotype. Early protection of cells might be achievable by supporting the cell's own self-preservation mechanisms including cell-cycle arrest. Conversely, once the danger is past, it may be important to rapidly reverse this process so that the adverse consequences including cell senescence and fibrosis are avoided. Thus, cell-cycle arrest activation and deactivation at critical clinical time points for a patient may prove to be targets of therapeutic intervention in the future.

CONCLUSIONS

It is now apparent that cell-cycle arrest has a 'dark side' where it heralds the onset of AKI and persistence of this signaling may

lead to maladaptive repair processes ultimately favoring fibrosis over regeneration. However, cell-cycle arrest also has a 'light side' in that its detection can be used as an early warning prior to actual damage. This warning may be quite useful in avoiding ongoing injury [40]. Intriguingly though, we might also be able to manipulate cell-cycle in ways that can foster the innate protective aspects of this response while avoiding the maladaptive aspects—turning the dark toward the light.

CONFLICT OF INTEREST STATEMENT

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