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RESEARCH ARTICLE

The role of IGFBP-1 in the clinical prognosis and pathophysiology of acute kidney injury

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Abstract

The ability to predict progression to severe acute kidney injury (AKI) remains an unmet challenge. Contributing to the inability to predict the course of AKI is a void of understanding of the pathophysiological mechanisms of AKI. The identification of novel prognostic biomarkers could both predict patient outcomes and unravel the molecular mechanisms of AKI. We performed a multicenter retrospective observational study from a cohort of patients following cardiac surgery. We identified novel urinary prognostic biomarkers of severe AKI among subjects with early AKI. Of 2,065 proteins identified in the discovery cohort, insulin-like growth factor binding protein 1 (IGFBP-1) was the most promising. We validated IGFBP-1 as a prognostic biomarker of AKI in 213 patients. In addition, we investigated its role in the pathophysiology of AKI using a murine model of cisplatin-induced AKI (CIAKI). Urinary IGFBP-1 concentration in samples collected from patients with stage 1 AKI following cardiothoracic surgery was significantly higher in patients who progressed to severe AKI compared with patients who did not progress beyond stage 1 AKI (40.28 ng/ml vs. 2.8 ng/ml, $P < 0.0001$) and predicted the progression to the composite outcome (area under the curve: 0.85, $P < 0.0001$). IGFBP-1 knockout mice showed less renal injury, cell death, and apoptosis following CIAKI, possibly through increased activation of the insulin growth factor receptor 1. IGFBP-1 is a clinical prognostic biomarker of AKI and a direct mediator of the pathophysiology of AKI. Therapies that target the IGFBP-1 pathways may help alleviate the severity of AKI.

NEW & NOTEWORTHY The ability to predict progression to severe AKI remains an unmet challenge. Early prognostic biomarkers of AKI hold promise to improve patient outcomes by early implementation of clinical therapy, as well as unravel the pathophysiological mechanisms of AKI. Here, we present a novel urinary biomarker, IGFBP-1, that predicts the progression to severe AKI following cardiac surgery. In addition, we show that IGFBP-1 mice are protected against CIAKI, suggesting a mechanistic role for IGFBP-1 in AKI.

acute kidney injury; biomarker; IGFBP-1; insulin-like growth factor binding protein 1

INTRODUCTION

Acute kidney injury (AKI) affects 10%–20% of hospitalized patients, including 50% of patients in the intensive care unit (ICU) (1, 2). Current definitions of AKI provide tools to diagnose and stage AKI but have significant limitations. The criteria used to define AKI depend only on urine output and creatinine values, which may not accurately reflect the severity or progression of AKI (3–5) and provide little guidance for management. Currently, the management of AKI is limited to the removal of the underlying etiology, avoiding secondary insults, and assessing the need for renal replacement therapy (RRT) (6). The discovery of novel prognostic biomarkers could enhance the ability to diagnose AKI, predict the progression and severity of AKI, help guide management, and enrich clinical trials by identifying patient populations at higher risk of developing severe AKI (7). Biomarkers also hold the promise of unraveling underlying pathophysiological mechanisms of AKI. Reverse translation of promising biomarkers discovered in the clinical setting could guide preclinical research to gain a more robust understanding of the pathophysiological mechanisms of AKI before heavy investments in human clinical trials (7–9).

Several biomarkers have shown promise for the early diagnosis and prediction of patients at high risk of progression to moderate or severe AKI (10–12). However, only two biomarkers

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are currently FDA-approved for clinical use. Urinary concentrations of neutrophil gelatinase-associated lipocalin (NGAL) are highly accurate [area under the curve (AUC): 0.998] at predicting progression to moderate or severe AKI within 72 h following cardiothoracic surgery in the pediatric population when collected at the time of cardiothoracic surgery (11); however, it is not approved for use in adults over the age of 21. Nephrocheck ([TIMP-2] × [IGFBP7]) is a point-of-care assay measuring the product of urinary concentrations of tissue inhibitor of metalloprotease 2 (TIMP-2) and insulin-like growth factor binding protein 7 (IGFBP-7) that is approved to predict the development of moderate to severe AKI (KDIGO stages 2 to 3) in critically ill patients when collected within 24 h of admission to the ICU (13, 14). However, [TIMP-2] × [IGFBP7] does not distinguish which patients will progress to the most severe stage of AKI or require RRT. Thus, there remains a pressing need to identify novel prognostic biomarkers of AKI to stratify the risk of progression for specific patients and to enrich clinical trials (7).

Insulin-like growth factor binding protein 1 (IGFBP-1) is a member of a highly conserved family of six proteins (IGFBP-1–6) that regulate cell growth, survival, and proliferation by sequestration of insulin-like growth factors (IGFs) or direct binding of its C-terminus arginine-glycine-aspartic acid (RGD) domain to integrin cell surface receptor (15, 16). IGFBP-1 is primarily synthesized in the liver; however, it is also thought to be expressed to a lesser degree in kidneys (17). Previous animal studies have explored the role of IGF-1 in AKI, but these studies did not address the effect of IGFBP-1 in modulating IGF-1 concentrations at the effector sites (18–20). IGFBP-1 has also been implicated in the pathology of chronic kidney disease (21) and glomerulopathy (22–24), mainly through binding of integrin receptors. However, the pathophysiological role of IGFBP-1 during acute kidney injury has not been investigated.

In our current study, we identified IGFBP-1 as a prognostic clinical biomarker of AKI and implicated a pathophysiological role for IGFBP-1 in an animal model of AKI. First, we performed a discovery analysis to identify candidate biomarkers that predict the need for RRT in a cohort of patients who developed severe AKI following cardiothoracic surgery. We found six candidate biomarkers in which the urinary concentration increased in patients who required RRT following cardiothoracic surgery compared with patients who did not develop AKI postoperatively. Of these six potential biomarkers, IGFBP-1 demonstrated the most profound increase among patients who progressed to require RRT. We then validated its prognostic ability in a larger cohort of patients. Finally, we demonstrate a pathophysiological role for IGFBP-1 during AKI by showing that the severity of AKI, cell death, and apoptosis is attenuated in IGFBP-1 knockout (KO) mice possibly through increased activation of the insulin growth factor receptor 1 (IGF-1r).

MATERIALS AND METHODS

Patient Selection and Sample Collection

Urine samples were obtained from a cohort of patients enrolled in the SAKInet consortium between 2008 and 2013. Sample collection was approved by the local Institutional Review Boards of each collecting center. Samples used in this cohort were collected at The Medical University of South Carolina (MUSC), Duke University, and George Washington University. Written informed consent was obtained from all subjects. Samples used in this study were deidentified and used under a nonhuman subjects' determination from the University of Arkansas for Medical Sciences Institutional Review Board. The selection of samples for this study is outlined in Fig. 1. Urine samples from 225 subjects met the inclusion criteria for this study (baseline creatinine < 3 mg/dL; development of KDIGO stage 1 AKI within 72 h of surgery; and urine sample collection within 72 h of surgery). All subjects were followed until hospital discharge or death. Data from 12 subjects were excluded due to insufficient samples to perform the analysis.

Discovery Analysis

In the discovery analysis, we performed a proteomic analysis of urine samples obtained from 30 patients with stage 1 AKI at the time of collection, which was within 24 h after surgery: 10 who required RRT within 10 days of surgery and 20 patients who did not progress beyond stage 1 AKI within 10 days after surgery and did not die within 30 days of surgery. The characteristics of these patients are shown in Table 1. Each subject requiring RRT was matched to two control subjects with the same surgery type and baseline serum creatinine within 0.2 mg/dL. The samples were then randomized into two blocks of 5 patients who required RRT and 10 matched controls. Candidate biomarkers were identified using LC-MS/MS, as described next. The 30 samples used for the discovery analysis were also later included in the validation study.

Liquid Chromatography/Tandem Mass Spectrometry for Proteomic Analysis of Human Urine Samples and Mouse Kidney Tissue Homogenates

Following sample collection, urine or mouse tissue homogenate samples were frozen at −80°C. Before LC-MS/MS, samples were thawed on ice. Protein concentration was

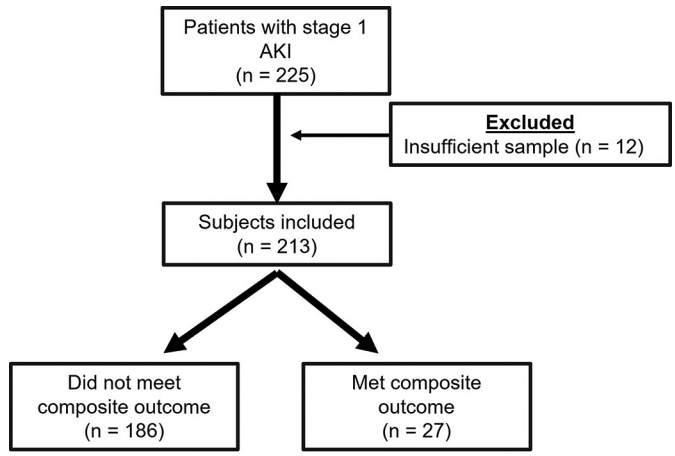

Figure 1. Flowchart of patient enrollment in the validation study. Samples from 225 patients with stage 1 AKI were collected within 72 h of cardiothoracic surgery. Twelve samples were insufficient for analysis, leaving a total of 213 patients for analysis of which 27 patients met criteria for inclusion in the composite primary outcome group. AKI, acute kidney injury.

Table 1. *Demographics for patients included in the discovery cohort*

	Controls ($n = 20$)	Cases ($n = 10$)
Female	7 (35%)	4 (40%)
Ethnicity (Caucasian)	14 (70%)	5 (50%)
Age, yr	69 ± 2	60 ± 7
Weight, kg	99 ± 5	108 ± 11
IABP	3 (15%)	1 (10%)
History of CHF	6 (30%)	5 (50%)
Previous cardiac surgery	4 (20%)	5 (50%)
Diabetes	12 (60%)	8 (80%)
COPD	1 (5%)	1 (10%)
Peripheral vascular disease	2 (10%)	4 (40%)
History of CVA	2 (10%)	0 (0%)
Emergent surgery	4 (20%)	1 (10%)
CABG	10 (50%)	5 (50%)
Valve surgery	6 (30%)	3 (30%)
CABG + valve surgery	4 (20%)	2 (20%)
Cardiopulmonary bypass	20 (100%)	10 (100%)
Bypass time, min	122 ± 12	185 ± 79
Baseline creatinine	1.3 ± 0.05 mg/dL	1.3 ± 0.25 mg/dL
Collection creatinine	2.0 ± 0.08 mg/dL	2.3 ± 0.59 mg/dL
Δ Creatinine	0.7 ± 0.05 mg/dL	1.0 ± 0.38 mg/dL
% Change creatinine	53 ± 3%	75 ± 20%
Time to collection, h	28.8 ± 2.4 h	26.4 ± 9.6 h
Maximum creatinine	2.0 ± 0.09 mg/dL	5.0 ± 1.7 mg/dL
RRT	0 (0%)	10 (100%)
30-day mortality	0	3 (30%)

CABG, coronary artery bypass graft; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CVA, cerebral vascular accident; Dz, disease; IABP, intra-aortic balloon pump; RRT, renal replacement therapy.

measured with QUANTTEST Red (Quantimetrix, Redondo Beach, CA) by assaying samples in duplicates in a 96-well plate. Proteins were digested using the Filter-Assisted Sample Preparation (FASP) protocol on YM-10 Microcon filter units. Proteins were denatured and alkylated, followed by digestion with trypsin. The peptides were separated online by reverse phase at 350 nL/min using a 5 × 0.2 mm guard column and a 100 × 0.1 mm analytical C18 BEH 1.7 μm column (Waters) with a nanoACQUITY UPLC system (Waters). Peptides were eluted onto the Thermo Scientific Fusion Tribrid mass spectrometer using a 120-min gradient from 98:2 to 40:60 buffer A:B ratio (buffer A = 0.1% formic acid, 0.05% acetonitrile; buffer B = 0.1% formic acid, 75% acetonitrile). Eluted peptides were ionized by electrospray (2.0 kV) followed by MS/MS analysis using collision-induced dissociation. Survey scans of peptide precursors from 300 to 1,500 mass-to-charge ratio (m/z) were performed at 500 K resolution (at 400 m/z) in the orbitrap with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1.6 Th with the quadrupole, higher energy collision-induced dissociation (HCD) fragmentation with a normalized collision energy of 30, and rapid scan MS^2 analysis in the ion trap. The MS ion count target was set to 10^4 and the maximum injection time at 35 ms. For each fragmentation cycle, a full MS scan was acquired once every 3 s, the nested MS^2 scans were performed at a maximum rate between the MS scans.

LC-MS/MS data were analyzed using MS-Amanda Proteome Discoverer (Research Institute of Molecular Pathology, Vienna, Austria; v. 2.1.0.81) and Mascot (Matrix Science, London, UK; v. 2.1.0.81). Search engines used the December 2015 version of uniprot human FASTA database with the addition of common contaminants using a semi-tryptic search with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.0 parts per million. Carbamidomethyl of cysteine was specified as a fixed modification. Pyro-Glu of glutamine, deamidation of asparagine, oxidation of histidine, and acetylation of the n-terminus were specified as variable modifications. Scaffold (v. 4.8.1; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >99.0% probability to achieve an FDR <0.1% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at >99.0% probability and contained at least three identified peptides. Protein probabilities were assigned by the ProteinProphet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Normalized intensity values for each protein were exported from Scaffold to Microsoft Excel. Mean values, fold change values, and P values using an unpaired t test assuming unequal variance were calculated.

Validation Analysis

Because urinary IGFBP-1 showed the largest fold increase (15-fold) of all potential biomarkers identified in patients who required RRT in the discovery analysis, we measured urinary IGFBP-1 concentrations in 213 subjects who developed AKI following cardiothoracic surgery to validate its potential as a prognostic biomarker of AKI (Fig. 1). The primary outcome used to validate the performance was the composite of 30-day mortality, the need for RRT, or progression to KDIGO stage 3 AKI by serum creatinine staging within 10 days of surgery.

Measurement of Urinary Biomarkers and Serum Creatinine Concentrations in Human Samples

Human urine IGFBP-1, NGAL, and [TIMP-2] × [IGFBP7] concentrations were measured using commercially available ELISA kits (RayBiotech, Norcross, GA). The urine creatinine concentrations were determined by kinetic Jaffe assay, using the Creatinine Reagent Set (Pointe Scientific, Canton, MI). All samples were assessed in duplicates.

Breeding and Genotyping of Wild-Type and IGFBP-1 KO Mice

B6.129X1-*Igfbp1*^{tm1Taub}/J (IGFBP-1 knockout with C57Bl/6J background) mice were obtained from The Jackson Laboratory (Bar Harbor, MN) and bred to maintain a homozygote colony of IGFBP1-KO mice. C57Bl/6J (wild-type) mice originally obtained from The Jackson Laboratory were used as controls. Offspring were weaned at 21 days of age and marked with numbered ear tags. For genotyping, a small piece of tail (<5 mm) was cut with sterile surgical scissors, whereas the mouse was immobilized by hand. Southern blot was performed to identify the presence of wild-type (550 bp) and KO (320 bp) alleles using the following primers: Common AAC AAC TGT GGG CAT TGT CA; Knockout TGG ATG TGG AAT GTG TGC GAG; and wild type AGC AGG CTG TGG ATG AGA CT.

Cisplatin-Induced AKI in Mice

Eight- to ten-week-old male mice underwent a single Cisplatin (20 mg/kg), or saline (control) injection administered intraperitoneal (IP) with a 1-mL syringe and 25-gauge needle. Analgesic (buprenorphine, 0.1 mg/kg via SQ injection) was administered after cisplatin injection if the animal showed signs of pain or distress. Seventy-two hours after the cisplatin or saline injections, mice were euthanized via overdose of carbon dioxide followed by cervical dislocation. Blood and kidneys were collected at the time of euthanasia.

Determination of Mouse Blood Urea Nitrogen and Creatinine Levels

Blood urea nitrogen (BUN) and creatinine were determined from serum collected at the time of euthanasia using diagnostic kits from Pointe Scientific (Canton, MI).

Periodic Acid-Schiff Histology of Mouse Kidney Tissue

Periodic acid-Schiff (PAS)-stained sections of kidney tissue were scored blindly by a renal pathologist in a semiquantitative manner. For each animal, at least 10 high-power fields of view (×400) were examined. The percentage of tubules that displayed cellular necrosis, loss of brush border, cast formation, vacuolization, or tubular dilation was scored as follows: 0 = no evidence, 1 = <25% involvement, 2 = 26%–50% involvement, 3 = >50% involvement.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay

Mouse kidney samples were embedded in paraffin, and 5-micron sections were prepared. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed and quantified, as previously described (25). Briefly, the samples were stained using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Cells were counterstained with 4′,6-diamidino-2-phenylindol (DAPI) to visualize cell nuclei and mounted under cover slips with a Prolong Antifade kit (Invitrogen, Carlsbad, CA), and images were acquired using an Olympus IX-81 inverted microscope (Olympus America, Center Valley, PA) equipped with a Hamamatsu ORCA-ER monochrome camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan).

Measurement of Caspase 3/7 Activity

Caspase 3/7 activity was measured in kidney tissue homogenates with Promega Caspase-Glo 3–7 Assay (Madison, WI) according to the manufacturer's protocol. All samples were run in triplicate and analyzed with a SpectramaxM5e Multimode plate reader (Molecular Devices, LLC, San Jose, CA).

Measurement of BCL-xl Protein Concentrations in Kidney Tissue Homogenates

BCL-xl protein concentrations were measured using a commercially available ELISA kit (Abcam, Boston, MA). Samples were run in duplicate on a 96-well plate. BCL-XL protein concentrations were normalized for the total protein concentration of the tissue homogenate. Total tissue homogenate protein concentrations were measured using the bicinchoninic acid (BCA) assay.

Measurement of Phosphorylated Protein Kinase B and Phosphorylated Focal Adhesion Kinase in Mouse Kidney Tissue Homogenates

Tissue concentrations of total protein kinase B (pan-AKT) and phosphorylated protein kinase B (p-AKT) were measured simultaneously in the same sample using a commercially available ELISA kit (RayBiotech, Norcross, GA). The measured OD450 for p-AKT was compared against pan-AKT to determine the percentage of p-AKT (p-AKT/pan-AKT × 100). Phosphorylated focal adhesion kinase (FAK) levels were measured using a commercially available ELISA kit (RayBiotech, Norcross, GA). For pan-AKT, p-AKT and phosphorylated focal adhesion kinase (p-FAK) samples were run in duplicates on a 96-well plate. Values were normalized for tissue homogenate protein concentration as determined by a BCA assay.

Statistical Analysis

Normalized intensity values (quantitative value) for each protein were exported from Scaffold to Microsoft Excel. Mean values, fold change values, and *P* values were assessed using an unpaired *t* test assuming unequal variance. We compared the mean spectral count values for each protein between the control and case groups. We set a *P* value threshold <0.01 to consider the proteins to be valuable prognostic biomarkers. Statistical analyses of the validation cohort were performed using IBM SPSS version 24 software and GraphPad Prism 9.2.0. For patient demographic and validation study data, an unpaired *t* test was used to compare parametric variables and an unpaired *t* test with Mann–Whitney *U* correction was used to compare nonparametric data between two groups. GraphPad Prism 9.2.0 was used to analyze data from animal studies. A one-way ANOVA with Tukey's post hoc analysis was used to compare parametric data when multiple groups were present. Data for histological scoring of mouse PAS staining were analyzed using a nonparametric Kruskal–Wallis with Dunn's multiple comparison test. Statistical significance was set at $P < 0.05$. Receiver operator characteristic (ROC) curves were used to quantify the ability of a biomarker to predict the primary outcome. Youden's test was used to set the optimal cutoff values for urinary IGFBP-1 and NGAL. The optimal cutoff value for [TIMP-2] × [IGFBP7] was set at 0.3, as is used commercially. Youden's test of our patient cohort also confirmed the optimal cutoff as 0.3 for [TIMP-2] × [IGFBP7]. We compared area under the curve (AUC) values of receiver operator characteristics (ROC) between biomarkers using GraphPad Prism 9.2.0. Independent ROC curves were compared with a one-tailed Hanley–McNeil analysis. Schematic figures were generated using BioRender.com.

Study Approvals

Written informed consent was obtained in accordance with the institutional review boards at the SAKInet institutions, as described previously (26). Permission to perform the studies described here was obtained from the IRB at the University of Arkansas for Medical Sciences (205605). Animal studies were approved by the Institutional Animal

Care and Use Committee and Research Safety Committee of the Central Arkansas Veteran's Healthcare System under project number 1448807-10.

RESULTS

Discovery Analysis

From the 30 human urine samples selected for the discovery cohort, we identified 2,065 high-confidence proteins of which 126 had *P* values of <0.01 for comparison between the groups. Patient demographics of the 30 subjects included in the discovery analysis are shown in Table 1. The urinary concentrations of six proteins were increased in subjects who needed RRT following cardiothoracic surgery (IGFBP-1, heme-binding protein 2, myoglobin, adipocyte-type fatty acid binding protein, thymosin β-4, and heart-type fatty acid binding protein), and 120 were decreased. Of the six proteins that increased, IGFBP-1 exhibited the largest fold increase in patients who required RRT (1 ± 0.4 vs. 15 ± 4.1, $P < 0.01$, Table 2, Fig. 2*A*) compared with patients who did not develop AKI (controls). Median amino acid coverage was 0% and 26%, respectively. Total amino acid coverage across all samples was 47%. We compared the number of peptides observed between controls and RRT. A larger number of peptides were seen in those patients who progressed to the composite outcome. Twenty-seven spectra for 12 unique peptide sequences were identified from IGFBP-1 (Fig. 2*B*).

Patient Characteristics in the Validation Cohort

Of the 213 patients included in the validation cohort, 27 met the primary outcome of stage 3 AKI, the need for RRT, or death (Fig. 1). There was no difference between the control group and patients who progressed to the primary end point regarding age, weight, gender, or ethnicity. There was no difference between controls and progressors regarding pre-existing medical comorbidities including diabetes, systolic heart failure with ejection fraction < 35%, or baseline serum creatinine. Surgical parameters including history of previous cardiac surgery, the need for pre-operative intra-aortic balloon pump (IABP), the mean cardiac bypass time, or type of surgical procedure were not statistically different between

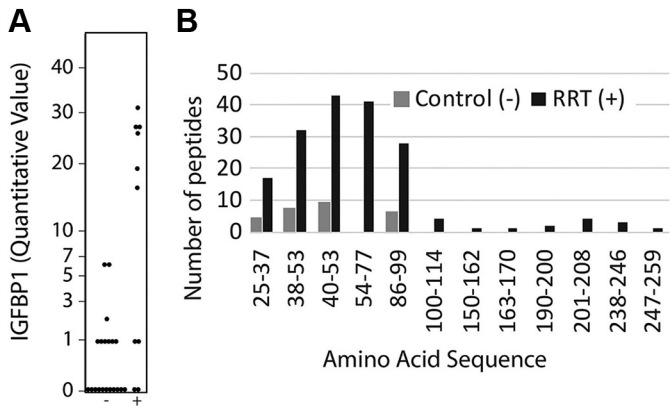

Figure 2. IGFBP-1 abundance and peptide sequences in the discovery proteomic analysis. The relative abundance of spectra for IGFBP-1 (*A*) is shown for urine samples from 20 control subjects who did not progress beyond AKI stage 1 (−) and 10 subjects who required renal replacement therapy (+). Quantitative value is a normalized representation of spectral counts. Each dot represents the normalized abundance of IGFBP-1 in urine for one subject. The number of peptides observed for each peptide sequence in control subjects and subjects who required renal replacement therapy are presented in *B*. AKI, acute kidney injury; IGFBP-1, insulin-like growth factor binding protein 1.

controls and patients who progressed to severe AKI. There was no difference in the average time of sample collection between the two groups (Table 3).

Measurement of Urinary Concentrations of IGFBP-1, NGAL, and [TIMP-2] × [IGFBP7]

Urinary IGFBP-1 concentrations were significantly higher in the patients who met the primary composite outcome compared with patients who did not progress beyond stage 1 or 2 AKI (134.2 ± 37.7 vs. 18.8 ± 8 ng/mL, $P < 0.0001$, $n = 213$) (Fig. 3*A*). Urinary concentrations of IGFBP-1 also increased progressively with each stage of AKI [stage 1: median 2.8 ng/mL, interquartile range (IQR) 1.34–10.9 ng/mL, 95% confidence interval (CI) 2.01–3.72 ng/mL, $n = 163$ vs. stage 2: median 2.39 ng/mL, IQR: 0.86–23.26 ng/mL, 95% CI: 0.95–22.74, $n = 22$ vs. stage 3: median 44.17 ng/mL, IQR: 9.74–208.8 ng/mL, 95% CI: 10.2–162 ng/mL, $n = 22$, stage 1 vs. stage 2, $P = 0.968$, stage 1 vs. stage 3, $P < 0.0001$, stage 2 vs.

Table 2. *Potential novel prognostic biomarkers of AKI*

Protein	Gene Name	Accession Number	Predicted Molecular Weight	% Protein Coverage	Exclusive Unique Peptides Observed	Total Spectra Observed	Control (−)	RRT (+)	*P* Value
IGFBP-1	IGFBP1	PO8833	28 kDa	47%	12	27	1 ± 0.4	15 ± 4.1	0.008
Heme-binding protein 2	HEBP2	Q9Y5Z4	23 kDa	53%	8	11	2.5 ± 0.7	10 ± 2.3	0.007
Myoglobin	MB	P02114	17 kDa	89%	27	75	13.1 ± 0.8	49 ± 8.8	0.003
Fatty acid binding protein (adipocyte)	FABP4	P15090	15 kDa	70%	11	31	8.0 ± 1.6	25 ± 4.5	0.004
Thymosin β-4	TMSB4X	P62328	5 kDa	93%	8	29	13.2 ± 2.0	29 ± 3.9	0.003
Fatty acid binding protein (heart)	FABP3	P05413	15 kDa	76%	10	50	22.1 ± 3.6	42 ± 5.0	0.005

Six potential urinary biomarkers were identified in the discovery cohort analysis which increased in patients that required RRT. IGFBP-1 showed the largest fold increase of the six biomarkers. AKI, acute kidney injury; IGFBP-1, insulin-like growth factor binding protein 1; RRT, renal replacement therapy.

Table 3. *Demographics for patients included in the validation study*

Parameter	Controls (186)	Outcome (27)	*P* Value
Age, yr	66.1 ± 0.88	69.9 ± 2.6	$P = 0.120$
Weight, kg	89.9 ± 1.54	87.5 ± 4.97	$P = 0.569$
Gender, % male	67.6 ± 3.4	58.6 ± 9.3	$P = 0.399$
Ethnicity, % Caucasian	69.2 ± 3.4	68.9 ± 8.7	$P > 0.999$
Diabetes, % patients	39.3 ± 3.6	41.4 ± 9.3	$P = 0.841$
Baseline creatinine, mg/dL	1.14 ± 0.02	1.28 ± 0.10	$P = 0.055$
Ejection fraction < 35%, % patients	33.5 ± 3.5	42.1 ± 8.1	$P = 0.352$
Previous cardiac surgery, % patients	15.7 ± 2.7	23.7 ± 6.9	$P = 0.345$
Preoperative IABP, % patients	9.6 ± 2.2	13.8 ± 6.5	$P = 0.507$
Cardiopulmonary bypass, % patients	86.7 ± 2.5	84.2 ± 5.9	$P = 0.796$
Bypass time, min	157.4 ± 5.1	177.1 ± 14.6	$P = 0.118$
Sample collection (hours postoperative), h	27.1 ± 0.9	28.7 ± 2.7	$P = 0.549$

There was no difference between patients that met the composite outcome compared with control samples regarding age, weight, gender, ethnicity, chronic comorbid conditions (diabetes, baseline creatinine, and congestive heart failure), surgical parameters (previous cardiac surgery, preoperative IABP, use of cardiopulmonary bypass, or bypass time), or time of sample collection. IABP, intra-aortic balloon pump. Data are expressed as value ± standard error of the mean (SE). A *P* value < 0.05 was considered statistically significant.

stage 3, $P < 0.001$) (Fig. 3*B*). There was no difference between the individual groups who comprised the composite primary outcome (stage 3 AKI by KDIGO creatinine median: 44.17 ng/mL, IQR: 9.75–208.8 ng/mL, 95% CI: 10.2–162.0 ng/mL, $n = 22$ vs. stage 3 AKI with RRT median: 81.1 ng/mL, IQR: 13.38–353.2 ng/mL, 95% CI: 10.2–365.3 ng/mL, $n = 14$ $P > 0.999$ vs. 30-day mortality median: 40.41 ng/mL, IQR: 9.72–333.1 ng/mL, 95% CI: 4.99–422.2 ng/mL, $n = 9$, $P = 0.907$). Please note that some subjects qualified for inclusion in the primary outcome cohort by meeting more than one inclusion criteria. Urinary concentrations of [TIMP-2] × [IGFBP7] (0.87 ± 0.1 vs. 1.92 ± 0.5, $P < 0.01$, $n = 154$) (Fig. 3*C*) and NGAL (82.8 ± 16.6 vs. 220.5 ± 101, $P < 0.05$, $n = 150$) (Fig. 3*D*) were also increased, but not to the degree of IGFBP-1 compared with the primary composite outcome.

Comparison of Urinary IGFBP-1 Concentrations against Creatinine

The prognostic ability of urinary IGFBP-1 concentration to predict the progression to severe AKI was determined by the AUC of the ROC. The AUC for urinary IGFBP-1 concentrations was 0.85 (95% CI: 0.77–0.91, $P < 0.0001$). Using an optimal set point for urinary IGFBP-1 concentration of 10.2 ng/mL, we were able to predict the progression to the primary outcome with a sensitivity of 76.9%, a specificity of 74.1%, and a likelihood ratio of 2.97 (Table 4). Similarly, the AUC of IGFBP-1/uCr was 0.83 (95% CI: 0.75–0.91, $P < 0.0001$). The ability of urinary IGFBP-1 to predict progression to the primary outcome was better than serum creatinine at the time of collection (AUC: 0.69, 95% CI: 0.57–0.80) and the change in serum creatine from time of collection (AUC: 0.70, 95% CI: 0.59–0.82) (IGFBP-1 vs. creatinine at collection, $P < 0.05$ and IGFBP-1 vs. delta creatinine, $P < 0.05$) (Fig. 4*A*).

Comparison of Urinary IGFBP-1 against Other Prognostic Biomarkers of AKI

In addition, we performed a direct comparison of urinary IGFBP-1 concentrations against two established prognostic biomarkers of moderate to severe AKI: urinary NGAL and [TIMP-2] × [IGFBP7]. Urinary IGFBP-1 concentrations (AUC: 0.85, 95% CI: 0.77–0.91, $P < 0.0001$, $n = 213$) accurately predicted the progression to the composite outcome. The values for AUC of IGFBP-1 were numerically higher compared with urinary concentrations of NGAL (AUC: 0.72, 95% CI: 0.60–0.83, $P < 0.05$, $n = 150$) and [TIMP-2] × [IGFBP7] (AUC: 0.71, 95% CI: 0.60–0.83, $P < 0.01$, $n = 154$) (Fig. 4*B*). Urinary IGFBP-1 concentrations were statistically a stronger predictor of the primary outcome compared with [TIMP-2] × [IGFBP7] ($P < 0.05$); however, the AUC for IGFBP-1 was numerically, but not statistically, different than NGAL ($P = 0.06$). [TIMP-2] × [IGFBP7] showed a small numerical increase in sensitivity (80% vs. 76.9%) compared with IGFBP-1, [TIMP-2] × [IGFBP7], but had a much lower numerical value for specificity (44% vs. 74.1%) and positive predictive value (PPV) (17.2% vs. 29.4%). IGFBP-1 displayed a numerically higher sensitivity (76.9 vs. 70.6%) and specificity than NGAL (74.1 vs. 66.2%). IGFBP-1 also showed a higher numerical value for PPV, negative predictive value (NPV), and likelihood ratio when compared with both NGAL and [TIMP-2] × [IGFBP7] (Table 4).

The Effects of IGFBP-1 KO on Renal Function following Cisplatin Injection in Mice

To determine the effects of IGFBP-1 on cisplatin-induced acute kidney injury (CIAKI) in mice, we measured serum creatinine and BUN levels. Wild-type (WT) mice with CIAKI showed a significant increase in serum creatinine levels when compared with saline-injected WT controls (0.3 ± 0.06 mg/dL vs. 1.5 ± 0.29 mg/dL, $P < 0.0001$, $n = 10–12$), whereas IGFBP-1 KO mice with CIAKI showed an attenuated rise in serum creatinine levels compared with WT mice with CIAKI (0.5 ± 0.08 mg/dL vs. 1.5 ± 0.29 mg/dL, $P < 0.001$, $n = 10–11$) (Fig. 5*A*). Similarly, WT mice with CIAKI showed a significant increase in BUN levels compared with WT controls (24.2 ± 0.6 mg/dL vs. 150.1 ± 13.3 mg/dL, $P < 0.0001$, $n = 10–12$). IGFBP-1 KO mice with CIAKI showed a blunted increase in BUN levels when compared with WT mice that received cisplatin (91.7 ± 15.8 mg/dL vs. 150.1 ± 13.3 mg/dL, $P < 0.001$, $n = 10–11$) (Fig. 5*B*).

The Effect of IGFBP-1 KO on Renal Tubular Damage

To examine the effects of IGFBP-1 KO on renal tubular damage, periodic acid Schiff (PAS)-stained sections of kidney tissue were examined and scored blindly by a renal pathologist.

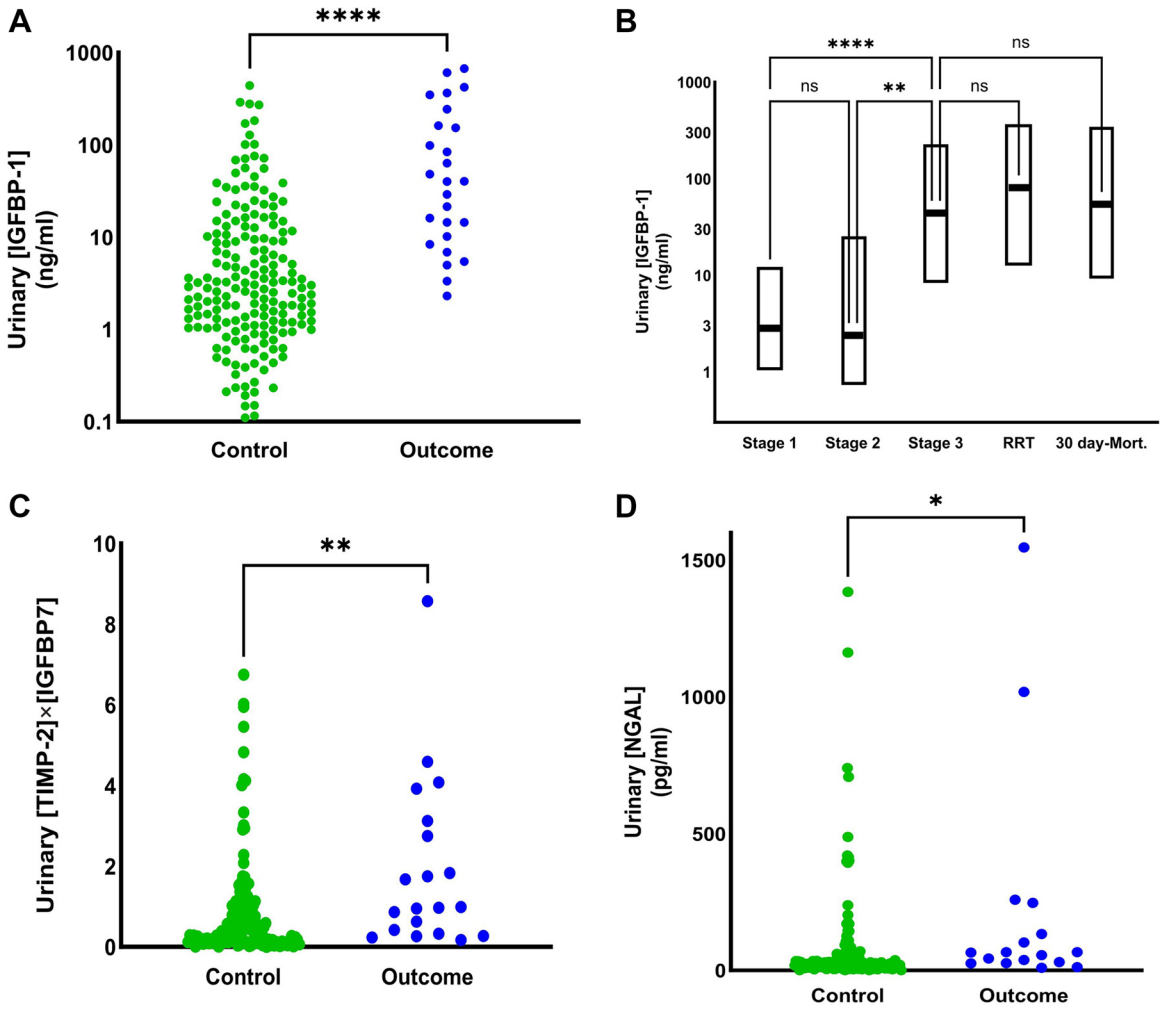

Figure 3. Urinary concentrations of urinary biomarkers. Shown are the measured concentrations of urinary IGFBP-1 (*A*), [TIMP-2] × [IGFBP7] (*C*), and NGAL (*D*) for patients who met the primary outcome compared with controls. Each individual data point represents a measured value (IGFBP-1 $n = 213$, [TIMP2] × [IGFBP7] $n = 154$, NGAL $n = 150$). Urinary concentrations of IGFBP-1 separated by stage of AKI, and each individual inclusion criteria of the composite outcome are shown in *B*. The horizontal bars represent the median value, and the surrounding boxes represent the interquartile range. The range of data for the individual groups is as follows (stage 1: 0.111–439.5; stage 2: 0.069–289.6; stage 3: 2.307–1,160; RRT: 2.307–673.9; 30-day mortality: 4.992–1,160). Please note that some patients qualified for primary outcome by more than one inclusion criteria. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, ns, not significant by unpaired *t* test (*A*, *C*, and *D*) or one-way ANOVA (*B*). AKI, acute kidney injury; IGFBP-1, insulin-like growth factor binding protein 1; NGAL, neutrophil gelatinase-associated lipocalin; RRT, renal replacement therapy; TIMP-2, tissue inhibitor of metalloprotease 2.

Saline-injected control WT mice (Fig. 6*A*) showed no renal tubular damage, whereas WT mice that received cisplatin (Fig. 6*B*) showed significantly increased renal tubular damage (mean pathology score: 0 ± 0 vs. 2.75 ± 0.08, $P < 0.0001$, $n = 12$) (Fig. 6*D*). IGFBP-1 KO mice injected with cisplatin (Fig. 6*C*) showed attenuated renal tubular damage compared with WT mice that received cisplatin (1.36 ± 0.36 vs. 2.75 ± 0.08, $n = 12$, $P < 0.05$) (Fig. 6*D*).

Effects of IGFBP-1 KO on Cell Death and Markers of Apoptosis

We next applied the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay to qualitatively and quantitatively assess the degree of cell death induced by cisplatin in mouse kidneys. TUNEL assay measures the presence of DNA 3′OH ends produced by DNA cleavage by endogenous DNases. The kidney is known to have high activity of DNase I that makes tubular epithelium sensitive to injury, particularly by cisplatin (27) and permits the use of TUNEL as a mechanistic and quantitative marker of irreversible cell death (28). In our experiments, TUNEL assay showed no irreversible cell death in control mice, markedly induced cell death in wild-type mice treated with cisplatin, and statistically significant (~threefold) mitigation of that injury in KO mice (Fig. 7, *A–D*). Because TUNEL does not distinguish between apoptotic and necrotic injury (28), we used caspase 3/7 activity as a proapoptotic marker and Bcl-xL as an anti-apoptotic marker to determine whether cisplatin injury and its amelioration were apoptotic by nature. Our experiments clearly showed cisplatin-induced apoptosis in WT mice and it was ameliorated in IGFBP-1 KO mice (Fig. 7, *E* and *F*). Taken together, these data indicate that inactivation of IGFBP-1 significantly mitigates kidney cell death and apoptosis induced by cisplatin.

Table 4. *Performance of prognostic biomarkers of AKI*

	IGFBP-1	NGAL	[TIMP-2] × [IGFBP7]
AUC	0.85	0.72	0.71
Optimal cut-off	10.2 ng/mL	37.6 ng/mL	0.3
Sensitivity	76.9%	70.6%	80%
Specificity	74.1%	66.2%	44%
Positive predictive value	29.4%	21.1%	17.2%
Negative predictive value	95.8%	94.6%	93.6%
Likelihood ratio	2.97	2.09	1.43
P value	<0.0001	<0.05	<0.01

Area under the curve (AUC) from the receiver operating characteristics, optimal cut-off values, sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio, and *P* value are shown for urinary concentrations of IGFBP-1, NGAL, and [TIMP-2] × [IGFBP7]. AKI, acute kidney injury; IGFBP-1, insulin-like growth factor binding protein 1; NGAL, neutrophil gelatinase-associated lipocalin; TIMP-2, tissue inhibitor of metalloprotease 2.

Evaluation of Changes in the Renal Proteome Following CIAKI

To determine if the development of CIAKI altered the expression of IGFBP-1 in the kidney, proteomic analysis was performed on mouse renal tissue homogenates. We compared protein expression between saline-injected control, saline-injected IGFBP-1 KO, wild-type CIAKI, and IGFBP-1 KO CIAKI mice (Fig. 8). We were unable to detect IGFBP-1 in renal tissue homogenates by proteomic analysis. However, changes in the renal proteome following CIAKI showed significant changes in expression of other known biomarkers of AKI in wild-type CIAKI mice including NGAL (23.94 ± 0.25 vs. 28.51 ± 0.68, $P < 0.0001$) and angiotensinogen (27.05 ± 0.09 vs. 30.95 ± 0.51, $P < 0.0001$), but not IGFBP-1 KO mice. We did not detect significant changes in IGFBP7 following CIAKI. The only other IGFBP detected was IGFBP-4, which was significantly decreased in IGFBP-1 KO mice following CIAKI (21.53 ± 0.09 vs. 20.49 ± 0.26, $P < 0.05$).

Measurement of Phosphorylated Protein Kinase B and Phosphorylated Focal Adhesion Kinase in Renal Tissue Homogenates

To further investigate the signaling mechanism of renal protection in IGFBP-1 KO mice, we measured concentrations of p-AKT and p-FAK in mouse renal tissue homogenates. IGFBP-1 modulates cellular signaling by one of two mechanisms: sequestration of IGF-1 or binding of its C-terminus RGD domain to integrin receptors. The immediate downstream effect of IGF-1 binding the IGF-1r is phosphorylation of AKT by phosphoinositide 3 kinase (PIP3K). Our data show a significant increase in the ratio of p-AKT to pan-AKT in IGFBP-1 KO mice compared with WT controls (4.04 ± 0.22% vs. 14.39 ± 3.83%, $P < 0.05$, $n = 7$–10) and cisplatin-injected WT mice (5.57 ± 0.25% vs. 14.39 ± 3.83%, $P < 0.05$, $n = 9$–10). Although there was a numerical increase in the ratio of p-AKT to pan-AKT in IGFBP-1 KO controls compared with WT controls, this difference was not statistically different (4.04 ± 0.22% vs. 11.47 ± 3.37%, $P = 0.11$, $n = 7$–11) (Fig. 9*A*). We found that total levels of p-FAK were reduced in IGFBP-1 KO controls (102.3 ± 21.96 vs. 48.65 ± 24.53 OD450/mg, $P < 0.05$, $n = 6$–7), WT CIAKI mice (102.3 ± 21.96 OD450/mg vs. 55.75 ± 13.91 OD450/mg, $P < 0.05$, $n = 7$–8), and IGFBP-1 KO CIAKI mice (102.3 ± 21.96 OD450/mg vs. 58.24 ± 20.16 OD450/mg, $P < 0.05$, $n = 7$–8) compared with WT controls (Fig. 9*B*). Collectively, these data suggest that attenuation of CIAKI in IGFBP-1 KO mice stems from increased activation of the IGF-1r.

DISCUSSION

In this study, we have identified IGFBP-1 as a prognostic biomarker to predict progression to severe AKI. Proteomic analysis of urine samples from a selected group of subjects enrolled in the SAKInet cohort identified IGFBP-1 as having

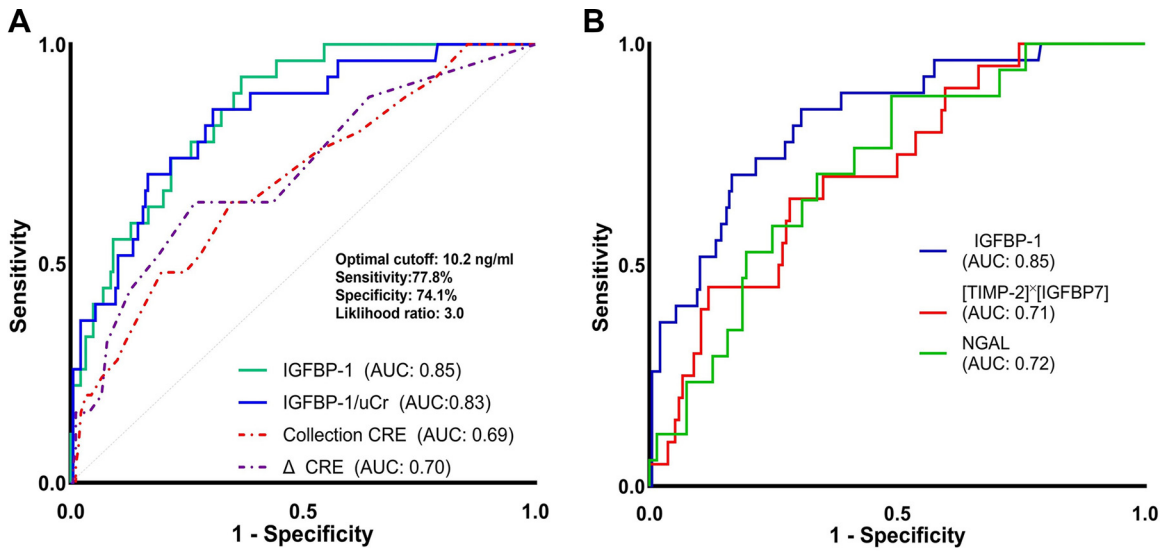

Figure 4. Receiver operating characteristics (ROCs) for biomarkers of AKI. Shown in *A* is the ROC comparison of urinary IGFBP-1 against the traditional clinical biomarker, creatinine. Values for the AUC, optimal cutoff, sensitivity, specificity, and likelihood ratio for IGFBP-1 are shown on the graph. The ROCs for IGFBP-1 ($n = 213$), [TIMP-2] × [IGFBP7] ($n = 154$), and NGAL ($n = 150$) are shown in *B*. AUC values for each biomarker are displayed on the graph. AKI, acute kidney injury; AUC, area under the curve; IGFBP-1, insulin-like growth factor binding protein 1; NGAL, neutrophil gelatinase-associated lipocalin; TIMP-2, tissue inhibitor of metalloprotease 2.

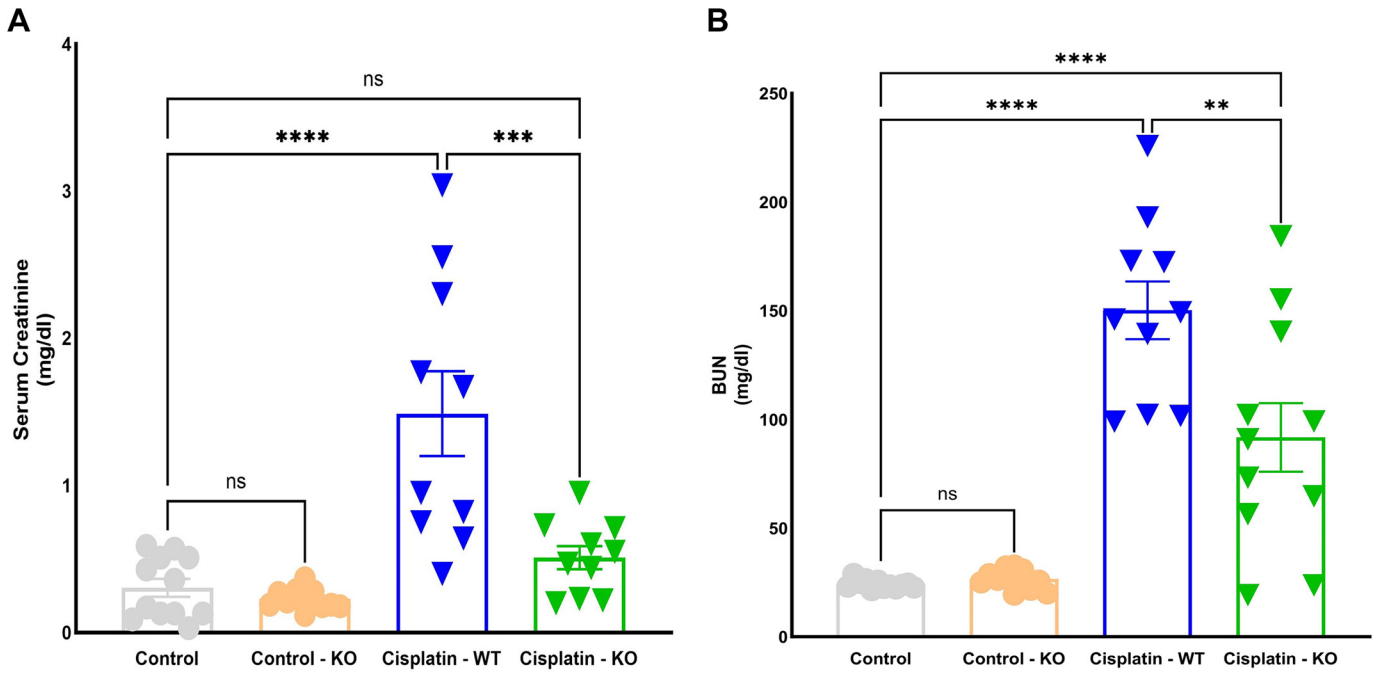

Figure 5. Effect of IGFBP-1 knockout on CIAKI. Serum creatinine values (*A*) and blood urea nitrogen (BUN) (*B*) measured from mouse serum following cisplatin or saline (control) injection are displayed. Each dot (control) or triangle (cisplatin injection) represents a measurement for an individual mouse. CIAKI, cisplatin-induced acute kidney injury; Cisplatin-KO, cisplatin injected IGFBP-1 knockout; Cisplatin-WT, cisplatin-injected wild type; Control, saline-injected wild type; Control-KO, saline-injected IGFBP-1 knockout; IGFBP-1, insulin-like growth factor binding protein 1. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant by one-way ANOVA.

the largest fold-increase among patients who required RRT within 10 days of surgery. In our previously published proteomic study, we observed that IGFBP-1 spectral counts were higher in subjects who developed severe AKI compared with controls (26). But in that study, there was limited coverage of the IGFBP-1 protein by LC MS/MS (27%), so it did not appear to be a strong predictor of severe AKI. Improved instrumentation allowed us to identify a more robust protein coverage (47%) of IGFBP-1 in the current study and increased confidence of its ability to predict progression to the composite outcome. The improved study design and data quality in this study identified IGFBP-1 as a biomarker that is likely to predict severe AKI.

There have been several promising prognostic biomarkers of AKI proposed; however, to date, no single biomarker has demonstrated the ability for widespread clinical implementation. Urinary concentrations of NGAL collected at the time of cardiothoracic surgery in pediatric patients are highly accurate (AUC: 0.998) at predicting AKI progression (11); however, its effectiveness in adult patients is less impressive (29, 30). [TIMP-2] × [IGFBP7] collected within the first 24 h of ICU admission can predict the progression to moderate or severe AKI (KDIGO stages 2 to 3) in critically ill patients within 72 h (13, 14). However, [TIMP-2] × [IGFBP7] does not distinguish which patients will progress to the most severe stage of AKI or require RRT. Due to its high false positive rate and several other factors, it is not recommended for use by The American Association for Clinical Chemistry (31) or the National Health Service in the United Kingdom (32). The furosemide stress test (FST), a test of renal tubular function, has been proposed to address this issue as it can predict progression to severe AKI and the future need for RRT with high sensitivity and specificity (33, 34), but its usefulness is limited as it cannot be followed serially due to the duration of the diuretic response. Despite significant progress in the field of prognostic biomarkers, it remains challenging to reliably predict the progression to severe AKI and the need for RRT.

Our validation studies demonstrated that the ability of urinary IGFBP-1 concentrations to predict the composite outcome is very good (AUC: 0.85). Urinary IGFBP-1 was superior to the traditional clinical biomarker, serum creatinine. In addition, IGFBP-1 was superior when compared against [TIMP-2] × [IGFBP7]. Although the AUC for IGFBP-1 was numerically higher than NGAL and there was a trend toward statistical difference ($P = 0.06$), the difference did not meet statistical significance. Our findings show that urinary IGFBP-1 was a stronger predictor of the progression to the composite outcome regarding numerical values of AUC, specificity, PPV, NPV, likelihood ratio, and *P* value of the independent ROC. Although [TIMP-2] × [IGFBP7] showed a mild numerical increase in sensitivity compared with IGFBP-1, IGFBP-1 showed higher numerical values of specificity, PPV, NPV, likelihood ratio, and *P* value. The slightly higher sensitivity of [TIMP-2] × [IGFBP7] is to be expected as both urinary NGAL[11 29] and [TIMP-2] × [IGFBP7] (13, 35) are designed to be collected before renal injury to predict the progression to moderate or severe AKI. However, our study was designed to detect progression to severe AKI in patients who had already developed stage 1 AKI. These differences in study design somewhat limit the direct comparisons between biomarkers and could explain the higher numerically higher sensitivity of urinary [TIMP-2] × [IGFBP7] compared with IGFBP-1.

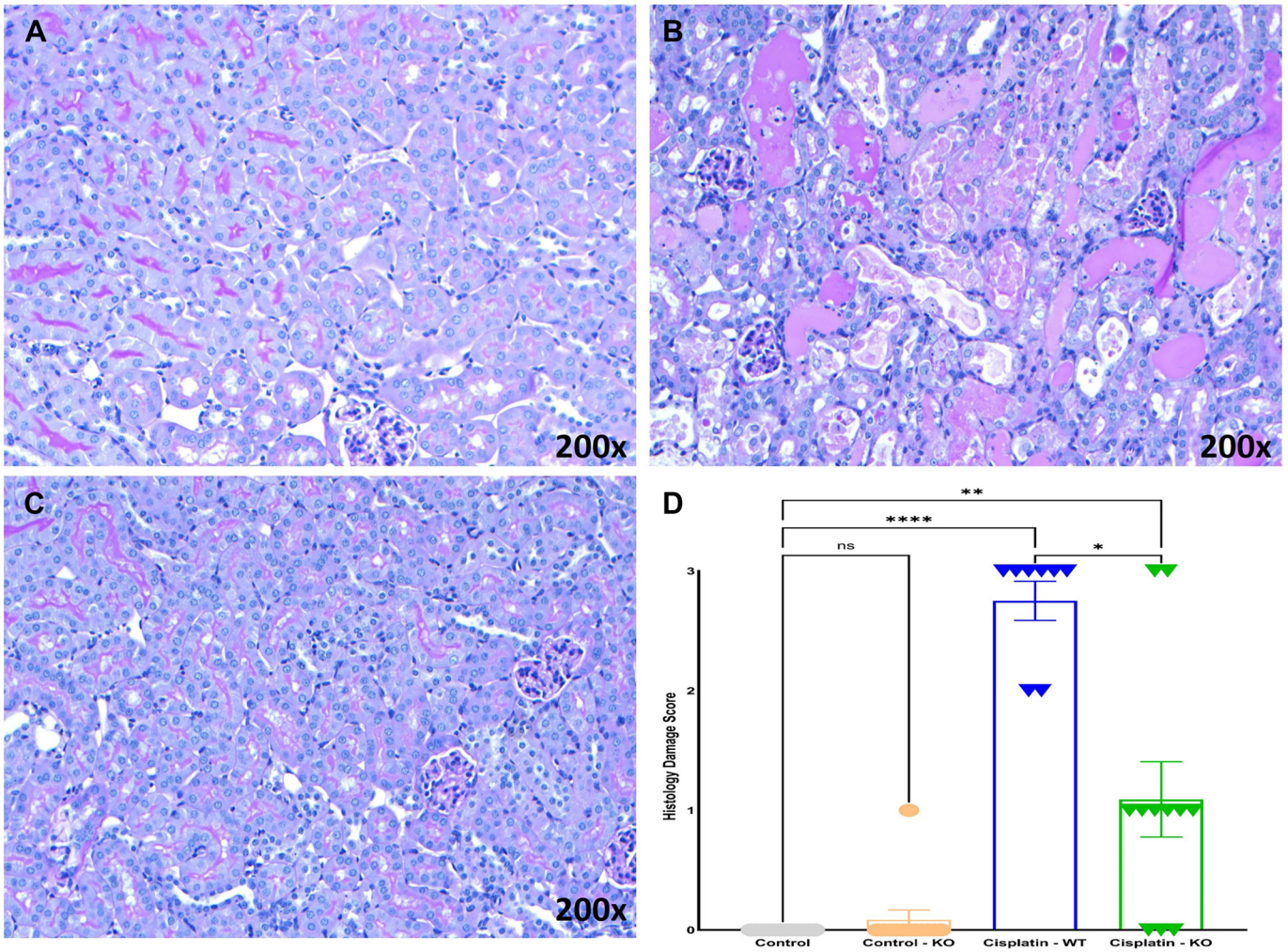

Figure 6. Effect of IGFBP-1 KO on renal tubular cell damage during CIAKI. Representative images of PAS staining of mice kidney tissue are shown at ×200 magnification. Wild-type sham mice are represented in *A*, wild-type mice that received cisplatin injection are found in *B*, and IGFBP-1 KO mice that received cisplatin injections are represented in *C*. Slides from each animal were scored blinding by a renal pathologist (*D*) for degree of tubular injury. Histology scoring was evaluated by Kruskal–Wallis with Dunn's multiple comparison test. Each dot (control) or triangle (cisplatin injection) represents scoring for an individual mouse. CIAKI, cisplatin-induced acute kidney injury; Cisplatin-KO, cisplatin-injected IGFBP-1 knockout; Cisplatin-WT, cisplatin-injected wild type; Control, saline-injected wild type; Control-KO, saline-injected IGFBP-1 knockout; IGFBP-1, insulin-like growth factor binding protein 1; KO, knockout; PAS, Periodic acid Schiff. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, ns, not significant.

In our study, urine concentration of IGFBP-1 in subjects who progressed to stage 2 AKI was not different from those who remained at stage 1. In contrast, concentrations were significantly increased in patients who progressed to stage 3 AKI by KDIGO creatinine criteria, needed RRT, or died within 30 days of surgery. This demonstrates that IGFBP-1 can discriminate patients who will reach moderate AKI from patients who will achieve more severe clinical outcomes. The ability to distinguish between subjects who will have moderate versus severe AKI will be important in guiding treatment, as well as enrollment and enrichment of clinical trials.

A lack of clinical therapies for AKI stems from a poor understanding of the molecular mechanism of AKI. Reverse translation of novel clinical biomarkers to preclinical animal studies could be a tool to shed light on the pathophysiology of AKI, ultimately leading to the development of new clinical therapies and diagnostic techniques (7–9). Several previously discovered biomarkers of AKI have also been implicated in the pathophysiology of AKI. NGAL is a 25 kDa protein of the lipocalin family. In the kidney, NGAL is basally expressed at low levels but is quickly upregulated within 3 h of the initial insult and readily detectable in the urine in both clinical studies and animal models AKI (36, 37). Mechanistically, it is believed that NGAL makes use of its ability to bind iron-siderophore complexes to regulate innate immunity (38, 39) and modulate function of iron-responsive genes (40, 41). Kidney Injury Molecule 1 (KIM-1) is a 38 kDa type I transmembrane glycoprotein with an extracellular immunoglobulin-like domain that is expressed at low basal levels in renal proximal tubule cells; however, it is dramatically upregulated following AKI (42–44). Rat models of AKI have shown increased KIM-1 following ischemia (42) and administration of nephrotoxins (45, 46). KIM-1 has been implicated to play an important role in proximal tubular cell recovery and regeneration by mediating phagocytosis of apoptotic bodies and cell debris (47–49). IGFBP-7 and TIMP-2 have been shown to play a role in cell cycle arrest in human microvascular cells (50) and cancer cell lines (51, 52), but

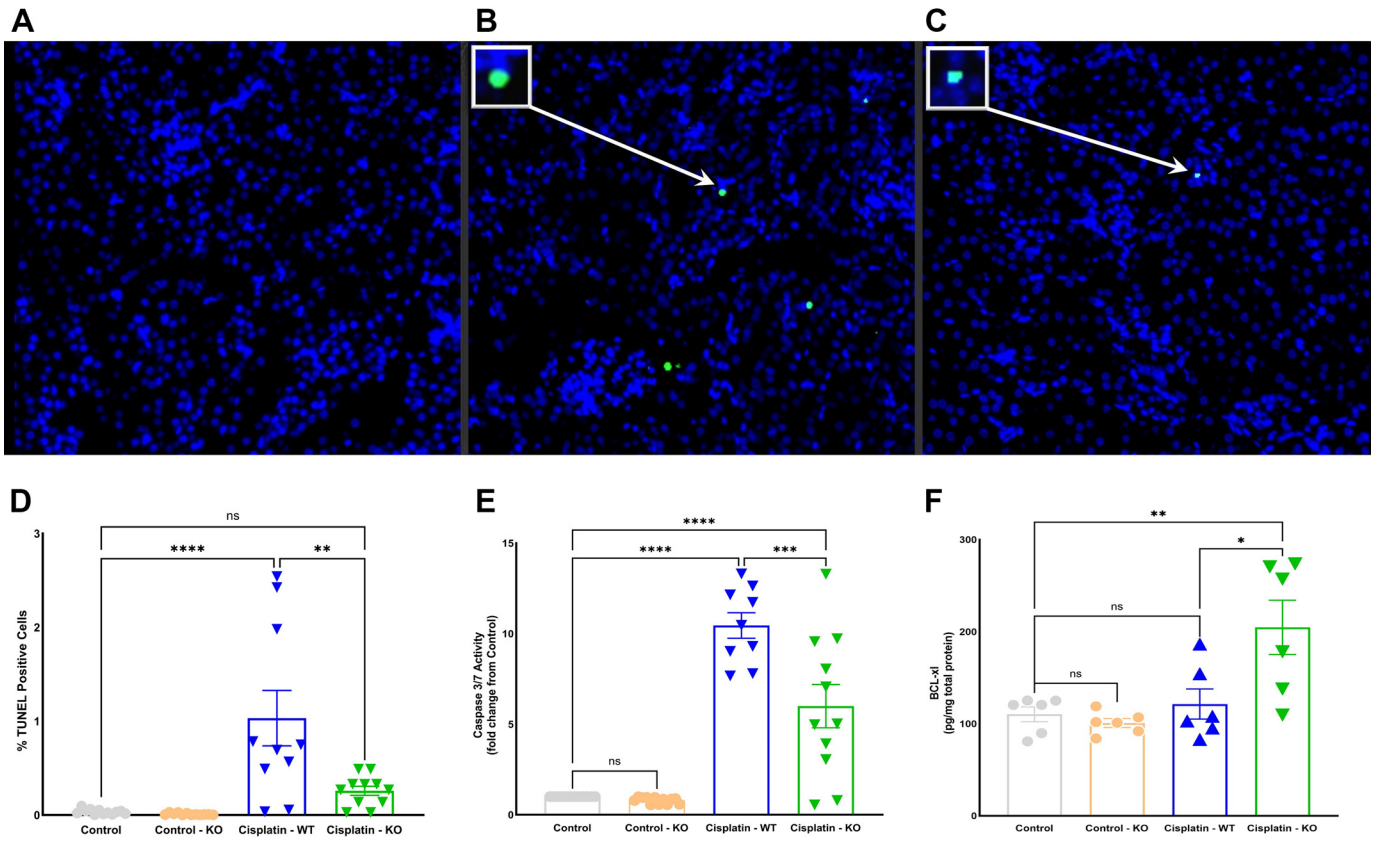

Figure 7. Effect of IGFBP-1 KO on cell death and apoptosis during CIAKI. Representative imagines of TUNEL staining from wild-type saline-injected mice (*A*), wild-type cisplatin-injected mice (*B*), and IGFBP-1 KO cisplatin-injected mice (*C*) are shown. The percentage of TUNEL positive cells (*D*), caspase 3/7 activity in renal tissue homogenates (*E*), and BCL-xl protein concentrations of renal tissue homogenates (*F*) is shown below the representative images. Each dot (control) or triangle (cisplatin injection) represents a measurement for an individual mouse ($n = 6$–12). CIAKI, cisplatin-induced acute kidney injury; Cisplatin-KO, cisplatin-injected IGFBP-1 knockout; Cisplatin-WT, cisplatin-injected wild type; Control, saline-injected wild type; Control-KO, saline-injected IGFBP-1 knockout; IGFBP-1, insulin-like growth factor binding protein 1; KO, knockout; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant by one-way ANOVA.

their mechanistic role during AKI has not been established (43). It should be noted that IGFBP7 shares only 20%–25% homology with IGFBPs 1–6 and binds IGF-1 and IGF-2 with much lower affinity, thus its function as a true IGFBP is debatable (53).

In parallel with our clinical data showing patients with higher levels of urinary IGFBP-1 suffer more severe AKI, our animal studies mirror these findings by showing that IGFBP-1 KO mice have attenuation of CIAKI. Our data implicate IGFBP-1 as a mediator of AKI severity in CIAKI. IGFBP-1 mice show a blunted decline in renal function, decreased renal tubular damage, and decreased cell death by apoptosis.

IGFBP-1 regulates cell signaling through two signaling mechanisms: sequestration of IGF-1 from binding the IGF-1r and binding of its C-terminus RGD domain to integrin receptors. The immediate downstream effect of IGF-1 binding the IGF-1r is phosphorylation of AKT by PIP3K. Likewise, the immediate downstream effect of the RGD domain of IGFBP-1 binding integrin receptors is phosphorylation of FAK by a non receptor-tyrosine kinase (SRC); thus, p-AKT and p-FAK can be used as surrogate markers of IGF-1r and integrin receptor activation, respectively (Fig. 10). In our studies, IGFBP-1 KO mice showed an increased ratio of p-AKT to pan-AKT, suggesting that the lack of IGFBP-1 to sequester IGF-1 from binding the IGF-1r enriches downstream anti-apoptotic mediators to attenuate renal injury. Conversely, p-FAK was decreased in IGFBP-1 KO controls, cisplatin-injected WT mice, and cisplatin-injected IGFBP-1 KO mice. The lack of p-FAK in IGFBP-1 KO mice is expected since the absence of IGFBP-1 removes the ligand for binding the integrin receptor. The decrease in p-FAK in WT mice with CIAKI is interesting, as activation of the integrin receptor is generally considered to worsen renal injury in other models of AKI (54, 55). However, to our knowledge, the role of p-FAK signaling in CIAKI has not been explored. One possible explanation for the decrease in p-FAK among WT mice with CIAKI is that our study was designed to look at only a single time point (72-h postinjection). It is possible that p-FAK formation occurs early in the onset of CIAKI and is depleted by 72 h. Another explanation could be that cisplatin injection has a direct effect on phosphorylation of FAK as cisplatin injection in cancer models has been shown to decrease total FAK expression and block FAK phosphorylation at specific tyrosine residues (56). Future studies will be needed to gain a firm grasp on the role of integrin receptor and p-FAK signaling in CIAKI.

Interestingly, our proteomic analysis of mouse renal tissue homogenates did not show an increase in IGFBP-1 expression following cisplatin injection. This finding suggests that IGFBP-1 is upregulated in other organs. The liver is known to

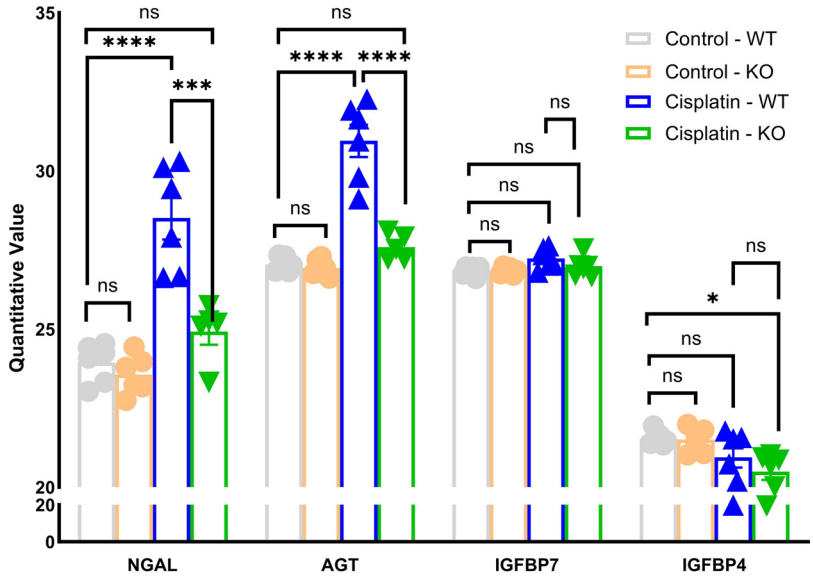

Figure 8. Proteomic analysis of mouse renal tissue homogenates following cisplatin injection. Changes in the renal proteome between saline-injected control mice (grey dots and columns), saline-injected IGFBP-1 KO control (orange dots and columns), cisplatin-injected wild type (blue dots and columns), and cisplatin-injected IGFBP-1 KO mice are shown. IGFBP-1 was not detected in the analysis. Known biomarkers of AKI including NGAL and AGT. IGFBP7 was not changed. IGFBP4 was significantly decreased in cisplatin-injected IGFBP-1 KO mice. AGT, angiotensinogen; AKI, acute kidney injury; IGFBP-1, insulin-like growth factor binding protein 1; IGFBP4, insulin-like growth factor binding protein 4; IGFBP7, insulin-like growth factor binding protein 7; KO, knockout; NGAL, neutrophil gelatinase-associated lipocalin. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$, ns, not significant by one-way ANOVA.

be the major site of IGFBP-1 synthesis in the body (17, 57), and given the size of IGFBP-1 (28 kDa), it is likely freely filtered from the serum into the tubular lumen. It is tempting to speculate that renal injury stimulates a renal-hepatic cross talk that upregulates IGFBP-1 in the liver, which is then secreted into the blood and freely filtered across the glomerulus to reach the tubular lumen.

As our studies showed attenuated CIAKI in IGFBP-1 KO mice, extrapolation of our animal studies to our clinical findings could suggest that patients with higher levels of urinary IGFBP-1 experienced more severe AKI due to increased sequestration of IGF-1 and subsequent downregulation of anti-apoptotic pathways. In addition, the downregulation of IGFBP-4 in renal tissue homogenates following CIAKI may implicate dynamic changes in other IGFBPs to compensate for the absence of IGFBP-1 in the knockout mice. Future studies should be focused on addressing this possibility.

Previous clinical trials have explored the administration of recombinant human IGF-1 as a treatment for AKI. However, these trials were ultimately unsuccessful. Hirschberg et al. (58) conducted a multicenter randomized, double-blinded, placebo-controlled trial in which 72 patients with AKI were treated with rhIGF-1 (35 patients) or placebo (37 patients). No

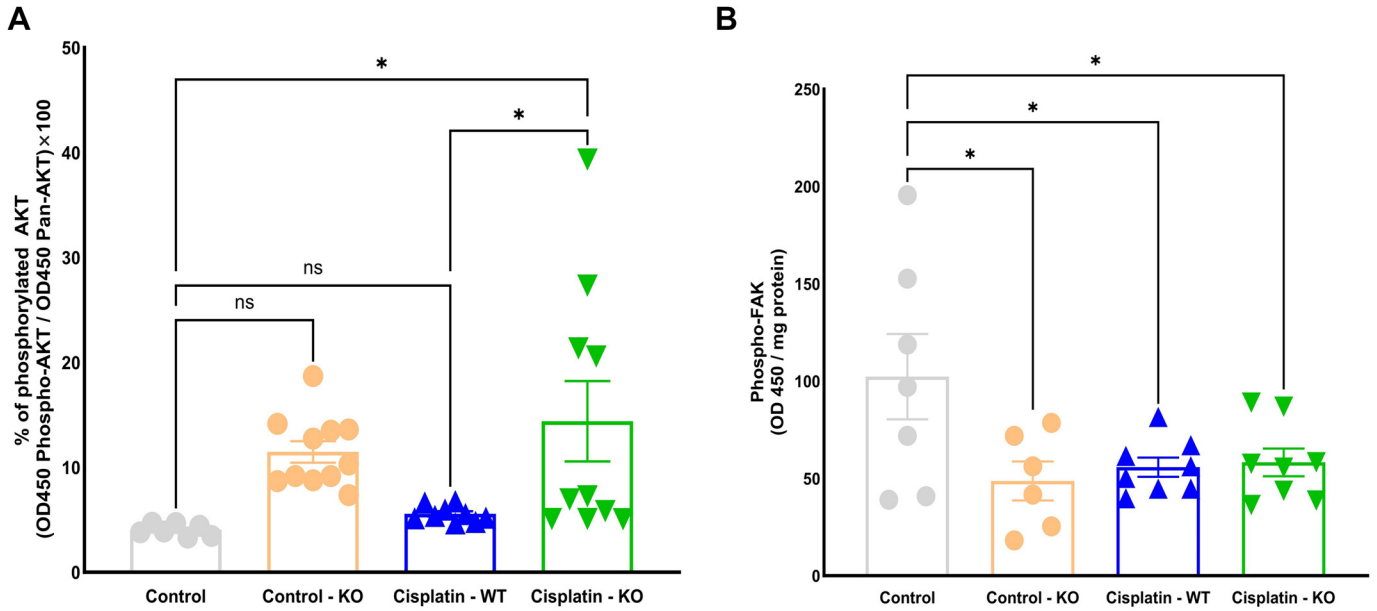

Figure 9. Measurement of phosphorylate AKT and FAK in renal tissue homogenates. The percentage of total AKT that is phosphorylated is displayed in *A*. The total concentration of p-FAK is displayed in *B*. Values are normalized for tissue homogenate protein concentrations. Each dot (control) or triangle (cisplatin injection) represents a measurement for an individual mouse. AKT, protein kinase B; Cisplatin-KO, cisplatin-injected IGFBP-1 knockout; Cisplatin-WT, cisplatin-injected wild type; Control, saline-injected wild type; Control-KO, saline-injected IGFBP-1 knockout; IGFBP-1, insulin-like growth factor binding protein 1; p-FAK, phosphorylated focal adhesion kinase. $*P < 0.05$; ns, not significant by one-way ANOVA.

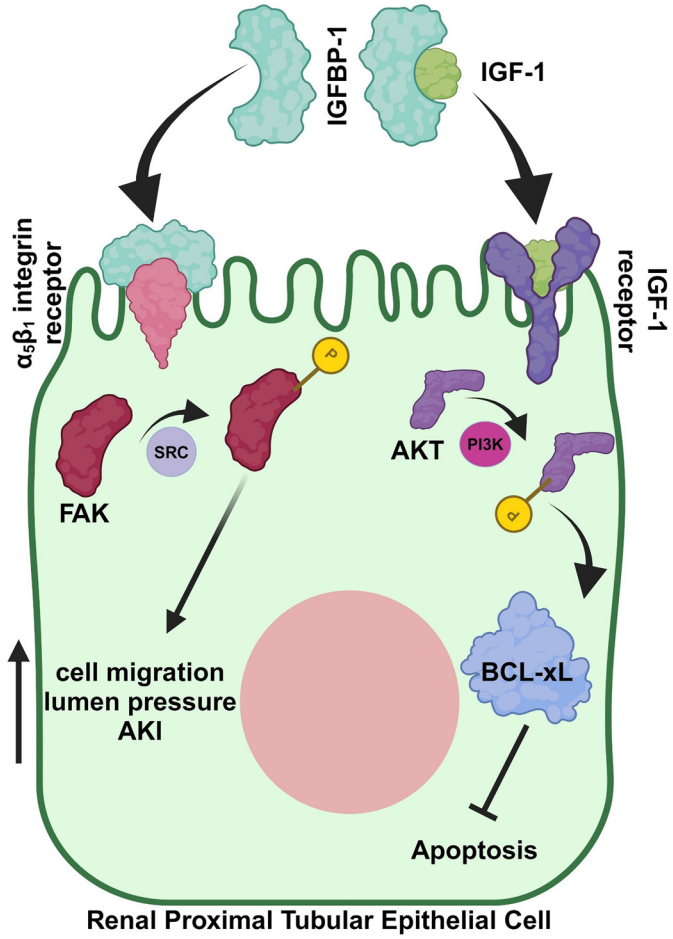

Figure 10. Schematic representation of proposed mechanism of IGFBP-1 signaling in AKI. IGFBP-1 modulates cellular signaling in renal proximal tubular epithelial cell by one of two mechanisms: sequestration of IGF-1 or direct binding of the C-terminus RGD domain to the $\alpha_5\beta_1$ integrin receptor. Binding of IGF-1 to its receptor activates a phosphorylation cascade through AKT via Phosphoinositide 3-kinase (PI3K) ultimately leading to increased synthesis of the anti-apoptotic BCL-xl protein. Binding of IGFBP-1 to the $\alpha_5\beta_1$ integrin receptor activates a separate phosphorylation cascade through FAK via SRC kinases ultimately leading to increased cell migration and increased luminal pressure worsening AKI. The decreased apoptotic markers, increased BCL-xl, and increased ratio of p-AKT in our studies suggest that IGFBP-1 KO mice are protected against CIAKI due to inability to sequester IGF-1 and subsequent increased activation of the IGF-1r. AKI, acute kidney injury; CIAKI, cisplatin-induced AKI; IGF-1r, insulin growth factor receptor 1; IGFBP-1, insulin-like growth factor binding protein 1; KO, knockout.

difference was found in the GFR, urine volume, serum markers of AKI, or the need for RRT between the groups (58). Hladunewich et al. (59) found that administration of rhIGF-1 did not improve GFR in patients with delayed graft function following renal transplant. A possible explanation for the failure of these trials is the lack of attention to the effects of IGFBP-1 on regulating IGF-1 signaling. In fact, a small study by Yamashita et al. (60) showed increased levels of serum IGFBP-1 in critically ill patients with AKI. The upregulation of IGFBP-1 during AKI could counteract the administration of recombinant human IGF-1, at least to some degree. Also, the IGF-1 independent effects of the upregulated IGFBP-1 through integrin receptor binding could have offset any potential therapeutic benefit.

A limitation of this study was the relatively small size of its cohort. However, the number of severe AKI events was large, thus maintaining adequate power. In the TRIBE (12) and SAPPHIRE (13) studies, a total of 22 and 18 subjects, respectively, reached the end point of severe AKI (stage 3 AKI) compared with 22 subjects who met this outcome in our cohort. By including subjects only after they developed stage 1 AKI, we were able to greatly enrich the cohort for outcomes like death, the need for RRT, and progression to stage 3 AKI. We also acknowledge that the 30 samples used in the discovery analysis were included among the 213 samples in the validation phase. However, this does not weaken the analysis since they were only used as a discovery analysis and exclusion of these samples from our validation study does not alter the conclusions. Finally, the mechanism of renal injury in our patient cohort is presumably different than the mechanism of renal injury in our animal model. A large portion of our patient cohort likely developed acute tubular necrosis from renal ischemia during cardiac surgery, whereas our animal model utilized cisplatin toxicity. However, due to the complex nature of AKI in the clinical setting, we cannot rule out contributions to AKI from other sources such as sepsis, cardiogenic shock, and contrast-induced nephropathy. Likewise, no one animal model can replicate the complex and heterogeneous nature of AKI pathophysiology. However, the fact that our studies implicate IGFBP-1 as a mediator of AKI across different species and mechanisms of injury shows the potential for IGFBP-1 to be a global mediator of all causes of AKI. Future animal studies should focus on the reproducibility of our findings across other models of experimental AKI, and human studies should validate urinary IGFBP-1 in patients with all causes of AKI.

In conclusion, urinary IGFBP-1 is highly predictive of severe AKI in subjects after cardiac surgery. It may identify those who will progress to require RRT thus facilitating the planning of clinical interventions (e.g., placing dialysis catheters and initiating RRT in a nonemergent manner) or avoiding the use of aggressive and invasive interventions in those who are not likely to progress to severe AKI. Moreover, it may help in identifying high-risk patients allowing for objective enrichment of study populations in AKI clinical trials. Perhaps, the most exciting finding is the fact that we have implicated a mechanistic role of IGFBP-1 during AKI, which could lead to a more robust understanding of the pathophysiological mechanism and the eventual development of novel therapeutic treatment targets.

DATA AVAILABILITY

All data presented in this article are available upon request to the corresponding author. Upon publication, proteomic data will be uploaded to the MassIVE data repository (University of California San Diego).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.H.H., N.K., C.H., L.A.J., and J.M.A. conceived and designed research; J.H.H., N.K., A.G.B., R.D.E., T.W.F., Y.H., C.H., K.G.H., K.L.R., and R.S.S. performed experiments; J.H.H., N.K., A.G.B., R.D.E., N.G., C.H., K.G.H., L.A.J., K.L.R., and J.M.A. analyzed data; J.H.H., N.K., A.G.B., R.D.E., N.G., C.H., L.A.J., and J.M.A. interpreted results of experiments; J.H.H., N.K., A.G.B., N.G., and J.M.A. prepared figures; J.H.H., N.K., A.G.B., and J.M.A. drafted manuscript; J.H.H., N.K., A.G.B., Y.H., C.H., L.A.J., and J.M.A. edited and revised manuscript; J.H.H., N.K., A.G.B., R.D.E., T.W.F., N.G., Y.H., C.H., K.G.H., L.A.J., K.L.R., R.S.S., and J.M.A. approved final version of manuscript.

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