Discovery of Novel Proteomic Biomarkers for the Prediction of Kidney Recovery from Dialysis-Dependent AKI Patients

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Key Points
- High throughput proteomics detected serum protein levels in patients with AKI-D who recovered kidney function.
- Novel predictive biomarkers of kidney recovery from patients with AKI-D were discovered.
- Potential biologic pathways associated with kidney remodeling, repair, and regeneration were suggested.

Abstract

Background AKI requiring dialysis (AKI-D) is associated with prolonged hospitalization, mortality, and progressive CKD among survivors. Previous studies have examined only select urine or serum biomarkers for predicting kidney recovery from AKI.

Methods Serum samples collected on day 8 of randomized RRT from 72 patients enrolled in the Veteran's Affairs/National Institutes of Health Acute Renal Failure Trial Network study were analyzed by the SOMAscan proteomic platform to profile 1305 proteins in each sample. Of these patients, 38 recovered kidney function and dialysis was discontinued, whereas another 34 patients remained on dialysis by day 28.

Results Differential serum levels of 119 proteins, with 53 higher and 66 lower, were detected in samples from patients who discontinued dialysis, compared with patients who remained on dialysis by day 28. Patients were classified into tertiles on the basis of SOMAscan protein measurements for the 25 proteins most differentially expressed. The association of serum levels of each protein with kidney recovery was further evaluated using logistic regression analysis. Higher serum levels of CXCL11, CXCL2/CXCL3, CD86, Wnt-7a, BTK, c-Myc, TIMP-3, CCL5, ghrelin, PDGF-C, survivin, CA2, IL-9, EGF, and neuregulin-1, and lower levels of soluble CXCL16, IL1RL1, stanniocalcin-1, IL-6, and FGF23 when classified in tertiles were significantly associated with better kidney recovery. This significant association persisted for each of these proteins after adjusting for potential confounding risk factors including age, sex, cardiovascular SOFA score, congestive heart failure, diabetes, modality of intensive dialysis treatment, cause of AKI, baseline serum creatinine, day 8 urine volume, and estimated 60-day mortality risk.

Conclusions These results suggest concerted changes between survival-related proteins and immune-regulatory chemokines in regulating angiogenesis, endothelial and epithelial remodeling, and kidney cell regeneration, illustrating potential mechanisms of kidney recovery. Thus, this study identifies potential novel predictive biomarkers of kidney recovery in patients with AKI-D.

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Introduction

AKI requiring dialysis (AKI-D) is associated with high mortality in patients who were hospitalized. Of those that survive, a fraction recovers kidney function, but most progress to CKD (1–3). Current assessment of kidney recovery primarily relies on urine output and measurement of serum creatinine to estimate GFR; however, these parameters are inconsistent and limited in predicting kidney recovery (4). Previous studies have examined the potential of decreased levels of select kidney injury biomarkers

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(5–7) or inflammatory biomarkers (8, 9) for the prediction of kidney recovery from AKI, including urine hepatocyte growth factor, urine neutrophil gelatinase-associated lipocalin (uNGAL), and plasma NGAL (pNGAL). Studies also found that urine insulin-like growth factor-binding protein 7, and tissue inhibitor of metalloproteinase 2 provided more accurate prediction of kidney recovery than other investigated urine AKI biomarkers (10, 11). Recent studies showed that post-AKI proteinuria was associated with kidney disease progression (12) and preadmission proteinuria was an independent risk factor for nonrecovery for AKI-D (13). The Translational Research Investigating Biomarker Endpoints for AKI study found that high postoperative plasma levels of vascular endothelial growth factor A isoform (VEGF) and placental growth factor were independently associated with reduced risk for AKI, prolonged AKI, and mortality, whereas high levels of the antiangiogenic marker soluble VEGF receptor 1 were associated with increased risk for these outcomes in patients after cardiac surgery (14). These studies suggest that serum/plasma/urine AKI or angiogenic biomarkers with use of appropriate mathematical models could provide prognostic information for the prediction of kidney function recovery. However, the performance of these biomarkers/models has not been well established or validated.

Using the slow off-rate modified aptamers scan (SOMAscan) proteomics platform, we recently identified fibroblast growth factor-23 (FGF23), tissue plasminogen activator (tPA), neutrophil collagenase (matrix metalloproteinase-8), soluble urokinase plasminogen activator receptor, and IL-6 as potential mortality-associated biomarkers in patients with AKI-D (15). In this study, we aimed to identify biomarkers for prediction of kidney function recovery by analysis of serum samples obtained on day 8 of randomized RRT from 72 patients with AKI-D enrolled in the Veteran’s Affairs/National Institutes of Health Acute Renal Failure Trial Network (ATN) study. Although most previous studies concentrated on kidney recovery within 60 or 90 days (5, 16), this study focused on kidney recovery within 28 days. Our study not only confirmed that lower serum levels of AKI biomarkers were associated with AKI recovery but also identified higher levels of survival-related proteins as novel biomarkers of kidney recovery.

**Methods**

**Study Design**

The ATN study was a prospective, multicenter clinical trial to evaluate strategies of intensive versus conventional RRT in patients who were critically ill with AKI-D; 1124 patients were enrolled and randomly assigned to intensive or conventional RRT in 27 Veterans Affairs (VA) and 12 academic medical centers across the United States. Outcomes included 60-day mortality, recovery of kidney function, and intensive care unit and hospital lengths of stay. Details of the study protocol including inclusion and exclusion criteria have been previously published (17, 18). Patients enrolled in the ATN study were critically ill adults (aged ≥18 years) who had AKI clinically consistent with acute tubular necrosis and failure of one or more nonrenal organ(s) (defined as a nonrenal sequential organ failure assessment [SOFA] score of ≥2) or sepsis. Patient consent for serum sample collection and at least one sample were obtained from 827 of the 1124 subjects who participated in the ATN study. A total of 819 patients provided samples on day 1 and 573 patients on day 8, with 565 patients contributing samples on both day 1 and day 8 of randomized RRT. Among the 626 patients who survived to day 28, 343 patients were dialysis independent on day 28, whereas the remaining 283 patients remained on dialysis. Selection of the samples used for this study was random, and the decision was made by the ATN Study coordinating center/biorepository, with constraints regarding survival status that were imposed. This post-hoc proteomic biomarker study of kidney recovery included day 8 serum samples from 72 randomly selected patients who either survived independent of dialysis (n=38) or survived dependent on dialysis (n=34) by day 28. We did not analyze urine samples because urine was not stored in the ATN study. Power analysis indicated that differentially expressed proteins could be identified with a power of >0.8. In this study, kidney function recovery was defined as alive and free of dialysis on day 28. Clinical data available through the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) data repository via a crosswalk file were then linked to the deidentified samples analyzed in this study. This post-hoc analysis was approved by the Salem Veterans Affairs Medical Center and Food and Drug Administration (FDA) Institutional Review Boards.

**SOMAscan Proteomic Profiling**

Quantitative proteome profiling of AKI samples was performed by SOMAscan assay (version 1.3k) developed by SomaLogic Inc. (Boulder, CO) as described previously (15, 19). The assay quantified 1305 low, middle, and high abundance proteins using single-stranded DNA slow off-rate modified aptamers (SOMAmers), with five calibration samples and two quality control samples for each assay. Briefly, serum samples (50 μl) were incubated with preimmobilized SOMAmers, which were subjected to a series of washes to remove nonspecific bindings. SOMAmers specifically bound to their cognate proteins were released and hybridized to custom DNA microarrays. The microarrays were scanned using an Agilent C scanner (G2505C, Agilent Technologies, Palo Alto, CA). The raw data were processed as described previously (15). Mainly, hybridization control normalization was applied to relative fluorescence units (RFUs) to remove variation introduced during the hybridization and scanning processes, followed by median signal normalization to eliminate intrarun bias, and finally calibration to account for inter-run differences.

**Olink Assay of Inflammatory Proteins**

The Olink inflammation panel consisting of 92 proteins was measured by Olink Proteomics (Boston, MA) using the Proximity Extension Assay technology, with consumption of 1 μl of each serum sample, as previously described (20). Quality control was performed by adding four internal controls into all samples and running external controls in every assay plate. Assay results were reported in arbitrary, relative units as Normalized Protein eXpression (NPX) on a log2 scale. More information regarding the Olink platform, NPX, assay validation data, and the full list of proteins in...
the panel is available at Olink’s website (www.olink.com). The correlation between SOMAscan and Olink data was assessed using multivariate correlation analysis in JMP (version 12.1.0, SAS Institute Inc., Cary, NC) to calculate the correlation coefficient and P value.

### Identification of Differential Proteins

Day 8 samples were stratified on the basis of dialysis status on day 28. Welch’s t test was performed for log-transformed SOMAscan RFUs to find significantly changed proteins between the dialysis-dependent and independent groups. A fold change of ≥1.2 and P<0.05 were set as significant changes as the criteria were evaluated and validated (15). The t test P values were also adjusted for this multiplex assay to calculate false discovery rate using the Benjamini and Hochberg method (21). For protein expression mean, SD, and fold changes, RFUs were used for SOMAscan data, whereas log-scale NPX (i.e., non-log transformed, linear NPX) for Olink data.

### Ingenuity Pathway Analyses

Ingenuity Pathway Analysis (IPA) software was used for gene ontology, pathways, and core analysis comparison of proteins with significant fold changes in patients who discontinued dialysis versus those who continued dialysis on day 28. A P value <0.05 was considered significant.

### Statistical Analysis of Proteins for Kidney Recovery

The top proteins with altered abundance (either higher or lower serum levels) were of interest in this study. Because the distributions of these proteins were relatively skewed, patients were classified into tertiles for each of these proteins, and the associations of categorized proteins with the kidney recovery response were further evaluated and the effects of elevated protein levels were quantified. Continuous variables were expressed as mean with SD or median (25th, 75th percentile), and categorical variables as %; CV-SOFA, cardiovascular sequential organ failure assessment; F, female; PO4, phosphate.

### Results

#### Patient Characteristics

We analyzed a total of 72 randomly selected samples obtained on day 8 of randomized RRT as part of the ATN study. Table 1 shows the characteristics of the patients stratified by the status of kidney function recovery by day 28 (i.e., dialysis on or off). Kidney function recovered (i.e.,...
survived without dialysis) in 38 (53%) patients, whereas the remaining 34 (47%) patients survived with dialysis by day 28. Thus, recovery rate of kidney function in this subset of patients that we studied was similar to that in the overall ATN study cohort in which 343 of 626 patients (55%) surviving to day 28 were dialysis independent on day 28. The number (percentage) of patients with or without recovered kidney function by day 28 was also similar in diabetes, CV-SOFA score ≥2, intensive dialysis treatment arm, and cause of AKI, with no statistically significant differences (P=0.05). Higher baseline SOFA scores (both overall and CV component) were associated with mortality; however, they were not associated with recovery of kidney function among survivors. The median baseline serum creatinine levels and estimated 60-day mortality risk scores were the same between the two groups. In addition, the median urine volume on day 8 was not significantly different (P=0.06) between the kidney-recovered group (81.5, 60–478 ml/day) and the nonrecovered group (59.5, 10–207 ml/day). These urine volumes were far less than that expected for kidney function recovery (>500 ml/day).

**SOMAScan Proteomic Profile of Day 8 AKI-D Serum Samples**

A volcano plot of SOMAScan analysis of 1305 serum proteins (Figure 1) demonstrates fold changes (FC; FC cutoff=1.2) and statistical significance (P<0.05) of those proteins that were higher (red dots) or lower (green dots) in day 8 serum samples from patients who discontinued dialysis compared with those who remained on dialysis by day 28. Although most serum proteins did not show a significant statistical difference (gray dots) between the two groups, 119 proteins showed statistically significant differences in serum levels, 53 of which were higher and 66 of which were lower in the group of patients who discontinued dialysis (Figure 1, Supplemental Table 1). Serum levels of mortality-associated proteins such as FGF23 (FC=−2.69, P<0.01) and IL-6 (FC=−2.44, P<0.009), AKI biomarkers NGAL (FC=−1.62, P<0.002) and chitinase-3-like protein 1 (YKL-40, FC=−1.44, P<0.04), and cardiac biomarker creatine kinase-MB (CK-MB, FC=−9.94, P<0.004) were lower on day 8 in the patients that recovered by day 28. In contrast, several growth and survival-related proteins were higher on day 8 in the patients that discontinued dialysis by day 28, such as tyrosine-protein kinase Fyn (FC=2.28, P<0.01) and BTK (FC=1.63, P<0.04), protein Wnt-7a (FC=1.83, P=0.003), Myc proto-oncogene protein (c-Myc, FC=1.62, P<0.0004), ghrelin (FC=1.42, P<0.005), platelet-derived growth factor C (PDGF-C, FC=1.40, P<0.005), survivin (FC=1.39, P=0.02), epidermal growth factor (EGF, FC=1.32, P=0.004), and neuregulin-1 (FC=1.31, P<0.03). The SOMAScan measurements of all 1305 proteins are presented in Supplemental Table 2.

**IPA Analysis of Biologic Function Changes Related to Tissue Repair**

IPA core analysis of proteins with significant changes in abundance revealed significant alterations in diseases and biologic functions on day 8 (Figure 2, Supplemental Table 3). The analysis indicated increased functions in cell-to-cell signaling and interaction, cellular growth and proliferation, cellular development, embryonic development, tissue morphology, tissue development, organismal development, molecular transport, and post-translational modification. The analysis also showed decreased function in cell death, connective tissue disorders, organismal injury and abnormalities, and inflammatory response and disease. The results suggest molecular pathways related to these biologic functions were already activated or suppressed on day 8, which potentially affected kidney recovery by day 28.

**Olink Verification of Top-Changed Proteins**

Of the top 25 proteins with significant changes identified from the SOMAScan assay (Table 2), CXCL11, IL-6, and FGF23 were also included on the Olink inflammation panel.
Consistent results were obtained for all three proteins between the SOMAscan and Olink assays. Strong positive correlations between the two assays were observed for CXCL11 and IL-6 with correlation coefficients of 0.86 ($P<0.0001$) and 0.89 ($P<0.0001$), respectively. SOMAscan and Olink intensities of FGF23 showed positive but moderate correlation with $r=0.47$ and $P<0.0001$ (Figure 3).

Association of Significantly Changed Proteins with Kidney Function Recovery

To establish a direct association between the serum levels of proteins that were significantly different in the two groups and the end points of recovered or nonrecovered kidney function, we stratified their serum levels by tertiles of top-changed proteins (i.e., Fyn, CXCL11, CXCL2/CXCL3, CD86, Wnt-7a, BTK, c-Myc, TIMP-3, CCL5, ghrelin, PDGF-C, survivin, CA2, IL-9, EGF, neuregulin-1, YKL-40, soluble CXCL16, NGAL, IL1RL1, stanniocalcin-1, IL-6, CA3, FGF23, and CK-MB) in day-8 samples. Higher serum levels for each of CXCL11, CXCL2/CXCL3, CD86, Wnt-7a, BTK, c-Myc, TIMP-3, ghrelin, CA2, IL-9, EGF, and neuregulin-1 on day 8 were associated with increased recovery rate of kidney function by day 28, with a statistically significant ($P<0.05$) as tested by the chi-square analyses (Table 2). Most significantly, 88% of the patients in the highest tertile of c-Myc levels recovered kidney function, whereas only 38% of the patients in the lowest tertile recovered kidney function. In contrast, higher serum levels of soluble CXCL16, stanniocalcin-1, and CA3 on day 8 were significantly ($P<0.05$) associated with decreased recovery rate of kidney function by day 28. Similarly, higher serum levels of the AKI biomarkers YKL-40, NGAL, IL-6, and FGF23, and cardiac biomarker CK-MB were associated with decreased rate of kidney recovery, albeit with less statistical significance (Table 2).

To further explore the relationships of serum levels of proteins with kidney recovery, logistic regression analysis was performed. Patients with upper serum levels for each of CXCL11, CXCL2/CXCL3, CD86, Wnt-7a, BTK, c-Myc, TIMP-3, CCL5, ghrelin, PDGF-C, survivin, CA2, IL-9, EGF, and neuregulin-1 had greater odds of kidney recovery compared with the patients with lower levels of each corresponding protein, and these associations remained significant ($P<0.05$) after multivariable adjustment for age, sex, congestive heart failure, diabetes mellitus, CV-SOFA scores, intensive dialysis treatment, cause of AKI, baseline serum creatinine, day-8 urine volume, and estimated 60-day mortality risk (Table 3). However, logistic regression analysis of all these confounding factors per se did not show any of them were significantly associated with kidney recovery (Supplemental Table 5). The association of higher serum levels of FYN with higher chance of kidney recovery was less significant ($P>0.05$) (Supplemental Table 6). Higher c-Myc levels were significantly associated with kidney recovery, with an approximately 56-fold increase in the chance of recovery compared with lower c-Myc levels (odds ratio=55.79, 95% CI, 5.53 to 562.99, $P=0.0007$). In contrast, for the proteins with lower serum levels on day 8, patients with the upper serum levels for each of soluble CXCL16, IL1RL1, stanniocalcin-1, IL-6, and FGF23 were unlikely to show kidney recovery by day 28 compared with those with the lower corresponding protein levels, and these associations remained significant ($P<0.05$) after multivariable adjustment (Table 3). The associations of the higher serum levels with lower chance of kidney recovery for proteins YKL-40, NGAL, CA3, and CK-MB were less significant after multivariable adjustment (Supplemental Table 6).

Discussion

Using routinely available clinical data including baseline eGFR, preadmission hemoglobin level, chronic liver disease, and age as the predictors, Lee et al. recently developed a predictive model for 90-day kidney recovery after AKI-D.
Approximately half of the patients died during the first 8 days in the hyperacute and acute phases of AKI (22). However, the model’s modest discrimination limits its clinical utility, and further studies are required to develop better models (16). This study is the first to use SOMAscan to discover proteomic biomarkers of kidney recovery from patients with AKI-D to develop such models. The VA/ National Institutes of Health ATN study enrolled the patients with AKI-D to develop such models. The VA/ National Institutes of Health ATN study enrolled the patients with AKI and started randomized RRT on day 1. Serum samples were collected on day 1 and/or day 8. Of the proteins identified from the SOMAscan assay that showed the greatest change, CXCL11, IL-6, and FGF23 were also verified by the Olink assay. Although a strong correlation between the two assays was observed for CXCL11 and IL-6 (r=0.85), FGF23 showed moderate correlation (r=0.47). In our previous analysis, the correlation between SOMAscan and ELISA for FGF23 was also moderate (r=0.61, P<0.0001) (15). SOMAscan, Olink, and ELISA assays are all affinity based and measure apparent quantitative changes as discussed previously (23); however, the affinity reagents of each assay might recognize a different region/epitope of a protein. Blood sample processing and storage could also differentially affect individual proteins under different preanalytical conditions (23). However, under standard operating procedures, the effects could be

| Protein | Tertile 1a (Low Levels) | Tertile 2a (Intermediate Levels) | Tertile 3a (High Levels) | Chi-Square P Value |
|---------|------------------------|---------------------------------|-------------------------|-------------------|
| FYN     | 11 (45.8)              | 11 (45.8)                       | 16 (66.7)               | 0.25              |
| CXCL11  | 6 (25.0)               | 15 (62.5)                       | 17 (70.8)               | 0.003             |
| CXCL2/CXCL3 | 8 (33.3)         | 13 (54.2)                       | 17 (70.8)               | 0.03              |
| CD68    | 9 (37.5)               | 11 (45.8)                       | 18 (75.0)               | 0.02              |
| WNT7A   | 10 (41.7)              | 9 (37.5)                        | 19 (79.2)               | 0.006             |
| BTK     | 9 (37.5)               | 11 (45.8)                       | 18 (75.0)               | 0.02              |
| c-Myc   | 9 (37.5)               | 8 (33.3)                        | 21 (87.5)               | 0.0002             |
| TIMP-3  | 9 (37.5)               | 10 (41.7)                       | 19 (79.2)               | 0.006             |
| CCL5    | 9 (37.5)               | 12 (50.0)                       | 17 (70.8)               | 0.07              |
| Ghrelin | 9 (37.5)               | 10 (41.7)                       | 19 (79.2)               | 0.006             |
| PDGF-C  | 9 (37.5)               | 13 (54.2)                       | 16 (66.7)               | 0.13              |
| Survivin| 9 (37.5)               | 12 (50.0)                       | 17 (70.8)               | 0.07              |
| CA2     | 10 (41.7)              | 10 (41.7)                       | 18 (75.0)               | 0.03              |
| IL-9    | 7 (29.2)               | 16 (66.7)                       | 15 (62.5)               | 0.02              |
| EGF     | 9 (37.5)               | 11 (45.8)                       | 18 (75.0)               | 0.02              |
| Neuregulin-1 | 9 (37.5) | 11 (45.8) | 18 (75.0) | 0.02 |

Proteins with lower serum levels in the recovery group

| Protein | Tertile 1a (Low Levels) | Tertile 2a (Intermediate Levels) | Tertile 3a (High Levels) | Chi-Square P Value |
|---------|------------------------|---------------------------------|-------------------------|-------------------|
| YKL-40  | 15 (62.5)              | 14 (58.3)                       | 9 (37.5)                | 0.18              |
| CXCL16, soluble | 18 (75.0)          | 13 (54.2)                       | 7 (29.2)                | 0.006             |
| NGAL    | 17 (70.8)              | 11 (45.8)                       | 10 (41.7)               | 0.09              |
| IL1R1   | 17 (70.8)              | 12 (50.0)                       | 9 (37.5)                | 0.07              |
| Stanniocalcin-1 | 19 (79.2) | 12 (50.0) | 7 (29.2) | 0.002 |
| IL-6    | 16 (66.7)              | 14 (58.3)                       | 8 (33.3)                | 0.06              |
| CA3     | 18 (75.0)              | 11 (45.8)                       | 9 (37.5)                | 0.02              |
| FGF23   | 17 (70.8)              | 11 (45.8)                       | 10 (41.7)               | 0.09              |
| CK-MB   | 15 (62.5)              | 15 (62.5)                       | 8 (33.3)                | 0.07              |

Proteins with higher serum levels in the recovery group

However, the model’s modest discrimination limits its clinical utility, and further studies are required to develop better models (16). This study is the first to use SOMAscan to discover proteomic biomarkers of kidney recovery from patients with AKI-D to develop such models. The VA/ National Institutes of Health ATN study enrolled the patients with AKI and started randomized RRT on day 1. Serum samples were collected on day 1 and/or day 8. Most patients who survived to day 8 continued to survive, and some recovered kidney function and discontinued dialysis. Using SOMAscan proteomic assays, we recently identified mortality-associated biomarkers, including FGF23, tPA, matrix metalloproteinase-8, and soluble urokinase plasminogen activator receptor in 100 serum samples collected on day 1. We also found that high serum levels of FGF23, tPA, and IL-6 were associated with mortality in 107 samples collected on day 8 (15). Because patients on day 1 were at hyperacute and acute phases of AKI, day-1 samples were more suitable for analysis of biomarker of mortality. However, the model’s modest discrimination limits its clinical utility, and further studies are required to develop better models (16). This study is the first to use SOMAscan to discover proteomic biomarkers of kidney recovery from patients with AKI-D to develop such models. The VA/ National Institutes of Health ATN study enrolled the patients with AKI and started randomized RRT on day 1. Serum samples were collected on day 1 and/or day 8. Most patients who survived to day 8 continued to survive, and some recovered kidney function and discontinued dialysis. Using SOMAscan proteomic assays, we recently identified mortality-associated biomarkers, including FGF23, tPA, matrix metalloproteinase-8, and soluble urokinase plasminogen activator receptor in 100 serum samples collected on day 1. We also found that high serum levels of FGF23, tPA, and IL-6 were associated with mortality in 107 samples collected on day 8 (15). Because patients on day 1 were at hyperacute and acute phases of AKI, day-1 samples were more suitable for analysis of biomarker of mortality. However, the model’s modest discrimination limits its clinical utility, and further studies are required to develop better models (16). This study is the first to use SOMAscan to discover proteomic biomarkers of kidney recovery from patients with AKI-D to develop such models. The VA/ National Institutes of Health ATN study enrolled the patients with AKI and started randomized RRT on day 1. Serum samples were collected on day 1 and/or day 8. Most patients who survived to day 8 continued to survive, and some recovered kidney function and discontinued dialysis. Using SOMAscan proteomic assays, we recently identified mortality-associated biomarkers, including FGF23, tPA, matrix metalloproteinase-8, and soluble urokinase plasminogen activator receptor in 100 serum samples collected on day 1. We also found that high serum levels of FGF23, tPA, and IL-6 were associated with mortality in 107 samples collected on day 8 (15). Because patients on day 1 were at hyperacute and acute phases of AKI, day-1 samples were more suitable for analysis of biomarker of mortality.
uniform for the samples from the same study cohort. A study showed that affinity-based proteomic approaches did not reveal any systematic effect of storage period on biobanked samples stored over a period of 13 to 17 years (24). In contrast, clinical factors such as disease status (e.g., AKI) and treatment might change the stability and structures of individual proteins, which in turn affect protein quantification. All these factors could potentially result in discordance between different assays.

Previous studies demonstrated that reduced levels of kidney injury biomarkers could serve as potential biomarkers for predicting kidney recovery. Plasma and urine levels of NGAL were decreased in patients with AKI who had recovering kidney function (5–7). In this study, we found that lower serum levels of AKI biomarkers NGAL and YKL-40 were associated, to a certain degree, with increased kidney recovery in patients with AKI-D, albeit with less statistical significance. Importantly, it was found that the association of lower serum levels of AKI mortality biomarker IL-6 with increased kidney recovery was statistically significant (p=0.05). The results suggest that AKI biomarkers have the potential to be predictive biomarkers of kidney recovery. However, these biomarkers need to be verified in larger clinical studies.

Inflammatory response is associated with phases of acute kidney injury and repair (25,26). Reduced levels of the inflammatory biomarker IL-18 were found to be indicative of kidney recovery (27). In this study, we found that lower serum levels of inflammation-related proteins (FGF23, soluble CXCL16, IL1RL1, and IL-6) on day 8 were associated with increased kidney recovery by day 28 and demonstrated that lower levels of these proteins were more significantly associated with kidney recovery than AKI biomarkers YKL-40 and NGAL. We also found that higher serum levels of CXCL11, CXCL2/CXCL3, CD86, CCL5, and IL-9 were significantly associated with kidney recovery. Chemokine CXCL11, CXCL2/CXCL3, and CCL5 target T lymphocytes, endothelial cells, monocytes, and macrophages during wound healing through their receptors on these cells (28). CXCL11 is a ligand of CXCR3, which has been shown to play roles in protecting the kidney from ischemia reperfusion injury by recruiting CXCR3+ natural killer T cells (29,30) and by promoting re-epithelialization in wound tissue repair (31). CXCL2 and CXCL3 are ligands of CXCR2, which also play important roles in wound healing (32). It has been shown that CXCL2/3-driven macrophage-myoﬁbroblast crosstalk promotes intestinal repair (33). Studies have shown that CD86 is required for a robust regulatory T-cell response during the recovery phase for lung tissue repair after inﬂuenza A virus clearance (34). Furthermore, IL-9 is required to promote tissue repair in the recovery phase of inﬂammatory lung (35) and inhibit early podocyte injury and progressive glomerulosclerosis (36). These ﬁndings suggest reduced inﬂammation and increased chemokines/cytokines responsible for recruiting tissue injury immune cells or protecting kidney cells from injury may be useful predictive biomarkers of kidney recovery, underlining potential mechanism(s) of renal regeneration and repair.

This study indicates that a large fraction of the proteins associated with kidney recovery in patients with AKI-D are related to cellular growth, survival, or proliferation. Higher serum levels of Wnt-7a and c-Myc on day 8 were associated with kidney recovery by day 28. Protein Wnt-7a is a ligand in the canonical Wnt/beta-catenin signaling pathway, which has been shown to be involved in renal tubular protection after AKI (37) and repair and regeneration after kidney injury (38). Wnt/β-catenin signaling regulates the expression of the key driver of angiogenesis, VEGF (39–41). The recent Translational Research Investigating Biomarker Endpoints for AKI study found that high postoperative plasma levels of VEGF A isoform and placental growth factor were associated with reduced risk for AKI and mortality (14). The present finding of high levels of Wnt-7a could coincide with VEGF effects on angiogenesis and endothelial remodeling, thus biomarkers associated with vascular regeneration could be potential biomarkers of kidney recovery (42). c-Myc is a transcription factor that activates the expression of growth-related genes (43). It is also well established that c-Myc is the major transcription factor...
## Table 3. Odds ratios and 95% confidence intervals of day 8 serum proteins in tertiles significantly associated with kidney function recovery by day 28 as analyzed by logistic regression

| Protein        | Tertile | Median (25th, 75th percentiles) | Univariate Analysis | Multivariable Analysis |
|----------------|---------|----------------------------------|---------------------|------------------------|
|                |         | (95% Confidence Interval) | Odds Ratio | P Value | Odds Ratio | P Value |
| CXCL11         | 1st     | 2532 (2060–3467)              | 1          | 1       | 11.51 (2.22 to 59.63) | 0.004   |
|                | 2nd     | 8658 (7016–10,109)             | 5.00 (1.45 to 17.27) | 0.01 | 1.93 (0.40 to 9.32) | 0.41    |
| CXCL2/CXCL3    | 1st     | 459 (406–512)                 | 1          | 1       | 5.00 (1.45 to 17.27) | 0.01    |
|                | 2nd     | 695 (643–766)                 | 2.36 (0.74 to 7.60) | 0.15 | 1.45 (0.37 to 5.78) | 0.60    |
| CD86           | 1st     | 1105 (1065–1127)              | 1.41 (0.45 to 4.46) | 0.56 | 1.45 (0.37 to 5.78) | 0.60    |
|                | 2nd     | 1539 (1365–1748)              | 5.00 (1.45 to 17.27) | 0.01 | 6.74 (1.47 to 30.80) | 0.01    |
| WNT7A          | 1st     | 270 (246–294)                 | 0.84 (0.26 to 2.68) | 0.77 | 11.6 (0.25 to 5.39) | 0.85    |
|                | 2nd     | 1005 (958–1106)               | 1.41 (0.45 to 4.46) | 0.56 | 0.92 (0.20 to 4.20) | 0.92    |
| BTK            | 1st     | 758 (673–837)                 | 5.32 (1.49 to 19.06) | 0.01 | 13.1 (2.40 to 71.52) | 0.003   |
|                | 2nd     | 356 (500–609)                 | 5.32 (1.49 to 19.06) | 0.01 | 5.48 (1.19 to 25.21) | 0.03    |
| c-Myc          | 1st     | 638 (556–683)                 | 0.83 (0.26 to 2.72) | 0.76 | 2.45 (0.42 to 14.40) | 0.32    |
|                | 2nd     | 863 (771–915)                 | 11.67 (2.70 to 50.49) | 0.001 | 55.79 (5.53 to 562.99) | 0.0007 |
| TIMP-3         | 1st     | 1046 (680–1381)               | 1.19 (0.37 to 3.79) | 0.77 | 1.15 (0.21 to 6.32) | 0.87    |
|                | 2nd     | 3706 (5328–9175)              | 6.33 (1.75 to 22.91) | 0.005 | 36.98 (4.31 to 317.19) | 0.001  |
| CCL5           | 1st     | 12,782 (10,444–16,950)        | 1.67 (0.53 to 5.27) | 0.38 | 2.38 (0.56 to 10.04) | 0.24    |
|                | 2nd     | 38,218 (28,916–49,428)        | 4.05 (1.21 to 13.54) | 0.02 | 8.64 (1.64 to 45.42) | 0.01    |
| Ghrelin        | 1st     | 1519 (1401–1707)              | 1.19 (0.37 to 3.79) | 0.77 | 2.00 (0.47 to 8.50) | 0.35    |
|                | 2nd     | 2852 (2506–3311)              | 6.33 (1.75 to 22.91) | 0.005 | 11.70 (2.21 to 61.77) | 0.004   |
| PDGF-C         | 1st     | 484 (411–529)                 | 1.97 (0.62 to 6.24) | 0.25 | 3.24 (0.73 to 14.44) | 0.12    |
|                | 2nd     | 648 (631–723)                 | 3.33 (1.02 to 10.90) | 0.05 | 5.12 (1.12 to 23.41) | 0.04    |
| Survivin       | 1st     | 1113 (1006–1220)              | 1.67 (0.53 to 5.27) | 0.38 | 1.33 (0.28 to 6.39) | 0.72    |
|                | 2nd     | 1515 (1342–1578)              | 4.05 (1.21 to 13.54) | 0.02 | 10.71 (1.94 to 59.27) | 0.007   |
| CA2            | 1st     | 260 (247–271)                 | 1.00 (0.32 to 3.15) | 1.00 | 0.94 (0.24 to 3.75) | 0.93    |
|                | 2nd     | 380 (318–431)                 | 4.20 (1.23 to 14.37) | 0.02 | 5.11 (1.14 to 22.98) | 0.03    |
| IL-9           | 1st     | 388 (371–415)                 | 4.86 (1.43 to 16.50) | 0.01 | 14.59 (2.30 to 92.51) | 0.004   |
|                | 2nd     | 571 (515–634)                 | 4.05 (1.21 to 13.54) | 0.02 | 10.38 (1.83 to 58.72) | 0.008   |
| EGF            | 1st     | 747 (655–866)                 | 1.41 (0.45 to 4.46) | 0.56 | 1.46 (0.30 to 7.11) | 0.64    |
|                | 2nd     | 1114 (1035–1346)              | 5.00 (1.45 to 17.27) | 0.01 | 12.06 (2.20 to 66.12) | 0.004   |
| Neuregulin-1   | 1st     | 253 (223–282)                 | 1.41 (0.45 to 4.46) | 0.56 | 3.43 (0.77 to 15.34) | 0.11    |
|                | 2nd     | 360 (333–408)                 | 5.00 (1.45 to 17.27) | 0.01 | 7.92 (1.61 to 38.90) | 0.01    |
| CXCL16, soluble| 1st     | 12,884 (10,813–15,447)        | 0.39 (0.12 to 1.34) | 0.14 | 0.38 (0.09 to 1.69) | 0.20    |
|                | 2nd     | 27,219 (21,844–29,434)        | 0.14 (0.04 to 0.49) | 0.002 | 0.09 (0.02 to 0.44) | 0.003   |
| IL1RL1         | 1st     | 10,425 (7549–12,137)          | 0.41 (0.13 to 1.35) | 0.14 | 0.41 (0.09 to 1.81) | 0.24    |
|                | 2nd     | 30,420 (21,322–38,962)        | 0.25 (0.07 to 0.83) | 0.02 | 0.11 (0.02 to 0.66) | 0.02    |
| Stanniocalcin   | 1st     | 2594 (1831–3082)              | 0.26 (0.07 to 0.94) | 0.04 | 0.13 (0.02 to 0.77) | 0.02    |
|                | 2nd     | 4166 (3851–5157)              | 0.25 (0.08 to 0.83) | 0.02 | 0.22 (0.05 to 0.99) | 0.05    |
promoting VEGFA gene expression (44), illustrating its role in cell growth and angiogenesis.

Coincidentally, higher serum levels of BTK, ghrelin, PDGF-C, survivin, EGF, and neuregulin-1 were associated with kidney recovery by day 28. BTK is a tyrosine-protein kinase and plays roles in B-cell development. PDGF-C, EGF, and neuregulin-1 are growth factors, and both EGF and neuregulin-1 belong to the epidermal growth factor family. Delayed recovery from AKI was found in mice with specific deletion of the EGF receptor in renal proximal tubule epithelial cells (45). Ghrelin is expressed in kidney and other organ tissues (46); however, ghrelin improves kidney function in mice with ischemic acute kidney failure (47) and reduces kidney tissue damage in rats (48), probably due to its anti-inflammatory and anti-oxidant effects (49). Increased expression of survivin in kidney epithelial cells was induced by AKI, and kidney recovery was markedly delayed in mice with renal proximal tubule-specific deletion of survivin (50), suggesting survivin plays a direct role in kidney recovery. Taken together, low levels of inflammation-associated proteins (e.g., FGF23, CXCL6), high levels of chemokines/cytokines for inflammatory tissue repair, and high levels of Wnt-7a, BTK, c-Myc, ghrelin, PDGF-C, survivin, EGF, and neuregulin-1 are likely to play concerted roles in the coordination and regulation of angiogenesis, endothelial and epithelial remodeling, kidney cell regeneration in the transition from inflammation to kidney recovery, and thus may be used as potential biomarkers for the prediction of kidney recovery.

This study had several limitations. First, the availability of randomized day-8 serum samples with secondary data analyses from the clinical trial study population was limited. This restricted the development of a predictive model. However, we addressed these shortcomings by using multiple statistical methods to attenuate potential biases. Second, both SOMAscan and Olink assays measured relative protein level changes, but not absolute protein concentrations in serum, and not all of the proteins discovered in the SOMAscan assay could be verified using Olink assays as many of them were not included on the Olink panel. Third, this is a biomarker discovery study for kidney recovery and there is a lack of an independent cohort of patients for validation of these novel biomarker candidates. The results from this study are informative for further verification in a larger cohort of samples. Finally, we were unable to follow the trajectory of protein changes over the course of kidney recovery due to lack of samples collected at later time points. Further studies specifically designed for this purpose may be able to address this issue. However, the novel biomarkers of kidney recovery discovered in our study warrant further independent validation in patients with AKI-D.

In conclusion, this study used a high-throughput proteomic technology to identify potential novel biomarkers of kidney recovery and defined potential biologic pathways associated with kidney remodeling, repair, and regeneration after AKI-D. Biomarkers involved in the progressive biologic processes of reducing inflammation, recruiting tissue repair immune cells, releasing growth factors, activating epithelial and endothelial remodeling, and increasing kidney cell growth and regeneration may be important targets for future studies to evaluate kidney function recovery.

Disclosures

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Research/FDA. J. Ma reports being a scientific advisor or member of the Editorial Board of American Journal of Physiology – Renal Physiology, Data and Safety Monitoring Board Member for the Clinical Trials network, the National Institute on Drug Abuse, and Statistician on the Editorial Board for Frontiers in Molecular Psychiatry. L.-R. Yu reports being a scientific advisor or membership as Member of the Editorial Board of Journal of Proteomics. P. Palevsky reports having consultancy agreements with Janssen Research & Development; reports being a scientific advisor or membership as Member of the Editorial Board of the Journal of Intensive Care Medicine, President and Member of the Scientific Advisory Board of the National Kidney Foundation, Member of the Quality, Safety and Accountability Committee of the Renal Physicians Association, Chair of the Medical Review Board for the Quality Insights Renal Network 4, and Section Editor, Renal Failure of UpToDate. R. Beger reports being a scientific advisor or member as Editor for Metabolomics Society and Scientific Reports and Coordinating Committee Member of the metabolomics Quality Assurance and quality Control Consortium (mQACC). All remaining authors have nothing to disclose. The ATN Study was conducted by the ATN Investigators and supported by the Cooperative Studies program of the Department of VA Office of Research and Development as CSP 530 and by the NIDDK under interagency agreement Y1-DK-3508-01.

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This article reflects the views of the authors and does not necessarily reflect those of the US FDA. Any mention of commercial products is for clarification only and is not intended as approval, endorsement, or recommendation. This manuscript was not prepared in collaboration with the ATN Study Investigators and does not necessarily reflect the opinions or views of the ATN Study, VA, or NIDDK.

Author Contributions
R. Beger, D. Portilla, and L.-R. Yu conceptualized the study; J. Daniels and L.-R. Yu were responsible for data curation; Z. Cao, J. Ma, and L.-R. Yu were responsible for formal analysis; D. Portilla, J. Sun, and L.-R. Yu, were responsible for funding acquisition; P. Palevsky, D. Portilla, and L.-R. Yu were responsible for the methodology; D. Choudhury, P. Palevsky, and D. Portilla were responsible for the resources; R. Beger provided supervision; Z. Cao and L.-R. Yu were responsible for the validation and for visualization; L.-R. Yu wrote the original draft; R. Beger, Z. Cao, D. Choudhury, J. Daniels, J. Ma, P. Palevsky, L. Pence, D. Portilla, L. Schnackenberg, and J. Sun reviewed and edited the manuscript.

Supplemental Material
This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl?doi=10.1038/kid.0002642011.-/DCSupplemental.

Supplemental Table 1. Proteins with significant changes in serum levels (1.2-fold and P<0.05) on day 8 as measured by SOMAscan assays in the patients who recovered (R) kidney function compared with those who did not recover (NR) by day 28.

Supplemental Table 2. All of the 1305 proteins measured by SOMAscan assays for day 8 serum samples from the patients who recovered (R) kidney function compared with those who did not recover (NR) by day 28.

Supplemental Table 3. Significantly altered biological functions or diseases revealed from Ingenuity Pathway Analysis.

Supplemental Table 4. All of the 92 proteins measured by the Olink assay for day 8 serum samples from the patients who recovered (R) kidney function compared with those who did not recover (NR) by day 28.

Supplemental Table 5. Logistic regression of kidney recovery on the baseline covariates.

Supplemental Table 6. Odds ratios and 95% confidence intervals of day 8 serum proteins in tertiles not significantly associated with kidney function recovery by day 28 as analyzed by logistic regression.

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