Urinary excretion of twenty peptides forms an early and accurate diagnostic pattern of acute kidney injury

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Early and accurate detection of acute kidney injury (AKI) is needed to prevent the progression to chronic kidney disease and to improve outcome. Here we used capillary electrophoresis–mass spectrometry to identify urinary peptides predictive of AKI in a training set of 87 urine samples longitudinally collected from patients in an intensive care unit. Within this patient cohort, 16 developed AKI while 14 maintained normal renal function. The sequence of twenty peptides significantly associated with AKI was identified. They were found to be degradation products of six proteins. These formed a diagnostic pattern. Peptides of albumin, α-1-antitrypsin, and β-2-microglobulin were upregulated but fragments of fibrinogen α and collagens 1 α(I) and 1 α(III) were downregulated in AKI. After cross-validation of the training set, a good diagnostic performance of the marker pattern was found with an area under the ROC curve of 0.91. This was confirmed in a blinded validation set of 20 patients in the intensive care unit and 31 allogeneic hematopoietic stem cell transplantation patients, of which 13 had and 18 had not experienced an episode of AKI. In comparison to more established markers of AKI such as serum cystatin C and urinary kidney injury molecule-1, interleukin-18, and neutrophil gelatinase associated-lipocalin, the proteomic marker pattern was found to be of superior prognostic value, detecting AKI up to 5 days in advance of the rise in serum creatinine.

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Acute kidney injury (AKI) is a common and serious clinical condition, especially in critically ill patients, increasing mortality independent of other factors.[1–3] Early and accurate detection of AKI is crucial to enable early therapeutic intervention and thereby to potentially improve the outcome.[4,5] In clinical practice, AKI is detected by changes in serum creatinine, and sudden increments of creatinine currently define AKI.[6] However, there are major limitations to the use of creatinine, as it is moderately accurate and enables AKI detection only at a late stage.[7–9] Therefore, several new biomarkers have emerged in the last 5 years, which promised earlier and more accurate AKI detection in initial studies, including serum cystatin C (S-CysC), kidney injury molecule-1 (KIM-1), interleukin-18 (IL-18), and neutrophil gelatinase-associated lipocalin (NGAL).[10–13] However, validation in independent studies and larger, less-specific patient cohorts presented conflicting results.[14–17] Combined patterns of these biomarkers have recently been suggested to perform better than single biomarkers.[13,17,18] The diagnostic accuracy in predicting AKI of such combinations is yet unknown, pinpointing the demand for novel, predictive, and AKI-specific biomarkers.

Proteomic techniques have already proven to be a valuable tool in the discovery of novel AKI markers.[19–23] These studies were, however, limited by small or selected patient cohorts, less information on the diagnostic value and/or identity of the biomarkers, and absence of blinded assessments.

The purpose of this study was to overcome these challenges in clinical study design and to map the urinary low-molecular-weight proteome of non-selected critically ill patients who either developed AKI or maintained their normal renal function by capillary electrophoresis–mass spectrometry (CE–MS). We defined a biomarker pattern based on naturally occurring polypeptides that allows accurate and early detection of AKI and assessed its clinical utility in two independent cohorts of patients with different underlying etiologies of AKI.

Table 1 | Characteristics of patients with and without AKI in the training and the two validation sets

	ICU training set				ICU validation set				HSCT validation set				Healthy controls
	AKI	Non-AKI	*P*	STD	AKI	Non-AKI	*P*	STD	AKI	Non-AKI	*P*	STD	
Patients/samples (*n*)	16/38	14/49			9/9	11/11			13/16	18/22			28
Age (years)	66 ± 13	69 ± 14	0.21	0.22	63 ± 15	68 ± 12	0.49	0.37	53 ± 13	51 ± 13	0.84	0.00	51 ± 11
Gender (female/male)	15/22	18/30	0.95		3/6	3/8	1.00		6/7	6/12	0.47		6/22
Diabetes mellitus, *n* (%)	6 (38)	4 (29)	0.61		3 (33)	2 (18)	0.85		ND	ND			0
Chronic kidney disease, *n* (%)	5 (31)	3 (21)	0.54		2 (22)	2 (18)	0.74		ND	ND			0
APACHE II score (points)	23 ± 5	24 ± 4	0.17	0.22	24 ± 3	23 ± 4	0.63	0.29	ND	ND			NA
S-creatinine at baseline (μmol/l)	88 ± 14	89 ± 12	0.85	0.08	93 ± 14	91 ± 15	0.78	0.14	90 ± 22	93 ± 24	0.36	0.13	97 ± 12[a]
Estimated glomerular filtration rate at baseline (ml/h per 1.73 m^2)	72 ± 18	71 ± 17	0.98	0.06	69 ± 17	71 ± 19	0.56	0.11	72 ± 27	75 ± 28	0.42	0.10	73 ± 15
Urine output at baseline (ml/day)	2.2 ± 0.6	2.4 ± 0.7	0.33	0.31	2.3 ± 0.5	2.4 ± 0.6	0.81	0.16	ND	ND			
Etiology of AKI (*n*)													
Ischemia	5				2				1				
Nephrotoxic	1	NA			0	NA			3	NA			NA
Sepsis	3				2				7				
Combination	7				5				2				
Worst AKI stage, *n* (I/II/III)	1/4/11				0/3/6				NA				
Renal replacement therapy, *n* (%)	8 (50)				4 (44)								
Oliguria, *n* (%)	1 (6)				1 (11)								

Abbreviations: AKI, acute kidney injury; APACHE II, Acute Physiology and Chronic Health Evaluation II; HSCT, hematopoietic stem cell transplantation; ICU, intensive care unit; NA, not available; ND, not determined; STD, standardized difference. Data are presented as mean ± s.d.

[a]S-creatinine at baseline was significantly lower in AKI cases and non-AKI controls of the ICU training set when compared with healthy controls (*P*=0.011 and *P*=0.007, respectively). These differences might be attributed to lower muscle mass, less physical mobility, and high rate of sepsis of ICU patients compared with healthy subjects.

RESULTS

For biomarker definition, we analyzed urine samples of 30 intensive care unit (ICU) patients, of whom 16 developed AKI (training set). Patients with and without AKI did not substantially differ with respect to demographic and clinical characteristics, as demonstrated in Table 1. In 14 patients, AKI was defined by the serum creatinine criteria, and in further 2 by serum creatinine and urine output criteria. Worst AKI stages I, II, and III were present in 1, 4, and 11 patients, respectively. One patient (6%) developed oliguria before the detection of AKI by Acute Kidney Injury Network criteria, and 8 patients (50%) required renal replacement therapy in the ICU training set. Initial urine samples of AKI patients were collected at 3.4 ± 1.0 days before the diagnosis of AKI by serum creatinine as reference standard, with a maximum of 5 days. Mean sample number per patient was 2.4 in the AKI and 3.5 in the non-AKI ICU group, with ranges of 1–5 and 1–6 days, respectively. Urine samples were subjected to CE–MS to identify novel biomarkers for AKI.

For multivariate statistical comparison of the data from patients with and without AKI, only polypeptides with identified peptide sequence were considered. Analysis resulted in the definition of 64 potential biomarkers with Benjamini and Hochberg adjusted *P*-values <0.05 (Supplementary Table S1 online). Initially, all peptides from this pool were employed for pattern establishment and their number was sequentially reduced by a take-one-out procedure. A total of 16 urinary peptides were combined using the support vector machine-based MosaCluster software[24] to generate an AKI-specific multimarker pattern. Sequence data indicated that the 16 AKI-specific urinary peptides were fragments of the serum proteins albumin, α-1-antitrypsin (AAT), β-2-microglobulin (B2M), and fibrinogen α chain (FIBA), and of the structural proteins collagen 1 α(I) (COL1A1) and collagen 1 α(III) (COL1A3). The characteristics of these peptides are presented in Table 2. All fragments from albumin, AAT, and B2M were consistently upregulated in AKI, whereas all peptides from COL1A1, COL1A3, and FIBA were markedly downregulated. Sequence alignment of the AKI-specific peptides with the corresponding parent proteins demonstrated that fragments from albumin and FIBA form clusters of overlapping sequences with predominantly identical N-terminus, and different C-terminus. Therefore, we searched for additional differentially regulated (Wilcoxon *P*-values <0.05) urinary peptides that originate from the same proteins and protein regions.

This approach resulted in the identification of four additional peptides that improved the performance of the support vector machine model from 73/87 to 82/87 correctly classified samples in the training set. Cross-validated classification (as described by Dakna *et al.*[25]) of the training samples with the final 20 peptide marker pattern yielded an area under the curve (AUC) value of 0.91 for AKI detection in receiver operating characteristic curve with the 95% confidence interval (CI) being 0.83–0.96. Sensitivity of 92% (95% CI 79–98%) and specificity of 90% (95% CI 78–97%) were achieved. As presented in the Supplementary Figure S1a online, for three AKI and three non-AKI ICU patients of the training set with consecutively daily urine samples available, the urinary peptide pattern consistently discriminated well between AKI and non-AKI over a time period of 4 days before AKI.

To validate reliability in AKI detection, the proteomic marker pattern was subsequently applied to 20 ICU patients of a blinded phase II study cohort. Patients of this ICU validation set did not significantly differ in their demographic and clinical data from the patients in the training set

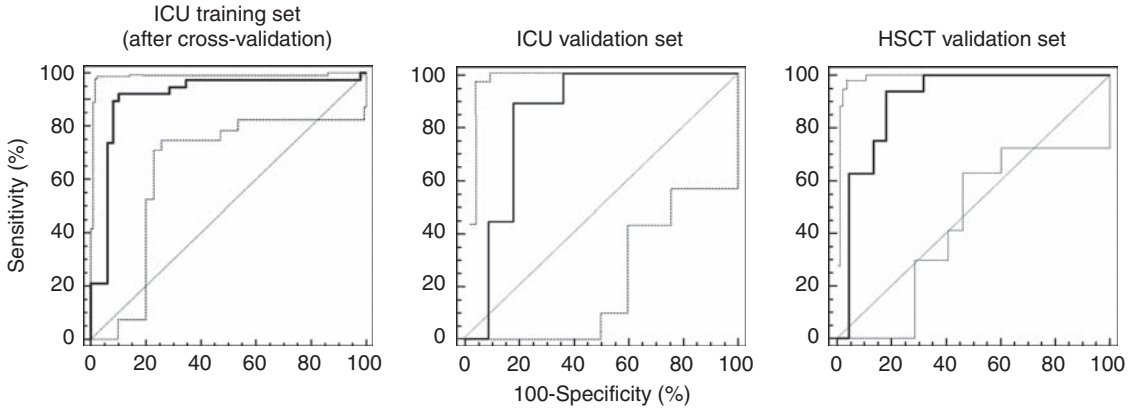
Table 2 | Characteristics of urinary polypeptides included in the AKI marker pattern

						Training set					
Mass (Da)	CE time (min)	Protein	Sequence[a]	AA	Remarks	Mean counts AKI	Frequency AKI	Mean counts non-AKI	Frequency non-AKI	*P*	AUC
1439.66	29.82	AAT	TIDEKGTEAAGAMF	363–376	- Fragments C-terminally from the extended loop containing the active site	752	0.50	148	0.27	0.0395[†]	0.64
912.54	20.06		KVVNPTQK	411–418		126	0.50	39	0.13	0.0057[†]	0.68
2080.99	20.20	ALB	DAHKSEVAHRFKDLGEEN	25–42	- N-terminal sequence of mature ALB	6580	0.44	729	0.36	0.0138[‡]	0.59
2356.16	19.52		DAHKSEVAHRFKDLGEENFK	25–44	- Fragments with identical N-terminus, but different C-terminus	2801	0.47	346	0.20	0.0109[†]	0.66
2427.19	19.58		DAHKSEVAHRFKDLGEENFKA	25–45		1512	0.45	339	0.15	0.0109[†]	0.66
2540.28	19.68		DAHKSEVAHRFKDLGEENFKAL	25–46		2820	0.47	654	0.23	0.0340[†]	0.64
2639.35	19.78		DAHKSEVAHRFKDLGEENFKALV	25–47		1371	0.22	247	0.09	0.0292[‡]	0.59
1081.66	20.73	B2M	IQRTPKIQV	21–29	- Fragments, but not full-length B2M, were found differentially regulated in AKI	896	0.45	207	0.18	0.0395[†]	0.63
1300.59	28.53		VSGFHPSDIEVD	47–58	- Peptides with overlapping sequence motifs	980	0.39	29	0.12	0.0165[†]	0.64
2603.30	20.07		LKNGERIEKVEHSDLSFSKDWS	60–81		20 452	0.45	5411	0.40	0.0226[‡]	0.58
2716.38	20.19		LLKNGERIEKVEHSDLSFSKDWS	59–81		144 368	0.63	24 126	0.52	0.0423[†]	0.65
1654.79	23.13	COL1A1	SpGEAGRpGEAGLpGAKG	522–539	- Peptides 522–539 and 798–810 also detected in plasma	911	0.68	2872	0.98	0.0004[†]	0.78
1737.78	31.00		TGSpGSpGPDGKTGPPGpAG	541–560		939	0.63	3298	0.82	0.0078[†]	0.73
1250.55	27.93		ApGDRGEpGPpGP	798–810		16 430	0.97	25 594	1.00	0.0083[†]	0.71
3359.57	31.90		PpGADGQPGAKGEpGDAGAKGD AGPpGPAGPAGPpGPIG	816–854		154	0.16	437	0.55	0.0106[†]	0.68
2942.30	22.23		ESGREGApGAEGSpGRDGSpGAK GDRGETGP	1011–1041		1934	0.68	3970	0.92	0.0085[†]	0.71
2007.94	22.10	COL1A3	DGESGRPGRPGERGLPGPPG	230–249		2781	0.82	5923	0.96	0.0047[†]	0.74
1825.78	19.41	FIBA	DEAGSEADHEGTHSTKR	605–621	- Fragments with identical N-terminus, but different C-terminus	511	0.37	1593	0.88	0.0004[†]	0.78
1882.80	20.14		DEAGSEADHEGTHSTKRG	605–622	- Peptides 605–622 and 605–628 also detected in plasma	11 553	0.87	25 962	0.90	0.0083[†]	0.72
2559.18	19.41		DEAGSEADHEGTHSTKRGH AKSRP	605–628		6342	0.61	5184	0.49	0.0399[‡]	0.58

Abbreviations: AA, amino acid; AAT, α-1-antitrypsin; AKI, acute kidney injury; AUC, area under curve; B2M, β-2-microglobulin; CE, capillary electrophoresis; COL1A1, collagen 1 α(I); COL1A3, collagen 1 α(III); FIBA, fibrinogen α chain.
[a]Lower case p indicates hydroxyproline.
[†]Benjamini and Hochberg adjusted *P*-values.
[‡]Unadjusted Wilcoxon *P*-values.

(Table 1). In all nine patients, AKI was defined by serum creatinine criteria. Data on worst AKI stages and required renal replacement therapy are presented in Table 1. Upon unblinding, 8 of 9 patients with AKI and 9 of 11 patients without AKI were correctly identified. As shown in Figure 1, this yielded an AUC of 0.84 (95% CI 0.61–0.96), a sensitivity of 89% (95% CI 52–98%), and a specificity of 82% (95% CI 48–97%). For further validation, the AKI-specific pattern was applied to a second validation set of 31 patients after allogeneic hematopoietic stem cell transplantation (HSCT) as different underlying etiology of AKI. Patients of the HSCT validation set with and without AKI also did not significantly differ in demographic and clinical characteristics (Table 1). In this cohort, 13 patients developed AKI. As indicated by an AUC of 0.90 (95% CI 0.76–0.97), a sensitivity of 94% (95% CI 70–99%), and a specificity of 82% (95% CI 60–95%), the AKI-specific biomarker pattern also performed well to detect AKI in this non-ICU validation cohort (Figure 1). Of note, urine samples in HSCT patients were spontaneously voided and not obtained from indwelling catheter as in ICU patients. This is of interest as urine collection from indwelling catheters may potentially alter the urinary proteome by protein adsorption or prolonged storage in the catheter collection bag. The validity of this biomarker pattern was further supported by the finding that none of the 28 healthy controls was diagnosed false positive for AKI.

To test whether the AKI-specific biomarker pattern performed differently at varying AKI stages, we compared ICU patients with AKI stages 1 and 2 ($n = 17$) with those in stage 3 ($n = 30$). Comparison of classification factors between these groups revealed no statistically significant differences of classification by the urinary peptide pattern depending on the severity of AKI (data not shown). To investigate how the AKI-specific biomarker pattern performed during the recovery phase of AKI compared with serum creatinine, the longitudinal course of classification by the AKI marker panel was monitored for three HSCT patients, for whom multiple measurements at the time of AKI were available. As presented in the Supplementary Figure S1b online, the AKI-specific biomarker pattern permitted earlier detection of AKI. The AKI-specific biomarker pattern dropped below the cutoff value for AKI diagnosis in parallel to the decrease of serum creatinine. This suggests an equivalent diagnostic value of both markers for AKI recovery.



Patient group	ICU training set	ICU validation set	HSCT validation set
Sample size case/control	38/49	9/11	16/22
AUC	0.91	0.84	0.90
s.d.	0.31	0.43	0.35
95% CI	0.83–0.96	0.61–0.96	0.76–0.97
Significance level *P*	0.0001	0.0004	0.0001

Figure 1 | Receiver operating characteristic (ROC) curves of the acute kidney injury (AKI)-specific biomarker pattern to detect AKI in the intensive care unit (ICU) training set (left), in the ICU validation set (middle), and in the hematopoietic stem cell transplantation (HSCT) validation set (right). The table presents the distribution of AKI patients in the different cohorts, areas under the ROC (AUC), and 95% confidence intervals (CI). The 95% CIs in the ROCs are indicated by dashed lines.

In Figure 2, the compiled CE–MS profiles of AKI marker distribution in patients with and without AKI of the ICU training, the ICU validation, and the HSCT validation sets were compared. Mean excretion levels of individual peptides in the compiled CE–MS spectra of case and control patients differed to some extent between the ICU and HSCT cohorts. This is not unexpected as etiology, comorbidity, and severity of illness vary in ICU and HSCT patients and indicate the ability of the AKI-specific marker pattern to differentiate between AKI and non-AKI in ICU and HSCT patients independently of these potential confounders.

The classification performance of urinary proteome analysis was compared with urinary NGAL, IL-18, and KIM-1, and the serum marker S-CysC. Receiver operating characteristic analysis on the ICU and HSCT validation sets revealed low classification accuracy for the proposed single AKI markers, not reaching statistical significance in any of these two cohorts (Figure 3). Even when both patient cohorts were combined, insignificant classification performance was obtained (data not shown).

In the following, we analyzed early diagnostic efficacy of the AKI marker pattern. For this purpose, the nine AKI patient samples of the ICU validation set were subdivided into available samples of days −5 and −4 (−5/−4) and days −3 to 0 (−3 to 0) before AKI. The 11 non-AKI samples of the ICU validation set served as reference group in both comparisons. As presented in Figure 4, the biomarker pattern performed equally well in the two subgroups, as indicated by an AUC for AKI detection of 0.86 (95% CI 0.59–0.97) in the −5/−4 group and 0.82 (95% CI 0.54–0.96) in the −3 to 0 group. Compared with the proteomic marker pattern, a statistically significant rise in the levels of proposed single AKI biomarkers was first detected for S-CysC at day −3, whereas NGAL showed only significance on day −2, and KIM-1 and IL-18 on days −1 and 0 before AKI (Supplementary Figure S2 online). In the case of the single AKI biomarkers, receiver operating characteristic analysis was extended to all samples of AKI and non-AKI ICU patients to obtain statistical confidence.

In 43 ICU patients, data on albuminuria before AKI episodes were available. The odds ratio to develop increased urinary excretion of albumin fragments when having demonstrated albuminuria was 1.2 (95% CI 0.8–1.7; $P = 0.72$). From 17 ICU patients with established AKI, serum creatinine values were available for >3 months of follow-up after AKI episode. In this subgroup, a downregulation of COL1A1 and COL1A3 fragments was associated with the development or progress of chronic kidney disease (CKD) subsequent to AKI with an odds ratio of 3.0 (95% CI 1.3–7.4; $P = 0.009$).

DISCUSSION

We developed a multivariate, AKI-specific biomarker pattern consisting of 20 urinary polypeptides. The pattern detects the development of AKI up to 5 days earlier than serum creatinine, the current standard reference for AKI detection. Our results were confirmed by validation in two independent, blinded ICU and cross-sectional HSCT patient cohorts. In addition, our data suggest that this AKI-specific biomarker pattern consistently discriminates well between AKI and

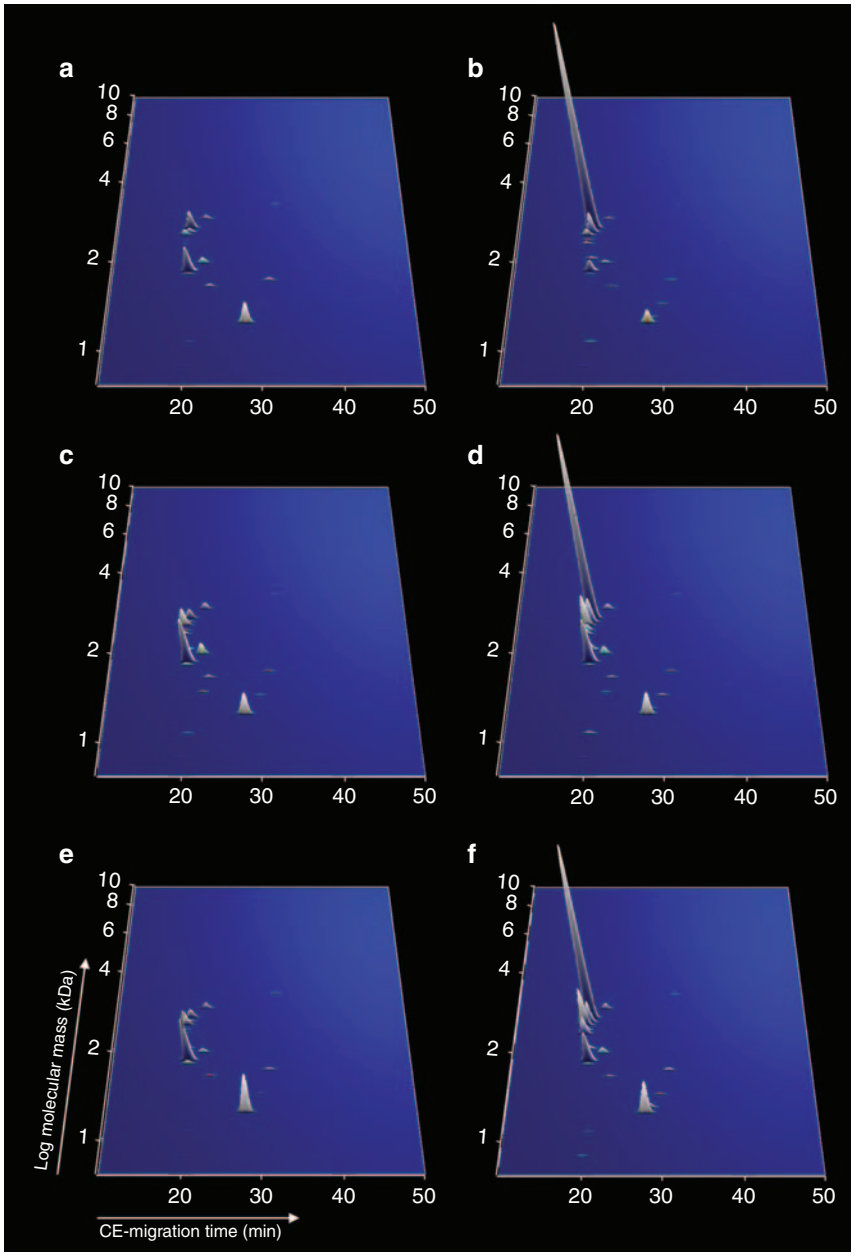
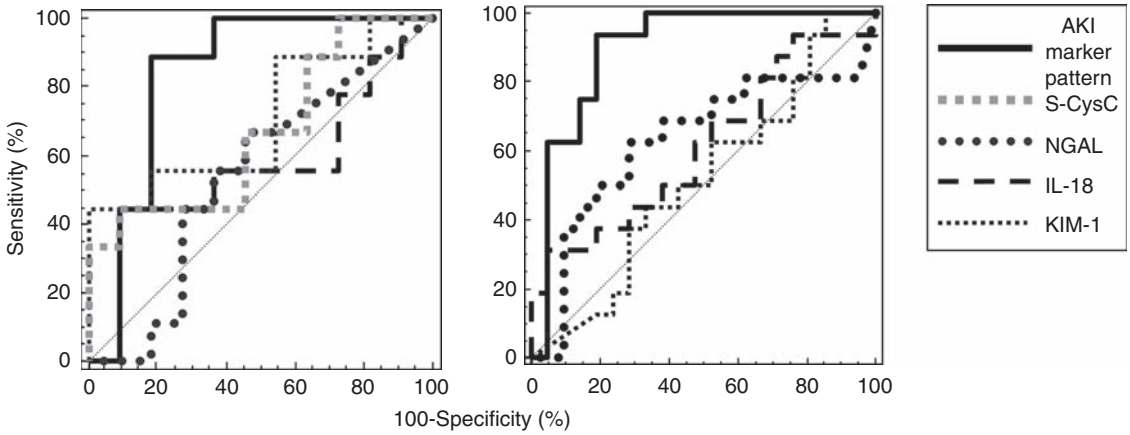


Figure 2 | Peptide pattern distinguishing patients with and without acute kidney injury (AKI). Compiled capillary electrophoresis–mass spectrometry (CE–MS) profiles of the 20 urinary polypeptides included in the AKI-specific biomarker pattern in the intensive care unit (ICU) training set (**a**, **b**), ICU validation set (**c**, **d**), and hematopoietic stem cell transplantation (HSCT) validation set (**e**, **f**). Polypeptide profiles of AKI patients are presented on the right, those of patients without AKI (non-AKI) on the left. Normalized CE-migration time (range 10–50 min) on the *x* axis is plotted against log molecular mass (range 0.8–10 kDa) on the *y* axis. Mean signal intensity of polypeptides is encoded by peak height.

non-AKI over a time period of several days before AKI, that the pattern does not change depending on the severity of AKI, and that it has equivalent diagnostic value for AKI recovery in comparison to serum creatinine.

The sequences of all 20 polypeptides were known and the fragments could be attributed to 4 serum and 2 structural proteins with a wide range of regulatory functions. So far, only full-length B2M and peptide fragments have repeatedly been reported as urinary biomarkers of AKI.[19–21,26] The alterations of these polypeptides identified in urine may be attributed to differences in production rates, increased assembly into filaments, increased proteolysis in the plasma or urine, renal dysfunction, or a combination of the above. This study does not permit to distinguish between these various mechanisms and might be an origin for systematic investigation of the pathophysiological implications of these markers.

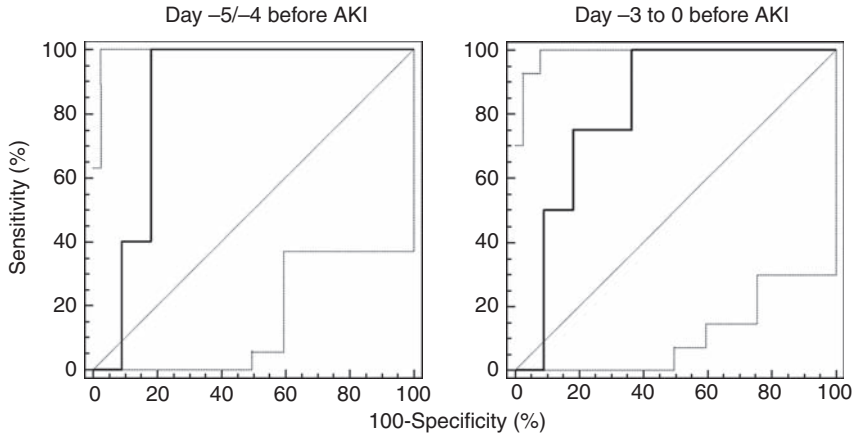
The most pronounced change detected in this study was the upregulation of urinary B2M fragments. Renal tubular epithelial cell injury is indicated by increased urinary



Biomarker / Study group	AKI marker pattern	S-CysC	NGAL	IL-18	KIM-1
ICU validation set (n=20)	0.84	0.67	0.54	0.57	0.71
HSCT validation set (n=38)	0.90	ND	0.63	0.61	0.52

ND, not determined

Figure 3 | Acute kidney injury (AKI) classification of the intensive care unit (ICU) and hematopoietic stem cell transplantation (HSCT) validation set based on either the AKI marker pattern or detection of levels of urinary neutrophil gelatinase-associated lipocalin (NGAL; in ng/mg U-Crea), urinary interleukin-18 (IL-18; in pg/mg U-Crea), urinary kidney injury molecule-1 (KIM-1; in pg/mg U-Crea), or serum cystatin C (S-CysC; in mg/l). Comparison of receiver operating characteristic (ROC) curves (upper panel) and table of ROC-derived area under the curve (AUC) values for classification of the ICU and HSCT validation sets with the AKI marker pattern or single AKI biomarkers (lower panel).



Patient group	Day –5/–4 before AKI	Day –3 to 0 before AKI
Sample size case/control*	5/11	4/11
AUC	0.86	0.82
s.d.	0.47	0.55
95% CI	0.59–0.97	0.54–0.96
Significance level P	0.003	0.025
Positive likelihood ratio	5.49	4.12

* The same non-AKI ICU samples were used as controls in both ROC comparisons

Figure 4 | Early diagnostic efficacy of the acute kidney injury (AKI) marker pattern at different days before AKI detection by serum creatinine. The nine AKI patient samples of the intensive care unit (ICU) validation set were subdivided into available samples of days −5 and −4 and days −3 to 0 before AKI. In both receiver operating characteristic (ROC) curves, the 11 non-AKI samples of the ICU validation set were used as negative reference group. As indicated by identical ROC curve characteristics, the AKI marker pattern performed equally well in both groups.

excretion of B2M.[27] After passage of the glomerular filter, B2M is taken up from the proximal tubule by megalin and cubilin-mediated endocytosis.[28] In experimental AKI, increased urinary B2M could be attributed to impaired megalin and cubilin expression.[29] Moreover, accelerated fragmentation of accumulated urinary B2M may occur because of an inflammation-induced increase in tubular proteolytic activity.[30] The same mechanism might also account for the observed upregulation of urinary albumin fragments in AKI patients, as albumin and most likely albumin fragments are also filtered by the glomerulum and reabsorbed by megalin and cubilin.[31] Increased levels of albumin fragments have already been described in nephrotic syndrome,[32] where fragmentation was primarily discovered not only in plasma but also in urine.

AAT is a major acute-phase protein with various anti-inflammatory and anti-apoptotic effects, as could be shown in an experimental model of renal ischemia/reperfusion injury.[33] In various animal models of AKI, cytokine-mediated proinflammatory responses have a major role in the pathophysiology of AKI.[34] Human AKI is associated with increased levels of proinflammatory cytokines and acute-phase proteins.[35,36] Although no previous data on urinary AAT and its fragments in AKI have yet been published, AAT fragments were detected in small amounts in plasma and urine of healthy individuals and in larger amounts in patients with nephrotic syndrome.[32] Fragmentation of AAT may occur in plasma during increased supply and degradation of AAT-protease complexes as well as in urine because of increased activity of microenvironmental proteinases, such as matrix metalloproteinase (MMP)-9, in response to tubular damage.[13,37,38] With respect to the observed decrease in collagen fragments, it was found that intact AAT affects remodeling of extracellular matrix (ECM) mainly through binding to elastase,[39] thereby preventing its capacity to cleave tissue inhibitor of metalloproteinases.[40] The balance between tissue inhibitor of metalloproteinase and MMPs is crucial in maintaining ECM homeostasis of constant assembly and disassembly.[41] A tissue inhibitor of metalloproteinase-mediated inhibition of MMP activity may therefore account for decreased urinary excretion of COL1A1 and COL1A3 fragments, as less collagen filaments from the ECM will be cleaved. This hypothesis is in line with observations that renal interstitial fibrosis is associated with reduced levels of MMPs and ECM accumulation in experimental AKI.[42–45] Furthermore, these histological findings are in accordance with recent clinical observations that AKI leads to CKD in a substantial proportion of patients.[46] Interestingly, we obtained a significant inverse correlation of lowered urinary excretion of collagen fragments and CKD development or progression post AKI.

Furthermore, AAT promotes thrombin-mediated conversion of fibrinogen to fibrin by inhibition of activated protein C.[47] This is in accordance with our observation of reduced levels of FIBA peptides. Shifting the balance toward coagulation, the inhibitory effects of AAT provide an explanation for decreased levels of activated protein C in AKI,[48,49] and microvascular fibrin deposits in experimental AKI.[50,51]

In summary, changes in the fragments of all six proteins constituting the biomarker pattern have a reasonable biological rationale, and some may not only be markers, but surrogates of underlying pathophysiology in AKI. In this context, the site where the AKI-specific peptide markers originate is of special interest. The detection of several of the urinary COL1A1 and FIBA fragments included in the AKI pattern in human plasma (as noted under the remarks in Table 2) at least proves that their occurrence is not restricted to urine alone and excludes the urinary bladder as possible site of their origin. Also of note is the finding that all AKI-relevant AAT peptides originate from the C-terminal region that contains the active site in an extended loop structure that is indicative for MMP cleavage in injured tissue.[52–55] An open question is, however, whether these peptides occur in the plasma because of release of proteolytic fragments from tissue or vascular endothelium into plasma/urine or because of tubular reabsorption of proximal tubular degradation products.

A decrease in the abundance of collagen fragments was already observed in proteomic studies investigating chronic kidney diseases by CE–MS.[56–58] As previously suggested, changes in the urinary proteome may reflect primary pathophysiological alterations as well as response of the organism to disease.[57] Although there are peptides generally affected by kidney injury, there are others that are more specific for particular disease processes, that is, tubular capillaritis, vasculitis, or IgA nephritis. Naturally occurring peptides resemble surrogates of specific proteases and are thus subject to a higher degree of variance than their protein counterparts. In this study, a partial synergism was observed between acute and chronic kidney failure, as both types lead to decreased excretion of peptides from type I collagens, which was attributed to altered ECM turnover and connected to increased risk of AKI patients to develop CKD.[59] Using CE–MS, it cannot be resolved whether the two diseases share the same underlying mechanism. However, a comparative analysis on peptides included in the AKI and CKD marker panels revealed that two of the five collagen 1 α(I) peptides in AKI similarly regulated in patients with macroalbuminuria. Not unexpectedly, another overlap between AKI and CKD exists for albumin-derived fragments. Therefore, in a future study it has to be determined to which extent pre-existing CKD interferes with AKI diagnosis by the proteomic marker panel.

We applied the currently widely accepted AKI definition given by Mehta *et al.*,[6] permitting comparison of our findings with other proteomic and biomarker studies.[10,12,13,15–17,22,23,26] The extend of the time frame obtained by early AKI diagnosis with the presented biomarker pattern substantially exceeds that of previous findings using only single biomarkers.[10,12] This is consistent with previous studies that showed that urinary NGAL, IL-18, KIM-1, and S-CysC permitted detection of AKI 12–36 h earlier than serum creatinine, but by far not as early as with our biomarker pattern, providing up to 5 days of valuable time

for clinical interventions before AKI defined by serum creatinine.[10–12,14,16,17,60,61] Although initial studies describing these single biomarkers reported good or excellent diagnostic accuracy, there have been conflicting results with poor and fair accuracy in some subsequent studies.[10–12,14–17,60,61] Although our study was not powered to test the accuracy of these single biomarkers, our findings of moderate diagnostic accuracy of urinary NGAL, IL-18, KIM-1, and S-CysC are consistent with some previous reports.[14–17,60]

Consideration of a biomarker as clinically relevant requires confirmation in independent cohorts of the target population.[62] We therefore analyzed samples from an unselected group of critically ill patients with several causes of multifactorial AKI. The accuracy of AKI detection in both ICU and HSCT validation sets demonstrated general applicability of the biomarker pattern independent of AKI etiology, comorbidity, severity of illness, medical treatment, and recruiting center. Because of the diversity of pathological processes leading to AKI, it is unlikely that a single marker is capable to serve as reliable predictor in every case. Hence, combining 20 biomarkers enables establishment of a broadly applicable pattern, compensating potential biological, pre-analytical, and analytical variability of single biomarkers.

In conclusion, the results presented in this study suggest that the identified AKI marker pattern enables accurate detection of AKI in patients 5 days before the rise of serum creatinine, providing a substantial time frame to initiate established therapeutic interventions to prevent AKI or its progression, potentially improving clinical outcome. As current MS technology is inappropriate for routine ICU use because of cost-intensive and time-consuming sample processing and analysis requirements, a future step is to switch to an alternative point-of-care analytical platform, allowing timely monitoring of patients in the ICU by the presented AKI-specific biomarker pattern.

MATERIALS AND METHODS

Patients

Adult patients at high risk for AKI admitted to three ICUs at the University Hospital Essen between January and October 2006 served as training set for the identification of early AKI markers and the establishment of the AKI marker panel. The high risk for AKI was defined by at least two of following criteria: age >80 years, diabetes, cardiogenic or hemorrhagic shock, severe sepsis or septic shock, nephrotoxic medication, rhabdomyolysis, intra-arterial contrast, decompensated liver cirrhosis, and coronary artery bypass grafting with heart valve replacement. The definitions are provided in the Supplementary Table S2 online. AKI was defined either by a serum creatinine increase of $\geqslant 50\%$ or $\geqslant 0.3$ mg/dl from stable baseline values within 48 h and/or by a reduction in urine output <0.5 ml/kg per hour for >6 h, and AKI stages as recently proposed.[6] We considered the baseline serum creatinine values as the first measurements performed after hospital admission. We excluded patients with (1) pre-existing advanced CKD as defined by an estimated glomerular filtration rate <30 ml/min per 1.73 m^2, (2) pre-renal azotemia defined as rapid reversibility of AKI following adequate fluid resuscitation, (3) post-renal AKI diagnosed by urinary tract obstructions in ultrasound, and (4) patients who developed AKI in $\leqslant 48$ h after admission to make early AKI detection meaningful. The glomerular filtration rate was estimated according to the recalculated equation derived from the Modification of Diet in Renal Disease study.[63] CKD was defined as an estimated glomerular filtration rate <60 ml/min per 1.73 m^2 present for >3 months before as well as at hospital admission, albuminuria as urinary albumin excretion $\geqslant 30$ mg/day, CKD development as a decrease in estimated glomerular filtration rate from >60 before hospital admission to <60 ml/min per 1.73 m^2 after hospital discharge, and CKD progress as deterioration by at least 1 CKD stage from prehospital admission to posthospital discharge. Patients not developing AKI ($n=14$) served as controls. In total, 82 patients were screened of whom 52 were excluded by the criteria described above. Daily urine and serum samples were collected between 0700 and 0800 hours starting from ICU admission until discharge. All urine samples were obtained from indwelling bladder catheters after emptying the urine collection bags at 0600 hours. In patients with AKI, 'day 0' was the day the AKI criteria were fulfilled for the first time. In controls, urine was analyzed from the samples collected for a maximum observation period of 6 days, starting from the first day of admission to ICU (49 samples in total). Urine sample were immediately frozen and stored at $-80\,^{\circ}C$ until analysis.

A second, blinded set of ICU patients was studied for validation of the AKI marker panel that consisted of all consecutive adults admitted to the ICUs between August and September 2007. In total, 51 patients were screened, of whom 20 were included in the ICU validation set. Inclusion and exclusion criteria were the same as described above.

A second validation set consisted of patients post HSCT, collected at the Hannover Medical School from February to May 2008. This cohort was assessed cross-sectionally, and samples were regarded AKI positive by a serum creatinine rise $\geqslant 50\%$ within 48 h. In the HSCT cohort and in a healthy control group ($n=28$), spontaneously voided second morning urine samples were collected.

The local ethics committees approved the study, and all subjects gave informed consent. The study adhered to the Helsinki Declaration.

CE–MS analysis

A 0.7 ml urine aliquot was thawed and diluted with 0.7 ml 2 M urea, 10 mmol/l NH_4OH containing 0.02% sodium dodecyl sulfate. Proteins >20 kDa were removed by ultracentrifugation through Centrisart filters (Sartorius, Goettingen, Germany) at 3000 r.c.f. until 1.1 ml filtrate was obtained. The filtrate was desalted on a PD-10 column (GE Healthcare, Uppsala, Sweden) equilibrated in 0.01% aqueous NH_4OH (Roth, Karlsruhe, Germany). Samples were lyophilized and stored at $4\,^{\circ}C$ until resuspension in high performance liquid chromatography (HPLC)-grade H_2O to a final protein concentration of 0.8 μg/μl before analysis. Analysis and data processing were performed in accordance with current guidelines for clinical proteome analysis[64,65] using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) on-line coupled to a Micro-TOF MS (Bruker Daltonic, Bremen, Germany).[66,67] Spectra were accumulated every 3 s over an m/z-range of 350–3000 Da. Details on accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE–MS method have been reported.[67]

Data processing

Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu.[68] The software employs probabilistic clustering and uses both isotopic distribution and conjugated masses for charge-

state determination of peptides/proteins. For peak definition, signals with $z>1$ observed in a minimum of three consecutive spectra with a signal-to-noise ratio of at least four were considered. The resulting peak list characterizes each polypeptide by its molecular mass, CE-migration time, and ion signal intensity (amplitude). Data were normalized using a calibration method for CE-migration time and ion signal intensity on the basis of 'internal standard' peptides, which we proved to be superior over creatinine normalization.[24,69] All peptides were deposited, matched, and annotated in a Microsoft SQL database (Unterschleissheim, Germany). Peptides were considered identical when mass deviation was <50 p.p.m. for small peptides or 75 p.p.m. for larger peptides and proteins. Deviation of migration time was <0.45 min.

Generation of AKI-specific polypeptide pattern

An AKI-specific polypeptide biomarker model was generated by combination of peptides that are differentially distributed between case and control samples using the support vector machine-based MosaCluster software.[24] In support vector machine, a sample is regarded as a p-dimensional vector with p being the number of peptides included in the pattern. The algorithm constructs a $(p\text{-}1)$-dimensional separation plane between case and control vectors. From all possible hyperplanes that separate cases and controls, the one with the largest distance to the nearest data points on both sides is selected. Classification is performed by determining the Euclidian distance of the data point to the maximal margin of the hyperplane and assignment of a positive or negative value depending on which side of the hyperplane, case or control, the vector is located.

Sequencing of peptides

Peptides were separated by nanoflow reverse phase-HPLC (Agilent 1100 (Agilent Technologies, Boblingen, Germany); flow split by tee to ~60 nl/min) and introduced into an electron transfer dissociation-capable Finnigan LTQ quadrupole linear ion trap (Thermo Scientific, Bremen, Germany) via nano-electrospray ionization as previously described.[70] The resulting tandem mass spectrometry data were submitted to MASCOT (Matrix Science, London, UK) for a search against human entries in the MSDB (Mass Spectrometry protein sequence DataBase) Protein Database with an accepted parent ion mass deviation of 50 p.p.m. and an accepted fragment ion mass deviation of 500 p.p.m. Only search results with a MASCOT peptide score >20 were included. An additional search was employed against the NCBI human non-redundant database using OMSSA (http://pubchem.ncbi.nlm.nih.gov/omssa/).

Enzyme-linked immunosorbent assay and laboratory tests

Assays for human NGAL and KIM-1 were from R&D Systems (Wiesbaden, Germany), and the human IL-18 enzyme-linked immunosorbent assay kit was from MBL International (Woburn, MA). Urinary samples were diluted 20-fold in calibrator diluents for measurement of NGAL or used undiluted for the IL-18 and KIM-1 assays. Enzyme-linked immunosorbent assay measurements were carried out according to the manufacturer's instructions. To eliminate the influence of variable urine flow, urinary levels of NGAL, KIM-1, and IL-18 were normalized to urinary creatinine. Serum creatinine was measured with an enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany), S-CysC by immunonephelometry (Dade Behring, Marburg, Germany).

Statistical analysis

Sensitivity, specificity, 95% CI, and AUC were calculated based on receiver operating characteristic analysis using MedCalc version 9.5.2.0 (MedCalc Software; Mariakerke, Belgium). After testing for normal distribution, continuous data were compared using Student's t-test or Mann–Whitney rank-sum test, and categorical data were compared using χ^2 or Fisher's exact test. A two-sided P-value of <0.05 was considered statistically significant. P-values were calculated using the log-transformed intensities and the Gaussian approximation to the t-distribution. To adjust for multiple testing, the false discovery rate was controlled according to Benjamini and Hochberg.[71]

DISCLOSURE

HM is founder and co-owner of Mosaiques Diagnostics, which developed the CE–MS technology and the MosaiquesVisu and MosaCluster softwares. JM and ES are employees of Mosaiques Diagnostics.

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SUPPLEMENTARY MATERIAL

Table S1. List of urinary peptides with Benjamini Hochberg-adjusted P-values <0.05 in a statistical comparison of AKI ($n=38$) and non-AKI ($n=49$) ICU patient samples.

Table S2. Definitions of potential risk factors for acute kidney injury.

Figure S1. Longitudinal course of AKI classification factors for (**A**) 3 ICU patients prior AKI (filled symbols) and 3 non-AKI ICU patients (open symbols) of the training set and (**B**) 3 patients of the HSCT validation set with post transplant AKI episode.

Figure S2. Early diagnostic efficacy of AKI classification for ICU patients by the proposed single AKI biomarkers serum cystatin C (S-CysC), urinary kidney injury molecule-1 (KIM-1), urinary interleukin-18 (IL-18) and urinary neutrophil gelatinase-associated lipocalin (NGAL).

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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