Chitinase-like Proteins are Candidate Biomarkers for Sepsis-induced Acute Kidney Injury*

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Sepsis-induced acute kidney injury (AKI) is a frequent complication of critically ill patients and leads to high mortality rates. The specificity of currently available urinary biomarkers for AKI in the context of sepsis is questioned. This study aimed to discover urinary biomarkers for septic AKI by contemporary shotgun proteomics in a mouse model for sepsis and to validate these in individual urine samples of mice and human septic patients with and without AKI. At 48 h after uterine ligation and inoculation of Escherichia coli, aged mice (48 weeks) became septic. A subgroup developed AKI, defined by serum creatinine, blood urea nitrogen, and renal histology. Separate pools of urine from septic mice with and without AKI mice were collected during 12 h before and between 36–48 h after infection, and their proteome compositions were quantitatively compared. Candidate biomarkers were validated by Western blot analysis of urine, plasma, and renal tissue homogenates from individual mice, and a limited number of urine samples from human septic patients with and without AKI. Urinary neutrophil gelatinase-associated lipocalin, thioredoxin, gelsolin, chitinase 3-like protein 1 and -3 (CHI3L3) and acidic mammalian chitinase were the most distinctive candidate biomarkers selected for septic AKI. Both neutrophil gelatinase-associated lipocalin and thioredoxin were detected in urine of septic mice and increased with severity of AKI. Acidic mammalian chitinase was only present in urine of septic mice with AKI. Both urinary chitinase 3-like protein 1 and -3 were only detected in septic mice with severe AKI. The human homologue chitinase 3-like protein 1 was found to be more excreted in urine from septic patients with AKI than without. In summary, urinary chitinase 3-like protein 1 and -3 and acidic mammalian chitinase discriminated sepsis from sepsis-induced AKI in mice. Further studies of human chitinase proteins are likely to lead to additional insights in septic AKI. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013094, 1–13, 2012.

Acute kidney injury (AKI) is a frequent and important complication of sepsis in critically ill patients (1, 2). Despite extensive research and advances in diagnostic tools, sepsis-induced AKI leads to high mortality rates (25–70%) (3, 4), especially in elderly patients (5–7). Early and specific diagnosis is therefore of benefit for prevention and targeted intervention (8). Recently, several biomarkers for AKI have been detected in urine such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), IL-18, sodium/hydrogen exchanger 3, and cystatin C. These have been associated with earlier diagnostic potential than serum creatinine (sCr), the standard surrogate marker for kidney function (9, 10). However, few studies have included septic patients with AKI (11). Although some of these markers hold promise for real-time indication and prognostic information of AKI (12, 13), their specificity for diagnosis of sepsis-induced AKI requires further study (10, 14–17).

Mouse models are a cornerstone in the preclinical quest for new sensitive and specific markers. Urine is an attractive biofluid for the discovery of renal markers because of its noninvasiveness and close proximity to the kidney. However, mouse urine proteome analyses have been scarcely performed (18–22). More specifically, gel-free proteomic approaches were only described in two studies that used ICAT or iTRAQ labeling (isobaric tags for relative and absolute quantification) (19, 21). In addition, the number of identified proteins in those studies was limited, confining our current knowledge of the urinary proteome of this frequently used mouse model for sepsis and to validate these in individual urine samples of mice and human septic patients with and without AKI. At 48 h after uterine ligation and inoculation of Escherichia coli, aged mice (48 weeks) became septic. A subgroup developed AKI, defined by serum creatinine, blood urea nitrogen, and renal histology. Separate pools of urine from septic mice with and without AKI mice were collected during 12 h before and between 36–48 h after infection, and their proteome compositions were quantitatively compared. Candidate biomarkers were validated by Western blot analysis of urine, plasma, and renal tissue homogenates from individual mice, and a limited number of urine samples from human septic patients with and without AKI. Urinary neutrophil gelatinase-associated lipocalin, thioredoxin, gelsolin, chitinase 3-like protein 1 and -3 (CHI3L3) and acidic mammalian chitinase were the most distinctive candidate biomarkers selected for septic AKI. Both neutrophil gelatinase-associated lipocalin and thioredoxin were detected in urine of septic mice and increased with severity of AKI. Acidic mammalian chitinase was only present in urine of septic mice with AKI. Both urinary chitinase 3-like protein 1 and -3 were only detected in septic mice with severe AKI. The human homologue chitinase 3-like protein 1 was found to be more excreted in urine from septic patients with AKI than without. In summary, urinary chitinase 3-like protein 1 and -3 and acidic mammalian chitinase discriminated sepsis from sepsis-induced AKI in mice. Further studies of human chitinase proteins are likely to lead to additional insights in septic AKI. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013094, 1–13, 2012.

1 The abbreviations used are: AKI, acute kidney injury; E.coli, Escherichia coli; NGAL, neutrophil gelatinase-associated lipocalin; TRX, thioredoxin; CHI3L, chitinase 3-like protein; CHIA, acidic mammalian chitinase; KIM-1, kidney injury molecule-1; sCr, serum creatinine; CFU, colony forming unit; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1; BUN, blood urea nitrogen; TDS, tubular damage score; S, septic; IA, iodoacetamide; TCEP, tris(carboxyethyl)phosphine; TEAB, tris(carboxyethyl)ammonium bicarbonate; EndoLys-C, Endoproteinase Lys-C; OPN, osteopontin; CATHL1, cathepsin L1; UT, uroteroglobin; SD, standard deviation.

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laboratory animal. With regards to the proteome analysis of sepsis-induced AKI, to the best of our knowledge, only one study has been reported on the discovery of urinary biomarkers (23). A two-dimensional DIGE of urine was used in a rat sepsis model, but again only detected a small number of differentially expressed proteins.

The present study describes a gel-free approach of the mouse urine proteome in a new experimental model for sepsis-induced AKI. Our aims were to discover candidate urinary biomarkers specific for sepsis-induced AKI by comparison of the urinary proteomes of aged female mice and without AKI, both before and after sepsis. Aged mice more closely resemble the human population at risk for septic AKI than the commonly used young animals, as AKI is more prominent and carries a worse prognosis in the aged (24). Validation of the discovered biomarkers was done by Western blot analysis of urine from young, aged and old female mice, representing adolescent, adult pre- and elderly post-menopausal women. Finally, selected candidate biomarkers were analyzed for the first time in urine of human septic patients with and without AKI. Our results indicate that chitinase-3 like proteins 1 and 3 (CHI3L1, -3) and acidic mammalian chitinase (CHIA) are candidate biomarkers for sepsis-induced AKI in mice and that CHI3L1 and CHIA hold promise as biomarkers or key proteins involved in human septic AKI.

**EXPERIMENTAL PROCEDURES**

**Experimental Model for Sepsis**

*Mice—*All mice were purchased from Harlan (Boxmeer, The Netherlands) and housed in a conventional animal facility. Before surgery, mice were kept at least 2 weeks in the animal facility to recover after transport. For the proteome analysis of urine by LC-MS/MS, pooled samples of aged (46 to 48 weeks) female C57BL/6 mice were used. For the validation of the selected proteins as markers for sepsis-induced AKI by Western blot analysis, individual urine samples of young (12 to 14 weeks), aged and old (70 to 72 weeks) female C57BL/6 mice were used. Old female C57BL/6 mice spontaneously develop postmenopausal glomerulosclerosis from 72 weeks onwards and are reported as model for glomerulosclerosis in aging women (25). All experimental procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

*Uterine Ligation and Inoculation of Bacteria Induces Sepsis—*Uterine ligation and inoculation of Escherichia (E.) coli or PBS was performed as previously described. Briefly, mice were anesthetized with isoflurane (Abbott Laboratories, Kent, UK) before a 1 cm-long horizontal incision after the left costal arch was made. The left uterine horn was ligated cranially to the bifurcation uteri with a 4–0 polyglactin thread (Vicryl, Ethicon inc., Gargrave, UK). The ligated horn was then punctured with a 32-gauge needle and the inoculum (20 µl) was injected. Fluid resuscitation (0.9% NaCl; 0.025 ml/g body weight) was performed after closure of the abdominal wall (two layers, muscle and skin). For control purposes, sham inoculation with sterile PBS was performed (Invitrogen, Merelbeke, Belgium). Young, aged, and old mice were sacrificed 48 h after inoculation (T1) of 10⁷ colony forming units (CFU) of *E. coli* O18:K1. This strain was originally isolated from a clinical case of human sepsis and has been used in various studies of sepsis in mice (26). An additional group of young mice underwent a surgical removal of the ligated left uterine horn (hysterectomy) at T1, under complete isoflurane anesthesia. Fluid resuscitation and closure of abdominal walls were performed as described. These mice and a control group of infected mice without hysterectomy were monitored until 7 days after inoculation at the latest.

**Clinical Observations and Bacterial Culture—*Mice were examined for generalized reactions such as awareness of the environment, activity and grooming, weakness and mortality. Rectal body temperature was measured every 4 h (model C20 type K; Comark Electronics, Beaverton, OR), as well as food and water uptake and body weight. The number of bacteria recovered from heart, liver, kidneys, spleen and uterus at T1 was determined after sterile homogenization and plating onto blood agar. Peritoneal swabs and blood were analyzed for the presence of bacteria.

**Measurement of Plasma Cytokines and Chemokines, Creatinine and Urea Nitrogen, and Urinary Albumin—**The amount of IL-1β, IL-6, IL-10, TNF, keratinocyte-derived chemokine (KC), and monocyte chemoattractant protein-1 (MCP-1) in the plasma was determined according to the protocol of the manufacturer with a murine cytometric bead assay (bfaclarity kit BioArray, Erembodegem, Belgium). The detection limits were 10 pg/ml for IL-1β, IL-6, TNF, and KC, and 20 pg/ml for IL-10 and MCP-1. Concentrations of creatinine in plasma (sCr) and urine; and of blood urea nitrogen (BUN) were determined with an improved Jaffe or Jung method (QuantChiTM creatinine or urea assay kit, BioAssay Systems, Hayward, USA), respectively. Urinary albumin was measured with an ELISA according to the manufacturer’s protocol (Mouse Albumin ELISA kit, Immunology Consultants Laboratory, Inc., Newberg, OR).

**Histology of Kidneys—**The 10% formalin-fixed, paraffin-embedded kidney sections (5 µm) were stained with hematoxylin-eosin. Histological changes in the cortex of the kidney and outer medulla were assessed by quantitative measurements of tissue damage (tubular damage score, TDS). Histological criteria for renal damage were tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation. The degree of kidney damage was estimated at 400x magnification using 10 randomly selected fields for each animal by the following criteria: 0, areas of damage ≤5% of tubules; 1–5, damage involving 6% to 10%, 11% to 25%, 26% to 45%, 46% to 75% and > 76% of tubules, respectively (27).

**Gel-free Proteomics of Urine of Mice Before and After Septic Insult**

*Urine Collection—*Mice were individually housed in metabolic cages (Tecniplast, Buguggiate, Italy) and urine was collected overnight, both before (−15 h to −3 h; T0) and after (36–48 h; T1) uterine ligation and inoculation. A 7 × stock solution of protease inhibitors in 1.5 ml PBS was prepared (Complete, Mini, EDTA-free, Roche Diagnostics, Mannheim, Germany) and urine was collected in a recipient containing 50 µl of this stock solution. After collection, a 1:7 ratio of the stock solution to urine was prepared according to the manufacturer’s protocol and consecutively centrifuged at 300 g at 4 °C for 5 min (min). The supernatant was subjected to an additional centrifugation at 2000 g for 10 min, then aliquoted (max. 1 ml) and immediately frozen at −80 °C. No more than two freeze-thaw cycles were allowed for each aliquot.

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2 Maddens, B., Vanholder, R., Chiers, K., Cauwels, A., and Meyer, E. (2011) Severity of sepsis-induced acute kidney injury in a novel mouse model is dependent on age. Submitted to Crit. Care Med.
Study Population for Urinary Gel-Free Proteomics

Variation in Renal Response After Experimental Sepsis—Renal histological changes consistent with AKI such as tubular cell vacuolization, and a significantly higher TDS were found in some mice at T1 after inoculation with E. coli (n = 5/12). Other infected mice had no light microscopic features of AKI at T1 (n = 7/12), nor was their TDS significantly different from PBS-inoculated control mice (Fig. 1). Consequently, septic mice were subdivided as only septic (S) or septic with AKI (S/AKI). Renal dysfunction was documented by significantly increased concentrations of sCr and BUN in S/AKI mice compared with S mice and PBS-inoculated mice (Fig. 1). Urine output during 12 h was significantly decreased in S/AKI mice at T1 when compared with the output before inoculation (T0) and to the output of S mice at T1 (supplemental Fig. S1). Urinary albumin to urinary creatinine ratios (Fig. 1D) and urinary albumin to urinary total protein ratios (not shown) of S and S/AKI mice at T1 were significantly increased compared with T0 and to ratios of PBS-inoculated controls at T0 and T1.

Inflammation is More Severe in Septic Mice With AKI Than Without AKI—Hypothermia was present at T1 in S/AKI mice, whereas the body temperature of S mice was not significantly different before (T0) and after inoculation (T1) (supplemental Fig. S2). The bacterial load of distant organs (CFU of E. coli/g organ) was significantly higher in S/AKI mice than in S mice (supplemental Fig. S3). No bacteria were present in blood or in organs of PBS-inoculated mice. Plasma concentrations of TNF, IL-6, KC, MCP-1, and IL-10 were significantly increased at T1 in infected mice compared with PBS-inoculated mice. With the exception of IL-10, these were also significantly increased in S/AKI mice compared with S mice (Fig. 2). Plasma IL-1β levels at T1 were below the detection limit in all mice (data not shown).

We investigated changes in the urinary proteome that could represent potential biomarkers or elucidate important mechanisms in sepsis-induced AKI by comparing urine from septic mice (S) with urine from septic mice with AKI (S/AKI). Separate pools of urine collected from S (n = 7) and S/AKI (n = 5) mice were prepared. The urine of S and of S/AKI mice was respectively pooled based on equal amounts of urinary protein from each individual mouse within its group. Comparative proteome analysis of pooled urine before (T0) and after (T1) inoculation was done within each group, as well as between the two different groups (T1 versus T0).
Discovery of Candidate Urinary Biomarkers by Gel-free Proteome Analysis

Endoproteinase Lys-C Digestion and Postmetabolic Labeling of Urinary Samples—One hundred and fifty micrograms of urinary proteins were used from each sample (S/H11001 AKI T0 and T1, and S T0 and T1), the volume was adjusted to 1 ml using 100 mM of Tris pH 8.7 (150 μg was approximately equal to a volume of 750 μl, hence 250 μl of the 100 mM Tris pH 8.7 was added). Alkylation of cysteines was carried out using 20 mM iodoacetamide (IA; Sigma-Aldrich, Steinheim, Germany) and 5 mM of triscarboxyethylphosphine (TCEP; ThermoScientific, Waltham, MA) for 30 min at 37 °C in the dark. Following alkylation, excess reagents were removed on a NAP-10 desalting column (Amersham Biosciences, Uppsala, Sweden) and proteins were collected in 1.5 ml of 50 mM tricarboxyethyl ammonium bicarbonate (TEAB; Sigma-Aldrich).

Prior to digestion, protein concentrations were measured again by the Bradford assay (BioRad Life Science, Nazareth, Belgium). Proteins were denatured at 95 °C for 10 min followed by cooling-down on ice for 15 min. To each sample, 2.5 μg of endoproteinase Lys-C (endoLys-C; Roche Diagnostics GmbH, Mannheim, Germany) was added and digestion was carried out overnight at 37 °C.

Labeling of peptides with stable isotopes was performed by propionylation as described (28). Briefly, peptides from S/H11001 AKI T0 and from S T1 were labeled with the light isotopic variant NHS-12C3-propionate and the peptides from S/H11001 AKI T1 and S T0 were modified with the heavy isotopic variant, NHS-13C3-propionate. Practically, we added 2 mg of the NHS-ester to 100 μg of digested proteins, the reaction lasted for 150 min at 37 °C. Excess reagent was neutralized by adding 60 μl of a 1 M stock glycine to the sample for 15 min at 25 °C. Removal of unwanted O-propionylation was performed by adding 20 μl of hydroxylamine (Sigma-Aldrich) for 15 min at 25 °C. Samples were then acidified to 1% trifluoroacetic acid f.c. (Sigma-Aldrich). Finally, 50 μg of each labeled sample was mixed according to the following scheme:

All of the mixtures were vacuum dried, redissolved in 100 μl of solvent A (0.1% trifluoroacetic acid in water/acetonitrile, 98/2 (v/v), water (LC-MS grade, Biosolve, Valkenswaard, The Netherlands) and acetonitrile (high-performance liquid chromatography (HPLC) grade, Baker, Deventer, The Netherlands)) and separated onto a reverse phase (RP)-HPLC column (2.1 mm internal diameter × 150 mm length) 300SB-C18, Zorbax®, Agilent, Waldbronn, Germany) using an Agilent 1100 Series HPLC system. Following a 10 min wash with HPLC solvent A, a linear gradient to 100% solvent B (0.1% trifluoroacetic acid in water/acetonitrile, 30/70 (v/v)) was applied over 100 min. Using Agilent’s electronic flow controller, a constant flow of 80 μl/min was used.
Peptides that eluted between 20 and 80 min were collected into 60 fractions (1 min each, containing ~80 μl) and fractions that were separated by 15 min were pooled in order to reduce the number of samples for LC-MS/MS analysis to 15. These were then vacuum dried and redissolved in 50 μl 2.5% acetonitrile. 

LTQ-Orbitrap Analysis—We analyzed the obtained fractions by liquid chromatography (LC)-tandem MS (MS/MS) on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. 2.5 μl of each sample was used per LC-MS/MS analysis using an Ultimate 3000 HPLC system (Dionex, Amsterdam, The Netherlands) in line connected to the LTQ Orbitrap XL mass spectrometer. Each sample was measured three times (technical replicates). Peptides were first trapped on a trapping column (PepMapTM C18 column, 0.3-mm inner diameter × 5 mm, Dionex), and following back-flushing from the trapping column (Reprosil-pur basic-C18-HD 5 μm (Dr Maisch), Ammerbuch, Germany), 100 μl × 20 mm, packed in house), the sample was loaded on a 75 μm inner diameter × 150 mm reverse-phase column (Reprosil-Pur basic-C18-HD 3 μm (Dr Maisch), packed in house). Peptides were eluted with a linear gradient of a 1.8% solvent B decrease (0.05% formic acid in water/acetonitrile (2:8, v/v)) increase per minute at a constant flow rate of 300 nl/min. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the six most abundant ion peaks per MS spectrum. Full-scan MS spectra were acquired at a target value of 1E6 with a resolution of 60000. The six most intense ions were then isolated for fragmentation in the linear ion trap with a dynamic exclusion time of 60 s. Peptides were fragmented after filling the ion trap with a maximum ion time of 10 ms and a maximum of 1E4 ion counts. From the MS/MS data in each LC-run, Mascot generic files (mgf) were created using the Mascot Distiller software (version 2.3.2.0, Matrix Science Ltd., London, United Kingdom). When generating these peak lists, grouping of spectra was performed with a maximum intermediate retention time of 30 s and maximum intermediate scan count of 5 used where possible. Grouping was done with 0.005 Da tolerance on the precursor ion. A peak list was only generated when the MS/MS spectrum contained more than 10 peaks, no de-isotoping was performed, and the relative signal to noise limit was set at 2. Such generated peak lists were then searched with Mascot using the Mascot Daemon interface (version 2.3, Matrix Science Ltd.).

Spectra were searched against the Swiss-Prot database (version 56.4) and taxonomy was set to Mus musculus (15988 entries). Enzyme was set to endolys-C. Variable modifications were set to pyroglutamyl formation of N-terminal glutamine and acetylation of the protein’s N terminus and methionine oxidation (to its sulfoxide form). Carbamidomethylation of cysteines were set as fixed modifications. Carbamidomethylated cysteines were set as fixed modifications. Mass tolerance of the precursor ions was set to ±0.05 Da and of fragment ions to ±0.5 Da. The peptide charge was set to 1+, 2+, or 3+ and one missed cleavage site was allowed. Also, Mascot’s C13 setting was to 1, and 13C3- or 13C3-N-propionylation were selected as the isotope labels (exclusive modifications) in the Mascot Distiller environment. Only peptides that were ranked one and scored above the identity threshold score set at 99% confidence were withheld (note that the false discovery rate was 2.95% as calculated by the method described by Käll et al. (29)). Identified peptides were quantified using Mascot Distiller Toolbox version 2.3.2.0 (Matrix Science) in the precursor mode. The software tries to fit an ideal isotopic distribution on the experimental data based on the peptide average amino acid composition. This is followed by extraction of the XIC signal of both peptide components (light and heavy) from the raw data. Ratios are calculated from the area below the light and heavy isotopic envelope of the corresponding peptide (integration method “trapezium,” integration source “survey”). To calculate this ratio value, a least squares fit to the component intensities from the different scans in the XIC peak was created. MS scans used for this ratio calculation are situated in the elution peak of the precursor determined by the Distiller software (XIC threshold 0.3, XIC smooth 1, Max XIC width 250). To validate the calculated ratio, the standard error on the least square fit has to be below 0.16 and correlation coefficient of the isotopic envelope should be above 0.97.

For the analysis of the differential data we only used those proteins that were identified in all three technical replicate analyses and validated them using the software tool Rover (30), which is supported from the ms-lims data platform (31). Because protein ratios were based on equal amount of urinary proteins and not on equal volumes of urine, correction for variation in diuresis (by means of urinary creatinine) was not standard procedure. Proteins were arbitrarily considered over- or underexpressed when ratios >2.0 or <0.5 were found, respectively (32). Of note is that correction of ratios for urinary creatinine did not affect marker selection approaches. The total list of identified peptides is available via the PRIDE data repository (http://www.ebi.ac.uk/pride/; accession number: 17750; username: review62103; password: k~nexH5; PRIDE Inspector WebStart URL: http://www.ebi.ac.uk/pride/do?accession =17750&username= review62103&password=k%7EnexH5).

Validation of Candidate Urinary Biomarkers for Sepsis-induced AKI

Western Blot Analysis of Urine, Plasma, and Renal Tissue Homogenates—For the validation of the discovered candidate biomarkers, Western blot analysis was performed on urine, plasma, and renal tissue homogenates from individual mice (n = 15–17 for each group). The inclusion of three different age groups was performed as part of the validation process. Laemmli buffer (33) was added to the supernatant after determination of the protein concentration using the Bradford method. A similar protocol was performed to prepare plasma samples for Western blot analysis. For the detection of their presence in the kidney, one half of the left and right kidney was combined and homogenized directly into lysis buffer (10 mM Tris HCl pH 7.4, 200 mM NaCl, 5 mM EDTA, 1 μM oxidized glutathione, 10% glycerol, and 1% Nonidet P-40) supplemented with protease inhibitors (0.15 μM aprotinin, 2.1 μM leupeptin, and 100 nM phenylmethylsulfonyl fluoride; Sigma-Aldrich). The lysate was centrifuged at 2000 × g and 4 °C for 30 min to remove debris and Laemmli buffer was also added to the supernatant after determination of the protein concentration. All urine, plasma, and kidney lysate samples were boiled for 15 min and loaded onto a polyacrylamide gel (12% and 18%). Following electrophoresis, proteins were transferred to a nitrocellulose membrane by semidry blotting. Membranes were blocked with Tris-buffered saline with 0.1% Tween-20 and containing 5% milk powder and then incubated with one of the following primary antibodies: against mouse NGAL (rabbit polyclonal), osteopontin (OPN, goat polyclonal), cathepsin L1 (CATHL1, rat monoclonal), urotoglobin (UT, rabbit polyclonal), gelsolin (rabbit polyclonal), thioredoxin (TRX, mouse monoclonal), sepiapterin reductase (SPR, C-terminal region), chitinase 3-like protein 3 (CHI3L3, rat monoclonal), chitinase 3-like protein 1 (CHI3L1, goat polyclonal), and acidic mammalian chitinase (CHIA, rabbit polyclonal); and against human NGAL (rabbit polyclonal), CHI3L1 (goat polyclonal), and CHIA (rabbit polyclonal), purchased from Abcam (Cambridge, UK; NGAL, UT, gelsolin, TRX, mouse monoclonal), sepiapterin reductase (SPR, C-terminal region), and redissolved in 50% acetonitrile.

References

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Results of the differential analysis of urinary proteomes in the experimental model. Ratios are shown of proteins in urine collected before sepsis (T₀) and at 48 h after infection (T₁) from mice which were either septic at T₁ (S) or were septic and developed AKI at T₁ (S+AKI).

| Numbers of proteins | S T₁/T₀ | S+AKI T₁/T₀ | S+AKI T₁/S T₀ |
|---------------------|---------|-------------|---------------|
| Identified           | 327     | 346         | 280           |
| Present in 3 technical repeats | 119     | 155         | 98            |
| Present after manual inspection of MS spectra | 101     | 133         | 87            |
| Range of protein ratios | 5000.1–1.2 | 714.3–0.2 | 20.6–0.2 |
| Proportion of proteins with ratio 0.5–2.0 | 1.0% | 4.5% | 67.8% |
| Proportion of proteins with ratio <0.5 | 0% | 1.5% | 4.6% |

**Human Urine Samples**—To verify the clinical potential of urinary NGAL, CHI3L1 and CHIA for diagnosis of septic AKI, we also collected human urine samples from 12 septic patients, with and without AKI, and from 2 healthy volunteers. Samples were collected at the intensive care unit of Ghent University Hospital under Ethical approval of the Institutional Review Board. Sepsis was defined and classified in accordance with the American college of Chest Physicians and the Society of Critical Care Medicine consensus (34). Patients were diagnosed with AKI when ≥ 1.5-fold increase in sCr from admission or known baseline values was present, in agreement with the AKIN criteria (35).

**Statistical Analysis**—All data were analyzed using the SPSS software (SPSS Inc, Chicago, IL, USA), at a global significance level of 0.05. Results were expressed as mean ± standard deviation (S.D.). Comparisons of data at T₁ between aged PBS-inoculated, S and S+AKI mice were made pair wise using the Kruskall Wallis test, with Bonferroni-adjusted comparison-wise post hoc test at a significance level of 0.017 (= 0.05/3). Data of T₀ and T₁ within one group of mice were compared using the Wilcoxon matched pairs test.

**RESULTS**

**Discovery of Candidate Urinary Biomarkers for sepsis-induced AKI**

Up-regulated Excretion of Urinary Proteins After Experimental Sepsis—Three-hundred and one different proteins were identified in the combined nondifferential analyses of urinary proteomes of S and S+AKI mice (T₁/T₀), of which 129 were not detected in urine before infection (T₀) (not shown). Eighty-six percent of these 129 proteins were annotated as intracellular proteins, and 12% as extracellular ones (universal Gene Ontology (GO) annotation terms) (36). Results of the differential analysis of urinary proteomes of S and S+AKI mice (T₁/T₀), and of S+AKI T₁/S T₀ are shown in Table I and in supplementary Table S1.

Selection Criteria for Candidate Urinary Biomarkers—Two approaches were combined to interpret the results of the differential analyses presented in Table I and to select for candidate markers with the highest potential for distinguishing between sepsis with AKI (S+AKI) and sepsis without AKI (S).

Approach 1, which consists of three filter components, filter 1 only considered proteins with a ratio > 9.3 (a cut-off value determined by the observed ratio of MUP5, as the MUP family consists of abundant urinary proteins that dominate the urinary protein content in healthy mice) in the S+AKI T₁/T₀ differential analysis (n = 79/133). Filter 2 excluded the proteins that were exclusively identified in the S T₁/T₀ analysis (n = 64/101). Filter 3 withheld those proteins with a S+AKI T₁/T₀ ratio > 2.0 (n = 24/87). This three-step filtering resulted in a list of proteins.

In the combined approach 1 bis, only proteins with a ratio (S+AKI T₁)/S T₁ > 2.0 (n = 24/87) were considered.

Table II shows the proteins selected by the combination of both approaches 1 and 1 bis for which a commercial antibody was available.

Approach 2 was based on the common proteins (n = 71) identified in the S+AKI T₁/T₀ and the S T₁/T₀ differential analyses. Here, we filtered that subgroup of common proteins (n = 13) with a (S+AKI T₁/T₀)/(S T₁/T₀) ratio > 1.0 (Fig. 3). Note that Fig. 3 shows the top 10 (n = 10/13) proteins of this selection.

Combining the protein list generated by approach 1 and 1 bis (Table II) with the proteins generated by approach 2 (Fig. 3), allowed us to finally select 8 proteins (supplementary Fig. S5) at the top of the common ranking based on the ratios of both S+AKI T₁/T₀ and S+AKI T₁/S T₁ and these were chosen for validation by Western blot analysis: neutrophil gelatinase-associated lipocalin (NGAL), thiroedxin (TRX), gelatinase-associated lipocalin (NGAL), thioredoxin (TRX), gelsolin, chitinase 3-like protein 3 (CHI3L3), sepiapterin reductase (SPR), osteopontin (OPN), cathepsin L1 (CATH L1), and urotoglobin (UT). A list of the peptide and protein ratios is provided in supplementary Tables S2A-C.

**Validation of Candidate Urinary Biomarkers for Sepsis-induced AKI**

**Preclinical Validation of Biomarkers in Experimental Sepsis**

Western Blot Analysis of Candidate Biomarkers in Urine—Septic mice of different ages were defined with AKI when renal histology lesions consistent with AKI, TDS ≥ 2 and sCr > 0.24 mg/dl were present. The cutoff for sCr was chosen based on a 1.5-fold increase in sCr compared with the mean sCr of PBS-inoculated mice (i.e. 0.16 mg/dl) in analogy with the Acute Kidney Injury Network staging criteria for human AKI (35). The differential presence of proteins was confirmed by Western blot analysis of individual urine samples at T₀ and from PBS-inoculated (T₀), septic (T₁) and septic mice with AKI (T₁) (Fig. 4A, 4C, and supplementary Fig. S4). Urinary CHI3L3 was only detected in septic mice with AKI (Fig. 4A), and not in...
TABLE II

Candidate urinary biomarkers for sepsis-induced acute kidney injury: selection approaches based on quantitative gel-free proteomics data. Accession numbers of identified proteins are shown as provided in the UniProt database. Data of the quantitative comparison of urinary proteomes collected before (T0) and at 48 h after infection (T1) from mice which were septic (S) or septic and developed AKI at T1 (S/H11001 AKI) are represented. Data are shown from proteins selected by approach 1 (n = 15, italics type font), approach 1 bis (n = 14, regular type font), and in both approach 1 and 1 bis (n = 10 bold type font) for which a commercial antibody is available. Blank fields link to proteins that were not detected in all three technical repeats of one analysis. ID: number of identified peptides, different peptides: number of identified unique peptides

| Protein                                      | Accession number | S+AKI T1/S+AKI T0 | Fold increase at T1 compared to S+AKI T0 | ID | Different peptides | Fold increase at T1 compared to S+AKI T0 | ID | Different peptides | Fold increase at T1 compared to S+AKI T0 | ID | Different peptides |
|----------------------------------------------|------------------|-------------------|------------------------------------------|----|--------------------|------------------------------------------|----|--------------------|------------------------------------------|----|--------------------|
| Neutrophil gelatinase-associated lipocalin   | P11672           | 3.7               | 38                                       | 5  |                     | 714.3                                    | 22 |                     | 120.9                                    | 16 |                     |
| Uteroglobin                                  | Q66318           | 5.5               | 16                                       | 3  |                     | 400.0                                    | 13 |                     | 2.4                                      | 5  |                     |
| Osteopontin                                  | P10923           | 5.3               | 179                                      | 13 |                     | 17.2                                    | 167|                     | 35.0                                    | 73 |                     |
| Thioredoxin                                  | P10638           | 2.5               | 15                                       | 3  |                     | 11.9                                    | 9  |                     | 30                                       | 6  |                     |
| Protein S100-A8                              | P27005           | 20.6              | 6                                         | 1  |                     | 212.8                                    | 43 |                     | 14.7                                    | 13 |                     |
| Parvalbumin alpha                            | P32848           | 17.7              | 32                                        | 11 |                     | 416.7                                    | 12 |                     | 500.0                                    | 11 |                     |
| Myoglobin                                    | P04247           | 15.7              | 11                                        | 4  |                     | 5.7                                      | 3  |                     | 14.1                                    | 7  |                     |
| Annexin A1                                   | P10107           | 7.1               | 3                                         | 1  |                     | 8.0                                      | 5  |                     | 43.1                                    | 4  |                     |
| Lactotransferrin                             | P08071           | 4.3               | 3                                         | 2  |                     | 2.7                                      | 5  |                     | 6.8                                      | 12 |                     |
| Apolipoprotein A-I                           | Q00623           | 4.0               | 5                                         | 2  |                     | 2.7                                      | 5  |                     | 6.8                                      | 12 |                     |
| Peptidyl-prolyl cis-trans isomerase A        | P17742           | 3.9               | 4                                         | 2  |                     | 27.9                                    | 6  |                     | 38.3                                    | 37 |                     |
| Meprin A subunit beta                        | Q61847           | 3.3               | 4                                         | 2  |                     | 23.1                                    | 4  |                     | 18.1                                    | 5  |                     |
| Cathespin D                                  | P18242           | 3.1               | 4                                         | 2  |                     | 23.1                                    | 4  |                     | 18.1                                    | 5  |                     |
| Calbindin                                    | P12658           | 3.1               | 6                                         | 1  |                     | 23.1                                    | 4  |                     | 18.1                                    | 5  |                     |
| Ig kappa chain C region                      | P01837           | 2.4               | 11                                        | 4  |                     | 14.4                                    | 20 |                     | 14.7                                    | 13 |                     |
| Ezrin                                        | P26040           | 2.4               | 20                                        | 5  |                     | 9.5                                      | 25 |                     | 500.1                                    | 11 |                     |
| Gamma-glutamyltranspeptidase 1              | Q05928           | 2.4               | 19                                        | 4  |                     | 15.2                                    | 13 |                     | 14.1                                    | 7  |                     |
| Lymphocyte antigen 6D                        | P35459           | 2.4               | 7                                         | 1  |                     | 11.6                                    | 9  |                     | 43.1                                    | 4  |                     |
| Na(+)H(+) exchange regulatory cofactor NHE-RF3| Q66L4            | 2.4               | 6                                         | 3  |                     | 10.5                                    | 13 |                     | 6.8                                      | 12 |                     |
| Insulin-like growth factor-binding protein 7 | Q61581           | 2.3               | 5                                         | 1  |                     | 11.3                                    | 4  |                     | 38.3                                    | 37 |                     |
| Serotransferrin                              | Q92111           | 2.3               | 85                                        | 13 |                     | 18.0                                    | 89 |                     | 38.3                                    | 37 |                     |
| Vitamin o-binding protein                    | P21614           | 2.2               | 13                                        | 5  |                     | 25.0                                    | 13 |                     | 37.9                                    | 11 |                     |
| Alpha-enolase                                | P17182           | 2.1               | 9                                         | 5  |                     | 14.5                                    | 11 |                     | 37.9                                    | 11 |                     |
| Transferrin                                  | P07829           | 2.1               | 6                                         | 1  |                     | 6.1                                      | 15 |                     | 38.3                                    | 37 |                     |
| Sepiapterin reductase                        | Q64105           | 3.5               | 3                                         | 1  |                     | 35.5                                    | 3  |                     | 38.3                                    | 37 |                     |
| Gelsolin                                     | P13020           | 3.4               | 5                                         | 2  |                     | 34.5                                    | 5  |                     | 38.3                                    | 37 |                     |
| Cathespin L1                                 | P06797           | 2.8               | 5                                         | 2  |                     | 24.8                                    | 5  |                     | 38.3                                    | 37 |                     |
| Chitinase-3-like protein 3                   | Q35744           | 2.1               | 9                                         | 3  |                     | 21.6                                    | 9  |                     | 38.3                                    | 37 |                     |
| Lipopoly saccharide-binding protein           | Q61805           | 117.6             | 3                                         | 1  |                     | 18.6                                    | 12 |                     | 38.3                                    | 37 |                     |
| Complement C3                                | P01027           | 19.6              | 12                                        | 8  |                     | 18.0                                    | 89 |                     | 38.3                                    | 37 |                     |
| Serotransferrin                              | Q92111           | 2.3               | 85                                        | 13 |                     | 18.0                                    | 89 |                     | 38.3                                    | 37 |                     |
| N-acetylgucosamine-6-sulfatase               | Q8BF64           | 18.2              | 9                                         | 5  |                     | 18.2                                    | 9  |                     | 38.3                                    | 37 |                     |
| Prothrombin alpha                            | P26350           | 15.7              | 3                                         | 1  |                     | 15.7                                    | 3  |                     | 38.3                                    | 37 |                     |
| Annexin A5                                   | P40836           | 13.0              | 10                                        | 3  |                     | 13.0                                    | 10 |                     | 38.3                                    | 37 |                     |
| UMP-CMP kinase                              | Q9DBBP5          | 12.9              | 3                                         | 1  |                     | 12.9                                    | 3  |                     | 38.3                                    | 37 |                     |
| Beta-2-microglobulin                        | P01887           | 11.5              | 3                                         | 1  |                     | 11.5                                    | 3  |                     | 38.3                                    | 37 |                     |
| kinesin-associated protein 3                 | Q70188           | 10.7              | 4                                         | 1  |                     | 10.7                                    | 4  |                     | 38.3                                    | 37 |                     |
| Destin                                       | Q9ROPS           | 10.4              | 3                                         | 1  |                     | 10.4                                    | 3  |                     | 38.3                                    | 37 |                     |
| Heat shock cognate 71 kDa protein            | P63017           | 9.8               | 4                                         | 2  |                     | 9.8                                     | 4  |                     | 38.3                                    | 37 |                     |

| 13C sample | 13C sample |
|------------|------------|
| S+AKI T0   | S+AKI T1   |
| S T0       | S+AKI T1   |
| S T1       | S+AKI T1   |
septic mice without AKI or in PBS-inoculated mice. Urine of septic mice with AKI contained higher amounts of NGAL, TRX (Fig. 4A) and SPR (supplemental Fig. S5) than urine of PBS-inoculated mice, septic mice without AKI, and mice recovering from sepsis after removal of the infected uterine horn (Fig. 4C). Old PBS-inoculated mice also excreted NGAL and TRX in their urine. An immunoreactive band of urinary gelsolin around 75 kDa was detected in young and aged septic mice with AKI, whereas only a band around 50 kDa was present in young and aged PBS-inoculated mice at T1, or before inoculation (T0).

In contrast, both bands were observed in urine of old mice, regardless the presence of sepsis or AKI. No clear patterns of cathepsin L1 and osteopontin were detected in urine, although septic mice showed bands with a stronger immunoreactive signal at the expected molecular weights (i.e. 37 and 32 kDa, respectively) (supplemental Fig. S5). Urinary uteroglobin was detected with a similar intensity in urine of all mice (supplemental Fig. S5).

Western Blot Analysis of Candidate Biomarkers in Renal Tissue Homogenates—To examine if the selected candidate biomarkers for sepsis-induced AKI originated from the kidney, Western blot analyses were repeated on renal tissue homogenates (Fig. 4B). Two bands of CHI3L3 were observed in all septic mice with AKI and not in young or aged septic mice without AKI or in PBS-inoculated controls. In contrast to

### Table 1: Proteins identified in both S+AKI T1/T0 and S T1/T0 and with a ratio of (S+AKI T1/T0)/(S T1/T0) > 1.0 (selection approach 2).

|        | YOUNG | AGED | OLD |
|--------|-------|------|-----|
| E. coli T1 | 0.22  | 0.16 | 0.16 |
| PBS T0   | 0.22  | 0.16 | 0.16 |

### Western Blot Analysis of Selected Candidate Biomarkers for Sepsis-induced Acute Kidney Injury in Individual Mouse Urine Samples and Renal Tissue Homogenates.

Representative samples of young (12 to 14 weeks), aged (46 to 48 weeks), and old (70 to 72 weeks) female C57BL/6 mice were investigated for the presence of neutrophil gelatinase-associated lipocalin (NGAL), thioredoxin (TRX), chitinase 3-like protein 3 (CHI3L3) and gelsolin in urine (A) and kidney lysates (B). Septic mice of different ages were defined with AKI when renal histology lesions consistent with AKI, tubular damage score (TDS) > 2 and serum creatinine (sCr) > 0.24 mg/dl were present. Western blot results are shown of urine from mice infected with E. coli, before (T0) and after inoculation (T1), as well as from mice after PBS-inoculation (T1). Removal of the infectious focus at 48 h after inoculation (d2) of E. coli resulted in recovery from sepsis and sepsis-induced AKI, with representative Western blot results of urinary biomarkers (C).
NGAL in urine, only a strong signal of the band around 20 kDa was detected in kidney lysates of septic mice with AKI. Similar intensities of gelsolin bands around 50 kDa were observed between age-matched mice. Kidneys of old mice and septic aged mice contained more TRX than young mice, whereas osteopontin was only present in kidney lysates of septic mice with AKI (supplemental Fig. S5).

**Mammalian Chitinase Family Members as Candidate Bio-markers for Septic AKI**—Because CHI3L3 was the most specific marker for sepsis-induced AKI, its presence in mouse plasma was examined by Western blot analysis (Fig. 5). Another protein member of the mammalian chitinase family is acidic mammalian chitinase (CHIA), which was detected in S+AKI T1/T0, because of its ratio < 9.3 (MUP5), it was formerly not selected as candidate marker. Based on the promising results of CHI3L3, we examined CHIA in urine and plasma of mice, as well as urinary chitinase 3-like protein 1 (Fig. 5). CHI3L1, -3, and CHIA were only present in plasma of septic mice and in urine of septic mice with AKI.

**Preliminary Clinical Validation of Urinary CHI3L1 and CHIA in Human Septic Patients With and Without AKI**—Urinary CHI3L1 was markedly more excreted in human septic patients with AKI than without AKI (Fig. 6; supplemental Fig. S6). In contrast, CHIA was less discriminative for the presence of AKI in the studied urine samples. In addition, NGAL was also investigated in urine from human septic patients and had at Western blot level another pattern than CHI3L1 (Fig. 6).

**DISCUSSION**

This study uses a gel-free proteomic approach to quantitatively compare the urinary proteome of septic mice with and without AKI, and to discover candidate urinary biomarkers for sepsis-induced AKI. Western blot analysis validated the LC-MS/MS data and confirmed that CHI3L1 and -3, and CHIA were able to discriminate septic mice with AKI from septic mice without AKI. Additionally, a preliminary study of CHI3L1 and CHIA in urine of human septic patients revealed that CHI3L1 holds promise for further evaluation as urinary biomarker for septic AKI.

Increased plasma levels of CHI3L1 and -3 were found in mice with sepsis compared with PBS-inoculated mice (Fig. 5). This is in agreement with increased plasma CHI3L1 concentrations in humans after endotoxin injection (37) and in patients with severe sepsis and septic shock (38). However, CHI3L1 and -3 were only detected in urine of septic mice with severe stages of AKI (TDS ≥4), indicating that their urinary presence is not merely the result of plasma filtration. In addition, septic mice with AKI had a different Western blot pattern of CHI3L3 in kidney homogenates than PBS-inoculated control mice and young and aged septic mice without AKI (Fig. 4B). Our results therefore strongly indicate that urinary CHI3L1 and -3 reflect an ongoing process in the kidneys of septic mice with severe stages of AKI. This elaborates the findings of a recent study which suggested that serum concentrations of CHI3L1 in human septic patients might be useful for severity grading of renal failure (38). Acidic mammalian chitinase (CHIA) was also only present in urine of septic mice with AKI (TDS ≥2) (Fig. 5), thus again suggesting a role as candidate biomarker for septic AKI. Interestingly, the presence of both CHIA and chitinase-like proteins in urine has never been related to kidney injury before (39).

Chitinases and chitinase 3-like proteins are members of the mammalian chitinase family and in contrast to CHIA, chitinase-like proteins have no chitin hydrolyzing activity exerting their biological effect through protein-protein or protein-carbohydrate interactions (40). Although CHI3L3 is mouse-specific, it is strongly related to human CHI3L1 and has consid-
CHI3L1 was only present after sepsis with severe AKI septic patients with AKI than without AKI (Fig. 6), whereas in mice, CHI3L1 was only present after sepsis with severe AKI (Fig. 5). However, results of a standardized mouse model without comorbidities cannot be extrapolated directly to septic patients. In contrast to the available renal histology in mice, no gold standard diagnostic criteria for intrinsic kidney damage in humans are available and the diagnosis of AKI is often made on clinical grounds such as increases of sCr (35). Furthermore, critically ill patients often have comorbidities leading to pre-existing renal disease before the septic insult. Elevated serum concentrations of CHI3L1 have been reported in humans with certain types of solid tumors (40), and other inflammatory conditions such as inflammatory bowel disease (47) and liver fibrosis (48). However, not all studied human patients with increased urinary CHI3L1 had recognizable comorbidities and at least no comorbidities in common. Although the results of this first proof-of-concept study are promising, the added value of measuring urinary CHI3L1 will only become clear when further study investigates its specificity for septic AKI, and associated sensitivity.

In contrast to CHIA and chitinase 3-like proteins, NGAL and TRX were also present in urine of septic mice without AKI (TDS≤1) (Fig. 4A, Table II). It is likely that sCr, urinary NGAL and TRX are proxies for other processes during septic AKI than chitinase 3-like proteins. NGAL has been recognized as a marker for AKI (49), but both NGAL and TRX have also been described as markers for sepsis (50, 51). This latter complicates their diagnostic value as urinary markers for AKI in a sepsis context. However, the amount of NGAL and TRX in urine of septic mice increased in parallel with sCr and TDS (Fig. 4A). One can argue that this increase is an indication of their sensitivity for real-time indication and prediction of AKI before histological damage occurs. Indeed, urinary NGAL has been described as useful in predicting AKI in septic humans and higher urinary NGAL concentrations were found in septic patients with AKI than without AKI (15). On the other hand, our results in mice question the added value of urinary NGAL and TRX for diagnosis of septic AKI compared with sCr increases because both NGAL and TRX rise in parallel with sCr. Furthermore, both NGAL and TRX were also excreted in urine of old PBS-inoculated mice (Fig. 4A), which complicates the discrimination of AKI from chronic kidney changes. Indeed, there is evidence that urinary NGAL is also a marker for chronic kidney disease (52). Although at the kidney level, NGAL was specifically detected in septic mice with AKI, TRX was also present in old PBS-inoculated mice (Fig. 4B). Increased TRX reflects inflammation and oxidative stress (51), which are key events in the aging kidney (25, 53), and a
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possible explanation for the latter finding. In summary, our results indicate that neither TRX nor NGAL are specific diagnostic markers for sepsis-induced AKI. However, their quantification in urine likely will indicate whether septic mice have AKI or not.

Different patterns of urinary gelsolin were observed before and after sepsis in young and aged mice, in contrast to old mice (Fig. 4A), thereby questioning its diagnostic value for AKI. Urinary full length gelsolin (82 kDa) has been described to discriminate between cisplatin- and gentamicin-induced AKI in rats (54). It is a remarkable finding that full length gelsolin levels increased in urine of septic mice, because plasma gelsolin has been reported to decrease in septic human patients (55). Both the full length and 50 kDa fragment of gelsolin were observed in large amounts in plasma of septic and PBS-inoculated mice (not shown). In contrast, we could not clearly detect full length gelsolin in kidney lysates, suggesting that its up-regulated excretion in urine probably is the result of plasma filtration through the glomerulus. Consequently, these findings might imply that both sepsis and the aging process in mice alter the glomerular permselectivity. Indeed, old female C57BL/6 mice spontaneously develop progressive glomerulosclerosis in the absence of recognizable associated diseases, in analogy with aging women (>65 years) (25). The different observations in old mice with regard to urinary gelsolin confirm the usefulness of including three different age categories throughout the validation process.

Shotgun proteomics in combination with differential labeling of peptides proves to be a more powerful method to unravel the urinary proteome and to discover candidate markers than the proteomic approaches of other rodent studies (19, 21, 23). We were able to detect the majority of previously described markers for AKI (supplemental Table S1). The final selection for subsequent validation was based on proteins which could potentially be produced by the kidneys, extrapolated from literature, and for which a commercially available antibody existed (20). Although it is a limitation of the study that the cutoff for candidate markers was based on the MUP, the selection approach resulted in promising candidate biomarkers for septic AKI. Surprisingly, we did not detect KIM-1, IL-18, or sodium/hydrogen exchanger-3 in urine of septic mice with AKI. A previous study in septic rats with acute renal failure could also not detect KIM-1 and suggested that this was because of their gel-based proteomic approach of urine (23). Remarkably, the urinary presence of KIM-1 in septic mouse models is rarely described. Technical reasons associated with intrinsic biochemical properties of mouse KIM-1 or species differences need to be elucidated before the specific role of KIM-1 in mouse sepsis-induced AKI can be further examined.

In conclusion, quantitative gel-free proteomics and Western blot analysis of urine of septic mice resulted in the discovery and validation of NGAL, TRX, gelsolin, CHI3L1 and -3, and CHIA as candidate biomarkers for sepsis-induced AKI. Urinary NGAL and TRX were increased in septic mice and paralleled sCr and renal histological damage. Acidic mammalian chitinase and chitinase 3-like proteins were only present in urine of septic mice with AKI and severe AKI, respectively. Urinary CHI3L1 was increased in human septic patients with AKI and not without AKI. Future studies will elaborate on the possible added value of urinary chitinase-3 like proteins in improving the specific diagnosis or severity staging of sepsis-induced AKI in combination with more early or predictive biomarkers such as NGAL.

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