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URINARY NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN, KIDNEY INJURY MOLECULE-1, UROMODULIN, AND CYSTATIN C CONCENTRATIONS IN AN EXPERIMENTAL RAT MODEL OF ASCENDING ACUTE KIDNEY INJURY INDUCED BY PYELONEPHRITIS

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Recent evidence suggests that neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), cystatin C (CysC), uromodulin (UMOD), and some interleukins (IL-6 and IL-18) can be considered as diagnostic markers of acute kidney injury (AKI). The aim of this study was to verify the applicability of four urinary (u) markers, namely uNGAL, uKIM-1, uCysC, and uUMOD, for the diagnosis of ascending AKI induced by bacterial pyelonephritis. The study included 30 female rats that were divided into three groups (n = 10 each) and were inoculated transurethrally with various doses of *Escherichia coli* to induce isolated pyelonephritis (group 1, 10⁵ CFU/ml), pyelonephritis-induced AKI (group 2, 10⁷ CFU/ml), or AKI and urosepsis (group 3, 10⁹ CFU/ml). The inoculate contained a highly virulent *E. coli* strain isolated from a patient with pyelonephritis. Urine samples were obtained prior to the inoculation and 7, 14, and 21 days thereafter. The concentrations of all assessed proteins were determined in the urine samples by ELISA. All the study groups showed elevated concentrations of uNGAL and uCysC at all study time points. The concentrations of uKIM-1 in group 1 were the same as that at the baseline, whereas it was elevated in groups 2 and 3 at all study time points. The concentrations of uUMOD in groups 1 and 2 tended to decrease with the time from inoculation, whereas it rapidly increased in group 3 at 21 days postinfection. uKIM-1 seems to be the only marker of ascending AKI associated with urinary tract infection. Elevated concentrations of uNGAL, uCysC, and uUMOD were found in both AKI and isolated pyelonephritis. Thus, it can be concluded that none of these markers can be used as a single diagnostic marker of ascending AKI, as it may produce false-negative results, leading to incorrect diagnosis, lack of adequate treatment, and increased mortality risk.

Key words: *acute kidney injury, kidney injury molecule-1, neutrophil gelatinase-associated lipocalin, uromodulin, pyelonephritis, cystatin C, Escherichia coli, novel biomarkers*

INTRODUCTION

In accordance with the Kidney Disease Improving Global Outcomes (KDIGO) criteria, serum creatinine (CreaS) concentration, a routinely determined laboratory parameter, is used as a primary diagnostic marker of acute kidney injury (AKI) (1). Usually, the determination of CreaS concentration is accompanied by the analysis of concentrations of nitrogen end products, namely urea and uric acid, as well as by the measurement of urine output. Unfortunately, elevated concentrations of CreaS and other metabolites are observed with some time lag, that is, after damage of active nephrons that result in a decrease in glomerular filtration rate (GFR) by approximately 50% (2). Moreover, elevated serum levels of these parameters do not necessarily reflect kidney dysfunction, as they may also be affected by patient's hydration status, age, sex, and diet (3). Therefore, neither CreaS nor other low-

molecular-weight nitrogen end products meet the criteria for accurate diagnostic markers of AKI (4). Considering the abovementioned limitation, the American Society of Nephrology in 2012 released guidelines for the identification of early markers of kidney damage, which will also enable to confirm the pathophysiological background of the developing AKI. Thus, research studies are being conducted on the accurate marker of early AKI, caused by diabetes 1% (5), sepsis 23%, septic shock 51% (6), myocardial infarction 10 – 20% (7), surgery 30% (8), chronic kidney disease 10%, and consumption of nephrotoxic substances for example aminoglycosides, including gentamycin 20 – 30% (9, 10).

For optimal assessment, the serum/urinary levels of new biomarkers of AKI should increase rapidly at early stages of the injury, prior to the decrease in GFR (11), and should not be affected by concomitant infections, diseases of the liver, and inflammatory conditions. Moreover, these markers should be

specific and enable to differentiate the etiology of AKI (12). Available evidence suggests that these criteria may be met by several proteins, including neutrophil gelatinase-associated lipocalin (NGAL) (13), kidney injury molecule-1 (KIM-1) (14, 15), cystatin C (CysC) (16, 17), and some interleukins (18, 19).

CreaS, currently considered as a biochemical surrogate marker of glomerular filtration, is increasingly being replaced by CysC in the evaluation of renal dysfunction. CysC is a low-molecular-weight polypeptide secreted by all nuclear cells and proximal tubular cells, and 98% of this polypeptide is resorbed from renal tubules. Increased concentration of uCysC was observed in the progression of diabetic nephropathy (20), damage of proximal tubules during various kidney diseases, and AKI of various etiologies (21). Although the level of CysC could be affected by some drugs (cyclosporine and corticosteroids), it is not affected by infection, diet, gender, and age.

The glycoprotein NGAL is the most extensively examined biomarker of renal function; it is produced by cells of the prostate, uterus, trachea, stomach, liver, lungs, and large intestine as well as by immune cells such as neutrophils and macrophages. NGAL was also demonstrated to be produced in kidneys by the ascending limb of Henle's loop and by intercalated cells of the collecting duct (22). The diagnostic role of NGAL was revealed in adult and pediatric AKI of various etiologies (ischemic, septic, toxic, posttransplant) and in other kidney diseases, for example, reflux nephropathy (23).

KIM-1 is an established marker of ischemia and proximal tubular injury (24). It was approved by the US Food and Drug Administration (FDA) as a biomarker of AKI in preclinical drug research (25). This protein was proved to be particularly useful in the diagnosis of AKI resulting from shock, surgery, or toxic damage and sepsis (26, 27).

UMOD is synthesized in the distal tubules and the ascending limb of Henle's loop and prevents adhesion of *Escherichia coli* to urinary bladder epithelium (28, 29). UMOD binds to type I fimbria (FimF protein) and affects bacterial adhesion to urinary bladder epithelium. The impairment of the protective mechanism promotes interaction of *E. coli* with the receptors expressed by epithelial cells, which leads to greater severity of urinary tract infection (UTI) (30, 31). Recent data show that UMOD also plays an important role in the immune response as a factor that binds to light chains of immunoglobulin, components of complement, and interleukins (32).

Kidney damage caused by hypoxia results in the increase in the concentration of KIM-1 after 1 hour, NGAL after 2 hours, and CysC after 24 hours, whereas CreaS concentration increases only on the third day after the damage (4). Thus, these findings confirm the usefulness of the new markers in the diagnosis of AKI as urinary disturbances of these markers correspond to the loss of structural integrity of the kidneys, thereby allowing the clinician to suspect AKI. In addition to the detection of early AKI, these parameters should also facilitate monitoring of its severity and establishing prognosis, for example, in patients in intensive care units (ICUs) who are at particular risk of developing AKI. It is also reported that approximately 80% cases of diagnosed AKI are due to ischemia occurring during surgery or after hemorrhage (33). In the remaining 20% of the cases, AKI develops primarily as a consequence of tubulointerstitial diseases, e.g., pyelonephritis, usually associated with a bacterial infection. Published evidence suggests that AKI may develop in even ~2–3% of adults with complicated pyelonephritis (34). The risk of kidney injury increases in patients with coexisting anatomic anomalies of the urinary tract and a history of long-term catheterization as well as in patients with immunosuppression or diabetes, pregnant women, and individuals with one kidney (35, 36).

Currently, many studies are being conducted on NGAL, KIM-1, CysC, and UMOD to confirm their role in the diagnosis of AKI

of various etiologies (37) and to reveal their diagnostic usefulness for monitoring the complications of diabetes (nephropathy) (38), diseases of the heart (39), or liver disturbances (40).

The aim of our study was to verify the usefulness of the urinary markers uNGAL, uKIM-1, uCysC, and uUMOD as the diagnostic biomarkers of bacterial-induced ascending AKI in an experimental rat model.

MATERIALS AND METHODS

Animals

The protocol of the study was approved by the First Local Bioethics Committee for Animal Experiments in Cracow (decision no. 133/2012 and 99/2014).

The study included 10-week-old female Wistar rats with approximately 200 g body weight ($n = 30$). During the entire experiment, the animals were kept under standardized conditions, with controlled light cycle (12 h/12 h) and unlimited access to water and food. The experiment was preceded by a 7-day quarantine period.

Study groups

The animals were divided into three groups, with 10 rats in each group. The rats from each group received *E. coli* suspension (500 μ l) through a sterile polyethylene catheter (1 mm \times 10 cm). The procedure was carried out under general anesthesia induced by pentobarbital administration (Morbital, Biowet, Pulawy, Poland; 20 mg/kg b.w, i.p.). Depending on the bacterial concentration in the suspension, the three groups were categorized as follows:

- pyelonephritis (group 1, 10^5 CFU/ml)
- pyelonephritis complicated with AKI (group 2, 10^7 CFU/ml)
- pyelonephritis complicated with AKI and urosepsis (group 3, 10^9 CFU/ml).

Criteria for the confirmation of pyelonephritis development:

- histopathological examination of kidney specimens was performed to reveal structural abnormalities and confirm the occurrence of the inflammatory process.

- criteria for diagnosing AKI in the study groups.

AKI was diagnosed under the following conditions:

- during the experiment, as compared to the control, there was significant decrease in 24-h diuresis, increase in CreaS, decrease in CreaU (urine creatinine), and decrease in CrCl (creatinine clearance);

- inflammatory infiltration was observed in the pelvis and medulla or in the pelvis, medulla, and cortex.

Urosepsis was diagnosed in group 3 under the following conditions:

- during the experiment, as compared to the control, FENa (fractional excretion of sodium) was significantly decreased due to vasodilatation, resulting from the presence of bacterial toxins and systemic cytokine action;

- except urosepsis, other reasons for the decrease in FENa were excluded (dehydration, fever, or loss of blood) (41).

The procedure of intraurethral infusion of *E. coli* followed the protocol described in detail by Lee *et al.*, (42), and in one of our previously published paper (41). To prevent the reflow of the fluid from the bladder after catheterization, the animals were kept under anesthesia for 4–6 h, in the position with the lower body part placed above the head.

Blood and urinary concentrations of the studied proteins were determined prior to the inoculation of the bacterial suspension (day 0) and constituted the baseline for further measurements. Because no significant intergroup differences in

the concentrations of the study markers were found at day 0, the results were pooled and the mean values for all 30 animals were considered as the baseline. This procedure enabled us to follow the 3R principle and to reduce the number of examined animals to a necessary minimum because of the application of the most accurate statistical methods.

Bacteria

E. coli isolate used to inoculate the study rats was obtained from a female patient with acute pyelonephritis. The strain expressed genes for the following virulence factors: fimH, papC and sfaD/E-encoding type 1 adhesin, and P and S fimbria. Further, the isolate harbored *iroN*, a gene encoding an iron ion receptor; salmochelin; and the *CNF1* gene for the cytotoxin tumor necrosis factor 1 (TNF-1).

Sampling and analysis of the material

Before induction of infection, on day 0 and on 7th, 14th, and 21st day after inoculation, blood from the tail vein was obtained for biochemical analysis.

Similarly, a 24-h urine collection was done prior to the inoculation (day 0) as well as on 7, 14, and 21 days postinoculation. On these days, the rats were kept in individual metabolic cages (Tecniplast, Italy). Urine samples were centrifuged (5 min at 2000 rpm), aliquoted, and used to determine uNGAL, uKIM-1, uCysC, uIL-6, and uMOD concentrations. Urinary concentrations of these markers were determined by ELISA by using commercially available kits: Rat IL-6, NGAL, KIM-1, Cystatin C, and Uromodulin ELISA Kit (Shanghai Sunred Biological Technology Co. Ltd., China). Sodium level in the blood and urine was measured using a flame photometer (model 450, Corning). CreaS and CreaU were estimated using the Cobas 6000 analyzer (biochemical module C501, Roche).

At day 21, after completing the last 24-h urine collection, the rats were euthanized with pentobarbital overdose (Morbital, Biowet, Pulawy, Poland; 100 mg/kg b.w., i.p.). Kidney and urinary bladder specimens were collected during nephrectomy and cystectomy, respectively, and used to prepare routinely stained histopathological slides.

Bladder and kidney specimens were washed in saline and then fixed for 24 h in 8% formalin in phosphate buffer solution (PBS, pH 7.4). The obtained fragments were then washed in running water for 2 hours and then dehydrated in increasing concentrations of ethanol (50 – 100%). Before embedding in paraffin specimens were performed by xylene solution. From the xylene mixture, the specimens were transferred to the mixture of xylene and paraffin in the ratio 1:1 and incubated at 37°C for 2 hours. The individual pieces of tissues were then transferred twice to clean paraffin and incubated at 62°C. After 2 hours, the specimens were embedded in paraffin blocks, and the solidified blocks were cut using a microtome. Finally, the block fragments were placed on glass slides and dried in an incubator at 37°C. To assess the severity of histological inflammation, the slide preparations were stained by the hematoxylin-eosin (HE) method. Ten consecutive fields of vision in particular specimens were analyzed using the Axiophot light microscope (Zeiss, Germany). During the microscopic analysis, the location and characteristics of neutrophil and mononuclear cell infiltrate were determined within the kidney parenchyma and urinary bladder wall.

Statistical analysis

Statistical analysis was carried out with Statistica 8 package (StatSoft). Results are presented as mean values and standard

deviation (SD). The results obtained at day 0, prior to transurethral inoculation with bacterial suspension, were considered as the baseline for the results obtained at days 7, 14, and 21. Intergroup comparisons of the study parameters were conducted with Fisher's ANOVA and HSD Tukey test. Results are presented in the tables (correlations) and on the graphs (other parameters), with whiskers reflecting the SD values. To evaluate the correlation between CrCl and all other biomarkers, Pearson's correlation coefficient was used. The intergroup differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Histopathological changes

Histopathological abnormalities were observed in 21 days of the experiment. No abnormalities were observed in the control group. Animals from group 1 presented with chronic pyelonephritis and tubulointerstitial nephritis, manifesting as a presence of low-grade lymphocytic infiltration. Inflammation was limited to the renal medulla. In specimens from group 2, chronic pyelonephritis was found, along with ulceration and tubulointerstitial nephritis with massive lymphocytic and granulocytic infiltration of both renal pelvis and medulla. Similar to group 1, however, the inflammation did not extend beyond the medulla. In rats from group 3, histopathologic examination revealed chronic pyelonephritis and tubulointerstitial nephritis involving both medulla and cortex of the kidneys, and characterized by massive lymphocytic and granulocytic infiltration (41) (*Fig. 1*).

Biochemical findings

In group 1, 24-h diuresis was significantly lower than the baseline only on day 14. In groups 2 and 3, 24-diuresis was significantly decreased on days 14 and 21. In groups 2 and 3, CreaS concentrations were significantly higher than the baseline on days 21 and 7, respectively. At all the analyzed time points, the concentrations of CreaU in groups 2 and 3 were significantly lower than the baseline. CrCl in groups 2 and 3 were significantly lower than the baseline on day 14 and on days 7 and 14, respectively. FENa in groups 1 and 3 was significantly lower than the baseline only on day 7 and on days 7 and 21, respectively. FENa in group 2 was significantly higher than the baseline on day 14. uIL-6 concentration in group 2 was significantly higher than the baseline on day 14, while in group 3, uIL-6 concentration was significantly higher than the baseline at all analyzed time points (*Table 1*).

Urinary neutrophil gelatinase-associated lipocalin

At all the analyzed time points, the concentrations of uNGAL in all groups (1, 2, and 3) were significantly higher than the baseline (23.09 ± 1.97 ng/ml). Moreover, we found statistically significant differences in uNGAL concentrations in groups 1, 2, and 3 (*Fig. 2*). The increase in uNGAL level would result not only from the enhanced release of this protein from hypoxic nephrons but also from its oversynthesis by immune cells migrating into the urinary tract during the course of bacterial inflammation.

Urinary kidney injury molecule-1

Regardless of the study time point, the concentrations of uKIM-1 in groups 2 and 3 were significantly higher than the baseline (436.6 ± 84.7 pg/ml). Moreover, statistically significant differences in uKIM-1 levels were found between groups 1, 2, and 3 (*Fig. 3*). These findings suggest that uKIM-1 may be an accurate diagnostic marker of AKI associated with UTI.

Urinary uromodulin

Our study showed a similar trend in the dynamics of uUMOD in groups 1 and 2. On the 7th and 14th day of the

experiment, the concentrations of uUMOD in both groups correlated inversely with the size of bacterial inoculum. Concentrations of uUMOD in the study groups were significantly higher than the baseline (0.65 ± 0.13 ng/ml) at

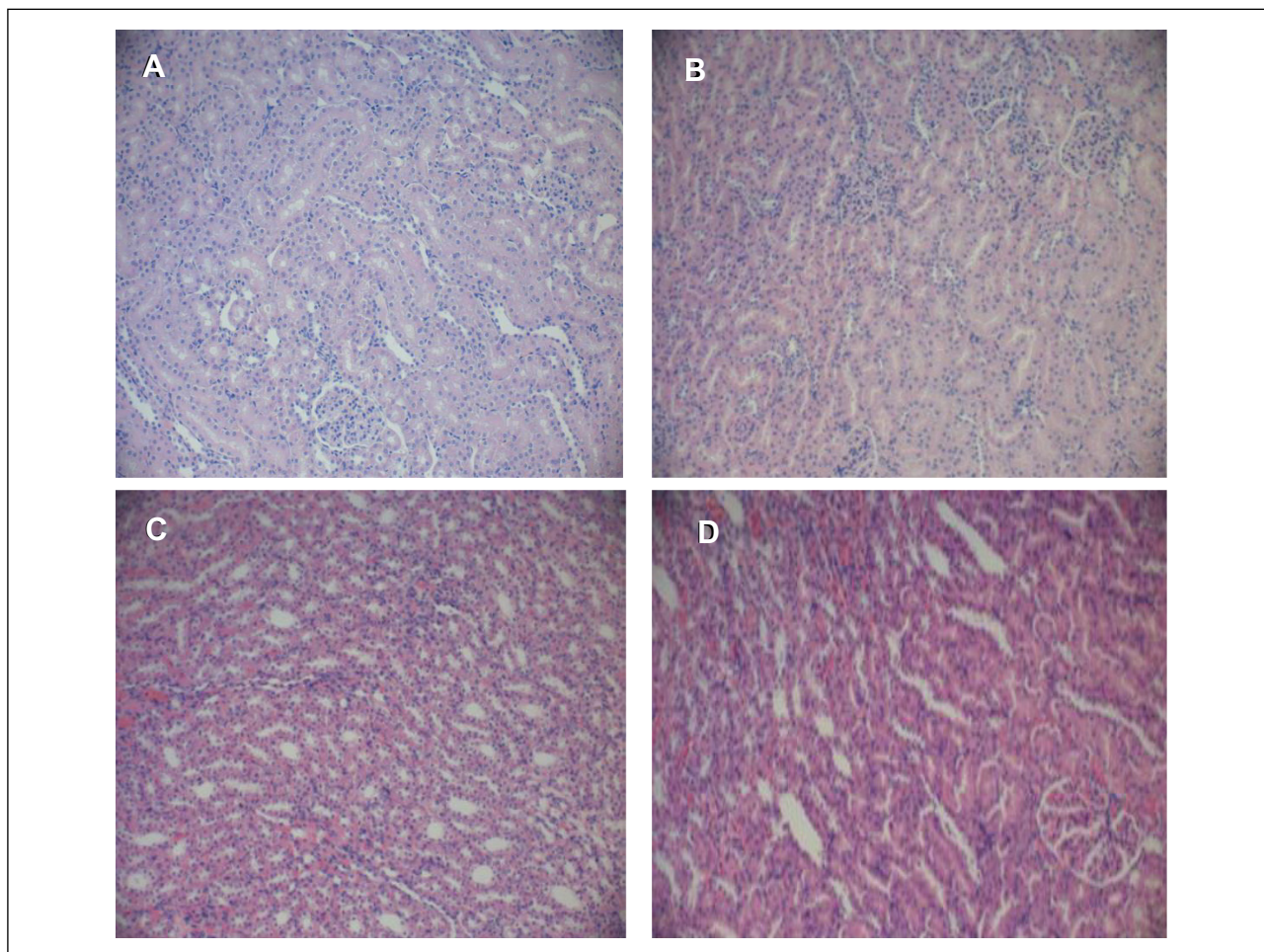


Fig. 1. Hematoxylin-eosin-stained slides of the kidney specimens from groups. (A) control, (B) 1 group, (C) 2 group, and (D) 3group, respectively.

Table 1. Values of 24-h diuresis, serum creatinine (CreaS), urine creatinine (CreaU), creatinine clearance (CrCl), fractional excretion of sodium (FENa), and urinary interleukin-6 (uIL-6) in the study groups.

	Group 1			Group 2			Group 3		
Parameter/day	day 7	day 14	day 21	day 7	day 14	day 21	day 7	day 14	day 21
24-h diuresis ml/24 h	15.7 ± 2.1	11.6 ± 2.2 [#]	16.6 ± 3.1	16 ± 3.85	8.7 ± 1.67 [#]	10.5 ± 4.6 [#]	15.1 ± 7.1	8.8 ± 3.76 [#]	9.5 ± 5.05 [#]
CreaS μmol/l	26.4 ± 4.3	31.6 ± 4	35 ± 11.2	32.4 ± 8	40.5 ± 19	32.6 ± 9.2 [#]	42 ± 13.1 [#]	39.5 ± 8	34.7 ± 22
CreaU μmol/24 h	62.6 ± 16	49.5 ± 12	60 ± 11.6	42.5 ± 12 [#]	34.6 ± 15 [#]	41.9 ± 16 [#]	44 ± 5.77 [#]	36.4 ± 8.7 [#]	40.6 ± 7.9 [#]
CrCl ml/min	1.7 ± 0.61	1.1 ± 0.31	1.27 ± 0.4	0.9 ± 0.25	0.7 ± 0.34 [#]	0.93 ± 0.3	0.77 ± 0.2 [#]	0.66 ± 0.2 [#]	0.99 ± 0.4
FENa %	0.09 ± 0.04 [#]	0.13 ± 0.09	0.1 ± 0.05	0.2 ± 0.07	0.47 ± 0.3 [#]	0.2 ± 0.17	0.07 ± 0.05 [#]	0.13 ± 0.05	0.09 ± 0.04 [#]
uIL-6 pg/24 h	15.5 ± 0.42	21.85 ± 6.5	17.53 ± 4.07	16.6 ± 2.15	55.7 ± 1.31 [#]	15 ± 2.6	23.2 ± 1.28 [#]	554 ± 19.25 [#]	17.87 ± 0.4 [#]

0 day control value: 24-h diuresis 17 ± 3.05 ; CreaS 30.1 ± 4.93 ; CreaU 64.7 ± 18.9 ; CrCl 1.36 ± 0.42 ; FENa 0.19 ± 0.07 ; uIL-6 13.8 ± 1.63 . # significantly different from the baseline - HSD Tukey Test.

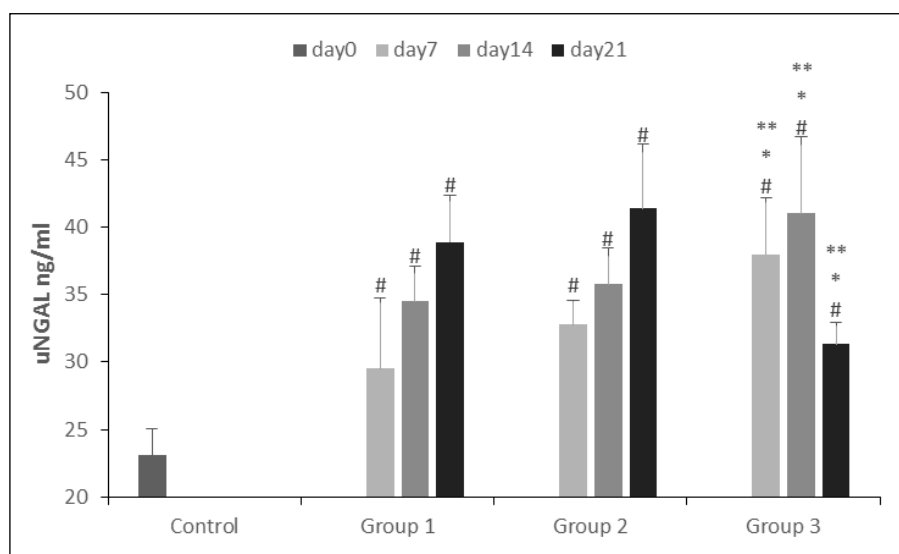


Fig. 2. Concentrations of urinary neutrophil gelatinase-associated lipocalin (uNGAL) (ng/ml) in the study groups.

significantly different from the baseline - HSD Tukey test; * significantly different from the result of group 1 at the same time point - HSD Tukey test; ** significantly different from the result of group 2 at the same time point - HSD Tukey test.

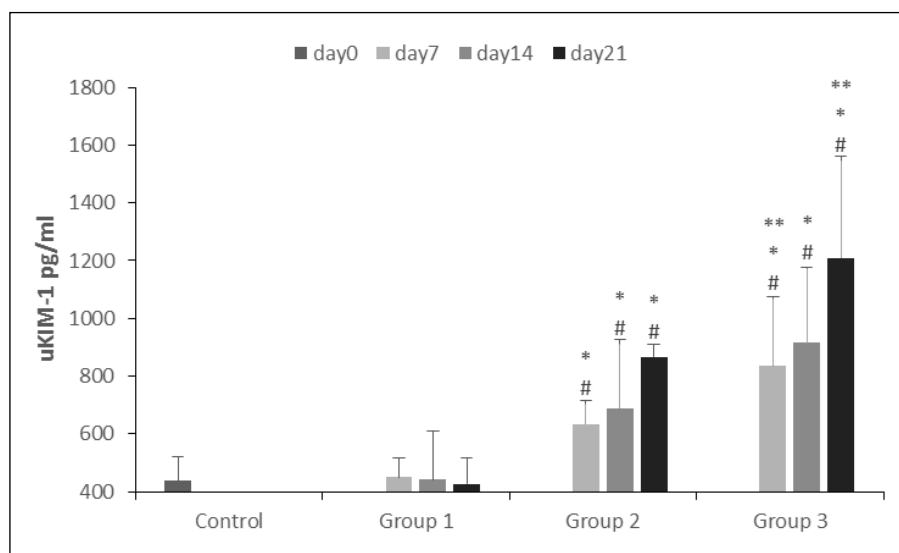


Fig. 3. Concentrations of urinary kidney injury molecule-1 (uKIM-1) (pg/ml) in the study groups.

significantly different from the baseline - HSD Tukey test; * significantly different from the result of group 1 at the same time point - HSD Tukey test; ** significantly different from the result of group 2 at the same time point - HSD Tukey test.

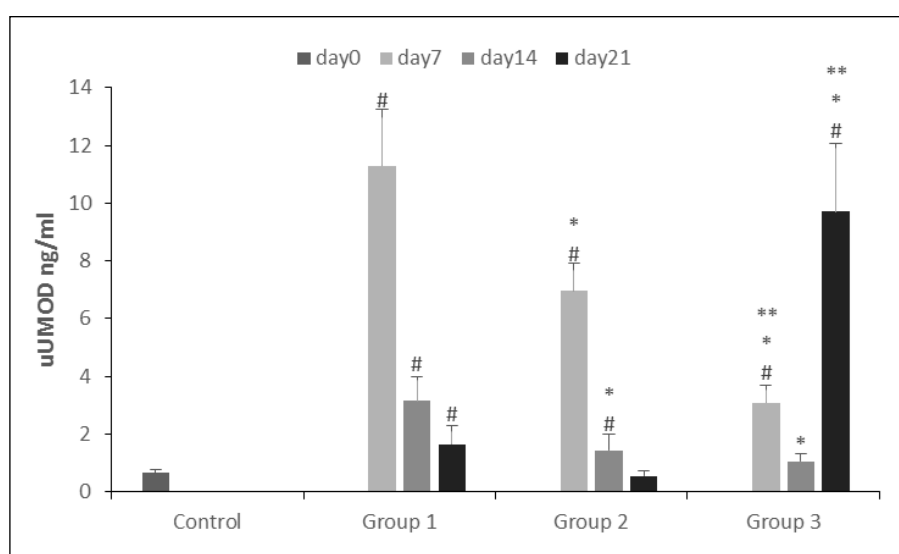


Fig. 4. Concentrations of urinary uromodulin (uUMOD) (ng/ml) in the study groups.

significantly different from the baseline - HSD Tukey test; * significantly different from the result of group 1 at the same time point - HSD Tukey test; ** significantly different from the result of group 2 at the same time point - HSD Tukey test.

various, but not all, study time points. The highest concentration of uUMOD was observed on day 7 in group 1. Moreover,

statistically significant differences in uUMOD levels were found between groups 1, 2, and 3 (Fig. 4).

Urinary cystatin C

Concentrations of uCysC in all the study groups were higher than the baseline ($1.83 \pm 0.23 \mu\text{g/ml}$). In group 3, the differences were statistically significant at all study time points, whereas in groups 1 and 2, significantly higher concentrations of uCysC were observed only on days 14 and 21. Moreover, statistically significant differences in uCysC levels were observed between groups 1 and 3 (Fig. 5). The results of our experiment suggest that disturbances in the uCysC level do not satisfy the criteria for an early diagnosis of AKI associated with lower UTI.

Correlation between urinary neutrophil gelatinase-associated lipocalin and creatinine clearance values

uNGAL increase was associated with decrease in CrCl, which was confirmed by the calculated correlations. The correlation between uNGAL and CrCl values was statistically significant only on days 14 and 21 of the experiment when the groups were combined ($P \leq 0.05$) (Table 2, Fig. 6).

Correlation between urinary kidney injury molecule-1 and creatinine clearance values

Increase in uKIM-1 concentration was associated with the decrease in CrCl, which was confirmed by the calculated correlations. The correlation between uKIM-1 and CrCl values was statistically significant on days 7, 14, and 21 when the groups were combined ($P \leq 0.05$) (Table 3, Fig. 7).

Correlations between uCysC and creatinine clearance

The increase of uCysC reflects a decrease in GFR and the involvement of renal parenchyma by a pathological process. It was confirmed by the observed correlation in our study. The correlation between uCysC and CrCl values was statistically significant only on days 14 and 21 when the groups were combined ($P \leq 0.05$) (Table 4, Fig. 8).

Correlation between uromodulin and creatinine clearance values

Our study revealing the lack of correlation between uMOD and CrCl, which demonstrates that uMOD may decrease in the initial stage of renal disease, even when CrCl is still normal. The

correlation between uMOD and CrCl values was statistically insignificant on all days and for all groups, and even when the groups were combined ($P > 0.05$) (Table 5).

Details related to the laboratory procedures, microbiological examination of urine, fractional excretion of urea (FEUrea), 24-h intake of water, and body temperature are provided in one of our recently published papers (41).

DISCUSSION

According to previous reports, high concentration of CreaS and low urine output observed during the course of AKI associated with upper UTI are consequences of renal hypoperfusion. Under physiological conditions, hemodynamics of glomerular are stable due to the renal autoregulation acting via extracellular ATP, P2 purinoceptors (P2Rs) and gap junctions (43). The renal hypoperfusion is mediated by proinflammatory cytokines (e.g., IL-1, IL-2, IL-6, and TNF- α) released from immune cells accumulated at the site of inflammation. Cytokines alter the glomerular arteriolar tone, thereby contributing to contraction of the afferent arterioles or dilation of the efferent arterioles. Therefore, in the early stage, the developed AKI takes the so-called 'pre-renal' form, which, however, transforms into the intrinsic, renal one; this is because the resultant reduction in filtration pressure, manifesting as a decrease in GFR and hypoxia, may also trigger changes in renal tubules. Thus, degradation of the actin cytoskeleton of epithelial cells, loosening of intercellular junctions, dysfunction of integrins, and desquamation of epithelial cells in the tubular lumen are observed (44, 45). From the pathophysiological point of view, UTI-induced AKI is therefore a heterogeneous entity, conditioned by complex pathomechanisms, initially leading to kidney hypoperfusion, supplemented by further development of kidney tubulopathy. Damage to the tubular lining enhances reabsorption of primary urine, which results in interstitial edema. Moreover, blocking of renal tubules with casts composed of UMOD and desquamated epithelial cells promotes an increase in intratubular hydrostatic pressure. This condition drastically deteriorates glomerular filtration, which further exacerbates and may also be reduced due to degenerative changes in the myocytes and vascular endothelium. Another mechanism predisposing to renal ischemia and resultant hypoxia and subsequent tubulopathy is a relative predominance of locally

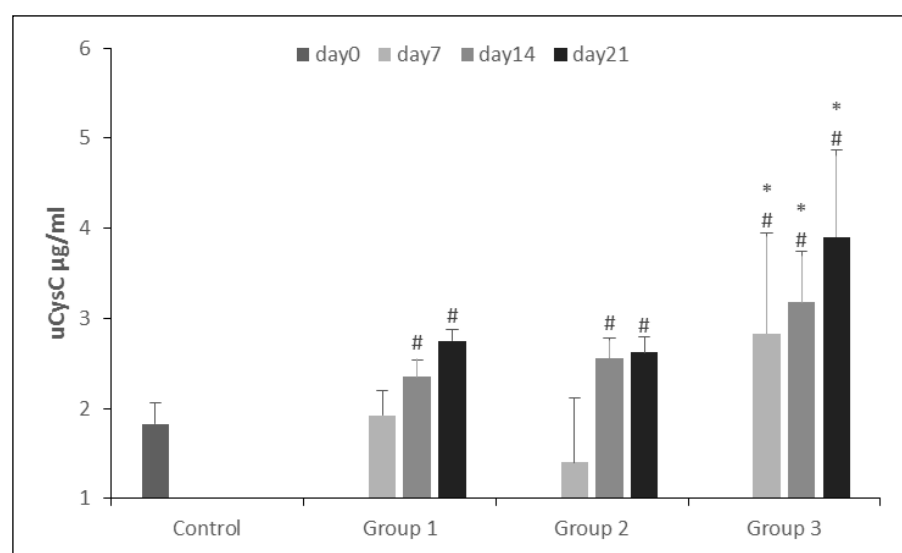


Fig. 5. Concentrations of urinary cystatin C (uCysC) ($\mu\text{g/ml}$) in the study groups.

significantly different from the baseline - HSD Tukey test; * significantly different from the result of group 1 at the same time point - HSD Tukey test.

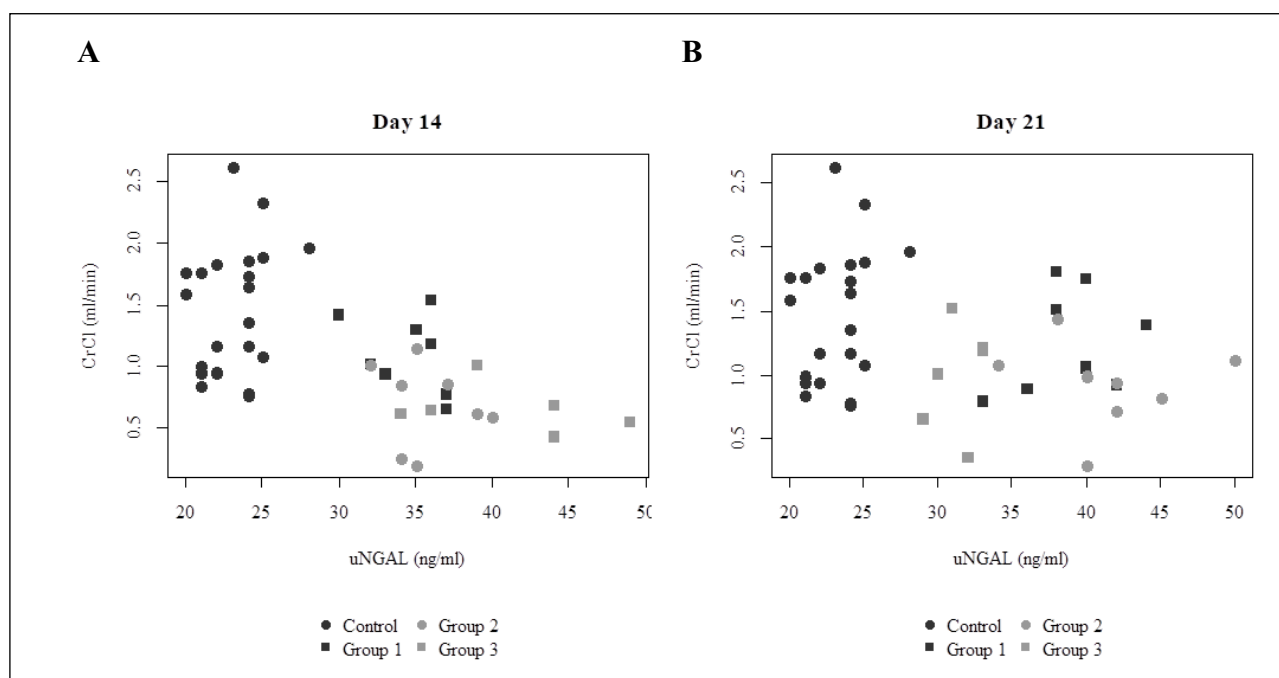


Fig. 6. Correlation between urinary neutrophil gelatinase-associated lipocalin (uNGAL) and creatinine clearance (CrCl) values on days (A) 14 and (B) 21 of the experiment.

Table 2. Correlation between urinary neutrophil gelatinase-associated lipocalin (uNGAL) and creatinine clearance (CrCl) values.

Day	Group	Correlation between uNGAL and CrCl values			
		Correlation factor	P value	Direction of relationship	Strength of relationship
0	All groups together	0.235	0.252	—	—
7	All groups together	−0.283	0.063	—	—
14	All groups together	−0.588	< 0.001	negative	strong
21	All groups together	−0.328	0.03	negative	medium

synthesized vasopressor compounds (endothelin and thromboxanes - TXA₂) over local vasodilator metabolites such as nitric oxide (NO) and prostacyclin (PGI₂) (46). The imbalance between the concentration of PGI₂ and TXA₂ is the cause of over-reactivity and vasoconstriction and excessive platelet activation (47).

Hypoxia is the principal factor upregulating NGAL and KIM-1 genes in the kidney. Hypoxia enhances synthesis of NGAL in many parts of the nephron. This suggests that the protein may exert a protective effect, probably promoting regeneration of damaged epithelium (48). Unfortunately, NGAL can also be synthesized by many extrarenal tissues as well as by immune cells (49). This may explain why in our study elevated concentrations of uNGAL were observed either in the group with pyelonephritis (group 1) or in both groups with AKI (groups 2 and 3). Histopathological examination demonstrated the presence of massive lymphocytic and granulocytic infiltrate in both lower and upper urinary tract (Fig. 1); therefore, we hypothesized that immune cells infiltrating the urinary tract might have been a source of uNGAL in all the study groups (Fig. 2). If this is true, the increase in uNGAL level would result not only from the enhanced release of this protein from hypoxic nephrons but also from its oversynthesis by immune cells migrating into the urinary tract during the course of bacterial

inflammation. Probably, the higher concentration of uNGAL in group 3 was due to the intensification of inflammation involving the kidneys. Contrary to groups 1 and 2, the inflammatory process also affected kidney's cortex, in addition to the pelvis and the core. uNGAL increase was also associated with decrease in CrCl, which was confirmed by the calculated correlations (Table 2, Fig. 6). Therefore, uNGAL is increasingly considered to be a marker of acute pyelonephritis (50, 51). The specificity and positive predictive value (PPV) of uNGAL as a marker of renal parenchymal inflammation caused by an infectious agent were estimated to be > 95% (52). However, in our experiment, elevated concentrations of uNGAL were observed both in the group with isolated pyelonephritis and in two groups with subsequently developed AKI. Therefore, we conclude that uNGAL is not an accurate diagnostic marker of bacterial ascending AKI. This statement is consistent with the results of Shavit *et al.*, who demonstrated that the level of uNGAL is strongly influenced by infection, and low predictive value of this parameter precludes its application for the detection of early AKI in patients with UTIs (53). Another useful parameter to distinguish pyelonephritis and AKI is uIL-6, which was also determined in our experiment. Tramma *et al.*, demonstrated that uIL-6 level increased in pyelonephritis (54, 55). In our study, uIL-6 increased only in groups with AKI (Table 1), and no

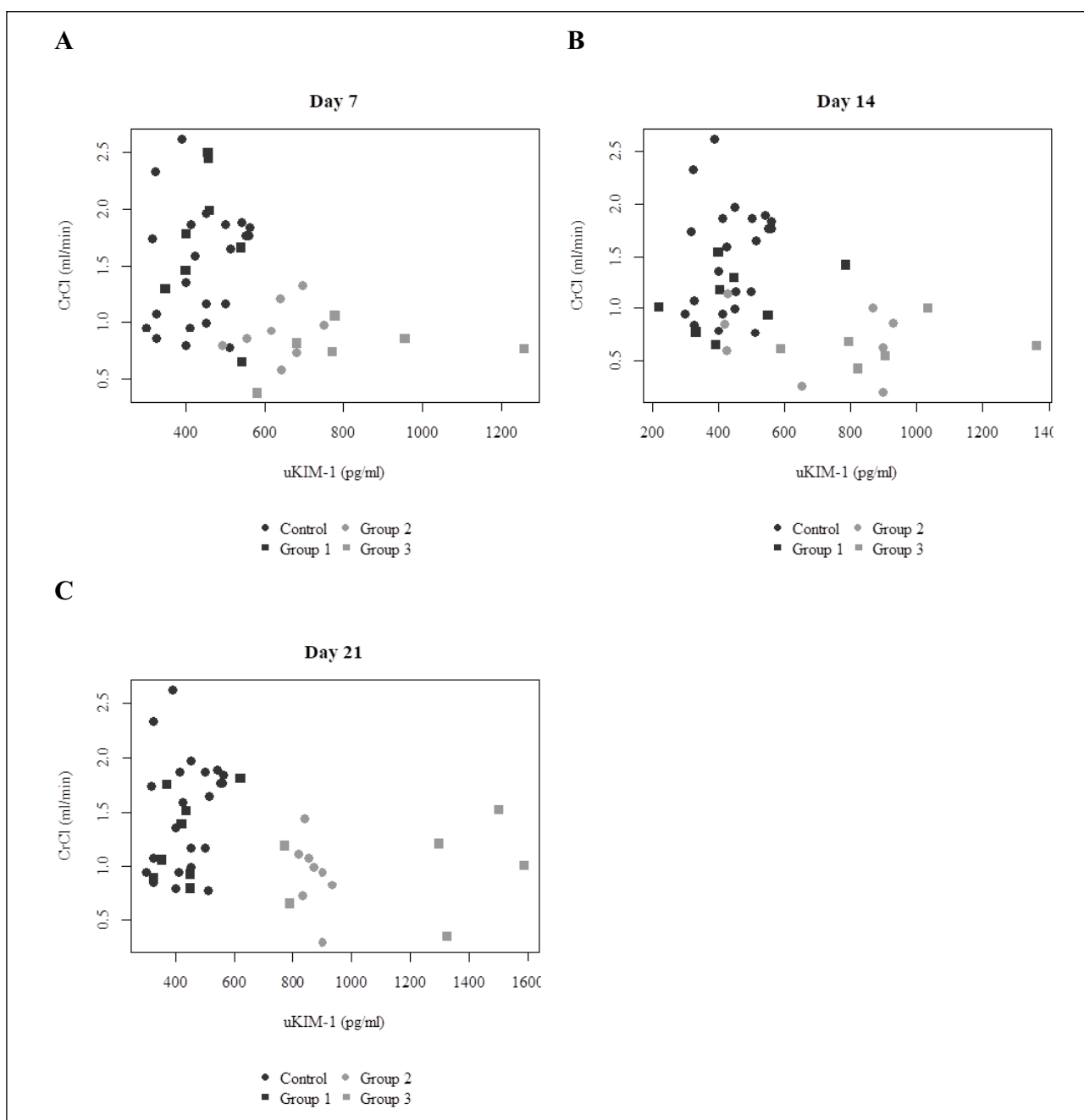


Fig. 7. Correlation between urinary kidney injury molecule-1 (uKIM-1) and creatinine clearance (CrCl) values on days (A) 7, (B) 14, and (C) 21 of the experiment.

change was observed in group 1 with isolated pyelonephritis; this finding confirms the usefulness of uIL-6 in the diagnosis of AKI. The above information also suggests that the use of uNGAL as a single biomarker for the diagnosis of ascending AKI poses the risk of obtaining a false-negative result, and as a consequence, it may cause a delay in the implementation of proper treatment, thereby resulting in high mortality. Moreover, monitoring of renal function should not be discontinued by measuring NGAL levels, as recent reports have demonstrated that in adult patients staying in ICU after surgery, NGAL serum level may be used as a marker for early AKI. As an early AKI marker in adult patients after surgery, the sensitivity and specificity of serum NGAL were estimated to be 90.2% and >89.5%, respectively (24). In pediatric patients, NGAL determined in urine, not in serum, seems to be a predictive factor

of AKI after cardiac surgery (22). Therefore, the diagnosis of AKI should be based ultimately on the compilation of information about the 'patient's condition (infection, surgery, poisoning, contrast administration, *etc.*) with the results of serum or urine NGAL level and a number of other diagnostic parameters, including urine IL-6, as recommended by KDIGO.

In contrast to NGAL, KIM-1 is not an inflammatory marker (56), and its increase is independent of infection (*e.g.*, UTI). This relationship was also confirmed in our experiment; while elevated levels of uKIM-1 were found in groups 2 and 3 (with induced AKI) at all study time points, the concentration of this marker in group 1 (with pyelonephritis) did not differ significantly from the baseline throughout the study duration (Fig. 3). Increase in uKIM-1 concentration was also associated with the decrease in CrCl, which was confirmed by the calculated correlations (Table 3, Fig. 7).

Table 3. Correlation between urinary kidney injury molecule-1 (uKIM-1) and creatinine clearance (CrCl) values.

Day	Group	Correlation between uKIM-1 and CrCl values			
		Correlation factor	P value	Direction of relationship	Strength of relationship
0	All groups together	0.168	0.455	—	—
7	All groups together	−0.385	0.01	negative	medium
14	All groups together	−0.389	0.009	negative	medium
21	All groups together	−0.315	0.037	negative	medium

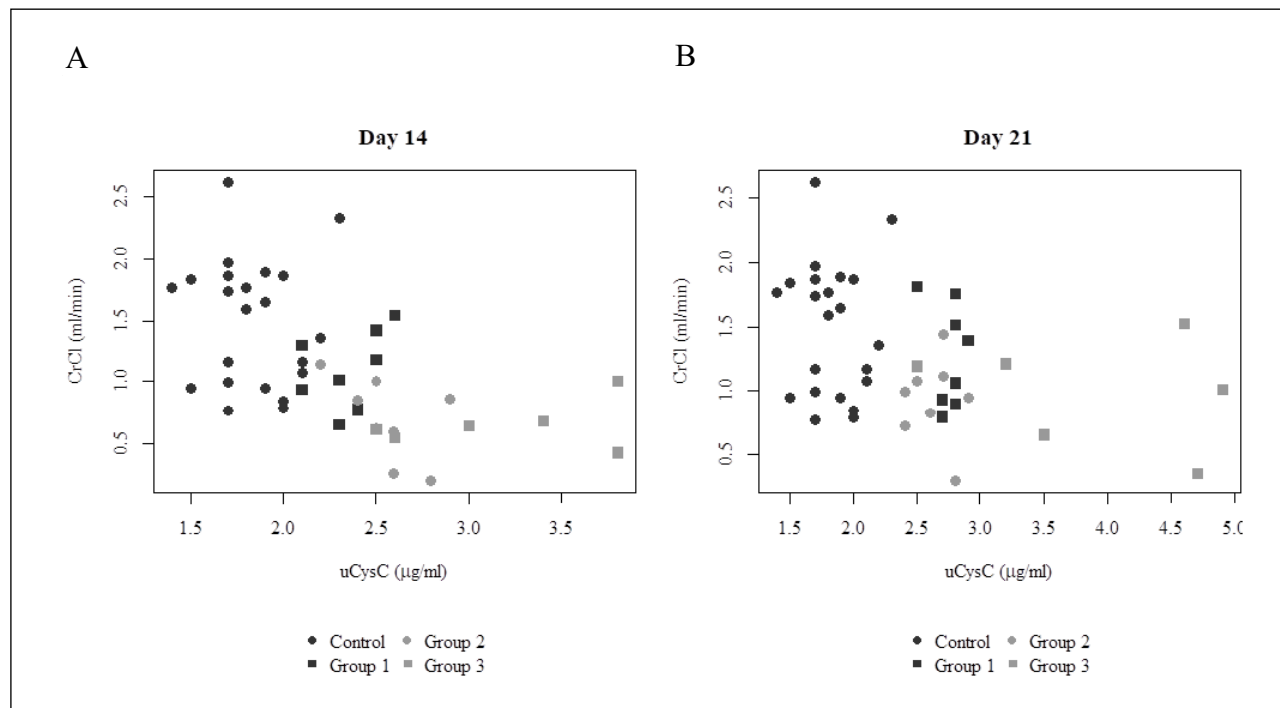


Fig. 8. Correlation between urinary cystatin C (uCysC) and creatinine clearance (CrCl) values on days (A)14 and (B) 21 of the experiment.

Table 4. Correlation between urinary cystatin C (uCysC) and creatinine clearance (CrCl) values.

Day	Group	Correlation between uCysC and CrCl values			
		Correlation factor	P value	Direction of relationship	Strength of relationship
0	All groups together	−0.069	0.761	—	—
7	All groups together	−0.114	0.461	—	—
14	All groups together	−0.549	< 0.001	negative	strong
21	All groups together	−0.382	0.011	negative	medium

Table 5. Correlation between urinary uromodulin (uMOD) and creatinine clearance (CrCl) values.

Day	Group	Correlation between uMOD and CrCl values			
		Correlation factor	P value	Direction of relationship	Strength of relationship
0	All groups together	0.047	0.863	—	—
7	All groups together	0.041	0.792	—	—
14	All groups together	−0.219	0.154	—	—
21	All groups together	−0.191	0.214	—	—

Furthermore, the concentrations of uKIM-1 in the group with pyelonephritis did not differ markedly from the levels of the protein observed in noncomplicated lower and upper UTIs, as reported by Urbaschat *et al.*, (57). These findings suggest that uKIM-1 may be an accurate diagnostic marker of AKI associated with UTI. This view was also confirmed by Petrovic *et al.*, who found significant differences in the concentrations of uKIM-1 in patients with UTI and AKI developed secondarily to this condition. Tu *et al.*, also showed the relationship between the high concentration of uKIM-1 and poor prognosis in septic AKI (58). Nevertheless, these authors also recommended to use additional markers of kidney function and emphasized that uKIM-1 should not be used as a single diagnostic marker of AKI (59). An increased number of evidence suggests that irrespective of its etiology, AKI should be diagnosed on the basis of both KIM-1 and NGAL assessment (57, 59–62). In our opinion, such approach would be particularly useful in patients with untreated asymptomatic bacteriuria, *e.g.*, in patients with diabetes, patients with polyneuropathy, patients who are chronically catheterized, and subjects with spinal injuries, with documented presence of bacterial virulence factors responsible for the colonization of the upper urinary tract. Analysis of both KIM-1 and NGAL levels would provide accurate information about the severity of UTI in those groups and would facilitate to introduce an appropriate treatment.

Because of its established role in the pathomechanism of UTI, another protein analyzed in our study was uMOD. Our study showed a similar trend in the dynamics of uMOD in groups 1 and 2. On the 7th and 14th day of the experiment, the concentrations of uMOD in both groups correlated inversely with the size of bacterial inoculum. Summing up, the larger the *E. coli* dose used to induce the UTI, the lower was the level of uMOD determined at the first and subsequent time points. This finding may have reflected a ‘depletion’ phenomenon associated with the ‘overactivity’ of UMOD exerting the nephroprotective effect against *E. coli*. In our opinion, a rapid increase in the concentration of uMOD observed on the 21st day of the experiment in group 3 might be a consequence of acute tubular necrosis (ATN) development and the resultant massive release of this protein from damaged nephrons (Fig. 4). Indeed, a review of previous studies investigating UMOD confirmed that this protein plays a regulatory role in both health (63, 64) and disease (65, 66). Polymorphism in the UMOD gene was shown to correlate strongly with the risk of chronic kidney disease (CKD) as well as with the outcome of this condition (67, 68), and reduced release of the protein, together with the abnormal level of CreaS, is known to precede the development of CKD and its diagnosis (69). Our study reconfirmed this finding by revealing the lack of correlation between uMOD and CrCl (Table 5), which demonstrates that uMOD may decrease in the initial stage of renal disease, even when CrCl is still normal. In many previous studies, a decrease in the uMOD level was associated with various conditions that affected the function and structural integrity of the kidneys (70–74). Unfortunately, a review of articles published after 2000 did not identify any previous studies analyzing uMOD concentrations in experimental models for ascending AKI. Therefore, we could not compare our findings to the results obtained by other authors. Nevertheless, the results of our experiment imply that the concentration of uMOD may serve not only as a useful prognostic factor but also as an additional diagnostic parameter to assess the risk for ascending AKI in patients with complicated UTI. A slight urinary increase of the parameter observed at early stages in our experiment may have reflected an impairment of the protective mechanism exerted by UMOD. It might also result from overdosing of bacteria used to induce the infection and/or from the involvement of renal parenchyma and inadequate synthesis of UMOD due to damage to distal tubules and Henle’s loops. A sudden increase in uMOD

concentration and a concomitant increase in KIM-1 level should be considered as a particularly unfavorable prognostic factor, as they may reflect a necrotic process occurring in the kidney. Unfortunately, our study was based on the histopathological assessment of routinely stained histopathological slides, and therefore, we were unable to confirm or exclude the presence of ATN. This justifies further research in this field by using more accurate techniques such as electron microscopy.

Urine of a healthy subject contains only a trace of CysC, and an increase in the concentration of this protein reflects a decrease in GFR and the involvement of renal parenchyma by a pathological process (75). This finding was also confirmed by the observed correlation between uCysC and CrCl in our study (Table 4, Fig. 8). The superiority of CysC compared to creatinine in kidney function estimation also results from the fact that elevated blood concentrations of CysC can be detected approximately 1.5 days earlier than a change in the creatinine level. This may be valuable for the diagnosis of AKI in some groups of patients, *e.g.*, in individuals after cardiac surgeries (76). However, the results of our experiment suggest that disturbances in the uCysC level do not satisfy the criteria for an early diagnosis of AKI associated with lower UTI. Similar to the uNGAL level, elevated concentrations of uCysC were found in all the experimental groups and at all study time points (Fig. 8). Histopathological analysis confirmed that the increase in the concentration of this marker coexisted with the dysfunction of CysC-resorbing tubules, which are damaged during the course of renal inflammation (41). Yim *et al.*, showed that serum CysC may be a useful marker for distinguishing between the lower and upper urinary tract inflammation (77), and Hassinger *et al.*, demonstrated the applicability of the protein as a risk marker of ischemic AKI (78). In our opinion, an accurate interpretation of the CysC level is central to determine the degree of kidney damage. Our experiment demonstrated that although elevated concentrations of uCysC were observed in all the study groups, the highest levels of the protein were found in the group with the most severe renal damage and urosepsis (group 3). The changes in uCysC concentration in group 1 (with pyelonephritis) and group 2 (with isolated ascending AKI) were not sufficiently different to establish the final diagnosis of AKI, but only suggested the presence of an inflammation in renal parenchyma. It should be stressed that the evident increase in the uCysC level in animals from group 3 resulted from the presence of urosepsis, which contributed to the largest damage of kidneys. This suggests that uCysC is more applicable in the detection of complicated AKI, and a rapid increase in the concentration of this marker may constitute an unfavorable prognostic factor, raising a suspicion of secondary urosepsis.

Our findings suggest that depending on the applied dose of bacterial inoculum, transurethral administration of *E. coli* may induce the symptoms of AKI or may induce a systemic infection. This observation seems to be clinically relevant. The results of our study underline the necessity of kidney function monitoring in patients with an asymptomatic bacteriuria threatening the development of interstitial nephritis (chronically catheterized individuals, persons with spinal cord injuries or polyneuropathy), who in line with current therapeutic guidelines, do not have to be subjected to antibiotic therapy to eradicate the infectious agent from the urinary tract. If a bacterial virulence factor responsible for kidney colonization is found in these patients, they would benefit from monitoring of all markers analyzed in this study. The use of a single marker is not the recommended method to diagnose AKI. A panel of specific parameters should be developed to help accurately diagnose AKI. Accurate interpretation of the results of both uNGAL and uKIM-1 concentrations would enable physicians to distinguish between individuals with pyelonephritis (high concentration of uNGAL

and low concentration of uKIM-1) as well as to identify the subjects at increased risk of AKI (high concentrations uNGAL accompanied with increase in uKIM-1). In turn, the risk of further progress in renal damage could be predicted on the basis of the changes in uMOD and uCysC levels. The lack of published reports related to issues discussed in our paper justifies further research, optimally with shorter intervals between the study time points and with more accurate diagnostic methods.

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