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ORIGINAL ARTICLE

Urinary HSP70 improves diagnostic accuracy for urinary tract infection in children: UTILISE study

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Abstract

Background The accuracy of conventional urinalysis in diagnosing urinary tract infection (UTI) in children is limited, leading to unnecessary antibiotic exposure in a large fraction of patients. Urinary heat shock protein 70 (uHSP70) is a novel marker of acute urinary tract inflammation. We explored the added value of uHSP70 in discriminating UTI from other infections and conditions confused with UTI.

Methods A total of 802 children from 37 pediatric centers in seven countries participated in the study. Patients diagnosed with UTI ($n=191$), non-UTI infections ($n=178$), contaminated urine samples ($n=50$), asymptomatic bacteriuria ($n=26$), and healthy controls ($n=75$) were enrolled. Urine and serum levels of HSP70 were measured at presentation in all patients and after resolution of the infection in patients with confirmed UTI.

Results Urinary (u)HSP70 was selectively elevated in children with UTI as compared to all other conditions ($p<0.0001$). uHSP70 predicted UTI with 89% sensitivity and 82% specificity (AUC = 0.934). Among the 265 patients with suspected UTI, the uHSP70 > 48 ng/mL criterion identified the 172 children with subsequently confirmed UTI with 90% sensitivity and 82% specificity (AUC = 0.862), exceeding the individual diagnostic accuracy of leukocyturia, nitrite, and leukocyte esterase positivity. uHSP70 had completely normalized by the end of antibiotic therapy in the UTI patients. Serum HSP70 was not predictive.

Conclusions Urine HSP70 is a novel non-invasive marker of UTI that improves the diagnostic accuracy of conventional urinalysis. We estimate that rapid urine HSP70 screening could spare empiric antibiotic administration in up to 80% of children with suspected UTI.

Keywords Children · Urinary tract infection · UTI · Heat shock proteins · HSP70

Introduction

The World Health Organization declared antimicrobial resistance one of the top ten threats to global public health [1]. Avoiding unnecessary antibiotic therapies is a key strategy to limit bacterial resistance. In children, suspected urinary tract infection (UTI) is one of the most common causes

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of empiric antibiotic therapy since the definitive diagnosis depends on urine culture results which are obtained with 24–48 h delay [2, 3]. The majority of these patients would not need antibiotic treatment. Saha et al. reported that urine culture did not confirm the diagnosis in 84% of patients who received empiric antibiotic therapy for presumed UTI [4].

Pediatricians often struggle with making the diagnosis of UTI because of the non-specificity of symptoms especially in infants [2, 3], frequent contamination of urine samples [5, 6], occasional non-invasive bacterial colonization of the bladder [7], and, most importantly, the limited specificity of conventional urinalysis by leukocyte microscopy and dipstick biochemical testing [2, 3]. Hence, there is a need for more sensitive and specific diagnostic biomarkers of UTI that can help in minimizing the number of unnecessary antibiotic therapies.

Heat shock protein 70 (HSP70) is a member of the heat shock protein family which is synthesized in response to hazardous factors, including infections, to protect the protein structure of the cells [8, 9]. HSP70 is released from macrophages exposed to bacterial products, especially Gram-negative bacteria such as *Escherichia coli (E. coli)* [10]. HSP70 is also secreted by tubuloepithelial cells under stress conditions [11].

In a recent pilot study in a small group of children, we found that the urine concentration of HSP70 increases in patients with UTI [12]. Here, we report the results of UTILISE, a large multicenter study specifically designed to validate the usefulness of HSP70 in urine and/or serum as a diagnostic biomarker distinguishing UTI from other infections and conditions.

Methods

Study protocol

The UTILISE (*U*rinary *T*ract *I*nfection and *L*evels of heat shock protein 70 *I*n children as a *S*ensitive marker for *E*xcluding other infections) study was a prospective observational study which enrolled pediatric patients (0–18 years old) with suspected UTI and four control groups in order to assess the performance of HSP70 in distinguishing UTI from other conditions in children. Inclusion criteria were defined for each study group below. The control groups included non-UTI infections, neurogenic bladder due to myelomeningocele, suspected vesicoureteral reflux (VUR) without current infection (sampling at time of voiding cystourethrography), and healthy children. Figure 1 summarizes algorithm for patient selection.

Ethics approval was obtained from the Ethics Committee of Istanbul University Istanbul Faculty of Medicine (2017/752), which was confirmed by the local Ethics Committees at all 37 participating sites. Written informed consent was obtained from the patients' parents. All data protection measures were in compliance with the EU General Data Protection Regulation (GDPR) and International Conference on Harmonization-Good Clinical Practice. Patient data were pseudonymized locally. Clinical, laboratory, treatment, and imaging data were recorded on a standardized form which was distributed along with the bagged sample tubes.

Definitions

Patients who applied to emergency room with symptoms suggestive of UTI (with or without fever) were assessed on presentation by physical examination, urinalysis, urine culture, white blood cell (WBC) count, and serum C-reactive protein (CRP) and creatinine. Leukocytosis was defined as a WBC count exceeding the age-related normal range [13]. Urinalysis findings taken to indicate UTI included the presence of microscopic leukocyturia and leukocyte esterase (LE) or nitrite test positivity [2]. Leukocyturia was defined as >5 leukocytes per high power field in centrifuged urine [2]. Urine sampling for bacterial culture was performed using collecting bag, mid-stream urine method, or bladder catheterization. Bacterial growth (of a single uropathogen) in urine cultures was considered significant at $\geq 10^5$ cfu/mL for samples obtained via collecting bag or mid-stream urine, and at $\geq 10^4$ cfu/mL for samples collected by catheterization [14, 15]. All patients suspected as having UTI by the participating centers were re-evaluated by two researchers in order to eliminate the differences between centers and to make the data uniform. UTI was considered confirmed in patients with clinical signs of infection, at least one urinalysis finding indicating UTI, and significant bacterial growth on culture [2]. Among the patients with suspected UTI, remaining patients without a urinalysis finding indicating UTI or significant bacterial growth on culture were classified as unconfirmed UTI. Pyelonephritis was defined by fever ≥ 38.5 °C, loin tenderness +/− rigors, leukocytosis, and elevated serum CRP.

Non-UTI infection group was composed of the patients with upper ($n=97$) and lower respiratory tract ($n=32$) infection, acute gastroenteritis ($n=24$), meningitis, and various other acute infectious conditions ($n=25$) such as parotitis, herpetic gingivostomatitis, EBV infection, viral rash, anal abscess, brucellosis, preseptal cellulitis, cholecystitis, dental abscess, lymphadenitis, and scarlet fever. These patients were identified based on clinical symptoms and diagnostic procedures appropriate for each of these diseases in the pediatric emergency room. Patients with fever of unknown origin and patients with co-morbidities were excluded.

Asymptomatic bacteriuria was diagnosed in children with a neurogenic bladder who exhibited $\geq 10^4$ cfu/mL bacterial growth of the same microorganism in two consecutive routine urine cultures obtained by clean intermittent

catheterization (CIC) in their elective outpatient visits [14, 15]. Bacterial contamination of urine was diagnosed in asymptomatic children who underwent urine collection prior to undergoing voiding cystourethrography for suspected vesicoureteral reflux. Bacterial contamination was defined by growth of a single microorganism at $< 10^5$ cfu/mL or the growth of a mix of two or more microorganisms in collecting bag or mid-stream urine in the presence of normal urinalysis findings [14, 15]. The healthy control group comprised children without any acute and chronic diseases or urinary tract abnormalities.

Study cohort

A total of 802 children were screened at 37 pediatric centers in seven countries. Two hundred eight individuals were excluded from further study because of non-compliance with the eligibility criteria shown in Fig. 1. The remaining 594 children were included as cases of confirmed UTI ($n = 191$), unconfirmed UTI ($n = 74$), non-UTI infection ($n = 178$), asymptomatic bacteriuria ($n = 26$), bacterial contamination ($n = 50$), and healthy controls ($n = 75$) (Fig. 1).

Laboratory analysis

Urine and venous blood samples were collected at the time of admission and after antibiotic treatment in the children with confirmed UTI once complete eradication was indicated by clinical and laboratory findings.

Blood samples were centrifuged at 2500 g (10 min) to separate serum. The serum and random urine samples were stored at − 80 °C until assayed. Samples from participating centers were sent on dry ice to the laboratory of Istanbul University Istanbul Faculty of Medicine where HSP70 was measured by ELISA following the manufacturer's instructions (Abbkine, Inc, China). HSP70 concentrations were expressed as ng/mL. The detection and quantification limits were set at < 0.05 ng/mL. The intra-assay and inter-assay coefficients of variation were 7.9% and 8.6%, respectively.

Procalcitonin in serum was measured centrally by ECLIA technology on a Roche Elecsys, Cobas e411 system. Serum creatinine and CRP were measured locally. Normal serum CRP and procalcitonin levels were accepted as < 5 mg/L and < 0.5 ng/mL, respectively. Estimated glomerular filtration rate (eGFR) was calculated with the Schwartz bedside equation [16].

Statistical analysis

Statistical analyses were performed using SPSS software (ver. 21.0; SPSS Inc., Chicago, IL, USA). The normality of the parameter distribution was tested using the Kolmogorov–Smirnov test. Descriptive analyses are presented as medians and interquartile range (*IQR*) for non-normally distributed and ordinal variables. Nonparametric tests

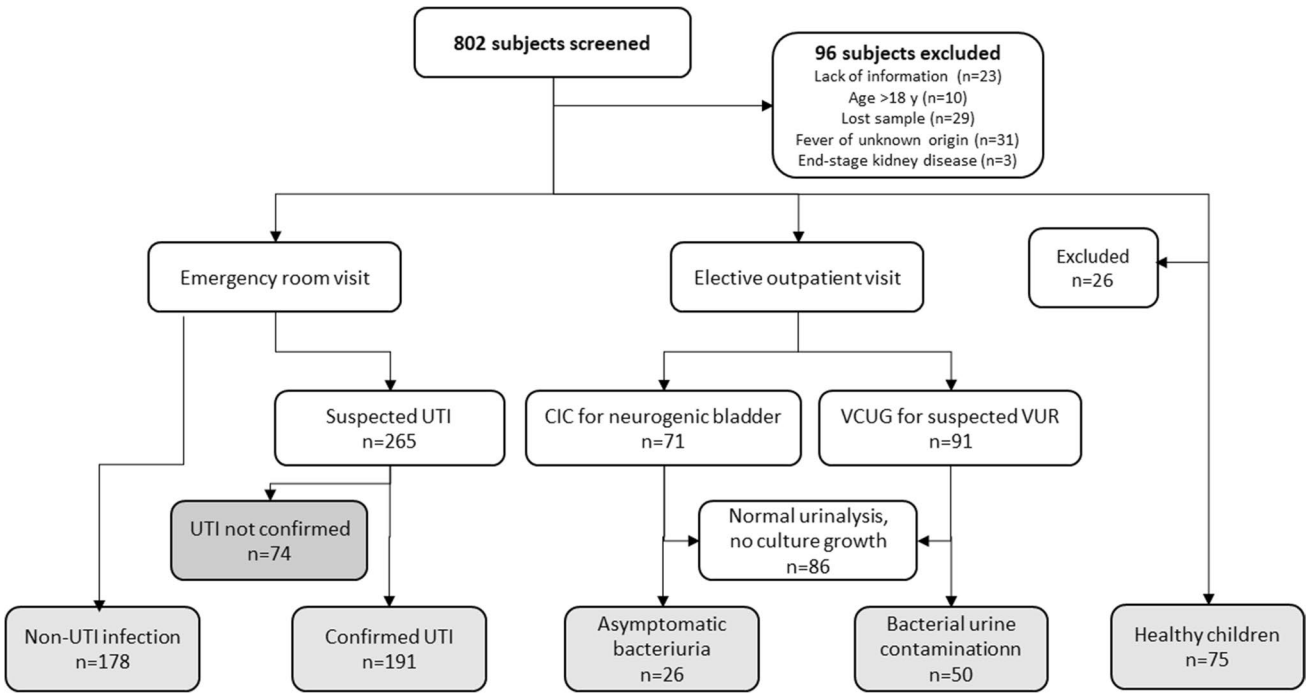


Fig. 1 Selection of subjects for analysis

(Mann–Whitney *U* test or Kruskal–Wallis test for more than two groups) were used for between-group comparisons. The Mann–Whitney *U* test was used to test the significance of pairwise differences, with the Bonferroni correction applied to adjust for multiple comparisons. Associations between variables were assessed by Spearman's correlation analysis. Chi-square or Fisher's exact test was used to compare proportions among groups as appropriate.

When investigating changes in urine level of HSP70 (uHSP70) according to nitrite positivity and *E. coli* urine culture growth, age effects were adjusted for using analysis of covariance (ANCOVA). Wilcoxon signed-rank test was used to compare HSP70 values before and after antibiotic treatment in the UTI group.

To calculate the sensitivity and specificity of uHSP70 at different cutoff values in detecting UTI, receiver operating characteristic (ROC) curve analysis was performed comparing patients with confirmed UTI to those with non-UTI infections, asymptomatic bacteriuria, contamination, and healthy controls. To verify the usefulness of uHSP70 relative to other urinalysis parameters in patients presenting with suspected UTI on admission to detect UTI, all patients with confirmed UTI were compared with unconfirmed patients who initially suspected UTI. In these children with suspected UTI, AUC values of individual conventional parameters of urinalysis alone and in combination with uHSP70 were compared.

Results

The clinical and laboratory characteristics of the patient groups and healthy controls are given in Table 1. There was a preponderance of girls among the subjects with UTI, asymptomatic bacteriuria, and bacterial contamination of urine samples. Fever was present in 62% of the children with confirmed UTI, 38% of patients with initially suspected but subsequently unconfirmed UTI, and 57% of the children with non-UTI infections. Pyelonephritis was diagnosed in 102 children with confirmed UTI (53.4%). The clinical signs and symptoms and laboratory data of the UTI group are summarized in Table 2. The mean duration of antibiotic treatment in this group was 8.7 ± 2.4 (range 4–21) days.

Serum HSP70 concentrations (sHSP70) did not differ between children with UTI, other infections, asymptomatic bacteriuria, contamination, and healthy children and did not change with antibiotic treatment in the UTI group (Table 1). By contrast, uHSP70 was significantly higher in the children with UTI compared to all other groups ($p < 0.0001$) and declined to the range of healthy children after resolution of the infection (Table 1). Normalization of uHSP70 to urine creatinine showed very similar findings (data not shown). uHSP70 concentrations were independent of age and eGFR.

In the confirmed UTI group, uHSP70 levels were not different between the patients who were nitrite positive and negative, leukocyte esterase positive and negative, and

Table 1 Demographic data and test results by condition

	UTI ($n=191$)	Non-UTI infection ($n=178$)	Asymptomatic bacteriuria ($n=26$)	Contamination ($n=50$)	Healthy controls ($n=75$)	p
Age (years)	6.4 (3.3–9.0)	6.6 (2.8–10.6)	7.5 (4.9–11.3)	7.4 (2.5–10.1)	7.6 (4.9–10.4)	0.108
% females	75	46	77	72	56	<0.0001
% eGFR < 90 mL/min/1.73 m^2	23	19	31	0	0	
Serum CRP (mg/L)	8.0 (1.2–61.6)	7.5 (2.0–28.0)	1.0 (0.3–2.5)	1.8 (0.3–3.2)	0.4 (0.3–2.2)	<0.0001
Serum procalcitonin (ng/mL)	0.03 (0.01–0.13)	0.05 (0.01–0.10)	0.01 (0.01–0.02)	0.01 (0.01–0.02)	0.01 (0.01–0.02)	<0.001
Serum HSP70 (ng/mL)	37.9 (32.4–63.7) (pre-treatment) 40.4 (33.2–65.9)[a] (post-treatment)	43.2 (33.4–77.3)	40.1 (34–81)	43.1 (35.4–97.7)	55.1 (41–89)	0.052
% with leukocyturia	86.9	11.2	38.5	0	0	<0.0001
% leukocyte esterase test positive	83.2	7.3	38.5	0	0	<0.0001
% nitrite positive	60.2	0	0	0	0	
Urinary HSP70 (ng/mL)	149.6 (70.1–198.7) (pre-treatment) 33.7 (30.8–39.7)[b] (post-treatment)	34.3 (31.8–44.0)	37.0 (31.4–49.3)	35.5 (31.5–41.3)	32.8 (29.4–40.1)	<0.0001

Data are given as percent or median (interquartile range) as appropriate. *UTI* urinary tract infection, *HSP70* heat shock protein 70, *CRP* C-reactive protein. [a]Difference pre-post treatment, p = NS. [b]Difference pre-post treatment, $p < 0.0001$

Table 2 Clinical findings, laboratory features, and urine culture results in 191 children with UTI

Clinical symptoms	*n* (%)
Fever	118 (61.8)
Dysuria	71 (37.2)
Abdominal pain	67 (35.1)
Nausea/vomiting	40 (20.9)
Urgency	32 (16.8)
Urinary incontinence	29 (15.2)
Loin pain	28 (14.7)
Irritability	28 (14.7)
Loss of appetite	24 (12.6)
Voiding frequency	20 (10.5)
Smell in urine	10 (5.2)
Macroscopic hematuria	5 (2.6)
Laboratory findings	
Leukocytosis	113 (59.2)
High C-reactive protein (> 5 mg/L)	102 (53.4)
High procalcitonin (> 0.5 ng/mL)	15 (7.9)
Urine nitrite positivity	115 (60.2)
Urine leukocyte esterase positivity	159 (83.2)
Pyuria on microscopy (> 5/hpf)	166 (86.9)
Urine culture results	
Escherichia coli	125 (65.5)
Klebsiella pneumonia	23 (12.0)
Enterobacter	7 (3.7)
Pseudomonas aeruginosa	5 (2.6)
Enterococcus	5 (2.6)
Others	26 (13.6)

also leukocyturia positive and negative ($p > 0.05$ for each) (Table 3). uHSP70 levels did not differ by the causative microorganism ($p = 0.174$) (Table 3). Patients with pyelonephritis tended to have higher uHSP70 levels than those with cystitis ($p = 0.06$), whereas sHSP70 did not differ ($p = 0.97$).

Comparative ROC analysis revealed that the optimal uHSP70 cutoff value for predicting UTI is 48 ng/mL. Using this cutoff, the presence of UTI (vs. non-UTI infection, asymptomatic bacteriuria, contamination, and healthy controls) was predicted with 89% sensitivity and 82% specificity (AUC = 0.934).

Table 3 Distribution of urine HSP70 concentrations stratified by other urine findings in confirmed UTI group

	n	Urine HSP70 (ng/mL)	*p*
Nitrite positive	115	155 (90–208)	0.097
Nitrite negative	68	134 (52–186)	
Leukocyte esterase positive	159	153 (77–199)	0.194
Leukocyte esterase negative	32	103 (51–199)	
Leukocyturia	166	151 (69–200)	0.917
No leukocyturia	12	141 (85–199)	
Escherichia coli	125	159 (94–200)	0.174
Other bacteria	66	99 (54–195)	

Data are presented as median (*IQR*)

Among the 265 patients with suspected UTI, the uHSP70 > 48 ng/mL criterion identified the 172 children with subsequently confirmed UTI with 90% sensitivity and 82% specificity (AUC = 0.862), exceeding the individual diagnostic accuracy of leukocyturia, nitrite, and leukocyte esterase positivity (Table 4). The combined consideration of uHSP70 and conventional urinalysis parameters increased the diagnostic performance to an AUC value of 0.874 (Table 4).

We explored whether the accuracy of UTI diagnostics could be further improved by adding serum biomarkers of bacterial infection to conventional urinalysis and uHSP70. The combination with serum CRP > 5 mg/L increased specificity to 93% but reduced sensitivity to 47% (AUC = 0.701). Adding serum procalcitonin > 0.5 ng/mL to the urine findings even increased specificity to 99% but reduced sensitivity to 8% (AUC = 0.534).

Discussion

In this study, we established the clinical usefulness of urinary HSP70 concentration as a biomarker of UTI in children. Since the information content of uHSP70 differs from that of conventional urinalysis parameters, its integration into the diagnostic workup maximizes the predictive power of initial urine screening.

HSP70 generation and release occur as part of the innate immune system's response to bacterial invasion [10]. Hence, we hypothesized that the urine concentration of HSP70 might signal host defense activation during invasive infections of the urinary tract and thus provides information that is partially complementary to conventional urinalysis. Indeed, we observed a selective increase of uHSP70 in children with confirmed UTI whereas the uHSP70 signal was absent in cases of asymptomatic bacteriuria and bacterial contamination, as well as non-UTI infections. uHSP70 concentrations were largely unrelated to urine leukocyte counts as well as nitrite and leukocyte esterase positivity, lending support to the notion that the biological information of uHSP70 may be additive to that of traditional urinalysis.

This was confirmed by the further analysis of children presenting suspected UTI. In this population, uHSP70 was the best single urinalysis parameter distinguishing UTI from other infections, being more sensitive than nitrite and leukocyte esterase positivity and more specific than leukocyturia and also nitrite positivity. The best overall test performance was achieved by combining uHSP70 with either leukocyturia, nitrite, or leukocyte esterase positivity. This combination

Table 4 Predictive value of individual and combined urine tests in patients presenting with suspected UTI

	AUC	Sensitivity (%)	Specificity (%)
Microscopy, WBCs	0.572	94.9	19.4
Nitrite test	0.677	62.8	72.5
Leukocyte esterase test	0.530	87.4	18.6
Urine HSP70 > 48 ng/mL	0.862	90.1	82.4
Leukocyte esterase test and nitrite test positive	0.642	54.4	73.9
Leukocyte esterase test and nitrite test and microscopy positive	0.622	47.6	76.7
Leukocyte esterase test or nitrite test positive	0.576	95.1	20.0
Leukocyte esterase test or nitrite test or microscopy positive	0.526	99.5	5.8
Microscopy positive and urine HSP70 > 48 ng/mL	0.849	84.8	84.9
Leukocyte esterase test positive and urine HSP70 > 48 ng/mL	0.830	78.7	87.3
Leukocyte esterase test or nitrite test or microscopy positive and urine HSP70 > 48 ng/mL	0.874	89.8	84.9

AUC area under curve, *HSP70* heat shock protein 70, *WBCs* white blood cells

of criteria yielded 15% false positive test results, reducing the number of patients unnecessarily receiving empiric antibiotic treatment for suspected UTI by 79%.

Our results suggest that the urine level of HSP70 is in better accordance with urine culture results than conventional urinalysis. Cell culture studies have provided evidence that HSP70 is actively secreted from baby hamster kidney (BHK-21) cells [17]. The superior value of HSP70 as a biomarker of UTI may be related to this direct release of HSP70 from kidney tissue in response to infectious agents, contrasting with the lower sensitivity and specificity of leukocyturia and nitrite positivity as indirect secondary response markers in invasive renal parenchymal infections. Also, HSP70 has been shown to be expressed by the epithelial cells of bladder [18]. uHSP70 levels were not significantly different between the patients with pyelonephritis and cystitis. Our findings suggest that bladder epithelial cells may produce an adequate immune response to an infectious agent by increasing HSP70 expression.

Bacterial growth in urine may indicate bacterial contamination of urine or asymptomatic bacteriuria which may be confused with UTI. Considering that leukocyturia is present in 38% of the patients in our asymptomatic bacteriuria group which consists of the patients using CIC, this could easily be interpreted as an actual urinary tract infection. Lack of increase in uHSP70 can be used to distinguish these conditions from UTI. Additionally, uHSP70 can be performed on urine specimens obtained by any of the techniques, including collection from a bag applied to the perineum.

Hence, addition of uHSP70 would substantially improve the diagnostic performance of initial urinalysis. The development of a rapid diagnostic test to identify uHSP70 concentrations exceeding the cutoff established in this study could help the discrimination of UTI from other, usually viral, causes of fever and facilitate the efforts to minimize unnecessary antibiotic therapies. The optimal cutoff value derived in our study was independent of age, sex, and kidney function. No adjustment of the urinary HSP70 concentration for urine dilution was required; normalization for urine creatinine concentration did not change diagnostic performance. Although uHSP70 elevations have been reported for children with type 1 diabetes mellitus and chronic kidney disease (CKD), the increases observed with these conditions were much less marked [19–23]. The average disease/control ratio of uHSP70 was 1.4 in diabetic children and 1.8 in those with CKD, compared to 4.5 in the UTI cohort studied here. Hence, the UTI cutoff established in this study would rarely be reached for these conditions in the absence of UTI.

Notably, adding information from serum biomarkers of bacterial infection (CRP, procalcitonin) to the combination of uHSP70 and conventional urinalysis maximized the diagnostic specificity of UTI in children with suspected UTI but led to a high false negative rate. This is explained by the dynamics of the systemic inflammatory response, which is frequently not yet established at the time of first presentation in patients with UTI. Hence, while a high CRP or procalcitonin level provides definitive confirmation of UTI in patients with high uHSP70, negative serum markers do not rule out the presence of a UTI.

The lack of a substantial systemic inflammatory response at this early stage may also explain why we did not observe any increase in serum HSP70 concentrations in children with UTI. sHSP70 has been found elevated in children with severe systemic infections such as meningococcal infection and septic shock [21, 24]. Heat shock proteins appear to be released by cells subjected to a bacterial infection primarily into the surrounding body fluids. For example, HSP72 was found increased in cerebrospinal fluid in patients with bacterial meningitis and in pleural exudates [25–27]. In UTI, it is conceivable that accumulation of HSP70 in urine would be more pronounced and precedes any "spill-over" into the circulation.

Repeated assessment showed complete normalization of uHSP70 at the time of resolution of the infection. This indicates that uHSP70 might be a useful non-invasive marker to monitor the response to antibiotic therapy and develop evidence-based concepts for the optimal duration of antibiotic administration in UTI.

In conclusion, uHSP70 is a novel non-invasive biomarker that facilitates the distinction of UTI from other infections. We estimate that its routine use would save unnecessary empiric antibiotic courses in up to 80% of those in whom the diagnosis of UTI is considered.

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Declarations

Conflict of interest The authors declare no competing interests.

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