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ORIGINAL ARTICLE

Biomarkers for febrile urinary tract infection in children

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Abstract

Background The sensitivity and specificity of the leukocyte esterase test for the diagnosis of urinary tract infection (UTI) are suboptimal. Recent studies have identified markers that appear to more accurately differentiate children with and without UTI. The objective of this study was to determine the accuracy of these markers, which included CCL3, IL-8, CXCL1, TNF-alpha, IL-6, IFN-gamma, IL-17, IL-9, IL-2, and NGAL, in the diagnosis of UTI.

Methods This was a prospective cross-sectional study to compare inflammatory proteins between urine samples from febrile children with a UTI, matched febrile controls without a UTI, and asymptomatic healthy controls.

Results We included 192 children (75 with febrile UTI, 69 febrile controls, and 48 asymptomatic healthy controls). Urinary proteins that best discriminated between febrile children with and without UTI were NGAL, a protein that exerts a local bacteriostatic role in the urinary tract through iron chelation; CCL3, a chemokine involved in leukocyte recruitment; and IL-8, a cytokine involved in neutrophil recruitment. Levels of these proteins were generally undetectable in asymptomatic healthy children.

Conclusions NGAL, CCL3, and IL-8 may be useful in the early diagnosis of UTI.

Trial registration ClinicalTrials.gov (NCT01391793)

Keywords UTI · Diagnostic accuracy · Biomarker · Infectious disease

Introduction

Urinary tract infections (UTI) are one of the most common bacterial infections in children, representing 5–14% of annual visits to emergency departments and 0.7% of visits to primary care pediatricians' offices [1]. The prevalence of UTIs in children presenting with either fever or urinary symptoms is 7.8% [2]. However, the diagnosis of UTI relies on the presence of bacteria cultivated from urine, which requires a minimum of 24 h. This delay in obtaining urine culture results forces clinicians to rely on imperfect screening tests at the point of care to determine which patients require treatment with antibiotics. Accurate screening tests are needed to prevent antimicrobial overuse. However, neither the leukocyte esterase test nor leukocyte count obtained using conventional urine microscopy, both of which are part of the routine screening tests for UTI, is sufficiently specific to serve as a screening test (specificities of 87% and 86%, respectively) [3]. This is illustrated by the significant rate of overuse of antimicrobials for UTI in children presenting to urgent care with findings consistent with a UTI [4]. One study found that 49% of children prescribed antimicrobials at the point of care did not have a UTI [5]. More specific markers of UTI would reduce such unnecessary antimicrobial use.

Compared to urinary leukocytes, urinary proteins involved in the innate immune response to a uropathogen are more likely to exhibit high specificity for UTI. We previously tested a panel of 53 candidate biomarkers and identified several that appeared to be highly specific for diagnosing UTI in children

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[6]. The goal of this study was to evaluate reproducibility of our prior findings and to ensure that levels of these biomarkers were undetectable or very low in healthy controls.

Methods

This was a prospective cross-sectional study of three groups of children: children with febrile UTI, febrile children without UTI, and healthy controls.

Children with UTI

Between October 2011 and August 2017, we enrolled children in a double-blind, placebo-controlled randomized trial [7] at the Children's Hospital of Pittsburgh, PA; Nationwide Children's Hospital in Columbus, OH; American Family Children's Hospital in Madison, WI; Children's National Health System in Washington D.C.; Hasbro Children's Hospital in Providence, RI; and Primary Children's Hospital in Salt Lake City, UT. The study was approved by the Institutional Review Boards of the respective institutions and registered at ClinicalTrials.gov (NCT01391793) prior to patient enrollment. Written informed consent was obtained from a parent of each enrolled child. Eligible children were aged 2 months to 6 years with a documented temperature of ≥ 38.3 °C (either documented in the emergency department or by parental report) who were treated for their first presumed UTI based on a urinalysis exhibiting pyuria (≥ 10 WBC/mm^3 in an uncentrifuged specimen, ≥ 5 WBC/hpf in a centrifuged specimen, or ≥ 1+ leukocyte esterase on dipstick). Children were excluded if they had received antibiotics within 7 days prior to enrollment or corticosteroids within 14 days prior to enrollment. For the present study, we included all males and a random sample of females who had a catheterized urine sample collected at the time of diagnosis and who had enough remaining urine for the tests planned in this study. We chose to use only those with a catheterized sample in order to avoid any potential misclassification bias caused by bacterial contamination of the urine sample. UTI was defined by growth of one or more uropathogens at ≥ 50,000 CFU/mL.

Febrile controls with suspected UTI

We enrolled febrile children less than 6 years of age in the Children's Hospital of Pittsburgh Emergency Department from April 8, 2018, through May 31, 2019. Children were eligible for enrollment if they had urine collected by catheterization, had both a urinalysis and urine culture sent to assess for UTI, had negative urine cultures, and had residual urine available for use in this study. Children with spina bifida, chronic medical conditions that could alter inflammatory response to UTI, and those who received antibiotics in the previous 15 days were excluded. For the present study, we selected children with < 1+ leukocyte esterase that were the best age and sex match to those in the UTI group. We excluded febrile children with a positive leukocyte esterase test and negative cultures because of the very small number of children in this category.

Healthy controls

We collected urine from healthy toilet-trained children who presented to one of three outpatient clinics in the Pittsburgh area for routine care between March 20, 2019, and May 24, 2019. Children were younger than 8 years of age, asymptomatic in the past week (i.e., no urinary symptoms, no fever, no upper respiratory tract symptoms), and not taking any systemic antimicrobials. We instructed parents to wipe the child's genitalia with three moist towelettes and to collect a midstream urine sample in a cup. A urine dipstick was performed on each sample.

Urine sample processing

Urine samples for all three groups were processed in an identical manner. Specifically, we filtered urine samples (Millex filters, Millipore) and stored them in cryovials at −80 °C. Processing generally occurred within 1 h of collection. However, if a delay was anticipated, samples were stored in a specimen refrigerator until filtering. While the majority of these samples did not undergo any freeze-thaw cycles prior to analysis for this work, nine samples in the febrile UTI group underwent one freeze-thaw cycle prior to this study.

Protein measurement

We assessed 10 candidate markers: CCL3, IL-8, CXCL1, TNF-alpha, IL-6, IFNgamma-, IL-17, IL-9, IL-2, and NGAL. These markers were measured in urine using a custom-made Bio-Rad human cytokine 9-plex plate (Hercules, CA) and Thermo Fisher's Rapid Human NGAL ELISA kit (KIT037; Waltham, MA). We included duplicates and control samples on each plate. We measured urine creatinine concentration using R & D Creatinine Parameter Assay Kit (KGE005; R & D Systems, Minneapolis, Minnesota).

Statistical methods

We used the t-test for normally distributed continuous data (the Kruskal-Wallis test for non-normally distributed continuous variables) and the Chi-square or Fisher's exact test for categorical variables. In order to adjust for multiple comparison, the resulting p-values were converted to *q*-values using the Benjamini-Hochberg correction [8]. To assess the discriminative power of each marker, we constructed a receiver

operating characteristic curve (ROC) using UTI vs. no-UTI status as the outcome and calculated the area under the curve (AUC). Sensitivities and specificities were calculated for the point on the ROC curve that maximized the Youden index (Youden index = sensitivity + specificity − 1). We utilized linear regression models to identify covariates (age, gender, race, isolated organisms, and time between fever and diagnosis) associated with biomarker levels among children with febrile UTI.

Results

Table 1 describes the demographic characteristics of the children in the three groups. As expected, children in the healthy control group were older, had a higher urine creatinine level, and had a higher proportion of urine collected using clean catch. There were no differences in either age, sex, or race between the febrile UTI and febrile control groups. All urine samples in both the febrile UTI and febrile control groups were collected by catheterization. The degree of leukocyte esterase present on urinalysis was higher in the febrile UTI group compared to both control groups. Similarly, the febrile UTI group had a higher proportion of samples with positive nitrites compared to both control groups (Table 1).

Biomarker levels, as well as their respective AUC, sensitivity, and specificity in discriminating UTI from no UTI are shown in Table 2. NGAL had the highest AUC (1.00), with a sensitivity of 1.00 and specificity of 0.97 (Table 2), a figure showing median urinary NGAL levels according to study group (Fig. 1). Five additional markers also had AUCs greater than 0.90: CCL3 (AUC: 0.98), IL-8 (0.97), CXCL1 (0.95), TNF-alpha (0.93), and IL-6 (0.92). All six markers with AUCs greater than 0.90 were highly specific, with specificities of 0.97 or greater. NGAL, CCL3, and IL-8 were the most sensitive markers and thus represent the best markers of UTI (Table 2). If we had used the previously published cutoff of 39,100 pg/mL [9] for NGAL rather than the cutoff suggested by the Youden index, the results would have been similar (sensitivity of 1.00 and specificity of 0.96). Normalization of protein marker levels by urine creatinine had little effect on the p-values or on the AUCs of the urinary markers (see Supplemental Table 1).

Escherichia coli was isolated on the majority (93%) of urine cultures in the febrile UTI group. Although the number of children with infections caused by organisms other than *E. coli* was small, NGAL levels did not differ significantly between children with *E. coli* UTIs and those with non-*E. coli* UTIs. In contrast, patients with non-*E. coli* UTIs had lower degrees of leukocyte esterase versus those with *E. coli* UTIs (Table 3).

We also evaluated whether there were any potential covariates affecting values of the top 3 performing biomarkers. Among children with febrile UTIs, there was no association between levels of NGAL, CCL3, or IL-8 and any of the covariates examined.

Discussion

In this study, we provide further data on the utility of NGAL, CCL3, and IL-8 in differentiating UTI from no UTI in febrile children presenting to the emergency department. To understand the implications of our findings, it is helpful to compare the accuracy of the markers identified in this work to the accuracy of leukocyte esterase in previous studies. We found that NGAL, for example, has a sensitivity of 1.0 and specificity of 0.97. In comparison, leukocyte esterase has a sensitivity of 0.79 and specificity of 0.87, while urinary white blood cell count has a sensitivity of 0.74 and specificity of 0.86 [3]. These data demonstrate the superior diagnostic accuracy of NGAL compared to the conventionally used point-of-care screening tests. These results are supported by a recent meta-analysis comparing NGAL and the leukocyte esterase test [10].

A role for the identified urinary markers in the diagnosis of UTI is supported by previous studies of these markers as diagnostic tools as well as their functions within the immune system. IL-8 is a key neutrophil chemoattractant in the urinary tract. It is secreted by the urothelium and responsible for directing the migration of neutrophils across the mucosa and into the urine [11]. IL-8 levels are elevated in adults and children with UTI [12, 13]. Further, IL-8 has also been shown to differentiate asymptomatic bacteriuria from febrile UTI in young children [14].

CCL3 (also known as MIP-1alpha) is a proinflammatory chemokine that plays a role in leukocyte recruitment [15, 16]. CCL3 is secreted by the urothelium in response to uropathogenic *E. coli* exposure [17]. While CCL3 has been studied as a marker of UTI in both mice and humans, results have been mixed. Some authors report increased levels of CCL3 in UTI [6], while others have not found a difference in urinary CCL3 levels in either mice [18] or humans during UTI [19]. Results have also been mixed regarding levels of CCL3 in bladder tissue of mice with UTI [20, 21]. Despite these mixed results, we found that CCL3 is very specific for UTIs. The reason behind these discordant findings is unclear and requires further investigation.

We found very high levels of urinary NGAL in children with UTI and negligible levels in febrile controls and healthy controls. Although the function of NGAL is not completely understood, it plays a role in sequestering iron required for bacterial growth within the urinary tract. NGAL is released from neutrophils and intercalated cells in the renal collecting duct in response to infection or cell injury [22]. Several studies [9, 23–26] and a recent meta-

Table 1 Demographic and clinical characteristics of included children

	Febrile UTI (A) n = 75	Febrile control with suspected UTI (B) n = 69	Healthy controls n = 48	p-value A vs. B	p-value All groups
Age in months at enrollment					
Median [IQR]	9.7 [5.7, 15.9]	14.0 [9.0, 20.0]	60.0 [48.0, 72.0]	0.06[a]	< 0.001[a]
Mean (sd)	12.5 (9.1)	14.7 (8.2)	64.0 (17.7)		
Gender—no. (%)					
Male	16 (21)	12 (17)	27 (56)	0.55	< 0.001
Female	59 (79)	57 (83)	21 (44)		
Race—no. (%)					
White	49 (65)	39 (57)	15 (31)	0.51	0.001
Black	19 (25)	24 (35)	30 (62)		
Other	7 (9)	6 (9)	3 (6)		
Fever within 24 h of presentation—no. (%)					
No	0 (0)	0 (0)	48 (100)	---	< 0.001
Yes	75 (100)	69 (100)	0 (0)		
Method of urine collection—no. (%)					
Catheter	75 (100)	69 (100)	0 (0)	---	< 0.001
Clean catch	0 (0)	0 (0)	48 (100)		
Leukocyte esterase—no. (%)					
Negative/trace	0 (0)	69 (100)	46 (96)	< 0.001	< 0.001
Small (+)	9 (12)	0 (0)	1 (2)		
Moderate (++)	13 (17)	0 (0)	1 (2)		
Large (+++)	53 (71)	0 (0)	0 (0)		
Nitrite—no. (%)					
Negative	42 (56)	69 (100)	48 (100)	< 0.001	< 0.001
Positive	33 (44)	0 (0)	0 (0)		
Creatinine					
Median [IQR]	41.0 [31.2, 55.8]	48.5 [29.0, 82.2]	89.0 [60.0, 121.5]	0.17[a]	< 0.001[a]

[a] Kruskal-Wallis test

analysis [10] have found that NGAL levels differ in children with and without UTI, which is consistent with our results. We also provide preliminary data that NGAL is superior to leukocyte esterase in detecting non-*E. coli* UTIs. In a previous study [27], we found that organisms other than *E. coli* are associated with lower levels of urinary leukocyte esterase and as such, screening using the leukocyte esterase test or the white blood cell count could miss infections caused by these organisms. The same finding was observed in the current study (Table 3). Although we had relatively few children with organisms other than *E. coli*, these children all had very high NGAL levels (lowest was 72,893 pg/mL). Thus, these data suggest that, by addressing an important shortcoming of the leukocyte esterase test, urinary NGAL promises to be a more accurate screening test for UTI. However, because the presence of urine leukocyte esterase was an inclusion criterion for patients in the febrile UTI group and its absence an exclusion criterion for patients in the febrile control group, we are unable to directly compare urine leukocyte esterase with any of the biomarkers in this study.

The results of this study vary slightly from our prior work [6]. While some markers performed similarly, including NGAL, CCL3, and CXCL1, others did not. In our prior work, IL-9 and IL-2 had the highest accuracies in differentiating UTI from no UTI (AUC of 0.93 and 0.89, respectively); in the current study, they were found to have lower AUCs (0.67 and 0.58). Notably, IL-9 and IL-2 are both involved in T-cell growth and differentiation whereas the other markers are involved in the innate immune response [28–30]. A potential explanation for differences in the two studies is the different study population; not all patients in our prior study had fever. Another difference between the two studies relates to sample collection; in the prior work, samples could have been collected by catheterization or clean catch, while all samples (except

Table 2 Mean biomarker levels (pg/mL) and their ability to discriminate children with and without urinary tract infection (UTI)

	Febrile UTI n = 75	Febrile controls n = 69	Healthy controls n = 48	Q value[a]	AUC[b]	Sensitivity[c]	Specificity[c]
Mean (sd) (pg/mL)							
NGAL	419,990.8 (134,209.6)	8,006.9 (40,666.9)	43,77.5 (21,401.1)	8.22E-52	1.00	1.00[d]	0.97[d]
CCL3	190.5 (290.9)	0.9 (4.8)	0.2 (0.3)	4.33E-07	0.98	0.92	0.99
IL-8	4580.3 (6747.0)	6.2 (7.3)	17.4 (41.5)	2.33E-07	0.97	0.92	1.00
CXCL1	57,408.2 (136,702.8)	124.7 (86.1)	182.0 (122.4)	8.33E-04	0.95	0.81	0.99
TNF-α	255.8 (379.1)	1.0 (5.0)	1.1 (7.6)	2.35E-07	0.93	0.87	0.97
IL-6	7168.8 (5153.2)	183.4 (1374.4)	8.6 (12.8)	5.66E-20	0.92	0.85	0.99
IFN-γ	2.9 (4.8)	49.4 (29.4)	24.2 (31.5)	1.52E-26	0.83	1.00	0.75
IL-17	66.7 (142.2)	0.6 (1.1)	0.3 (0.4)	2.42E-04	0.81	0.73	1.00
IL-9	37.4 (56.0)	18.6 (13.3)	26.8 (8.3)	7.99E-03	0.67	0.68	0.75
IL-2	0.7 (1.0)	0.9 (0.5)	0.8 (0.8)	8.36E-02	0.58	0.67	0.75

[a] Q value of marker in discriminating febrile UTI from febrile controls; Q value represents p value corrected for multiple comparison

[b] Area under the receiver operator curve of marker in discriminating febrile UTI from febrile controls

[c] Sensitivity and specificity of marker in discriminating febrile UTI from febrile controls. These values were determined using cutoff that maximized the Youden index J

[d] Using cutoff of 71,079

for healthy controls) in this work were collected by catheterization. While these discrepancies suggest that different markers may be better suited for detection of UTI depending on the specific population being studied, there is a need for these results to be validated in large, well-designed studies to rigorously validate the predictive accuracy of these candidate biomarkers.

As a case-control study, our study suffered from the limitations of this type of study design. One limitation is that accuracy values tend to be exaggerated, especially as all children with UTI had pyuria and all febrile controls lacked pyuria. However, case-control studies are an indispensable early phase of biomarker development. Another limitation is potential misclassification. Asymptomatic bacteriuria, although possible, is very unlikely [31] in this cohort because all patients were symptomatic to the extent that clinicians tested and treated for UTI, and all had pyuria. As with most studies on pediatric UTI, a small proportion of children categorized as having a UTI may have actually had contamination of the sample during collection. We attempted to minimize this by only using catheterized samples for children in the febrile UTI and febrile control groups, by requiring pyuria, and by using established cutoffs of both colony count and pyuria to define a UTI. Additionally, because healthy children do not routinely undergo bladder catheterization, children in the healthy control group were older than those in the febrile UTI group. Prior work has demonstrated a very small, but significant, difference in NGAL levels based on age [32]. However, as the patients in our febrile UTI group had NGAL levels that were 96 times higher than the healthy controls, the very small effect of age on NGAL levels likely does not affect the results of this work. Further, some biomarkers within this study (e.g., NGAL) are also markers of acute kidney injury (AKI). Therefore, the predictive ability of NGAL for UTI may be lower than presented here in children with AKI.

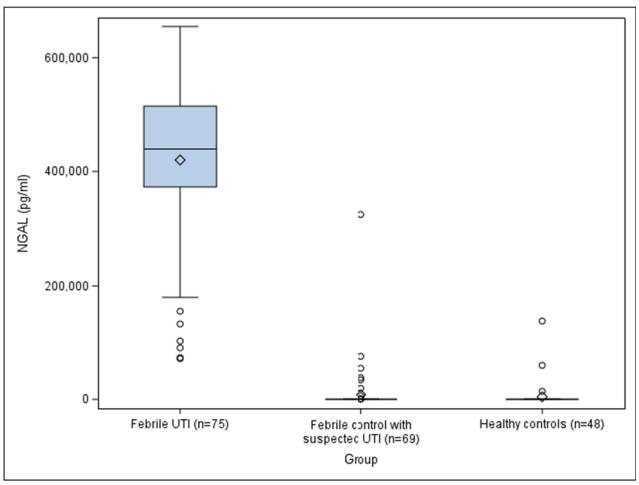

Fig. 1 Box and whisker plot of urinary NGAL level by group. Dots represent outliers. Diamonds represents the group mean

Finally, a small number of urine samples had undergone a freeze-thaw cycle prior to this work, potentially decreasing the levels of the biomarkers of interest in those samples. However, all samples that had undergone a prior freeze-thaw cycle were in the febrile UTI group. As we still found differences in biomarker levels between the groups, it is unlikely that the additional freeze-thaw cycle in a small number of urine samples impacted our results.

Table 3 NGAL and leukocyte esterase in children with non-*E. coli* UTI

	With *E. coli* n = 70	Without *E. coli* n = 5	p
NGAL, pg/mL—mean (SD)	425,748.4 (131,660.8)	339,384.1 (159,696.0)	0.17
Leukocyte esterase—no. (%)			
Small	6 (9)	3 (60)	0.01[a]
Moderate	13 (19)	0 (0)	
Large	51 (73)	2 (40)	

[a] Fisher's exact

The majority of studies to date that have investigated urine markers of UTI have not included a control group of healthy children. The addition of a healthy control group in this early stage of biomarker research is necessary to ensure that the signal seen in children with UTI is different from that seen in healthy children. We believe this is the first study with relatively large numbers of both healthy and febrile controls.

In conclusion, this study provides preliminary evidence to support a role for measurement of urinary NGAL, CCL3, and IL-8 in differentiating febrile children with and without UTI. Because of their high specificity, these markers, if confirmed by future studies, could be useful in reducing unnecessary antimicrobial use for children with presumed UTI. Furthermore, the high sensitivity of the markers could be useful also in reducing delays in treatment of UTIs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00467-021-05173-x.

Code availability Code is available upon request.

Author contribution Dr. Shaikh conceptualized and designed the study, collected data, interpreted the data, drafted the initial manuscript, and reviewed and revised the manuscript. Dr. Forster interpreted the data and revised the manuscript. Hui Liu performed the initial statistical analysis and reviewed and revised the manuscript. Marcia Kurs-Lasky verified the statistical analysis and reviewed and revised the manuscript. All authors gave final approval of the version to be published and agree to be accountable for all aspects of the work.

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Data availability Data is available upon request.

Declarations

Ethics approval The study was approved by the IRBs of the respective institution.

Consent to participate All patients were consented prior to enrollment.

Conflict of interest The authors declare no competing interests.

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