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Article

Urine Biomarkers Individually and as a Consensus Model Show High Sensitivity and Specificity for Detecting UTIs

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Abstract: We aimed to determine if infection-associated urine biomarkers can differentiate true urinary tract infection (UTI) from non-UTI controls. Midstream clean-catch urine samples were collected from asymptomatic volunteers and symptomatic subjects > 60 years old diagnosed with presumptive UTI in a specialty setting. Microbial identification and density were assessed using multiplex PCR/pooled antibiotic susceptibility test (M-PCR/P-AST) and standard urine culture (SUC). Three biomarkers (NGAL, IL-8, and IL-1β) were measured in the same urine specimens. Definitive UTI cases were symptomatic and had positive microorganism detection by SUC and M-PCR, while definitive non-UTI cases were asymptomatic volunteers regardless of microbial detection. We observed a strong positive correlation ($R2 \approx 1$) between microbial density and the biomarkers NGAL, IL-8, and IL-1β. Biomarker consensus criteria of two or more positive biomarkers had sensitivity 90.2%, specificity 91.2%, positive predictive value (PPV) 91.7%, negative predictive value (NPV) 89.7%, accuracy 90.7%, positive likelihood ratio of 10.28, and negative likelihood ratio of 0.11 in differentiating definitive UTI from non-UTI cases, regardless of microbial density. NGAL, IL-8, and IL-1β showed a significant elevation in symptomatic cases with positive microbe identification compared to asymptomatic cases with or without microbe identification. Biomarker consensus exhibited high accuracy in distinguishing UTI from non-UTI cases.

Keywords: urinary tract infection (UTI); diagnostic testing; urine biomarkers; neutrophil gelatinase-associated lipocalin (NGAL); interleukin (IL)-8, IL-1β

1. Introduction

The use of standard urine culture (SUC) that detects and quantitates classical uropathogens has been in use for over 100 years to confirm the diagnosis of an active urinary tract infection (UTI).[1] However, SUC has several limitations. SUC uses specific media and conditions that result in cultivating easy-to-grow microbes, like *Escherichia coli (E. coli)*. However, non-*E. coli* pathogens including fastidious microbes reported as important emerging uropathogens, are rarely grown.[2–4] Recent studies using more sensitive culture techniques, such as enhanced-quantitative urine culture (EQUC), and culture-free methods, such as gene sequencing and MALDI-TOF, have led to the discovery of

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the uromicrobiome, which is present even in asymptomatic individuals.[2–4] In addition, these studies have increased awareness of many additional clinically relevant microbial species, such as gram-positive organisms, fastidious microbes, and fungi, which can contribute to urinary microbiome dysbiosis in symptomatic subjects.[5]

Another disadvantage of SUC is the length of time necessary to perform the testing, which creates a delay before patients can be treated with a results-guided antimicrobial. This may result in an over-reliance on empiric therapy whereby the clinician overtreats patients lacking a true infection or treats them with the wrong antibiotic. These delays further increase the risks for poor outcomes including urosepsis. These challenges have encouraged the development of molecular tests, such as multiplex PCR (M-PCR), which have demonstrated better detection of non-*E. coli* and polymicrobial UTIs and a faster turnaround time for results compared to SUC.[6–10]

The presence of a urinary microbiome along with the high prevalence of asymptomatic bacteriuria[11–15] underscores the need for clinically relevant diagnostic tests that are both sensitive and specific for UTIs in symptomatic patients. While the identification of uropathogens in symptomatic patients is a strong indicator of infection, there remain questions about whether detected microbes are associated with a UTI. Also, symptom elucidation can be problematic in pediatric patients or in patients who are cognitively impaired. For example, in the long-term care setting there are both high rates of asymptomatic bacteriuria (up to 50%)[14] and cognitive impairment.

With that in mind, there have been hundreds of studies looking at biomarkers as a potential tool for the identification of urinary tract infections.[16] The innate immune system in the urinary tract consists of both resident and recruited cells expressing a variety of pattern recognition receptors that detect pathogens early and rapidly trigger a pro-inflammatory immune response to aid in bacterial clearance.[17,18] Uroepithelial cells secrete bacteriostatic agents, such as neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2.[19–21] In addition, resident and recruited immune cells secrete pro-inflammatory cytokines, such as interleukins (ILs), including IL-8, also known as chemokine (C-X-C motif) ligand 8 (CXCL8),[17,22–26] and IL-1β[22,27,28], to further promote the response until the microbial threat is resolved.[17,18,29] Soluble infection-associated biomarkers, such as NGAL, IL-8, and IL-1β, are detectable in urine and studies have demonstrated the association of these urinary biomarkers with the presence of a clinically diagnosed UTI.[16,30,31] Using such biomarkers individually, or in combination, provides strong evidence of immune response to uropathogens in the urinary tract at the time of urine collection.

This study aimed to determine whether an individual biomarker or a set of biomarkers can differentiate symptomatic subjects with microbes detected in the urine from asymptomatic subjects either with microbes detected (asymptomatic bacteriuria) or without microbes detected in the urine to support the clinical diagnosis of UTI.

2. Materials and Methods

Study design and participants

Results from biomarker analyses, M-PCR/P-AST tests and standard urine culture (SUC) included in this study were obtained from urine samples from both asymptomatic and symptomatic cohorts. Urine samples from the asymptomatic cohort were collected between 2/28/2023 and 3/22/2023 from adult volunteers for a prospective observational study. All subjects were 60 years of age or older and provided written informed consent (Western IRB 20230847) prior to enrollment. Subjects who were pregnant, taking antibiotics for a UTI, or taking steroids were excluded. All consenting subjects completed the validated American English Acute Cystitis Symptom Score (ACSS) baseline questionnaire and provided a midstream clean-catch urine specimen.[32] All 228 asymptomatic subjects had an ACSS sum score of ≤ 4 for the FDA-defined UTI symptoms (urinary frequency, urinary urgency, dysuria, and suprapubic pain) with no single symptom score > 1 and were included as the asymptomatic cohort.

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The symptomatic cohort consisted of 583 subjects. Urine samples were collected in the Pathnostics' clinical laboratory between 01/17/2023 and 04/24/2023 through a de-identified biorepository of adult patients with UTI symptoms and a presumptive diagnosis of UTI from a specialty urology setting. These patients were 60 years of age or older and presented to healthcare providers in urology offices from 39 U.S. states with ICD-10-CM codes consistent with UTI. The Western IRB deemed the biorepository-obtained specimens exempt from review.

All urine specimens were collected via the midstream clean catch/voided method.

The Guidance® UTI M-PCR/P-AST assay (Pathnostics in Irvine, CA) includes susceptibility testing for 19 antibiotics, semi-quantification of 27 distinct uropathogenic species and 3 bacterial groups, as well as identification of 32 antibiotic-resistance genes and the ESBL phenotype. The test was performed as described previously: the first step involves DNA extraction from the subject's urine sample using King Fisher/MagMAX™ automated DNA extraction instrument and the MagMAX™ DNA Multi-Sample Ultra Kit (Thermo Fisher, Carlsbad, CA) per the manufacturer's instructions. Extracted DNA was mixed with a universal PCR master mix and amplified using TaqMan technology in a Life Technologies 12K Flex 112-format Open Array System (Thermo Fisher Scientific, Wilmington, NC). Probes and primers were used to detect 26 bacteria/bacterial groups, fastidious and non-fastidious, and four yeast species[7–9] listed below:

Classical uropathogens: *Candida albicans, Candida glabrata, Candida parapsilosis, Citrobacter freundii, Citrobacter koseri, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii, Pantoea agglomerans, Proteus mirabilis, Providencia stuartii, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Streptococcus agalactiae,* and *Enterobacter group [including Klebsiella aerogenes* (formally known as *Enterobacter aerogenes)* and *Enterobacter cloacae]*.

Emerging uropathogens: *Acinetobacter baumannii, Actinotignum schaalii, Aerococcus urinae, Alloscardovia omnicolens, Candida auris, Corynebacterium riegelii, Gardnerella vaginalis, Mycoplasma hominis, Ureaplasma urealyticum,* coagulase negative *staphylococci* group (CoNS) (including *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus lugdunesis, and Staphylococcus saprophyticus),* and Viridans group *streptococci* (VGS) (including *Streptococcus anginosus, Streptococcus oralis,* and *Streptococcus pasteuranus*).

Results of the P-AST portion of the test, an antibiotic resistance and sensitivity assay which accounts for bacterial interactions, were not included in this analysis.

Standard Urine Culture (SUC)

The SUC method was performed as previously described.[8] Briefly, urine was vortexed, and a sterile plastic loop (1 µL) was used to inoculate blood agar plates. A sterile plastic loop (1 µL) was used also to inoculate colistin and nalidixic acid agar/MacConkey agar (CNA/MAC) plates, one loop-full of urine on the CNA side of the plate and another full loop-full on the MAC side of the plate. All plates were incubated at 35° C in 5% CO_2 for ≥ 18 hours and then examined for evidence of growth. Per standard operating procedures plates with < 10,000 CFU/mL were reported as normal urogenital flora.[33] For plates with growth (≥ 10,000 CFU/mL), the quantity and morphology of each organism were recorded. The maximum readable colony count using the 1 µL loop is > 100,000 CFU/mL. Colony counts were performed on blood agar plates. Species identification and colony counts were performed on CNA/MAC plates. Pathogen identification was confirmed with the VITEK 2 Compact System (bioMerieux, Durham, NC).

Enzyme-Linked Immunosorbent Assay (ELISA)

Urine levels of NGAL, IL-8, and IL-1β were analyzed according to the manufacturer's instructions, using ELISA kits from R&D Systems/Bio-Techne (Minneapolis, MN), including human Lipocalin-2 / NGAL Quantikine ELISA Kit (Catalog number SLCN20), human IL-8 / CXCL8 Quantikine ELISA Kit (Catalog number S8000C), and human IL-1β / IL-1F2 Quantikine ELISA kit (Catalog

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number SLB50). OD readings at 450nm and 540nm, respectively, were measured on an Infinite M Nano + microplate reader (TECAN, Switzerland).

Statistical analysis

Participant demographics and ICD-10-CM code breakdown were described by summary statistics (e.g., mean and standard deviation (SD) for continuous variables such as age, count, and percentage for categorical variables such as sex and ICD-10-CM code). A probit regression was fitted and plotted to describe the relationship between the density of organisms detected and the positivity (proportion of samples from symptomatic and asymptomatic cohorts with biomarker levels above the threshold) for each biomarker. Measures of biomarker clinical performance characteristics (sensitivity, specificity, positive predictive value, negative predictive value, accuracy, positive likelihood ratio, and negative likelihood ratio) were calculated using the exact method. All data analyses were performed using R 4.2.2 (https://www.r-project.org/).

To evaluate the ability of the biomarkers to differentiate UTI from non-UTI conditions, such as asymptomatic bacteriuria, we defined "Definitive UTI cases" and "Definitive non-UTI cases." Definitive UTI cases were defined using the current standard of care diagnostic criteria of symptoms/clinical presentation combined with the presence of microorganisms in the urine above a certain density threshold and being positive by *both* SUC and M-PCR ("Both Detected"). Definitive non-UTI cases were defined as asymptomatic subjects regardless of the presence of detectable microbes in the urine.

Although 100,000 CFUs/mL by SUC is typically considered diagnostically significant in the US, clinical reviews and guidelines, as well as our data (publications in preparation) suggest a microbial density threshold of 10,000 cells/mL or CFUs/mL is more clinically relevant.[34–39] Thus, we performed analyses using both microbial density thresholds of positivity: Criterion 1 (10,000 cells/mL by M-PCR or CFUs/mL by SUC) and Criterion 2 (100,000 cells/mL by M-PCR or CFUs/mL by SUC).

Criterion 1 definitions

Definitive UTI cases

Symptomatic cases where M-PCR detected bacterial counts of ≥ 10,000 or yeast counts > 0 cells/mL and SUC detected bacterial counts of ≥ 10,000 or yeast counts > 0 CFUs/mL.

Definitive Non-UTI cases

All asymptomatic cases regardless of microbe identification and density.

Criterion 2 definitions (Supplemental Data)

Definitive UTI cases

Symptomatic cases where M-PCR detected bacterial counts of ≥ 100,000 or yeast counts > 0 cells/mL and SUC detected bacterial counts of ≥ 100,000 or yeast counts > 0 CFUs/mL.

Asymptomatic Cohort

All asymptomatic cases regardless of microbe identification and density.

We previously demonstrated a 1:1 linear correlation between cells/mL reported by M-PCR and CFU/mL reported by SUC (publication in preparation). Statistical analysis between symptomatic and asymptomatic specimens used a Proportion Z-test. Statistical difference was defined as $p < 0.05$.

Biomarker Thresholds

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Thresholds previously reported in literature were used to determine positive and negative results for the biomarkers[18,19] (Table 1). Consensus biomarker positivity was defined as ≥ 2 of the 3 biomarkers measuring at or above their respective cutoff values.

Table 1. Biomarker Positivity Cutoffs.

Biomarker	Cutoff
NGAL	≥ 38.0 ng/mL
IL-8	≥ 20.6 pg/mL
IL-1β	≥ 12.4 pg/mL

3. Results

Demographics

A total of 811 unique subjects' urine specimens, 583 from the symptomatic cohort and 228 from the asymptomatic cohort were analyzed. The subjects in the symptomatic cohort trended slightly older [mean 76.6, median 76.3, range 60.0 – 99.0 years] than subjects in the asymptomatic cohort [mean 68.8, median 67.5 years, range 60.0 - 94.0]. There were also a greater proportion of females in the symptomatic cohort (68.3%, n = 398) than in the asymptomatic cohort 55.7% (n = 127). Most symptomatic subjects had an ICD-10-CM code (https://www.icd10data.com) of N39.0 for Urinary tract infection, site not specified (81.8%, n = 534) (Supplemental Table S1). The asymptomatic cohort specimens were collected from volunteers from the general population and therefore, had no ICD-10-CM codes.

Training set

There are many urine biomarkers with the potential for differentiating UTI from non-UTI conditions according to published literature[16]. A set of five candidate markers (MMP-9, NGAL, IL8, IL6, IL1-β) were evaluated using 100 consecutive cases obtained from Urology/Urogynecology practices from patients symptomatic for UTI. Cases dual-positive by M-PCR and SUC were considered "true positive" and cases dual-negative by M-PCR and SUC were considered "true negative". Sensitivity and Specificity results from this test cohort are summarized in Supplemental Table S2.

From this set of five preliminary candidates, three biomarkers with the highest sensitivity (NGAL, IL-6 and IL-1β) were selected to be validated for accuracy against the cases described above in Demographics.

Correlation relationships between biomarker percent positivity and microbial density

First, we examined the correlation between biomarker positivity and microbial density in both urine samples from patients symptomatic and asymptomatic subjects (Figure 1). Each probit regression had an $R^2 \approx 1$ and a p-value of < 0.0001 for all biomarkers in the symptomatic cohort and a p-value < 0.05 for all biomarkers in the asymptomatic cohort, indicating that the correlation between microbial density and biomarker positivity is statistically significant.

Although the symptomatic and asymptomatic cohorts both exhibited a strong positive correlation between biomarker positivity and microbial density, the biomarker proportion positivity was considerably higher across all microbial densities in symptomatic subjects relative to asymptomatic subjects (Figure 1 A – C).

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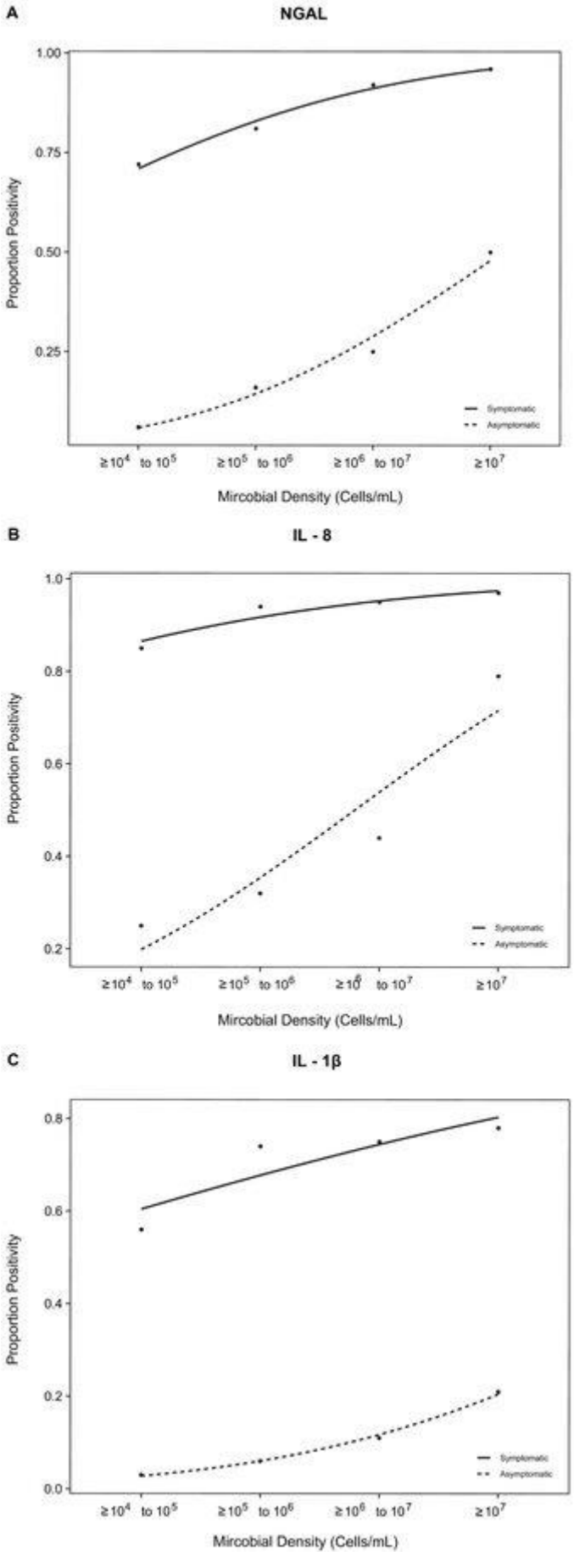

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Figure 1. Positive Correlation Between Microbial Density and Biomarker Positivity. The probit regression lines demonstrate a significant positive correlation between urine microbial density and NGAL (A), IL-8 (B), and IL-1β (C) positivity in both symptomatic (solid line), and asymptomatic (dashed line) subjects. Each data point indicates the proportion of biomarker positivity (x-axis) for urine specimens at each of the semi-quantitatively reported microbial densities ($\geq 10^4$ to 10^5, $\geq 10^5$ to 10^6, $\geq 10^6$ to 10^7, and $\geq 10^7$) presented along the y-axis. A probit regression analysis line is shown connecting the data points.

Levels of all three biomarkers (NGAL, IL, and IL-1β) are significantly lower ($p < 0.0001$) among all asymptomatic cohort specimens, regardless of the presence of detectable microorganisms (Definitive non-UTIs), compared to the symptomatic cohort specimens with microorganisms detected by both SUC and M-PCR (Definitive UTIs) (Figure 2, Table 2).

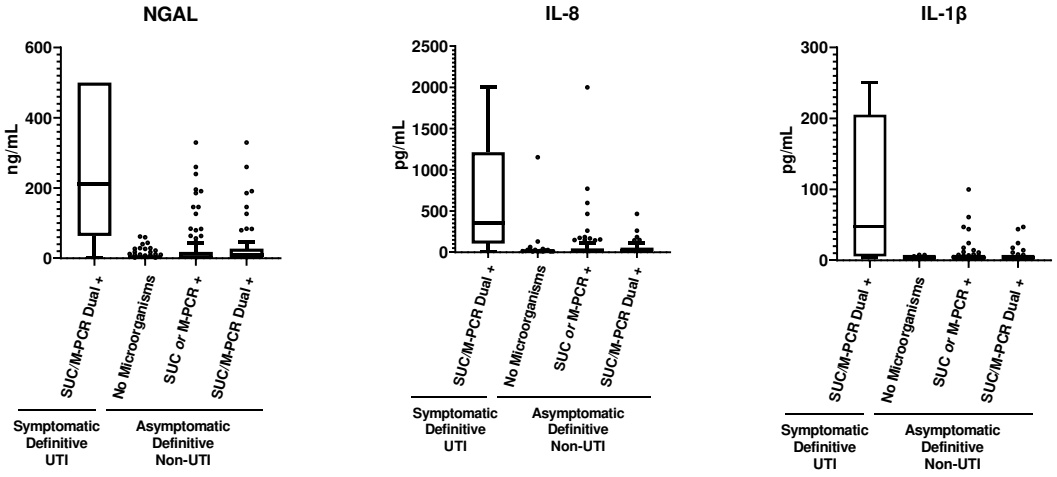

Figure 2. Biomarker Levels are Low in Definitive non-UTIs Regardless of Microbial Detection. Tukey boxplots extending to the 1st and 3rd quartiles with a line at the median indicate the distribution of biomarker (NGAL, IL-8, and IL-1β) levels among each group presented on the x-axis. Biomarker measurements are plotted along the y-axis with each point representing the measurement for a single urine specimen. Groups presented on the x-axis for comparison include "Definitive UTIs" cases (specimens from symptomatic subjects in which microorganisms are detected by both M-PCR and SUC at $\geq$ 10,000 cells/mL or CFUs/mL respectively), and "Definitive non-UTI" cases (asymptomatic cohort specimens). The "Definitive non-UTI" cases are further divided by microbial detection category: no microbes, microbes detected by SUC *or* M-PCR, and microbes detected by both SUC *and* M-PCR (Dual +).

Table 2. Descriptive Statistics of Biomarker Values for the Definitive UTI and Definitive non-UTI Cohorts. .

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NGAL	**Definitive UTI (Symptomatic)**	**Definitive Non-UTI (Asymptomatic)**		
		No Microbes	With Microbes	
	SUC and M-PCR +		SUC or M-PCR +	SUC and M-PCR +
n	351	110	118	51
Minimum	0.16	0.16	0.16	0.16
25th percentile	64.64	0.16	0.16	0.16
Median	211.09	0.16	0.16	9.51
75th percentile	500	0.16	17.65	27.04
Maximum	500	61.99	329.41	329.41
Mean	251.83	4.22	24.44	36.52
SD	193.96	11.33	56.79	70.24
SE	10.35	1.08	5.23	9.84
Lower 95% CI	231.47	2.08	14.08	16.76
Upper 95% CI	272.19	6.36	34.79	56.27
IL-8	Definitive UTI (Symptomatic)	Definitive Non-UTI (Asymptomatic)		
		No Microbes	With Microbes	
	SUC and M-PCR +		SUC or M-PCR +	SUC and M-PCR +
n	351	110	118	51
Minimum	0	0	0	0
25th percentile	109.12	0	0.38	0.15
Median	355.32	0.34	10.39	14.58
75th percentile	1206.36	3.07	46.98	52.46
Maximum	2000	1152.75	2000	466.02
Mean	693.47	15.57	61.65	46.53
SD	713.79	110.47	208.6	81.16
SE	38.1	10.53	19.2	11.36
Lower 95% CI	618.54	-5.31	23.62	23.7
Upper 95% CI	768.4	36.44	99.69	69.35
IL-1β	Definitive UTI (Symptomatic)	Definitive Non-UTI (Asymptomatic)		
		No Microbes	With Microbes	
	SUC and M-PCR +		SUC or M-PCR +	SUC and M-PCR +
n	351	110	118	51
Minimum	3.9	3.9	3.9	3.9
25th percentile	5.67	3.9	3.9	3.9
Median	47.07	3.9	3.9	3.9

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75th percentile	204.32	3.9	3.9	3.9
Maximum	250	7.68	99.84	47.09
Mean	93.4	3.98	6.56	6.72
SD	97.81	0.53	11.69	8.8
SE	5.22	0.05	1.08	1.23
Lower 95% CI	83.14	3.88	4.42	4.25
Upper 95% CI	103.67	4.08	8.69	9.2

We then compared the positivity of the individual biomarkers and combinations of biomarkers against symptomatic Definitive UTI cases using two microbial density detection thresholds: criterion 1 (10,000 cells/mL by M-PCR and CFUs/mL by SUC) and criterion 2 (100,000 cells/mL by M-PCR and CFUs/mL by SUC). All asymptomatic cases were considered Definitive non-UTI cases.

Criterion 1, Biomarker Positivity Performance Using Microbial Density Detection ≥ 10, 000 cells/mL by M-PCR and CFUs/mL by SUC.

Definitive UTI Percentage

Of 583 symptomatic subject specimens, bacterial detection ≥ 10,000 by both M-PCR (reported in cells/mL) and by SUC (reported in CFUs/mL) occurred in 351 specimens. These 351 specimens were considered Definitive UTI cases. The 228 asymptomatic subject specimens were considered Definitive non-UTI cases regardless of microbial detection. Therefore, using this criterion, the Definitive UTI percentage was 60.6%. It is worth noting that more than half of the asymptomatic group (52%, n = 119) had detectable microorganisms in the urine > 10,000 cells/mL by M-PCR or CFUs/mL by SUC (asymptomatic bacteriuria), and 22% had microbial detection at densities > 10,000 cells/mL and CFUs/mL by both SUC and M-PCR (n = 51) (Supplemental Figure S1, Supplemental Table S11).

Biomarker and microbial comparison defined by each biomarker

NGAL was positive (≥ 38 ng/mL) in 82.6% of definitive UTI cases and negative in 90.8% of Definitive non-UTI cases (Table 3A). IL-8 was positive (≥ 20.6 pg/mL) in 91.2% of Definitive UTI cases and negative in 76.8% of definitive non-UTI cases (Table 32B). IL-1β was positive (≥ 12.4 pg/mL) in 69.8% of definitive UTI cases and negative in 97.9% of Definitive non-UTI cases (Table 3C).

Table 3A. NGAL Positivity Contingency Table for Criterion 1

	Definitive UTI	**Definitive non-UTI**	*Total*
NGAL Positive	290 (50.1%)	21 (3.6%)	*311 (53.7%)*
NGAL Negative	61 (10.5%)	207 (30.2%)	*268 (46.3%)*
Total	*351 (60.6%)*	*228 (39.4%)*	579 (100%)

Table 3B. IL-8 Positivity Contingency Table for Criterion 1

	Definitive UTI	**Definitive non-UTI**	*Total*
IL-8 Positive	320 (55.3%)	53 (9.1%)	*373 (64.4%)*
IL-8 Negative	31 (5.4%)	175 (35.8%)	*206 (35.6%)*
Total	*351 (60.6%)*	*228 (39.4%)*	579 (100%)

Table 3C. IL-1β Positivity Contingency Table for Criterion 1

	Definitive UTI	**Definitive non-UTI**	*Total*
IL-1β Positive	245 (42.3%)	7 (1.2%)	*252 (43.5%)*

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IL-1β Negative	106 (18.3%)	221 (38.2%)	*327 (56.5%)*
Total	*351 (60.6%)*	*228 (39.4%)*	579 (100%)

A statistical analysis summary of the three biomarkers is listed in Table 4. IL-8 had the highest sensitivity (91.2%) while IL-1β had the highest specificity (96.9%).

Biomarker Performance Characteristics for Differentiating Definitive UTIs from Definitive non-UTIs			
≥ 10,000 Cells/mL and CFUs/mL	**NGAL*** **	**IL-8*** **	**IL-1β*** **
Sensitivity (95% CI)	82.6% (78.2%, 86.4%)	91.2% (87.7%, 93.9%)	69.8% (64.7%, 74.6%)
Specificity (95% CI)	90.8% (86.3%, 94.2%)	76.8% (70.7%, 82.1%)	96.9% (93.8%, 98.8%)
Positive Predictive Value (95% CI)	93.2% (89.9%, 95.8%)	85.8% (81.8%, 89.2%)	97.2% (94.4%, 98.9%)
Negative Predictive Value (95% CI)	77.2% (71.7%, 82.1%)	85.0% (79.3%, 89.5%)	67.6% (62.2%, 72.6%)
Accuracy (95% CI)	85.8% (82.7%, 88.6%)	85.5% (82.4%, 88.3%)	80.5% (77.0%, 83.6%)
Definitive UTI Percentage	60.6%	60.6%	60.6%
Positive Likelihood Ratio (95% CI)	8.97 (5.95, 13.52)	3.92 (3.09, 4.98)	22.74 (10.93, 47.3)
Negative Likelihood Ratio (95% CI)	0.19 (0.13, 0.29)	0.12 (0.09, 0.15)	0.31 (0.15, 0.65)

Table 4. Biomarker performance comparisons in the presence of microorganisms detected at both ≥ 10,000 cells/mL by M-PCR and ≥ 10,000 CFUs/mL by SUC.

***The Proportion Z-test comparison of sensitivity: p-value <0.0001

Biomarker and microbial comparison defined by either "Consensus" or "All three biomarkers"

"Consensus" is defined as two or more biomarkers meeting or exceeding their respective positivity thresholds (Table 1). Consensus positivity occurred in 84.0% of Definitive UTI cases and consensus negativity occurred in 91.2% of Definitive non-UTI cases (Table 5A). All three biomarkers were positive in 66.1% of Definitive UTI cases and negative in 97.4% of Definitive non-UTI cases (Table 5B).

Table 5A. Biomarker Consensus Positivity Contingency Table for Criterion 1

	Definitive UTI	**Definitive non-UTI**	*Total*
Consensus Positive	295 (50.9%)	20 (3.4%)	*315 (54.4%)*
Consensus Negative	56 (9.7%)	208 (35.9%)	*264 (45.6%)*
Total	*351 (60.6%)*	*228 (39.4%)*	579 (100%)

Table 5B. All Three Biomarkers Positivity Contingency Table for Criterion 1

	Definitive UTI	**Definitive non-UTI**	*Total*
All Three Positive	232 (40.1%)	6 (1.0%)	*238 (41.1%)*
Less than Three Positive	119 (20.6%)	222 (38.3%)	*341 (58.9%)*
Total	*351 (60.6%)*	*228 (39.4%)*	579 (100%)

A summary of the statistical analysis for the biomarker combinations is listed in Table 6. The consensus criteria of at least two biomarkers meeting or exceeding the positivity threshold performed well in terms of both sensitivity and specificity (84.9% and 91.2%, respectively). Although the combination of all three biomarkers being positive had the highest specificity (97.4%), it had lower sensitivity (66.1%).

Table 6. Biomarker "Consensus" and "All three biomarkers" performance comparisons in the presence of microorganisms detected at both ≥ 10,000 cells/mL by M-PCR and CFUs/mL by SUC.

Definitive UTI versus Definitive non-UTI

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≥ 10,000 Cells/mL and CFUs/mL	"Consensus"	"All three Biomarkers"
Sensitivity (95% CI)	84.0% (79.8%, 87.7%)	66.1% (60.9%, 71.0%)
Specificity (95% CI)	91.2% (86.8%, 94.6%)	97.4% (94.4%, 99.0%)
Positive Predictive Value (95% CI)	93.7% (90.4%, 96.1%)	97.5% (94.6%, 99.1%)
Negative Predictive Value (95% CI)	78.8% (73.4%, 83.6%)	65.1% (59.8%, 70.2%)
Accuracy (95% CI)	86.9% (83.8%, 89.5%)	78.4% (74.8%, 81.7%)
Definitive UTI Percentage	60.6%	60.6%
Positive Likelihood Ratio (95% CI)	9.58 (6.29, 14.6)	25.12 (11.36, 55.51)
Negative Likelihood Ratio (95% CI)	0.17 (0.11, 0.27)	0.35 (0.16, 0.77)

***The Proportion Z-test comparison of sensitivity: p-value <0.0001

4. Discussion

M-PCR has been previously demonstrated to be superior to SUC at detecting non-*E. coli* microorganisms and/or polymicrobial infections.[6–10] Failure to detect and correctly identify these organisms can result in many UTIs remaining undiagnosed and untreated or being sub-optimally treated based on culture results, potentially prolonging symptoms in patients and resulting in potential complications. To provide clarity on whether M-PCR-detected microorganisms are clinically and diagnostically relevant, it is useful to have biomarkers indicating active immune responses to infection, rather than solely relying on the presence of microorganisms. This study examined three potential biomarkers for UTI in Definitive UTI cases, along with Definitive non-UTI cases that included asymptomatic bacteriuria, to evaluate the usefulness of biomarkers in differentiating UTI from non-UTI patients.

The three biomarkers selected for this study, NGAL, IL-8, and IL-1β, are integral in the innate response to pathogens in the urinary tract.[31,40–44] As with any measurement of biomarkers, care in selection, interpretation, and use must be taken. For example, NGAL can be secreted both in response to infection and impaired renal function.[16,45] Additionally, many biomarkers, are impacted by intrinsic host factors such as age, sex, urinary abnormalities, genetic polymorphisms, and comorbid conditions which affect expression levels and responses to infection.[46–48]

To determine if these three biomarkers were sensitive and specific indicators for UTIs, this study measured them in both Definitive UTI cases (symptomatic cases, diagnosed in a Urology/Urogynecology specialty setting, with uropathogens identified above threshold values by both SUC and M-PCR) and in Definitive non-UTI control cases (asymptomatic based on FDA-defined criteria included in a Symptom Score Analysis)). The Definitive non-UTI cases included asymptomatic individual with detected microbes (asymptomatic bacteriuria). Previous studies had reported the presence of asymptomatic bacteriuria at lower prevalence and primarily in post-menopausal women (up to 5% of healthy pre-menopausal women, up to 25% of post-menopausal women, and up to 1% of healthy adult males)[11–15] [13–16] In this study, more than half of this control group (52%, n = 119) had had microbial detection at densities ≥ 10,000 cells/mL by either SUC *or* M-PCR, and 22% had microbial detection at densities ≥ 10,000 cells/mL by both SUC *and* M-PCR (n = 51)(Supplemental Figure S1, Supplemental Table S11). This relatively high prevalence of microorganisms in urine specimens from our asymptomatic cohort underscores the importance of practicing diagnostic stewardship such as implementing clinical testing only for the indicated population of symptomatic cases of presumed UTI and the value of having these types of biomarkers.[49]

The biomarkers exhibited excellent specificity (>75% individually and > 90% for consensus) indicating that urine specimens positive for infection-associated biomarkers are highly likely to be associated with cases of active UTIs. There was also a strong correlation between microbe density and rising positivity levels, with high positivity levels in symptomatic patients appearing even at 10,000 cells/mL and CFU/mL in symptomatic patients. Positivity levels for asymptomatic cases remained low even at 100,000 cells/mL and CFU/mL, though there was some increase observed with rising microbe density.

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The consensus biomarker positivity criteria showed very high sensitivity and specificity for UTIs, with specificity and NPV above 90%. This makes it a valuable tool to differentiate true UTI cases from asymptomatic bacteriuria and other false-positive differential diagnoses when establishing an objective "truth" for the comparison of existing and novel diagnostic test methods. This is especially important since the current "gold standard" test, SUC, is known to have significant limitations, including low sensitivity for non-*E. coli* organisms and polymicrobial infections, making it an unreliable source of diagnostic "truth."

The measurement of urinary biomarkers, individually or in combination, may also prove valuable as a supportive tool for the clinical diagnostic workup of suspected UTIs, especially in patients unable to clearly communicate their symptoms, such as pediatric patients and patients with cognitive impairment. Leukocyte esterase (LE) dipstick analysis is often employed in clinics as part of the diagnostic workup for UTI, even though the specificity is usually too low to be useful as an individual test (specificity range 9-59%, PPV is 86% and NPV is 72%.).[31,42,43,50] In contrast, the biomarker consensus criteria has a sensitivity of 84.0%, a specificity of 91.2%, a PPV of 93.7%, and an NPV of 78.8%, using a microbial density threshold of $\geq$ 10,000 cells/mL or CFUs/mL. It also has a sensitivity of 90.2%, a specificity of 91.2%, a PPV of 91.7%, and a NPV of 89.7% using a higher microbial density threshold of $\geq$ 100,000 cells/mL or CFUs/mL (Supplemental Data).

5. Conclusions

Using symptomatic subjects' urine specimens in which SUC and M-PCR results agreed on the presence of uropathogens, we demonstrated the association of NGAL, IL-8, and IL-1β, with Definitive UTI cases. A consensus criterion with $\geq$ 2 of the biomarkers meeting the positivity thresholds showed a good balance of sensitivity (90.2%), specificity (91.2%), and accuracy (90.7%). Therefore, this biomarker consensus is an excellent supportive diagnostic tool for resolving the presence of active UTI, particularly if SUC and M-PCR results disagree. These biomarkers can be used as an important supplemental tool to determine if a case is a UTI when the microbial detection and identification diagnostic test has significant limitations in sensitivity or when it is unclear whether the detected microorganism(s) can cause disease.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplemental Table S1: ICD-10-CM codes for the symptomatic cohort; Supplemental Table S2: Sensitivity and specificity of candidate biomarkers; Supplemental Table S3A: NGAL and IL-8 Positivity Contingency Table; Supplemental Table S3B: NGAL and IL-1β Positivity Contingency Table; Supplemental Table S3C: IL-8 and IL-1β Positivity Contingency Table; Supplemental Table S4: Combination of two biomarkers performance comparisons in the presence of microorganisms detected at both ≥ 104 cells/mL by M-PCR and ≥ 104 CFUs/mL by SUC; Supplemental Table S5A: NGAL Positivity Contingency Table for Criterion 2; Supplemental Table S5B: IL-8 Positivity Contingency Table for Criterion 2; Supplemental Table S5C: IL-1β Positivity Contingency Table for Criterion 2; Supplemental Table S6: Biomarker performance comparisons in the presence of microorganisms detected at both ≥ 105 cells/mL by M-PCR and ≥ 104 CFUs/mL by SUC; Supplemental Table S7A: Biomarker Consensus Positivity Contingency Table for Criterion 2; Supplemental Table S7B: All Three Biomarkers Positivity Contingency Table for Criterion 2; Supplemental Table S8: Biomarker "Consensus" and triple combination performance comparisons in the presence of microorganisms detected at both ≥ 105 cells/mL by M-PCR and CFUs/mL by SUC; Supplemental Table S9A: NGAL and IL-8 Positivity Contingency Table; Supplemental Table S9B: NGAL and IL-1β Positivity Contingency Table; Supplemental Table S9C: IL-8 and IL-1β Positivity Contingency Table; Supplemental Table S10: Biomarker combination performance comparisons in the presence of microorganisms detected at both ≥ 105 cells/mL by M-PCR and CFUs/mL by SUC; Supplemental Figure S1: Asymptomatic Microbial Density; Supplemental Table S11: Asymptomatic Subjects with Positive Microbial Detection (> 10,000 CFU or cells/mL for bacteria/bacterial groups).

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