Differentiating Asymptomatic Bacteriuria From Urinary Tract Infection in the Pediatric Neurogenic Bladder Population: NGAL As a Promising Biomarker

Sudipti Gupta, PhD,[1,2] Janae Preece, MD,[3] Andria Haynes, RN,[1] Brian Becknell, MD, PhD,[4] and Christina Ching, MD[1,2]

[1]Division of Urology, Nationwide Children's Hospital, Columbus, Ohio; [2]Center for Clinical and Translational Research, Nationwide Children's Hospital, Columbus, Ohio; [3]Department of Urology, Children's Hospital of Michigan, Detroit, Michigan; [4]Division of Nephrology, Nationwide Children's Hospital, Columbus, Ohio

Objective: To evaluate whether urinary antimicrobial peptides (AMPs) can discriminate between asymptomatic bacteriuria (ASB) and urinary tract infection (UTI) in pediatric patients with neurogenic bladder (NGB). **Design/Methods:** Bladder urine was collected from pediatric patients (≤18 years old) with NGB without augmentation cystoplasty. Patients were divided into the following groups based on symptomatology and results of urinalysis/urine culture: (a) UTI, (b) ASB, and (c) sterile. Urine AMPs β defense 1 (BD-1), neutrophil gelatinase-associated lipocalin (NGAL), cathelicidin (LL-37), hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP), and human α defensin 5 (HD-5) were compared between groups by enzyme-linked immunosorbent assays. In addition, urines from pediatric controls without NGB or UTI were also analyzed. Significance was determined using Student's *t* test for parametric or Mann-Whitney *U* test for nonparametric data. A *p* value of <.05 was considered significant. **Results:** Thirty-six patients with NGB from a spinal dysraphism were evaluated: UTI, *n* = 6; ASB, *n* = 18; sterile, *n* = 12. These groups did not differ significantly by age but did significantly differ by gender (*p* = .0139). NGAL significantly differed between UTI and ASB groups (median 38.5 ng/mg vs 15.5 ng/mg, respectively; *p* = .0197) with a sensitivity and specificity of 82.4% and 83.3%, respectively. HIP/PAP, BD-1, HD-5, LL-37, and NGAL levels were all significantly higher in sterile NGB urines compared to 17 non-NGB pediatric controls (*p* < .0001, *p* = .0020, *p* = .0035, *p* = .0006, and *p* = .0339, respectively). **Conclusion:** All five urinary AMPs evaluated were significantly elevated in NGB patients compared to controls. NGAL levels may help differentiate between UTI and ASB in pediatric NGB patients. **Key words:** *antimicrobial peptides, asymptomatic bacteriuria, myelomeningocele, NGAL, spinal dysraphism, urinary tract infection*

Spinal dysraphisms, comprised primarily of myelomeningocele (MMC), are the leading cause of neurogenic bladder (NGB) in the pediatric patient population.[1] Patients with NGB are at particularly high risk of developing urinary tract infections (UTI).[2-4] It is estimated that patients with MMC will have at least one UTI by 15 months of age.[5] This can result in significant morbidity, with MMC patients more likely to develop urosepsis compared to an age-matched group of non-MMC children with a history of a single symptomatic UTI.[3] Patients with NGB are predisposed to UTIs due to incomplete bladder emptying and neurogenic bowel dysfunction that also occur as a result of their spinal cord abnormality.[6] In addition, these patients often require frequent instrumentation of their urinary tract and/or have urinary tract reconstructions that may put them at risk for recurrent infections.[7,8]

These same risk factors for UTI predispose patients with NGB to a positive urine culture regardless of symptoms. A study of MMC children on clean intermittent catheterization (CIC) found that 70% of urine samples demonstrated growth of >10,000 colony-forming units (CFU) of bacteria.[9] The presence of urinary bacteria that does not cause symptoms is termed *asymptomatic bacteriuria* (ASB).[10] In general, a clinical UTI and ASB differ due to the presence or absence (respectively) of symptoms such as dysuria, urinary urgency

Corresponding author: Christina Ching, MD, Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205; phone: 614-722-6630; email: christina.ching@nationwidechildrens.org

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and frequency, urinary incontinence, abdominal and/or flank pain, and/or fever. Although there are specific situations in which ASB requires treatment,[11,12] the general consensus is not to treat ASB as antibiotic therapy adds little clinical benefit to the patient and instead significantly contributes to the development of antibiotic-resistant bacteria.[12-14]

Unfortunately, distinguishing clinically significant bacteriuria (infection) from ASB may not be straightforward, particularly in pediatric patients with NGB. This population may lack the normal UTI symptoms due to impaired sensation resulting from their neurologic lesion or limitations in their ability to vocalize symptoms due to their age or the presence of a cognitive delay.[15] In addition, urine samples in children with NGB often are ordered without valid clinical indication and/or in the setting of nonspecific findings, further contributing to the confusion in interpreting their urine culture results.[16-18]

Such difficulties in differentiating UTI from ASB may result in overtreatment of positive urine cultures in this population. Even without the confounding considerations of patients with NGB, one meta-analysis found that 45% of over 4,000 cases of ASB were treated inappropriately.[19] When physician residents were provided with clinical vignettes of UTI and ASB, only 34% of respondents correctly identified ASB, and even then 47% of respondents proposed treating with antibiotics.[20] Such studies identify a significant clinical deficit in the appropriate diagnosis and treatment of ASB and the necessity for better differentiators of true UTI versus ASB.

Antimicrobial peptides (AMPs) are innately expressed cationic proteins with known bactericidal and bacteriostatic activities. They represent an important part of the innate immune response to infection[21,22] and have been evaluated for their diagnostic potential in children with UTI, but they have not yet been evaluated in patients with NGB.[23] Therefore, the present study sought to identify urinary AMPs that might differentiate between those with UTI and ASB in a pediatric population of NGB. Specifically, we hypothesized that certain AMPs are differentially expressed between these patient populations and would be markers of true UTI.

Methods

Neurogenic bladder patient group

With Institutional Review Board approval, pediatric patients (≤18 years old) with a history of NGB due to spinal dysraphism were recruited, consented via legal guardian, and assented when appropriate, for urine collection at time of routine renal ultrasound (RUS) or urodynamic study (UDS). Only catheterized urine samples were collected. As is per protocol for these studies, urine was obtained by catheterization during UDS regardless of normal bladder management and during RUS only if catheterization is the normal patient routine for urination. An aliquot of urine underwent immediate urinalysis and culture by hospital laboratory core facilities. The remaining urine was processed and stored at -80°C after addition of a protease inhibitor (Assay Assure, Sierra Molecular, Incline Village, NV), as previously described.[24]

NGB patients were divided into the following groups based on symptomatology and results of urinalysis/urine culture: (a) UTI, (b) ASB, and (c) sterile. At time of urine collection, patients were asked for UTI symptoms such as fever >38°C, abdominal pain, new back pain, new or worsened incontinence, pain with catheterization or urination, and malodorous/cloudy urine. Patients were classified as UTI if they had two or more symptoms with >10 white blood cells per high-power field (HPF) on urinalysis and ≥50,000 CFU of bacteria in accordance with previously published data.[25-27] Patients were classified ASB if they had fewer than two symptoms and ≥10,000 CFU of bacteria on urine culture regardless of urinalysis.[9,27] Patients were considered sterile if they had <10,000 CFU of bacteria on urine culture and ≤10 white blood cells (WBC) per HPF. Patients who had <10,000 CFU of bacteria on culture and >10 WBC per HPF were excluded primarily because the number of patients that fit this criteria was too low for analysis ($n = 2$).

Electronic medical charts were reviewed for clinical history and imaging. Patients were excluded if they had an augmentation cystoplasty as this could serve as a confounder for bacteriuria and affect AMP expression. Patients catheterizing via appendicovesicostomy channel

were included, because it was not felt that the appendicovesicostomy represented a segment of bowel experiencing significant urinary dwell time that might affect AMP expression or significantly differed from patients catheterizing per urethra in regard to risk of bacteriuria. Samples with incomplete urinalysis or urine culture data were also excluded.

Non-neurogenic bladder patient control group

Control urine samples were obtained from a group of de-identified pediatric patients being seen in the Nephrology Clinic for issues such as hypertension and microhematuria without NGB. Samples were collected via free void or bagged specimen and evaluated by dipstick urinalysis. Patients were excluded if there was clinical concern for UTI and/or if their urinalysis was leukocyte esterase and/or nitrite positive.

Sample analysis

Enzyme-linked immunosorbent assays (ELISA) were run on cell-free supernatants for the following AMPs: β defense 1 (BD-1) (Peprotech, Rocky Hill, NJ), neutrophil gelatinase-associated lipocalin (NGAL) (Hycult Biotech, Plymouth Meeting, PA), cathelicidin (LL-37) (Hycult Biotech, Plymouth Meeting, PA), hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP) (Fisher Scientific, Pittsburgh, PA), and human α defensin 5 (HD-5) (USCN Life Sciences, Atlanta, GA), as previously described. Samples were normalized to urine creatinine (UCr) by colorimetric assay (Oxford Biomedical Research, Rochester Hills, MI) and reported as a ratio to UCr. Samples were restricted to a single freeze/thaw cycle before analysis.

Results were compared between NGB groups and between sterile NGB samples and non-NGB controls. Combined test results were only considered positive if all components were positive. D'Agostino-Pearson omnibus normality test was performed to determine whether samples were parametric. Depending on whether the samples were found to be parametric or not, intergroup comparison was performed utilizing Student's *t* test or Mann–Whitney *U* test and intergroup correlation was calculated using Pearson correlation coefficients or Spearman's rank. Multiple group comparison was performed utilizing one-way analysis of variance or Kruskal-Wallis test. Receiver operator characteristics (ROC) curves were generated when appropriate and area under the curve (AUC) was calculated. Sensitivity and specificity were used to determine the optimal threshold value. Optimal threshold values were calculated from the ROC at the angular point closest to coordinates (0.100). A p value of $<.05$ was considered significant. GraphPad Prism 7.0 (GraphPad Software Inc, La Jolla, CA) was used for these analyses.

Results

Thirty-six NGB patients were included in the study. The median age of patients included was 7.8 years (mean, 9.1; range, 0.4-18.0 years). The majority (63.9%) of patients were female. NGB groups included 12 sterile patients, 18 ASB patients, and 6 UTI patients. Demographics of the NGB patients are listed in **Table 1**. The groups did significantly differ in regard to gender but not age.

NGAL significantly differed between ASB and UTI patients (median, 15.5 ng/mg vs 38.5 ng/mg, respectively; $p = .0197$) (**Figure 1**). An ROC curve demonstrated a sensitivity and specificity of 82.4% and 83.3%, respectively, at an optimal threshold value of <31.62 ng/mg for differentiating ASB from UTI urines. For comparison, the sensitivity and specificity of leukocyte esterase on urinalysis is listed in **Table 2**. While the sensitivity of NGAL was higher than presence of trace and small leukocyte esterase, it was not higher than presence of moderate or large leukocyte esterase. NGAL had a lower specificity than any amount of leukocyte esterase. Combining presence of large leukocyte esterase and NGAL ≥31.62 ng/mg increased the specificity of distinguishing UTI from ASB to 97.2% but decreased the sensitivity to 73.3%. There was no significant difference between any of the NGB groups in regard to BD-1 ($p = .4619$), HD-5 ($p = .2557$), LL-37 ($p = .5617$), or HIP/PAP ($p = .7592$) (**Figure 1**). There was no correlation between any AMP expression and NGB patient age, sex, bladder management with CIC, or presence of appendicovesicostomy.

Table 1. Patient demographics of neurogenic bladder (NGB) patient groups

Demographics	Sterile (n = 12)	ASB (n = 18)	UTI (n = 6)	p
Median age, years (mean; range)	5.4 (6.9; 2-15.8)	9.5 (9.7; 0.4-17.6)	12.7 (10.9; 1.2-18.0)	.2074
Male, %	8 (67)	5 (28)	0 (0)	.0139
Spinal dysraphism, %				
Myelomeningocele	8 (67)	18 (100)	6 (100)	
Lipomyelomeningocele	2 (17)			
Tethered cord	1 (8)			
Caudal regression	1 (8)			
Patients with VUR, %	3 (25)	2 (11)	1 (17)	.6150
High grade (renal units)	1	1	0	
Presence of hydronephrosis, %	3 (25)	5 (28)	0 (0)	.3622
Bilateral	2	2		
SFU grade 1 (renal units)	3	6		
SFU grade 2 (renal units)	0	1		
SFU grade 3 (renal units)	2	0		
SFU grade 4 (renal units)	0	0		
Presence appendicovesicostomy, %	0 (0)	4 (22)	2 (33)	.1431
Bladder management, %				
CIC	8 (67)	17 (94)	6 (100)	.0595
Freely voiding	4 (33)	1 (6)	0 (0)	

Note: CIC = clean intermittent catheterization; VUR = vesicoureteral reflux.

BD-1, HD-5, LL-37, HIP/PAP, and NGAL levels were all significantly higher in sterile NGB samples compared to our non-NGB control cohort (p = .0020, p = .0035, p = .0006, p < .0001, and p = .0339, respectively) (**Figure 1**). Upon subgroup analysis comparing AMP expression between freely voiding children in the sterile NGB group and those in the control group, BD-1 and HIP/PAP were still significantly elevated (p = .0127 and p = .0023, respectively) (**Figure 2**). In our non-NGB control cohort, there was a significant inverse correlation between BD-1 and age (r = -0.623, p = .009, nonparametric) and NGAL and male gender (r= -0.578, p = .020, nonparametric).

Discussion

Treatment for a presumed UTI is one of the most common reasons for antibiotic prescription with significant ramifications for antibiotic overuse and rising bacterial antibiotic resistance rates.[28,29] High rates of antibiotic use in NGB patients have resulted in significant multidrug-resistant (MDR) organism carriage and infection in this particular population.[30] A recent study by Ortiz et al found that in children with UTI, those with MMC had double the rate of MDR organisms compared to those without MMC (21% vs 10%; p < .01).[3] While patients with NGB are at increased risk of UTI, they also commonly have ASB,[10] likely contributing to inappropriate antibiotic exposure.

The current gold standard for detecting UTI involves a patient having urinary symptoms and/or fever and defining features on urinalysis and urine culture.[27,31] The diagnostic accuracy of urinalysis has been questioned, however, and urine culture takes time to result (up to 48 hours) and cannot differentiate causes of bacteriuria.[31,32] Neither test can aid in the diagnosis of ASB versus UTI in patients with vague or atypical symptoms, such as in NGB patients. As a result, opportunity

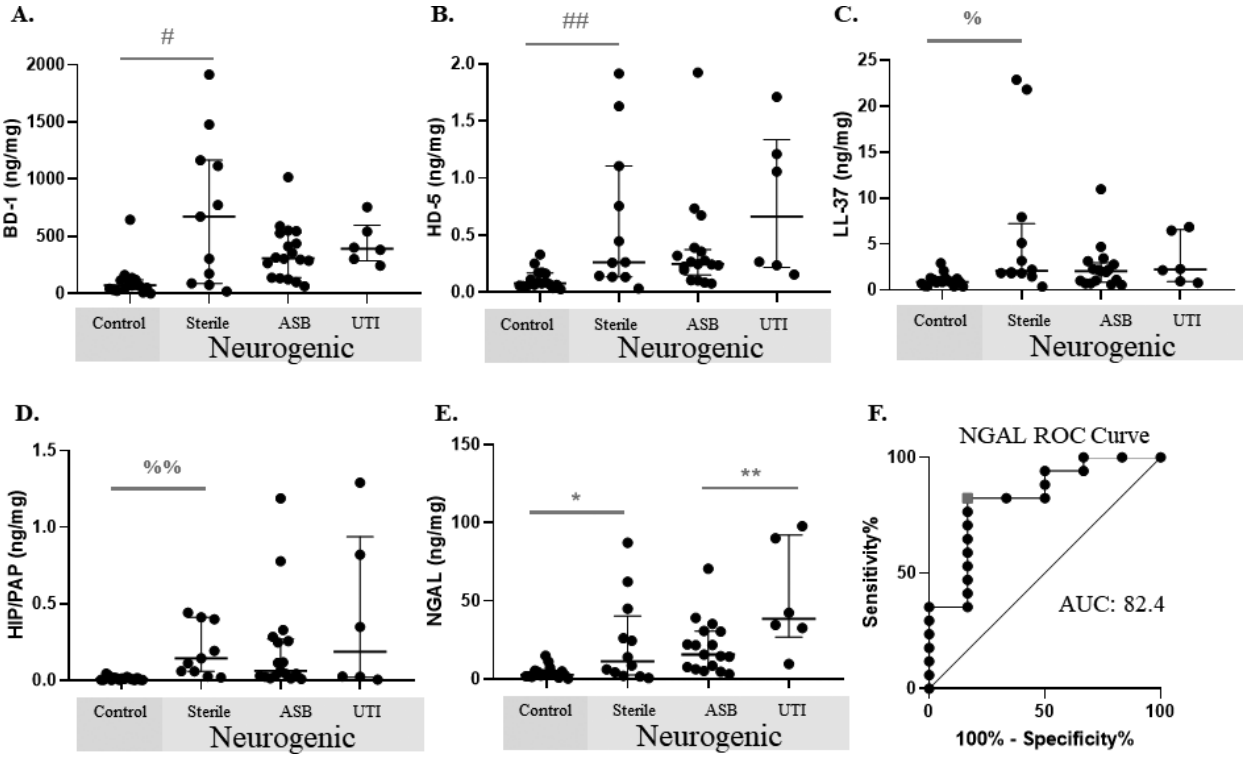

Figure 1. (A-E) Urinary antimicrobial peptide (AMP) levels (β defense 1 [BD-1], human α defensin 5 [HD-5], cathelicidin [LL-37], hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein [HIP/PAP], neutrophil gelatinase-associated lipocalin [NGAL]) in neurogenic bladder (NGB) group patients (Neurogenic) and non-NGB controls (Controls) on ELISA. # $p = .0020$. ## $p = .0035$. % $p = .0006$. %% $p < .0001$. *$p = .0339$. **$p = .0197$. (F) NGAL receiver operator characteristics (ROC) curve with area under the curve (AUC) between asymptomatic bacteriuria (ASB) and urinary tract infection (UTI) NGB patients.

Table 2. Sensitivity and specificity of leukocyte esterase (LE) in differentiating asymptomatic bacteriuria from urinary tract infection

	Sensitivity (%)	**Specificity** (%)
LE ≥ trace	44.4	100.0
LE ≥ small	66.7	100.0
LE ≥ moderate	83.3	83.3
LE ≥ large	88.9	83.3

exists for improved diagnostic techniques in this population of patients.

AMPs are a collection of innate proteins induced during infection with roles in host defense against UTI.[21,22] Their potential as biomarkers of UTI has been attractive to investigators due to their relative abundance in the urinary tract and ease of detection. Prior studies have specifically focused on identifying AMPs that are significantly evaluated in the setting of positive urine culture. In adults, human β defensin 2 was found to be significantly elevated in the urine of culture positive patients compared to those who were culture negative.[33] In children, HIP/PAP was significantly higher in urine from children with culture proven UTI versus uninfected controls.[34] Urine HD5 and human neutrophil peptides 1-3 (HNP1-3) have been found significantly elevated in both adults and children with culture proven UTI compared to those with negative cultures despite symptoms.[23,33,35]

Fewer studies have specifically evaluated the ability of AMPs to distinguish between UTI and ASB. Studies that exist focus primarily on elderly patients who, similar to NGB patients, have high rates of bacteriuria.[36] Heparin-binding protein (HBP), which has demonstrated high sensitivity

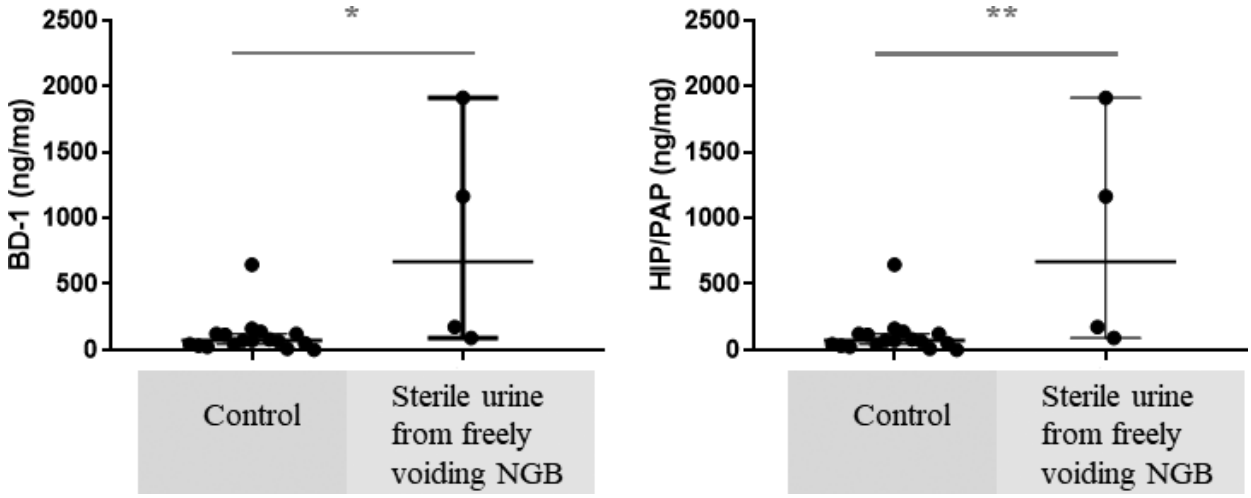

Figure 2. Urinary β defense 1 (BD-1) and hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP) ELISA levels between sterile urine from freely voiding neurogenic bladder (NGB) patients compared to non-NGB control patients. *p = .0127. **p = .0023.

and specificity in distinguishing children with febrile UTI from those without,[37] was found to identify ASB in a population of nursing home patients with high sensitivity (96%) but low specificity (33%). Urine HBP was better at differentiating those with pyelonephritis from patients with ASB, compared to distinguishing those with cystitis from ASB, but still with only a 49% positive predictive value.[38] Forster et al found NGAL significantly elevated in CIC-dependent NGB pediatric patients with UTI compared to those with sterile urine or ASB. The investigators report a particularly high negative predictive value for NGAL of 97% in ruling out UTI.[39]

Like Forster et al, we found significantly higher urinary levels of NGAL in NGB children with clinical UTI as compared to those with ASB. We were unable, however, to identify a difference in NGAL expression between patients with sterile urine and those with UTI. Differences between our study and Forster et al include our exclusion of augmentation cystoplasty patients and an overall smaller number of patients studied. In addition, Forster et al evaluated patients on whom urine culture was sent as "part of clinical care," which may include patients on whom UTI diagnosis was specifically indicated as compared to our study that obtained urine only at time of routine follow-up imaging with urine studies and symptomatology only ascertained for study purposes. As a result, it is possible that there was a difference in severity of our patients with UTI compared to those being clinically evaluated for UTI such as in other studies in which NGAL levels have been evaluated.[39-41] This may also explain the overall lower values for NGAL we observed compared to Forster et al. We did find that we could increase the specificity of NGAL to distinguish UTI from ASB when used in combination with the presence of large leukocyte esterase. Prior studies of AMPs in diagnosing UTI have similarly been able to increase their diagnostic specificity when combining test results, such as using HD5 levels in conjunction with leukocyte esterase or combining HD5 and HNP1-3 levels.[23] This would suggest the potential for improved diagnostics using a panel of tests versus any test in isolation.

An unexpected outcome of our study was the finding that all included AMPs were elevated in our sterile NGB population when compared to healthy controls. Our control samples were de-identified precluding specific comparison of patient demographics, but our findings are not completely unexpected given patients with NGB have known urothelial abnormalities.[42] Given many of the AMPs evaluate are urothelially derived,[22] our findings are further

confirmation of urothelial abnormalities that may be related to the pathology of NGB and thus deserve continuing evaluation in this patient population.

There are several limitations to our study. The number of patients evaluated in each group was small and may impact the ability of the other AMPs studied to differentiate between ASB and UTI samples. As previously noted, this could also explain why there was no difference in AMP expression between sterile NGB and UTI NGB urine samples. We also observed significant differences in gender between our NGB groups, with gender known to influence NGAL expression.[43] We did not, however, find that NGAL correlated with NGB sex in this study and thus do not feel gender impacted the effect we observed. Finally, our non-NGB control group was de-identified and thus was not matched to our NGB group for age and gender. As a result, it is hard to know exactly how to interpret our findings that AMPs in sterile NGB patients was significantly higher than in non-NGB patients.

Conclusion

Urinary NGAL may aid in differentiating between UTI and ASB in pediatric patients with NGB. Urinary AMPs may have other utilities in NGB urine even in the absence of infection that require further investigation. This suggests a broader role for AMPs in this patient population outside of acute infection.

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