Identification of Urinary CD44 and Prosaposin as Specific Biomarkers of Urinary Tract Infections in Children With Neurogenic Bladders

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ABSTRACT

PURPOSE: Distinguishing urinary tract infection (UTI) from urinary tract colonization (UTC) in children with neurogenic bladders who require clean intermittent catheterization (CIC) is challenging. Our objective was to identify urinary proteins to distinguish UTI from UTC in CIC-dependent children that have potential to serve as objective markers of UTI.

EXPERIMENTAL DESIGN: A total of 10 CIC-dependent children were included in the mass spectrometry analysis (UTI = 5, UTC = 5). Quantitative profiling of urine proteins with isobaric protein labeling was performed using tandem mass spectrometry. Candidate markers were normalized using a collective mixture of proteins from all samples. Relative quantitative abundance of proteins across all samples were compared. Proteins with >50% change in the average abundance were identified as proteins of interest, which were then measured using enzyme-linked immunosorbent assay (ELISA) in an additional 40 samples (no growth = 10, UTC = 15, UTI = 15).

RESULTS: Mass spectrometry revealed 8 differentially expressed proteins. Of these, apolipoprotein D, alpha-amylase 2B, non-secretory ribonuclease, CD44 antigen, and prosaposin were measurable by ELISA. Concentrations of both CD44 and prosaposin were significantly higher in UTI, with area under the curves (AUCs) of 0.72 and 0.78, respectively.

CONCLUSION: Urinary CD44 and prosaposin are candidate markers that may assist with the diagnosis of UTI in CIC-dependent children.

KEYWORDS: neurogenic bladder, urinary tract infection, biomarkers

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Introduction

Children with neurogenic bladders frequently have positive urine cultures. However, determining when a positive urine culture represents at urinary tract infection (UTI) as opposed to urinary tract colonization (UTC) can be difficult.[1] Indeed, as more than half of urine cultures from children with neurogenic bladders are positive in the absence of symptoms,[2] a positive urine culture is not diagnostic of a UTI in these children. Furthermore, there is no widely accepted definition of UTI in children with neurogenic bladders,[3] which contributes to the significant variability in the way that clinicians both diagnose and treat suspected UTIs in this population.[3-5] As there is no defintion of UTI in these children, clinicians rely on their assessment of symptoms to diagnose a UTI. However, while the combination of some symptoms is generally considered diagnostic of a UTI, such as fever, abdominal pain, and vomiting in the absence of another source, other symptoms, such as increased incontinence or discomfort with catheterization, are less clear. Indeed, in a series of adults with neurogenic bladder, symptoms were found to be non-specific for UTI.[6]

There is an absence of accurate biomarkers that have good predictive accuracy for UTI in children with neurogenic bladders. While specific urinalysis (UA) parameters have good sensitivity and specificity for the diagnosis of UTI in the general pediatrics population,[7,8] UA results are less useful in diagnosing UTIs in patients with neurogenic bladders.[9] This is likely related to the fact that components of the UA are either markers of inflammation (ie, pyuria, leukocyte esterase) or indicative of the presence of an organism (eg, nitrites) and are not specific for UTI in this population. A more accurate and objective method to diagnose UTIs in this population is needed. Indeed, there is a rapidly rising trend in antibiotic-resistant organisms cultured from urine in children with neurogenic bladders.[10] Although this is multifactorial, one reason behind trend is the large number of antibiotics that these children receive. Identification of a biomarker that is both sensitive and specific for UTIs in this urologically complex patient population would improve the ability to appropriately prescribe antibiotics.

The objective of this pilot study was to identify novel markers of UTI in children with neurogenic bladders. An ideal marker of UTI in children with neurogenic bladders will be specific for infection, rather than generalized inflammation. However, a

targeted approach to identifying novel markers of UTI is limited by the small number of candidate proteins. Therefore, we performed a pilot study consistent of an unbiased analysis of the urine of children with neurogenic bladder who have either UTI or UTC to identify novel candidate markers of UTI.

Material and Methods

Patients

This pilot study is a secondary analysis of patients, and residual urine samples, enrolled in other work designed to determine the predictive accuracy of a single protein in diagnosing UTI.[11] All patients with neurogenic bladders who were actively performing clean intermittent catheterization (CIC), were followed at our center, and had a urine culture sent as part of clinical care from August 1, 2015 through November 1, 2016 were eligible to participate in the original study. This included patients presenting for urodynamics, who have routine urine cultures sent at our institution, and those presenting for evaluation of potential UTI. Patients who met inclusion criteria (ie, presence of a neurogenic bladder, actively performing CIC) were initially identified in the electronic health record (EPIC™, Verona, WI) using the International Classification of Diseases, 9th revision codes for neurogenic bladder or spina bifida. Following initial identification, the research team then performed a manual review of each patient's medical record to confirm that inclusion criteria were met. All eligible patients were then flagged in VigiLanz™ (VigiLanz Corporation, Minneapolis, MN), a real-time lab monitoring software that sent an automatic notification via e-mail to the research team when a flagged patient had a urine sample sent to the clinical laboratory. The residual urine was then obtained for use in this work. Patients were excluded for the following reasons: if they met the KDIGO criteria for acute kidney injury,[12] had signs or symptoms of sepsis[13] at the time of presentation, or if they were dialysis-dependent. The study was approved by the Institutional Review Board.

Patients included in this study were drawn from the original cohort, who were identified as described above. Patients with UTI were chosen to be included in the mass spectrometry phase of this study if they had sufficient residual urine for this analysis, met our criteria for UTI (described below), and were confirmed to have unequivocal UTI on manual chart review. Five patients with UTI were randomly chosen from those who met these criteria. All patients with UTC were eligible to be included if they had sufficient urine for this analysis. Of the patients with UTC who met these criteria, those closest in age to the patients with UTI were selected. The charts of these 5 patients with UTC were then reviewed to ensure that there were no symptoms concerning for UTI.

Samples

Urine samples were obtained for use in this work within 10 hours of initial collection, during which time they were kept in a refrigerator. Samples were then centrifuged at 12 000 r/min for 10 minutes at a temperature of 4°Celsius. No additives were used at any point during sample processing. The supernatant was then aliquoted into 2-mL microcentrifuge tubes. As the volume of urine available per patient varied, the number of aliquots saved varied, from 1 to 9 aliquots per patient. Samples were each labeled with a unique stick-on barcode and frozen immediately at −80°C. All samples were processed and stored following the same protocol. Samples had not undergone any freeze-thaw cycles at the time of analysis.

Definitions

Patients were included in the UTI group if they met all 3 of the following criteria: (1) growth of greater than or equal to 50 000 colony forming units per milliliter (cfu/mL) of a known uropathogen from urine culture, (2) more than 10 urinary white blood cells/high-powered field in a spun urine specimen, and (3) 2 or more of the following signs and symptoms: fever greater than 38°C, abdominal pain, new back pain, new or worsened incontinence, pain with catheterization, or malodorous or cloudy urine. This is an adapted definition of UTI from that originally published by Madden-Fuentes and McNamara,[3] with the modification of changing the colony-count for UTI from 100 000 to 50 000 cfu/mL in accordance with the American Academy of Pediatrics guidelines for diagnosis of UTI.[14] Patients with a positive urine culture who did not meet these criteria were included in the UTC group. The no growth group includes all patients with negative urine cultures. We excluded all patients whose urine cultures grew either fungi or a mixture of unidentified organisms.

Candidate marker identification

Five patients with unequivocal UTIs and 5 patients with UTC with positive urine cultures and no clinical symptoms were included in the initial identification of candidate markers. Sample volumes were reduced with a 3-kDa filter into 1× Laemmli buffer. The protein concentrations were measured using the Pierce 660 nm Protein Assay kit (Thermo Scientific) as this assay is both compatible with higher concentrations of detergents and maintains a greater linear range than standard Coomassie dye-binding assays (eg, Bradford assay). The samples were then run in a 1D, 1.5 cm 4% to 12% Bis-Tris gel using MOPS (3-(morpholino)propanesulfonic acid) running buffer. The regions of each lane between the well and the dye front were excised for trypsin digestion. The resulting peptides were extracted and the control lanes pooled together. The recovery of the digested peptides in each sample was determined via Nanodrop analysis. The resulting peptides from each sample were tagged with the indicated iTRAQ reagent using the vendor instructions. Samples for each iTRAQ set were mixed 1:1:1:1 based on the peptide Nanodrop reading and loaded onto a Sciex 5600+ nanoflow LC-mass spectrometry system, as

Table 1. Patient demographics.

	PATIENTS IN MASS SPECTROMETRY GROUP		PATIENTS IN ASSAY GROUP		
	UTC (n = 5)	UTI (n = 5)	No growth (n = 10)	UTC (n = 15)	UTI (n = 15)
Mean age (years)	9.5 (7.5)	7.1 (7.3)	11.8 (7.4)	9.5 (5.4)	10.5 (6.6)
Male	3 (60)	1 (20)	9 (90)	6 (40) *	8 (53)
White	4 (80)	5 (100)	9 (90)	12 (80)	12 (80)
Myelomeningocele	2 (40)	3 (60)	4 (40)	6 (40)	5 (33)
Anorectal malformation	2 (40)	1 (20)	2 (20)	4 (27)	4 (27)
Tethered cord	0 (0)	0 (0)	1 (10)	2 (13)	1 (7)
Mitrofanoff	1 (20)	1 (20)	2 (20)	5 (33)	5 (33)
Bladder augmentation	0 (0)	1 (20)	0 (0)	2 (13)	1 (7)

Abbreviations: UTC, urinary tract colonization; UTI, urinary tract infection.
Data presented as n (%) unless otherwise specified.
*$P < .05$ compared with no growth.

previously described.[15] Each 4-plex set contained one common control sample made from an equal protein mixture of all samples in the cohort. Measuring protein ratios against a single common control then allows for cross comparison of the relative protein levels among all the samples in the cohort. ProteinPilot software (Sciex) was used to identify the proteins and determine the relative quantitation from each run. A merged search of all runs identified the full scope of proteins detected across all groups. The ProteinPilot data were then processed through Protein Alignment Template software from Sciex. Proteins with a significant fold-change between the UTI and UTC groups (those at >50% change in the average abundance across groups) were identified as proteins of interest. Operators were blinded to sample group until the analysis stage, when unblinding was necessary to interpret the results.

Protein validation

The remaining samples in the initial cohort, excluding those used in the protein identification stage, were used to measure the proteins of interest, including non-secretory ribonuclease (RNase2) (MyBiosource LLC, San Diego, CA), prosaposin, CD44, and Apolipoprotein D (Lifespan Biosciences, Seattle, WA). Commercially available assay kits were used to measure the candidate proteins in the remaining samples. Urine creatinine, measured by nephelometry, was used for standardization. For values below the limit of detection (LOD) for each of the proteins, we imputed a standardized value of $(\sqrt{(2)} \times \mathrm{LOD})/2$.

Statistical analysis

Categorical variables were compared by chi-square or Fisher exact test, as appropriate. Normally distributed continuous variables were compared with either Student *t* test or analysis of variance (ANOVA) with post hoc Tukey as appropriate. Non-normally distributed variables, such as the biomarker values, were compared with the Kruskal-Wallis test. Receiver operating characteristic (ROC) curves were generated for the biomarkers of interest and used to select cut-off thresholds to the outcome of UTI, and associated sensitivity and specificity. All statistical analysis was done using R (version 3.2.5)[16] with package pROC.[17] All data from this work is available by request from the authors.

Results

Five patients with UTI and 5 patients with UTC were included in the mass spectrometry analysis, while 10 patients with no growth, 15 with UTC, and 15 with UTI were included in the assay analysis. There were no differences in age, sex, race, etiology of neurogenic bladder, presence of Mitrofanoff, or bladder augmentation between those with UTC and UTI in the mass spectrometry analysis. In the assay analysis, there was a higher proportion of males in the no growth group compared with the UTC group. There were no other differences between groups (Table 1).

More than 200 proteins were identified using mass spectrometry. From this protein set, 100 proteins were consistently detected and quantified in at least 3 of the 5 patients from both groups and were subjected to *t* test analysis to establish the significance of the change (Supplemental Figure S1). There were a few examples of proteins that were detected in 3 or more of one group and less than 3 in the other group; however, in each case, the levels of the protein detected in the group of 3 were well outside of a significance *P* value of .05. After removing contaminating hemoglobin and several immunoglobulin proteins likely linked to minor blood contamination and/or inflammatory response, 8 proteins were identified with $P < .05$ as proteins of interest based on at least a 0.5-fold increase or

Protein of Interest	Mean log UTC	p-value for UTC	Mean log UTI	p-value for UTI	Log ratio UTI/UTC
Haptoglobin	-0.06	0.60	0.17	0.02	0.23
Apolipoprotein D	0.20	0.01	-0.001	0.99	-0.20
a-amylase 2B	0.22	0.02	0.004	0.93	-0.22
Inter-a-trypsin inhibitor heavy chain H4	0.11	0.12	-0.11	0.04	-0.23
RNase2	0.22	0.08	-0.12	0.04	-0.34
CD44 antigen	0.22	<0.01	-0.09	0.35	-0.31
LFABP	-0.46	0.01	-0.12	0.60	0.34
Prosaposin	0.38	<0.01	0.13	0.21	-0.26

Red; increase in relative abundance of protein: Blue; decrease in relative abundance of protein: Green; statistical significance.

Figure 1. Heat map of the eight proteins that are differentially regulated in either UTI or UTC in the mass spectrometry analysis. Red represents upregulation and blue represents downregulation. More intense color represents more up or down regulated proteins.
LFABP indicates liver fatty acid–binding protein; RNase2, non-secretory ribonuclease; UTC, urinary tract colonization; UTI, urinary tract infection.

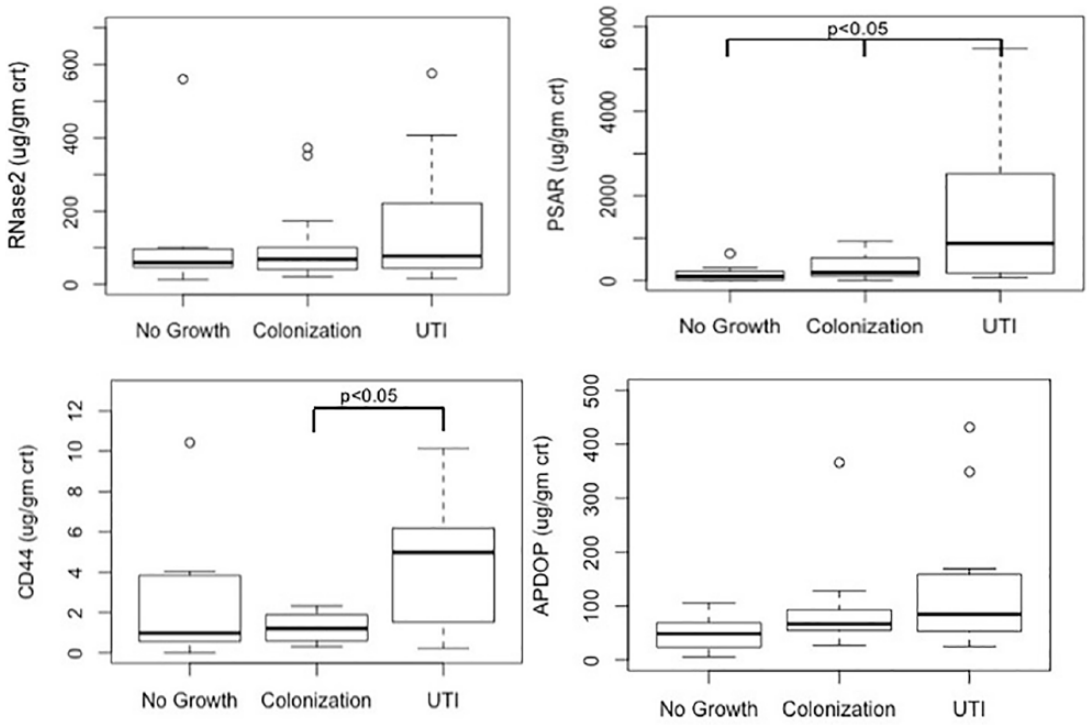

Figure 2. Box plot of normalized biomarker values as measured by assay. CD44 concentrations are significantly higher in patients with UTI compared with UTC, and prosaposin concentrations are significantly different between all groups. RNase2 and APDOP concentrations were not significantly different between groups.
APDOP indicates apolipoprotein D; PSAR, prosaposin; RNase2, non-secretory ribonuclease; UTC, urinary tract colonization; UTI, urinary tract infection.

decrease in the 2 test groups. These included haptoglobin, apolipoprotein D, alpha-amylase 2B, inter-a-trypsin inhibitor heavy chain H4, RNase2, CD44 antigen, liver fatty acid–binding protein, and prosaposin (Figure 1).

Of the 8 proteins of interest identified by mass spectrometry, 5 were measured by assay in an additional set of samples: apolipoprotein D, alpha-amylase 2B, RNase2, CD44, and prosaposin. Alpha-amylase 2B was not able to be analyzed as all samples had a value below the LOD. There were no significant differences between either normalized or non-normalized concentrations of either RNase2 or apolipoprotein D (Figure 2). Normalized prosaposin concentrations were significantly higher in UTI compared with no growth (P=.003) and UTC (P=.05). Normalized CD44 was significantly higher in UTI compared with UTC (P=.02), although there was no difference in normalized CD44 concentrations between UTI and no growth (Figure 2). Non-normalized prosaposin was significantly higher in UTI compared with both no growth (P=.02) and UTC (P=.05).

Table 2. AUCs, thresholds, sensitivity, and specificity of RNase 2, apolipoprotein 2, prosaposin, and CD44 for diagnosis of UTI.

	AUC	THRESHOLD (MG/G CREATININE)	SENSITIVITY	SPECIFICITY
RNase 2	0.55 (0.35-0.76)	125	0.40 (0.07-0.67)	0.84 (0.24-0.96)
Apolipoprotein D	0.62 (0.42-0.81)	81	0.53 (0.27-0.80)	0.76 (0.28-1.00)
Prosaposin	0.78 (0.60-0.95)	716	0.62 (0.39-0.92)	0.96 (0.52-1.00)
CD44	0.72 (0.52-0.92)	4	0.60 (0.20-0.87)	0.96 (0.30-1.00)

Abbreviations: AUC, area under the curve; UTI, urinary tract infection.

There was no difference in non-normalized CD44 between groups.

The area under the curves (AUCs) for both RNase2 and apolipoprotein D show that they are poor candidates for markers for UTI (RNase2: 0.55 [0.35-0.76]; apolipoprotein D: 0.62 [0.42-0.81]). However, both CD44 and prosaposin displayed AUCs that suggest that they have moderate utility in distinguishing UTC from UTI (CD44: 0.72 [0.52-0.92]; prosaposin: 0.78 [0.60-0.95]). At the identified cut-offs, both CD44 and prosaposin have poor sensitivity, but good specificity, for the outcome of UTI (Table 2).

Discussion

Here, we used mass spectrometry to identify novel urinary proteins that are candidate markers for UTI in children with neurogenic bladder. Of the 5 differentially expressed proteins that were measured in the urine samples, 2—CD44 and prosaposin—show potential to serve as markers to aid in the differentiation between UTI and UTC. Three of the proteins identified as proteins of interest were not included in the validation phase due to limitations in the amount of urine available. Liver fatty acid–binding protein was identified as a protein of interest, but not included as it is a known marker of tubular injury,[18] and therefore had the potential to confound our results. Similarly, we chose not to further investigate inter-a-trypsin inhibitor heavy chain H4 as it is also elevated in patients with decreased renal function.[19] Urine haptoglobin has been reported to be elevated in multiple conditions, including lupus nephritis,[20] diabetic nephropathy,[21] and urothelial cancer.[22] Given the presence of haptoglobin in multiple other conditions, it likely would not be a specific marker in UTI and therefore was not chosen as a protein of interest.

CD44, a type I transmembrane glycoprotein whose main ligand is hyaluronic acid,[23] is constitutively expressed in almost all tissues.[24] While initially believed to play a role in lymphocyte homing,[25] it has since been found to have multiple functions, including lymphocyte activation, cell-to-cell adhesion, hyaluron metabolism, embryonic development, cellular adhesion and migration, lymphocyte and monocyte proliferation and activation.[26-30] In this capacity, CD44 serves a role in the innate immune system, through both defense against pathogens[31] and stimulation of antimicrobial peptide production.[32] In addition, CD44 may also play a role in the pathogenesis of certain infections, including UTI. Indeed, *Escherichia coli* binds to CD44 in a hyaluronic acid–mediated mechanism and through this interaction invades urothelial cells.[33] Furthermore, CD44 knockout mice demonstrate increased bacterial clearance following transurethral inoculation compared with wild type, with no difference noted in neutrophil accumulation or activity.[33] However, the increase seen in urinary CD44 in this work is likely related to the fact that CD44 is constitutively expressed on the urothelial cells.[33] As one of the responses of the bladder to infection is exfoliation of superficial urothelial cells,[34] it is likely that the increased concentration of urinary CD44 seen in patients with UTI in this work is a result of the exfoliated urothelial cells.

Prosaposin, a parent protein for the saposins, is a glycoprotein. Together with the saposins, it has multiple functions in the body including glycosphingolipid transport, facilitation of sphingolipid hydrolysis,[35] activation of glycosphingolipid synthesis,[36] as well as development and homeostasis of the male reproductive organs.[37] Prosaposin also exists as a secretory protein and has been described in milk, cerebrospinal fluid, and seminal fluid.[35] While the majority of the function of prosaposin, and the associated saposins, occurs within the context of the neurological system, the saposins also play a role within the immune system. Indeed, the saposins play a critical role in activation of invariate natural killer T cells through assisting access of CD1d cells to microbial molecules.[38] Furthermore, CD1d-mediated natural killer T-cell activation has been implicated in the host response to *E coli* UTIs.[39] Taken together with our data shown here, this suggests that prosaposin may facilitate the innate immune response to UTIs, thus partially explaining its role as a potential marker of UTI.

Two proteins, apolipoprotein D and RNase2, studied within the confirmation arm of our work did not show differences between UTC and UTI. Apolipoprotein D has many functions, but has been most widely studied within the context of lipid metabolism and neurological disease.[40] However, it may also play a role in modulation of inflammation in the setting of infection.[41] Apolipoprotein D is a lipocalin[40] and as such may bind ligands with implications in the innate immune system. However, the known ligands for apolipoprotein D consist mainly of lipids within high-density lipoproteins or plasma membranes.[42] Accordingly, most of the known functions of apolipoprotein D involve the neurological system.[42] There is scant literature on the utility of this protein in the setting of infection. It is possible that apolipoprotein D is present in the urine of patients with neurogenic bladder due to its role within the neurological system and underlying etiology of neurogenic bladder.

RNase2, also known as eosinophil-derived neurotoxin, is a protein found in granules within eosinophils. While initially described in the urine, RNase2 was later found to have the same structure as eosinophil-derived neurotoxin,[43] a protein that is upregulated by Th2-driven allergic inflammation or parasitic infection.[43] Furthermore, RNase2 has anti-viral activity, specifically against single-stranded RNA viruses,[44] and can induce the migration of dendritic cells, but not neutrophils, monocytes, or T-cells.[45] While urinary RNase2 has been described in allergic[46] and parasitic diseases,[47] it has not been described in the context of bacterial infections. The presence of this protein in the urine of our patients may be related to dendritic cell migration. While there is no statistical significance in this protein, there were several patients in the UTI group with elevated levels of RNase2 although no differences in the median concentration. Further work is needed to understand the role, if one exists, of RNase2 in UTIs.

Both prosaposin and CD44 have low sensitivities, but good specificities, for UTI. This suggests that the greatest utility of these markers is not in identifying children who may have an infection; rather, they may serve to identify children who likely do not have an infection. There is a need for specific markers of UTI in this population as the currently used markers of UTI (ie, pyuria, leukocyte esterase, and nitrites) have greater sensitivity than specificity.[9] A specific marker will have greater utility in this population to identify children who can be safely monitored off of antibiotic therapy. As the rate of antibiotic-resistant organisms is increasing at a significantly faster rate in children with neurogenic bladder compared with those without neurogenic bladder,[48] there is a great need to identify children who do not require antibiotics. Both prosaposin and CD44 may serve as specific markers for UTI in children with neurogenic bladders, but future work is needed in larger cohorts to determine an appropriate cut-off and associated predictive accuracy.

We report conflicting results between the identification and validation arms of this work. While we report significant elevations in both prosaposin and CD44 in UTI compared with UTC, results from mass spectrometry suggest that these proteins are increased in UTC when compared with UTI. One possible explanation is the different methods of normalization. Samples were loaded into the mass spectrometer based on total protein concentration. While the literature is mixed on the presence of proteinuria in cystitis, there is data to suggest the presence of tubular proteinuria in pyelonephritis.[49] It is likely that most patients in the identification arm had pyelonephritis, as they were chosen based on their unequivocal presentation for UTI. Therefore, these patients likely had a higher degree of proteinuria, leading to a smaller amount of urine used for the analysis. Conversely, urine was loaded into the confirmatory assays based on volume, not concentration. Therefore, it is possible that tubular proteinuria in our patients with UTI led to these conflicting results. Indeed, our enzyme-linked immunosorbent assay (ELISA) results do display an increase in both prosaposin and CD44 in UTC when compared with children with no growth on urine cultures; this increase was further magnified in the UTI group with added specificity, suggesting their utility as specific markers of UTI in children with neurogenic bladders.

One of the main limitations in this work is the lack of a standardized definition of UTI in patients with neurogenic bladders. There is significant variation in the clinical management of bacteriuria in this patient population, as well as the definition of UTI used in research.[3] Therefore, we have chosen a definition that appears in the literature for use in research, but realize that this is still an imperfect definition. To mitigate some of the potential for misclassification bias, we performed an additional review of the patients' charts to ensure that there was no ambiguity around the classification of either infection or colonization. However, the combination of the definition that we used plus clinical acumen is still imperfect, and thus, the lack of a validated way to differentiate UTI from colonization remains a limitation of this work. Other limitations include the limited number of patients used in both arms of this work, and the inability to better match patients with UTI and UTC in the mass spectrometry arm. Furthermore, we did not perform stability studies on the proteins of interest and are unable to determine whether or not prolonged storage could affect these levels. However, as all urine samples used in this work were stored for the same amount of time, we anticipate this likely did not affect our results of the differentially upregulated proteins. A final limitation is the use of urine creatinine for normalization. We used urine creatinine to standardize the proteins for urine concentration, which may not be the ideal method of standardization in this cohort. Given the differences in body habitus and anthropomorphic measurements in this population, the production of serum creatinine is variable,[50] which directly impacts the utility of urine creatinine as a method of normalization. Therefore, the use of urine creatinine may be confounding our results.

Conclusions

In this pilot work, we show that urinary prosaposin and urinary CD44 concentrations are increased in children with neurogenic bladders who have UTI compared with those with UTC and that prosaposin is elevated in children with UTI compared with negative cultures. Urinary prosaposin and CD44 may help identify children with neurogenic bladders at the point of care who do not require antibiotics for treatment of UTI, although further work is needed to validate these results.

Author Contributions

CSF conceptualized and designed the study, analysed the assay data, drafted the initial version of the manuscript, and revised the manuscript. WD and KG assisted with study design, performed the mass spectrometry and analyzed the associated data, assisted with data interpretation, and critically reviewed the manuscript. MB assisted with study design, performed the ELISAs, assisted with data interpretation, and critically reviewed the manuscript. PD assisted with study design, data analysis and interpretation, and critically revised the manuscript. All authors approved the final manuscript.

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