



A Novel Proteomics Approach to Identify Serum and Urinary Biomarkers and Pathways that Associate with Lower Urinary Tract Symptoms in Men and Women: Pilot Results of the Symptoms of Lower Urinary Tract Dysfunction Research Network (LURN) Study

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OBJECTIVES

To assess the feasibility of a novel proteomics approach to identify biomarkers associated with lower urinary tract symptoms (LUTS) within serum and urine, because many clinical factors contribute to LUTS in men and women. These factors confound clinicians' abilities to reliably evaluate and treat LUTS. Previous studies identified candidate LUTS biomarkers, but none are clinically utilized.

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METHODS

Eighteen male and 18 female symptoms of lower urinary tract dysfunction research network (LURN) observational cohort study participants with LUTS (measured on the LUTS Tool questionnaire) were randomly selected. Twelve male and 12 female controls with minimal or no LUTS were recruited and matched for clinico-demographic characteristics. The SomaScan Assay (SomaLogic) was used to measure the abundance of 1305 proteins contained within urine and serum. Statistical analyses were performed to evaluate reproducibility of assays, compare protein abundances, and estimate effect size.

RESULTS

SomaScan assay results were more reproducible in serum than in urine. Within serum, there were many more differentially abundant proteins between cases and controls in males than in females. An enrichment/pathway analysis of the affected proteins in male and female subjects demonstrated that the enriched Gene Ontology processes were related to prostate morphogenesis in men and growth and inflammation in women.

CONCLUSION

The pilot study results support that the etiology and pathophysiologic mechanisms underlying LUTS may be sex-specific. While further studies involving larger numbers of subjects are warranted, our results support the feasibility of a novel proteomic approach to identify biomarkers for diagnostic classification of LUTS. UROLOGY 129: 35–42, 2019. © 2019 Elsevier Inc.

Lower urinary tract symptoms (LUTS) are common among men and women and have a significant impact on quality of life.^{1,2} They may be managed with either medical or surgical therapies. However, data indicate that many patients who experience LUTS are refractory to these therapies. For example, it has been shown that only 13%-33% of patients remain compliant with their medical therapies in a 12-month time period.^{3,4}

One potential reason for treatment-refractory LUTS may be due to their multifactorial etiology. For example, many pathologic conditions have been associated with LUTS, including but not limited to metabolic syndrome, inflammation, benign prostatic hyperplasia (BPH) in men, pelvic organ prolapse in women, hormone dysregulation, sleep-disordered breathing, and excessive fluid intake.⁵⁻⁷ The multitude of processes that contribute to LUTS have confounded clinicians' abilities to reliably evaluate and quantify their presence. While previous studies have identified candidate biomarkers designed to accomplish this purpose,⁸⁻¹³ no individual biomarker exists for diagnostic, prognostic, or predictive use in the clinical setting. This is likely related to the fact that most biomarker candidates were identified using a targeted approach evaluating only a limited panel of proteins, few have routinely been validated, and the associations between the biomarkers were only applicable to a subset of patients with specific LUTS (eg, overactive bladder symptoms). Therefore, novel biomarkers that can reliably distinguish LUTS are greatly needed.

The lack of diagnostic biomarkers may be related to several factors, including a lack of: (1) understanding of the proper media (eg, plasma, urine, etc.) for reliable identification of clinically useful biomarkers; (2) knowledge of the relevant biologic pathways affected in LUTS; and (3) understanding of multiple subtypes of LUTS (eg, overactive bladder, benign prostatic hyperplasia, etc.) that can be represented by different biomarkers. The SomaScan assay is a commercially-available test that measures a relatively large panel of biomarkers representative of many

different cellular pathways; it therefore represents a high throughput and relatively unbiased platform. Although the SomaScan assay has been utilized in multiple biomarker studies of common complex diseases as a means of identifying novel proteins,^{14,15} it has never been applied toward patients with LUTS.

The symptoms of lower urinary tract dysfunction research network (LURN) were established in 2012 to identify clinically important subtypes of patients with LUTS and improve the measurement of LUTS.¹⁶ The main goals of the LURN Biomarker Pilot project were: evaluate SomaScan reproducibility by assessing blind duplicates of the samples; estimate the number and effect size of the differentially abundant proteins in LUTS patients versus controls; determine the proper media for measuring proteins related to LUTS (plasma, urine, or both); and determine the feasibility of a larger-scale biomarker project of LUTS.

METHODS

We previously described the inclusion and exclusion criteria for the LURN Observational Cohort Study that enrolled 545 women and 519 men.¹⁷ Briefly, cohort study participants were ≥18 years, reported having urinary symptoms based on the LUTS Tool¹⁸ (Version 1.0. Copyright 2007 by Pfizer, Inc. used with permission) and did not have chronic prostatitis/chronic pelvic pain syndrome, interstitial cystitis/bladder pain syndrome, pelvic malignancy, active urinary tract infection, recent urologic procedures, and/or neurologic disorders. Eighteen male and 18 female participants that described having at least one urinary symptom "often" or "almost always" on the LUTS Tool were randomly selected from the enrolled subjects (cases), such that 3 males and 3 females from each of the 6 participating LURN institutions were included. This inclusion criterion allowed for participant selection from a pool of 98% of male and 95% of female LURN Observational Cohort participants, as the majority reported at least one moderate to severe symptom, and various LUTS were uniformly distributed across the institutions, comprising a representative pool for random selection. Importantly,

random selection of the patients from the above pool was performed with investigators blind to symptoms and demographics. It has been well-recognized that the presence and severity of LUTS is associated with comorbidities, including diabetes, increasing age, and increased body mass index (BMI). We did not want to identify a panel of proteins associated solely with these comorbidities. To prevent this, the cases' demographics (ie, age, race), BMI, and diabetic status were documented and used to recruit frequency matched controls. Twelve female and 12 male controls with minimal or no LUTS (none of LUTS Tool symptoms occurred more than "rarely") were recruited and matched for demographics, BMI, and diabetic status. Exclusion criteria for controls were the same as those for LURN cases. Therefore, controls were not necessarily healthy but did not have LUTS. Histograms illustrating the frequency matching of the controls and cases are presented in [Supplemental Figures 1 and 2](#).

From the abovementioned cases and matched controls, one case and one control of each sex was randomly selected for a total of 4 participants used for additional SomaScan reproducibility testing. For this pilot study, to test reproducibility, duplicate samples of each type (urine and plasma) were obtained from these 4 randomly selected patients and were processed by the SomaScan. Of the other 56 patients in this pilot study, only one sample of each type was processed. Therefore, a total of 72 plasma samples and 72 urine samples were analyzed with SomaScan at SomaLogic, Inc.

Plasma and Urine Collection

Biospecimens were previously obtained from all subjects enrolled in the LURN Observational Cohort; specimen collection protocols have been described.¹⁷ Briefly, blood specimens for plasma were collected in an ethylenediaminetetraacetic acid (EDTA) tube; within 2 hours, these samples were centrifuged at 300xg at room temperature, aliquoted, then frozen to -80°C . For urine collection, participants were asked to provide clean-catch midstream voided specimens. Urine specimens were inverted several times to ensure uniform suspensions, aliquoted, and stored at -80°C . Pilot study matched control samples were obtained and processed at each site in an identical manner as the Observational Cohort (case) participants. All specimens were stored at the NIDDK Biorepository and sent to SomaLogic for analysis.¹⁵

SomaScan Assay

SomaScan has been described. It measures 1305 proteins using highly specific "Slow Off-Rate Modified Aptamer" (SOMAmer) reagents.¹⁵

Data Analysis Methods

Data were first analyzed according to the SomaLogic protocol.¹⁵ Further data analyses were performed using MATLAB 2017a (MathWorks Inc, Natick, MA). Data for plasma and urine were analyzed separately. Reproducibility of SomaScan was evaluated by calculating standard deviations, means, and coefficient of variations (COVs) for protein abundances across the duplicate samples for each of 1305 proteins from the 4 participants for whom blind duplicate samples were analyzed. The data for females and males were analyzed separately by comparing protein abundances in subjects with LUTS and controls.

To identify differentially abundant proteins between cases and controls, as well as their effect size, we compared protein

abundance in cases with mean protein abundance and standard deviation in controls by using the following equation:

$$Z_{ij} = \frac{(\log A_{ij} - (\log A_{cj}))}{\text{std}(\log A_{cj})}$$

where A_{ij} – abundance of protein j in the sample from LUTS subject i ; A_{cj} – abundance of the same protein j in the sample from control c ; Z_{ij} – standardized logarithmic abundance of protein j in LUTS subject i .

Since protein abundances follow a log-normal distribution, the logarithmic scale was used.¹⁹ Standardization was performed by comparison with means and standard deviations of controls rather than the whole population. To determine differentially abundant proteins in cases with LUTS, we averaged the Z score (Z_{ij}) across all female and male LUTS subjects separately, resulting in Z_{fj} and Z_{mj} – average abundances of protein j in male and female subjects with LUTS. Proteins were considered differentially abundant if $|Z_{fj}| > 1$ or $|Z_{mj}| > 1$. In other words, we defined proteins that were both over- and under-abundant by having an averaged Z score above and below one standard deviation difference. In addition, we identified proteins for whom standard deviation of abundances across female and male subjects with LUTS was substantially higher than that of controls: $\text{std}(Z_{ij}) > 3$.

Proteins that were substantially overabundant or substantially underabundant compared with controls were assessed further in pathway analysis. Differentially abundant proteins were imported into MetaCore (Clarivate Analytics; <https://portal.genego.com>) to examine the intersection between these proteins and existing Gene Ontology (GO) networks.

RESULTS

Plasma

[Table 1](#) shows baseline characteristics of male and female cases and controls. The plasma and urine samples from each of these participants were analyzed separately by SomaScan analysis. All duplicate samples from one female subject failed to meet quality control and were removed from the analysis. Reproducibility of the serum assays is illustrated by histograms of COVs for 1305 proteins in male and female subjects (cases and controls of each sex; [Supplemental Figure 3](#)). The mean values of the COVs were within the 0.044 to 0.047 interval, which is in agreement with the previously-reported mean COV of 0.046.¹⁵

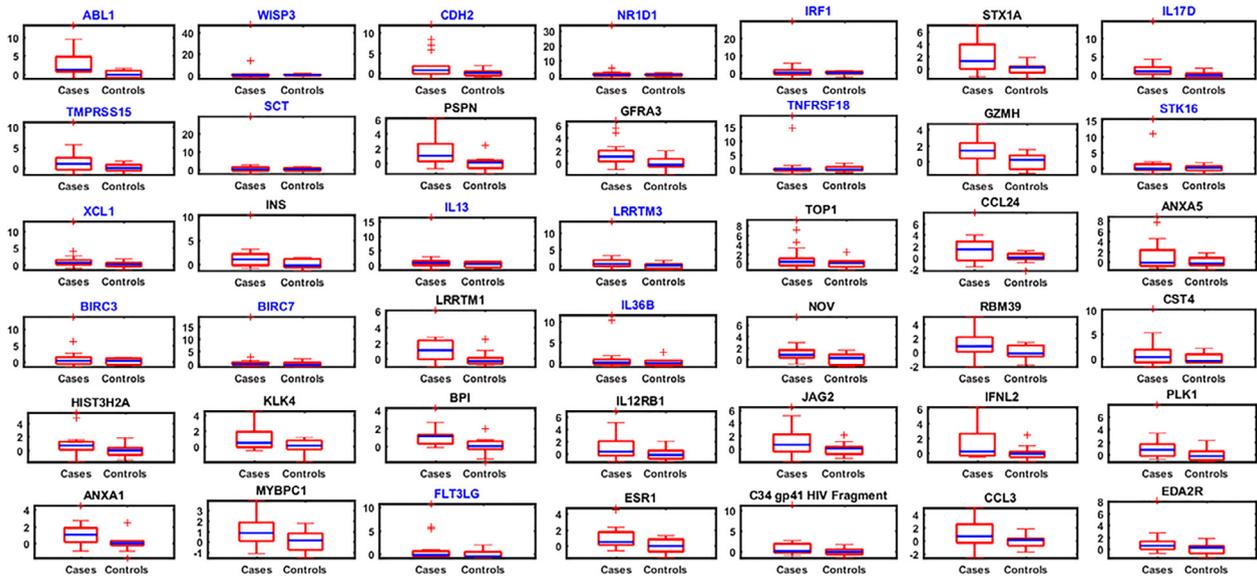
[Supplemental Figure 4](#) demonstrates the histograms of the mean standardized logarithmic abundance of all of the 1305 proteins for female and male LUTS cases. The histogram for male cases contains multiple differentially abundant proteins with >1 , >2 , and >3 standard deviations above or below the mean (ie, $|Z_{mj}| > 1$, >2 , >3). [Supplemental Table 1](#) provides the numbers of proteins that were significantly over- and underabundant (defined by $|Z_{fj}| > 1$ or $|Z_{mj}| < -1$) among female and male LUTS cases. The number of differentially abundant proteins was higher in male than female cases. Specifically, 297 proteins were affected (ie, either over- or underabundant or had standard deviation above 3 standard deviations of the controls) within plasma samples of male subjects with LUTS and 54 in plasma from female subjects with LUTS. Only 27 proteins were common between sexes, representing about 10% of total proteins affected in LUTS males and 50% of proteins affected in LUTS females. [Figures 1a and 1b](#) depict box plots of the abundances of differentially abundant upregulated proteins in females and male

Table 1. Baseline characteristics of male and female case and control subjects included in the biomarker pilot study

		Case		Control	
		Male	Female	Male	Female
Age	Age<45	2 (11.1%)	3 (16.7%)	1 (8.3%)	4 (33.3%)
	Age 45-<55	6 (33.3%)	3 (16.7%)	6 (50.0%)	1 (8.3%)
	Age 55-<65	3 (16.7%)	5 (27.8%)	2 (16.7%)	3 (25.0%)
	Age 65+	7 (38.9%)	7 (38.9%)	3 (25.0%)	4 (33.3%)
Race	African American	4 (22.2%)	4 (22.2%)	2 (16.7%)	2 (16.7%)
	White	14 (77.8%)	14 (77.8%)	10 (83.3%)	10 (83.3%)
Obesity	BMI <30	12 (66.7%)	7 (38.9%)	7 (58.3%)	6 (50.0%)
	BMI 30+	6 (33.3%)	11 (61.1%)	5 (41.7%)	6 (50.0%)
Diabetes	No diabetes	13 (72.2%)	16 (88.9%)	10 (83.3%)	11 (91.7%)
	Diabetes	5 (27.8%)	2 (11.1%)	2 (16.7%)	1 (8.3%)
How often did you urinate too frequently?	Often or almost always	13 (72.2%)	17 (94.4%)	0 (0.0%)	0 (0.0%)
How many times did you urinate during waking hours?	11 or more times per day	2 (11.1%)	6 (33.3%)	0 (0.0%)	0 (0.0%)
How many times did you wake up because you needed to urinate?	3 or more times a night	10 (55.6%)	6 (33.3%)	0 (0.0%)	0 (0.0%)
How often have you had the feeling your bladder was not empty after urinating?	Often or almost always	8 (44.4%)	6 (33.3%)	0 (0.0%)	0 (0.0%)
How often have you had a trickle or dribble at the end of your urine flow?	Often or almost always	7 (38.9%)	10 (55.6%)	0 (0.0%)	0 (0.0%)
How often have you had a sudden need to rush to urinate?	Often or almost always	7 (38.9%)	8 (44.4%)	0 (0.0%)	0 (0.0%)
How often have you had a delay before you start to urinate?	Often or almost always	4 (22.2%)	2 (11.1%)	0 (0.0%)	0 (0.0%)
How often did your urine flow start and stop while urinating?	Often or almost always	5 (27.8%)	1 (5.6%)	0 (0.0%)	0 (0.0%)
How often did you strain to urinate or strain while you were urinating?	Often or almost always	0 (0.0%)	1 (5.6%)	0 (0.0%)	0 (0.0%)
How often have you had a weak urine stream?	Often or almost always	6 (33.3%)	3 (16.7%)	0 (0.0%)	0 (0.0%)
How often have you had splitting or spraying of your urine stream?	Often or almost always	2 (11.1%)	5 (27.8%)	0 (0.0%)	0 (0.0%)
How often have you had a sudden need to rush to urinate for fear of leaking urine?	Often or almost always	4 (22.2%)	11 (61.1%)	0 (0.0%)	0 (0.0%)
How often have you had pain or discomfort in your bladder area?	Often or almost always	2 (11.1%)	3 (16.7%)	0 (0.0%)	0 (0.0%)
How often have you had a burning feeling when you urinate?	Often or almost always	1 (5.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
How often did you leak urine?	Often or almost always	2 (11.1%)	14 (77.8%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine just after you have finished urinating?	Often or almost always	3 (16.7%)	2 (11.1%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine in connection with a sudden need to rush to urinate?	Often or almost always	4 (22.2%)	11 (61.1%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine in connection with laughing, sneezing, or coughing?	Often or almost always	0 (0.0%)	11 (61.1%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine in connection with physical activities, such as exercising or lifting heavy objects?	Often or almost always	0 (0.0%)	8 (44.4%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine when you are sleeping?	Often or almost always	0 (0.0%)	4 (22.2%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine during sexual activity?	Often or almost always	0 (0.0%)	3 (16.7%)	0 (0.0%)	0 (0.0%)
How often have you leaked urine for no reason?	Often or almost always	0 (0.0%)	4 (22.2%)	0 (0.0%)	0 (0.0%)

Abbreviation: BMI, body mass index.

Differentially Abundant Proteins:(Z>1) – Female



Differentially Abundant Proteins: (Z>1) – Male

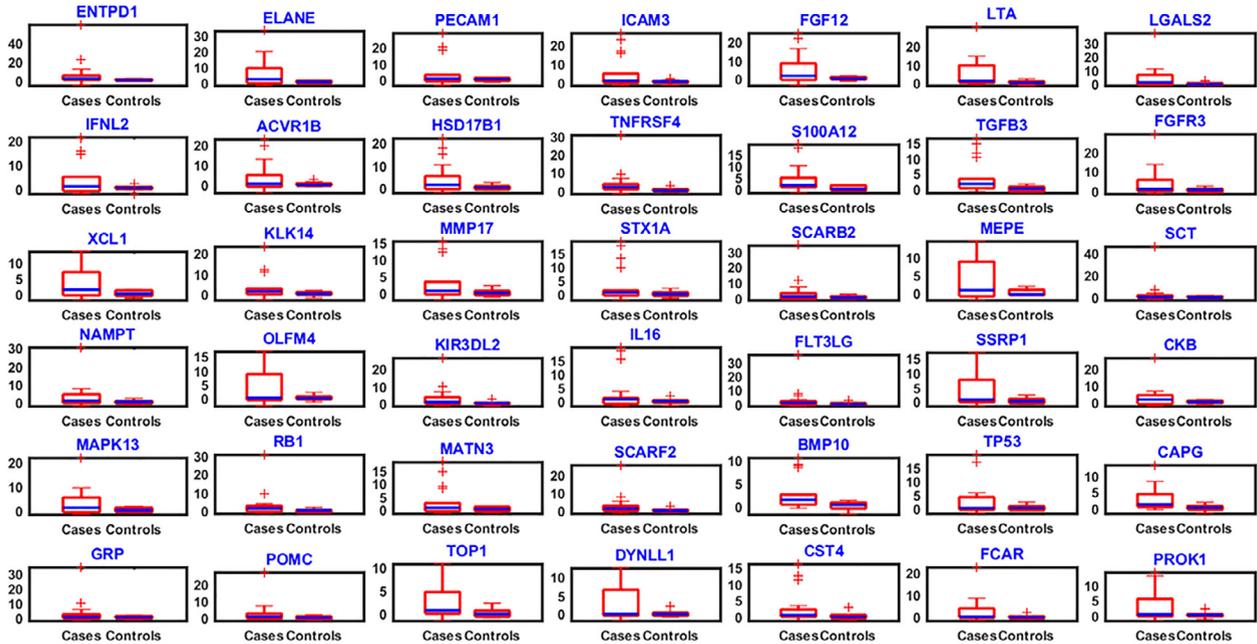


Figure 1. Comparisons of protein levels between cases and controls. Proteins were considered over- or underabundant by having an averaged Z score above or below one standard deviation difference. **Figures 1a** and **1b** demonstrate differentially abundant proteins in female and male subjects, respectively. (Color version available online.)

subjects. The entire lists of proteins for female and male subjects are shown ([Supplemental Tables 2](#) and [3](#)).

Next, an enrichment/pathway analysis of the affected proteins in male and female subjects was performed using MetaCore. **Figure 2** presents the results of enrichment analysis for proteins affected in male LUTS subjects. Interestingly, 5 out of the top 10 significantly enriched GO (<http://geneontology.org>) processes for men were related to prostate morphogenesis and growth. **Figure 3** presents the results of enrichment/pathway analysis for proteins affected in female LUTS subjects. In females, the top enriched GO processes were related mostly to regulatory and ion

transport pathways. There were no overlaps between the identified pathways in female and male LUTS subjects. Importantly, since no information on sex status was provided, the MetaCore software determined the relevant pathways blindly to sex.

Urine

Similar analyses were performed for urine samples. The reproducibility of SomaScan was significantly lower for urine compared with plasma, with mean COV values ranging from 0.138 to 0.229. Histograms illustrating the distribution of the COVs are presented in [Supplemental Figure 5](#).

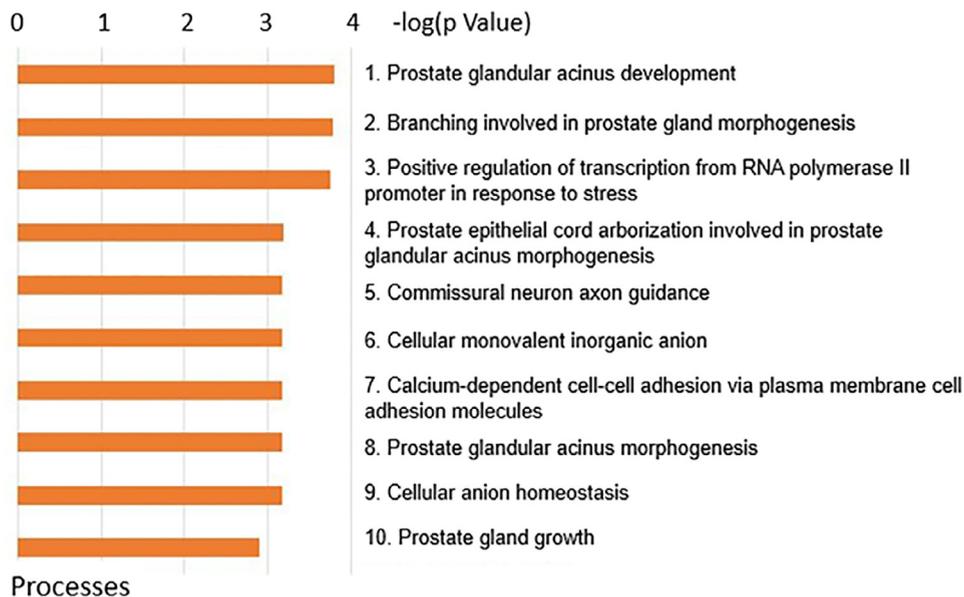


Figure 2. Enrichment/pathway analysis, including the top 10 over- and underabundant proteins in male plasma samples. (Color version available online.)

Supplemental Table 4 demonstrates the numbers of differentially abundant (upregulated and downregulated) proteins in urine, as well as proteins with high standard deviations of abundances in the urine samples of female and male LUTS subjects. In male participants, there were a 3-fold lower number of differentially abundant proteins in urine compared with plasma. However, in female LUTS participants, affected proteins were 2-fold higher in number in urine compared with plasma. There was minimal overlap between affected proteins in male and female LUTS urine samples.

The lists of affected proteins in female and male LUTS urine samples were also submitted for enrichment/pathway analysis.

Pathways enriched with affected proteins in male and female LUTS urine are presented in the Supplemental Figures 6 and 7. Several of the enriched pathways have been implicated in immune response and inflammation.

DISCUSSION

Ideally, a biomarker or panel of biomarkers would provide objective and measurable entities that could define the presence of a condition (diagnostic biomarker), its

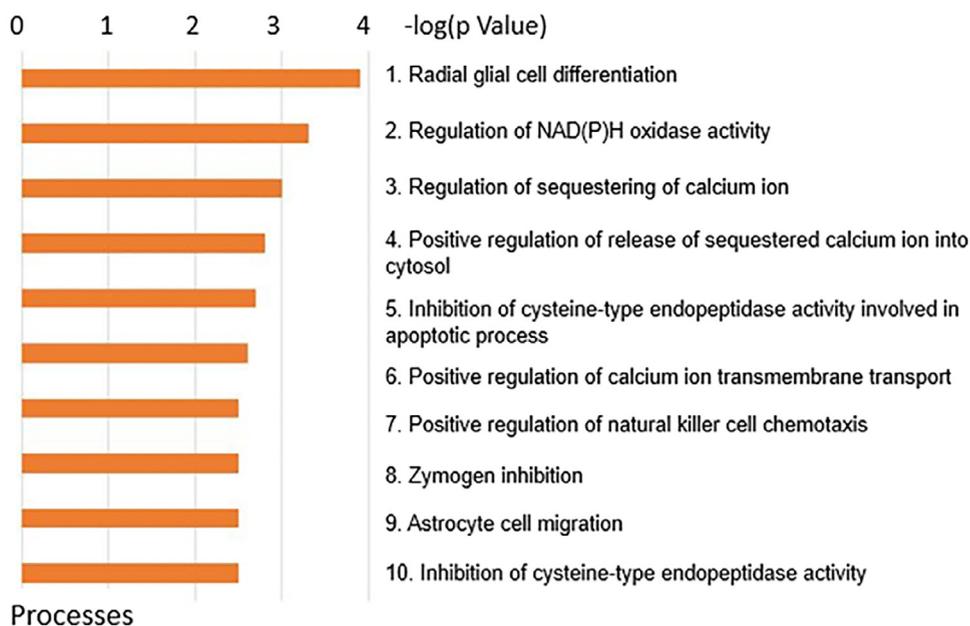


Figure 3. Enrichment/pathway analysis, including the top 10 over- and underabundant proteins in female plasma samples. (Color version available online.)

severity and progression (prognostic biomarker), or the response to a particular treatment (predictive biomarker). Identification of panels of biomarkers could provide additional insights into the patient-specific pathophysiologic mechanisms underlying LUTS in men and women, and thus could pave the way to therapeutic advances. Finally, a panel of biomarkers could also provide a tool for clinicians to “personalize” treatment strategies for their patients in order to initiate more effective treatments and monitor clinical response. In this study, we have shown that performing the SomaScan panel of biomarkers is feasible and therefore could be used in future large-scale studies of LUTS in men and women.

The vast majority of published studies that have attempted to identify biomarkers for LUTS have targeted only a limited set of potential candidate proteins. For example, candidate biomarkers, including nerve growth factor, C-reactive protein, prostaglandin E2, and brain-derived growth factor have been studied in patients with overactive bladder phenotypes compared with controls. However, many of these are cross-sectional association studies and demonstrate conflicting results.¹³ Similarly, proteomic studies conducted in humans and animals with bladder outlet obstruction have identified several candidate proteins that may be differentially expressed, including optineurin, thioredoxin, prehaptoglobin, and Kallikrein-related peptidase 2 (KLK2).²⁰ However, the results of these studies have not been reproduced and, therefore, require further validation; future studies that use panels of biomarkers and can be rigorously validated will be extremely important. The present pilot study used a relatively unbiased approach incorporating over 1300 proteins. In addition, while all cases reported LUTS, the results were not dependent on a single phenotype, such as overactive bladder or bladder outlet obstruction. The results using this novel and somewhat unrestricted approach demonstrated the SomaScan can distinguish proteins that are differentially abundant in male and female cases with LUTS compared with controls.

The results of the present study also compared the abundances of proteins contained within plasma and urine. The reproducibility and performance of the SomaScan assay were significantly better in plasma compared with urine (as measured by the COVs). Specifically, the reproducibility in plasma was even better than previously reported.²¹ Therefore, based upon the current assay limitations, plasma appears to be a better medium than urine for identifying proteins related to LUTS. This does not imply urine does not contain relevant biomarkers for this purpose. In fact, many previous studies have successfully used urine as a substrate to identify and compare biomarkers in LUTS patients versus controls. However, our data demonstrate SomaScan performs better in plasma than in urine.

One of the stated goals of this pilot study was to estimate the sample size required for a larger-scale biomarker study. The aim of the larger study would be to determine if there are biomarker signatures defining different

subtypes of LUTS based on unsupervised clustering of the biomarker data. Unlike traditional hypothesis testing, there are no standard methods for estimating sample size for a cluster analysis study. Here, we used our previously established approach²² for estimating sample size in clustering studies using simulation based on the results of the pilot project ([Supplemental Material](#)). Our sample size estimate results suggest a sample size of 220 patients with LUTS (110 male and 110 female cases) would provide a misclassification error below 0.7% for females and below 1% for males. We also plan on performing the SomaScan Assay in 30 non-LUTS female and 30 non-LUTS male controls, which would be matched for age, race, obesity, and diabetic status frequency to the cases. Control data will be used for standardizing protein abundances in LUTS subjects prior to clustering.

The present study was designed as a pilot study and therefore the results should only be interpreted as preliminary. Nevertheless, it is interesting that there was little overlap between differentially abundant proteins in female and male LUTS patients, suggesting that the etiology of LUTS may be significantly different based upon sex. Furthermore, both for males and females, the majority of affected proteins demonstrated not only differential abundance, but also much higher standard deviations relative to controls. This observation corroborates the hypothesis of the heterogeneity and multiple etiologies of LUTS. In other words, because there were not sufficient numbers of any single LUTS phenotype represented in this relatively small cohort, large variations in protein abundances were expected. The enrichment pathway analysis suggests that the pathways involved in men may be directly related to the prostate. This is supported by a vast literature supporting a prostocentric etiology of LUTS and suggests that proteins associated with prostate growth may be good candidate biomarkers of male LUTS. In comparison, similar enrichment/pathway analysis in women supports a larger role of dysregulation of ion transport and inflammation. Again, many studies have identified inflammatory pathways that contribute to female LUTS, including another pathway analysis specifically focused on overactive bladder where the majority of data came from female subjects.¹³ While the proteins and pathways identified in this study implicate a role for inflammation and tissue growth in LUTS, they do not imply causation. If validated as biomarkers, future studies specifically evaluating their role in LUTS should be performed.

There are several limitations of this pilot project that deserve mention, including the relatively small sample size. Cohorts involving larger numbers of cases and controls are required to validate these findings. In addition, further evaluation of how differentially abundant proteins correlate with known specific LUTS phenotypes (eg, storage and voiding symptoms) should be performed. The SomaScan assay was specifically used because it includes >1300 proteins involved in relevant pathways to LUTS, including inflammation and aging. While this is a large protein panel, it is by no means exhaustive. Therefore,

many candidate biomarkers associated with LUTS may have been missed. Future studies that evaluate even large panels of proteins and a larger number of participants may be considered.

CONCLUSION

Overall, the results of this pilot study identified plasma as suitable media for SomaScan to measure proteins related to LUTS in men and women. In addition, we were able to determine the number and effect size of biomarkers among cases with LUTS compared with controls. Our preliminary results suggest that the proteins and pathways that were significantly affected in LUTS cases were different among men and women. This supports that the etiology and pathophysiologic mechanisms underlying LUTS may be sex-specific. While further studies involving larger numbers of subjects are warranted, our results support the existence of biomarkers for diagnostic classification of LUTS. Importantly, this pilot study lays the necessary foundations and sample size estimates for a larger-scale study.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.urology.2019.03.014>.

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