Experimental Physiology

https://ep.msubmit.net

EP-SC-2020-088450R1

Title: A pilot study to investigate the associations of urinary concentrations of urinary NO, ATP and derivatives with overactive bladder symptom severity

Authors: Sepinoud Firouzmand
John S. Young

Author Conflict: John Young: JSY has previously received funding from Ferring Pharmaceuticals and TENA.

Running Title: Urinary NO, ATP and derivatives in overactive bladder

Abstract: Overactive bladder (OAB) is a highly prevalent symptom complex characterized by symptoms of urinary urgency; increased frequency; waking to void (nocturia) - with or without urge incontinence and in the absence of proven infection or other obvious pathology. The underlying pathophysiology of idiopathic OAB is not clearly known and the existence of several phenotypes has been proposed. Current diagnostic approaches are based on discordant measures, suffer from subjectivity and are incapable of detecting the proposed OAB phenotypes. NO, ATP and their metabolites have previously been shown to underlie the perception of bladder fullness, with their release modifying the pathological perception of urgency. Therefore, in this study we assessed the concentration of NO, ATP and associated metabolites in the urine of 113 consented participants recruited from the general population. Recruited participants completed a questionnaire to measure the severity of OAB-associated urinary symptoms and provided a mid-stream urine sample. Following identification of infection and

hematuria using microbiology and microscopy, 95 samples were subjected to assays to measure NO, NO2-, NO3-, ATP, ADP and creatinine. There was no correlation between [NO/Cr], [NO2-/Cr] or [NO3-/Cr] and overall OAB symptom severity. [ATP/NO], [ADP/NO] and a combination of these, [ATP/Cr*ADP/Cr]/[NO/Cr], correlated with OAB symptom severity; with [ATP/Cr*ADP/Cr]/[NO/Cr] also correlating with the severity of urinary frequency and urgency. This study adds to a growing literature that demonstrates the potential of urinary biomarkers and provides a foundation for a larger, longitudinal study.

New Findings: What is the central question of this study? Are the urinary levels of NO and ATP, and their metabolites, associated with the symptom severity of overactive bladder? What is the main finding and its importance? The urinary ratios of [ATP/NO], [ADP/NO] and a combination of these, [ATP/Cr*ADP/Cr]/[NO/Cr] were correlated with overall OAB symptom severity; with the latter also correlating with the severity of urinary frequency and urgency symptoms individually. Together these data reveal changes in urothelial signalling that accompany the transition from physiology to pathology.

Dual Publication: No

Funding: Institute of Biomedical and Biological Sciences: John S. Young, N/A

Title Page

A pilot study to investigate the associations of urinary concentrations of urinary NO, ATP and derivatives with overactive bladder symptom severity

Sepinoud Firouzmand PhD[a], John S. Young PhD[a,*]

[a] School of Pharmacy & Biomedical Sciences, University of Portsmouth, St. Michael's Building, White Swan Road, Portsmouth, UK, PO1 2DT.

* Corresponding Author: John S. Young

Corresponding author's contact details:

E: john.young@port.ac.uk

T: +44 (0)23 9284 3564

Address: School of Pharmacy and Biomedical Sciences, University of Portsmouth, St. Michael's Building, White Swan Road, Portsmouth, UK, PO1 2DT

Keywords: ATP, ADP, NO, overactive bladder, urine, biomarker

Word count of text (including abstract): 2859 words

Word count of abstract: 235

List of abbreviations[1]: please see footnote

Declaration of interest: SF declare no conflicts of interest. JSY has previously received funding from Ferring Pharmaceuticals and TENA.

[1] List of abbreviations: ADP - Adenosine diphosphate; ATP - Adenosine triphosphate; β-actin - Beta actin; DO - Detrusor overactivity; ICIQ-OAB - International consultation on incontinence questionnaire - overactive bladder; OAB - Overactive bladder.

New Findings

What is the central question of this study?

Are the urinary levels of NO and ATP, and their metabolites, associated with the symptom severity of overactive bladder?

What is the main finding and its importance?

The urinary ratios of [ATP/NO], [ADP/NO] and a combination of these, [ATP/Cr*ADP/Cr]/[NO/Cr] were correlated with overall OAB symptom severity; with the latter also correlating with the severity of urinary frequency and urgency symptoms individually. Together these data reveal changes in urothelial signalling that accompany the transition from physiology to pathology.

Abstract

Overactive bladder (OAB) is a highly prevalent symptom complex characterized by symptoms of urinary urgency; increased frequency; waking to void (nocturia) - with or without urge incontinence and in the absence of proven infection or other obvious pathology. The underlying pathophysiology of idiopathic OAB is not clearly known and the existence of several phenotypes has been proposed. Current diagnostic approaches are based on discordant measures, suffer from subjectivity and are incapable of detecting the proposed OAB phenotypes. NO, ATP and their metabolites have previously been shown to underlie the perception of bladder fullness, with their release modifying the pathological perception of urgency. Therefore, in this study we assessed the concentration of NO, ATP and associated metabolites in the urine of 113 consented participants recruited from the general population.

Recruited participants completed a questionnaire to measure the severity of OAB-associated urinary symptoms and provided a mid-stream urine sample. Following identification of infection and hematuria using microbiology and microscopy, 95 samples were subjected to assays to measure NO, NO_2^-, NO_3^-, ATP, ADP and creatinine.

There was no correlation between [NO/Cr], [NO_2^-/Cr] or [NO_3^-/Cr] and overall OAB symptom severity. [ATP/NO], [ADP/NO] and a combination of these, [ATP/Cr*ADP/Cr]/[NO/Cr], correlated with OAB symptom severity; with [ATP/Cr*ADP/Cr]/[NO/Cr] also correlating with the severity of urinary frequency and urgency.

This study adds to a growing literature that demonstrates the potential of urinary biomarkers and provides a foundation for a larger, longitudinal study.

Introduction

Overactive bladder (OAB) is defined by the presence of bothersome symptoms of urinary urgency, increased frequency, nocturia, with or without urge incontinence in the absence of proven infection or other obvious pathology (Drake, 2014).

The initial evaluation of idiopathic OAB includes the assessment of the severity of OAB characteristic symptoms and the exclusion of disorders that present with some overlapping symptoms. However, there is no consensus regarding the symptom severity and/or symptom combination for OAB diagnosis or to define treatment response. This is considered as a challenge for interpretation, replication or clinical implementation of observed findings from studies to date (Lightner et al., 2019). In addition, the identification of subgroups of patients presenting with distinct combinations of OAB characteristic symptoms and/or bothersome severities, suggest the existence of several OAB phenotypes. This hypothesis is further supported through the observation of discrepancies in the clinical validity and benefits of several OAB diagnostic approaches that have been thus far utilized. For instance, the presence of urodynamically-demonstrable detrusor overactivity (DO) was only observed in half of the patients presenting with OAB characteristic symptoms, while DO was present in two-thirds of those without any OAB symptoms (Rovner and Goudelocke, 2010). OAB is a symptom complex and exists in several phenotypes. Considering this, the conventional approach of dichotomous participant stratification utilized in preliminary biomarker discovery studies needs to be replaced. The most suitable alternative would be to identify biomarkers that are specific to OAB phenotypes based on individual OAB characteristic symptoms and bothersome severities.

Urinary biomarkers are proposed to be valuable tools in identifying OAB phenotypes and for the selection of more effective targeted therapies (Antunes-Lopes and Cruz, 2019). A growing number of studies have shown the ability of the urothelium to release various chemical factors including nitric oxide (NO) and ATP (Birder and de Groat, 2007), where the release is considered as being in a bidirectional manner into the bladder's mucosal layer and lumen (urine) (Yoshida et al., 2006). It was suggested that changes in their released levels may modulate bladder sensory signaling pathways and if their altered urinary levels correspond to the severities of OAB symptoms, then they may be used as non-invasive OAB biomarkers. In a study by Munoz and colleagues (2011), the urinary ratio of ATP/NO was significantly higher in overactive bladders from diabetic rats compared to underactive or control bladders (Munoz et al., 2011). Therefore it was proposed that the urinary ATP/NO ratio may be a sensitive clinical biomarker to characterize the extent of bladder dysfunction. These observations prompted us to undertake a pilot study to investigate the associations of urinary concentrations of urinary NO, ATP and their metabolic derivatives with OAB bladder symptom severity in 113 participants. We aimed to identify whether these urinary surrogates could be used as markers of OAB severity and potentially as means for phenotyping and monitoring therapeutic response.

Materials and Methods

Ethical Approval

All participants recruited to this study provided informed consent in writing. The work was approved by the NHS Research Ethics Committee South Central, reference 13/SC/0501 and the Ethics Committee of the School of Pharmacy and Biomedical Sciences at the University of Portsmouth. The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database. The privacy rights of human subjects were observed at all times.

Participants

Participant recruitment and methods performed on data and samples are together summarized in Figure A1. One hundred and thirteen volunteer participants were recruited (2014-2016) according to the inclusion and exclusion criteria (see below) to this study. Consented participants were asked to complete the ICIQ-OAB questionnaire and to provide a fresh urine sample. Urine samples were immediately placed on ice for transport to the University of Portsmouth, where they were processed as described below.

Inclusion Criteria: Male or female participants aged ≥18 years and able to give informed consent for participation in the study.

Exclusion Criteria: Male or female participants aged ≤18; taking any medication for OAB; unable to give informed consent; diagnosed with a neurologic disease (stroke, MS, Parkinson's disease, spinal cord injury); with a history of uterine, cervical, vaginal or urethral cancer; history of cyclophosphamide use or any type of chemical cystitis; history of benign or malignant bladder tumors; have had Botulinum toxin injections into the bladder, neuromodulation or augmentation cystoplasty.

Urine pathology tests

Microscopic examination, dipstick urinalysis and chromogenic urinary tract infection (UTI) medium tests were immediately performed on a small proportion of each collected urine sample. According to the performed tests, 10 participants were diagnosed as having a yeast/bacterial infection or hematuria and four did not meet the inclusion criteria and therefore these participants were excluded from the study. The remaining urine samples (n=95) were utilized for biomarker analyses.

Following urine pathology tests, the remainder of the urine sample was centrifuged (at 4000 rpm, 10 mins, at 4°C), separated into cell pellet and supernatant and stored at -80°C before being subjected to biomarker analyses.

Biomarker assays

Creatinine (Cr) Assay

The concentration of urinary (cell-free component) Cr was measured using the Cayman Creatinine (urinary) Colorimetric Assay Kit (CAY500701, Cambridge Bioscience, UK), following the manufacturer's instructions. All urinary biomarker values were normalized to corresponding Cr concentrations.

ATP Assay

The concentration of urinary (cell-free component) ATP was measured using ENLITEN® ATP Assay System Bioluminescence Detection Kit (FF2000, Promega, UK) according to the manufacturer's instructions. Briefly, 40µL of each standard and urine samples, in duplicates, were aliquoted into 96 half-well plates (781610, BRANDplates®, pureGrade™, UK). The

luminescence was measured (POLARstar Optima microplate reader, BMG LABTECH, UK) before and after the addition of luciferin-luciferase (40µL) mixture. Calculated ATP concentrations were normalized to their corresponding urinary creatinine values (ATP/Cr).

Nitric Oxide (NO) measurement

NO has a short half-life and is converted rapidly to its oxidation products Nitrite (NO_2^-) and Nitrate (NO_3^-). The urinary (cell-free component) concentrations of NO_2^- and NO_3^- were measured using Nitric Oxide Analyzer (Sievers (NOA™ 280i), Analytix, UK). To measure [NO_2^-], the reducing agent iodide in glacial acetic acid was used to convert NO_2^- to NO (at ambient temperature). Under these conditions only NO_2^- and S-Nitrosothiol (if present) would be reduced to NO for measurement. To measure NO_3^-, Vanadium (III) Chloride in 1N hydrochloric acid was used to convert NO_3- to NO (at 95^oC). Under these conditions not only would the NO_2^- and S-Nitrosothiol be reduced to NO but also the Nitrate. Therefore, the total [NO] is the same as the [NO_3^-] in this experimental situation. Hence, [NO_3^-] was calculated by deducting the [NO_2^-] value from the total [NO] value. The NO, NO_2^- and NO_3^- concentrations were normalized to their corresponding urinary creatinine values (i.e. NO/Cr, NO_2^-/Cr and NO_3^-/Cr).

Statistical Analysis

Correlation analysis

For each of the analyses made, the characteristics of participants (including age, gender and urinary symptom score profiles) are summarised in Table A1. D'Agostino-Pearson normality test (where appropriate) was performed on all the generated data. Pearson product-moment correlation coefficient (parametric) or Spearman's rank correlation coefficient (non-parametric) were used for correlation analyses on untransformed data. GraphPad

Prism 8.0.0 software was used for all the analyses and the preparation of lin-log correlation graphs. Statistical significance was observed when the p-value was ≤0.05.

Results

The urinary concentrations of NO, NO_2^-, NO_3^-, ATP and ADP were measured in 95 human urine samples. Concentrations were standardized to their corresponding urinary creatinine concentrations and their relationships with participants' OAB-associated urinary symptoms and age were investigated (Table 1).

There was no correlation between [NO/Cr], [NO_2^-/Cr] or [NO_3^-/Cr] and overall OAB symptom severity (i.e. total ICIQ-score) (Table 1; Figure 1Aa). There was a positive correlation between [NO_2^-/Cr] and the severity of urinary frequency (Table 1) but there were no other associations between [NO/Cr], [NO_2^-/Cr] or [NO_3^-/Cr] and the severity of individual urinary symptoms that define OAB (Table 1).

In order to address the hypothesis that NO in combination with ATP (i.e. the ATP/NO ratio) may be a sensitive clinical biomarker to characterize the extent of bladder dysfunction (Munoz et al. 2011), this ratio was correlated with OAB symptom severity. There was a positive correlation between [ATP/NO] and total ICIQ-score, urinary frequency but also age (Table 1; Figure 1Aa). Given that the concentration of the hydrolysis product of ATP, ADP, correlated with total ICIQ-score and a positive correlation between [ATP/Cr] and [ADP/Cr] (Figure 1Ba) was observed, the combined concentrations of ATP, ADP and NO (i.e. [ATP/Cr*ADP/Cr]/[NO/Cr]) were correlated with OAB symptom severity and a positive relationship was observed (Figure 1Bb). [ATP/Cr*ADP/Cr]/[NO/Cr] was also positively correlated with urinary frequency and urgency but not nocturia or incontinence severity (Table 1).

The influence of gender on the relationships between urinary concentrations of chemicals and total ICIQ-scores was investigated (Figure A2). In all comparisons, the concentrations of

biomarkers / biomarker combinations overlap. The small sample sizes of gender groups prohibit further analysis.

Discussion

Overactive bladder (OAB) is a symptom complex with a profound impact on many aspects of sufferers' quality of life (Milsom et al., 2012). Our understanding of the pathophysiological mechanisms underlying its development is limited but an association with multiple factors has led to the notion of the existence of several OAB phenotypes (Peyronnet et al., 2019); potentially identified by specific urinary biomarkers (Antunes-Lopes and Cruz, 2019). Therefore, in this study we aimed to investigate the validity of urinary biomarkers in human participants with mild to moderate OAB symptoms, in order to address the utility of these urinary biomarkers in early detection.

As urgency is the key OAB symptom (Abrams et al., 2012), we focused on chemicals thought to initiate it: ATP and NO, released from the urothelium, and shown to mediate the afferent signaling system (Birder and Andersson, 2013); in turn responsible for the perception of bladder fullness in health and its upregulated state of urgency in disease.

It was hypothesized that urinary [NO] would negatively correlate with OAB symptom severity, given evidence from animal studies that (i) NO increased the interval between bladder contractions without changes in contraction amplitude (Ozawa et al., 1999), (ii) a NO scavenger stimulated overactivity (Pandita et al., 2000), and (iii) mucosa-derived NO release was reduced in disease models (Munoz et al. 2011). In our study, urinary [NO/Cr], [NO_2^-/Cr] or [NO_3^-/Cr] did not correlate with overall OAB symptom severity (Table 1). This disparity is likely due to differences in experimental design; as previous studies used animal models of disease rather than humans or biomarkers of human bladder function.

We did, however, observe an altered [ATP/NO] with bladder dysfunction, as has been observed previously in animal studies (Munoz et al. 2011). In our study, we observed a

positive correlation between [ATP/NO] and both total ICIQ symptom score and the severity of urinary frequency. Utility of this combination is improved when the urinary concentration of ADP, the product of ATP hydrolysis, is included: there were positive correlations between [ATP/Cr*ADP/Cr]/[NO/Cr]) and OAB symptom severity, urinary frequency and urgency. To our knowledge, this is the first study in humans to demonstrate the utility of these biomarker concentrations. We hypothesize that OAB is characterized by an increase in urothelial ATP release - as observed by increased urinary [ATP] in OAB (Silva-Ramos et al., 2013); interstitial cystitis (Sun et al., 2001); and benign prostatic hypertrophy (Silva-Ramos et al., 2016) - and a small decrease in urothelial NO release. Measuring a combination of urinary biomarkers provides a more sensitive measure of small changes in bladder function attributable to disease onset.

It should be noted that by recruiting participants from the general population, we observed a range of symptom scores with the majority of participants exhibiting mild symptoms. In some ways this is advantageous, as biomarkers/biomarker combinations specific to pathogenesis or early onset offer the most utility to the scientific and clinical community. Future studies should, however, recruit participants across the entire range of the OAB symptom spectrum.

The relationships between urinary concentrations of putative biomarkers and OAB symptom severity appear similar between the gender groups (Figure A2). Small sample sizes of gender groups limit the conclusions that can be drawn from these data. Future studies should therefore aim to recruit an equal number of participants from both genders in order to more fully probe potential gender differences in urinary biomarkers.

By ruling out similarly presenting conditions at the point of recruitment (see 'Exclusion criteria') and by performing urine pathology testing, we had hoped to recruit participants with a spectrum of lower urinary tract symptoms attributable to OAB. We acknowledge, however, that the ICIQ-OAB questionnaire lacks the ability to differentiate between different types of incontinence such as urge urinary incontinence, mixed urinary incontinence or stress incontinence. Although all the participants in this study with incontinence (i.e. an incontinence score of >0) had other OAB characteristic symptoms, more needs to be done in the future to differentiate between different types of incontinence. Furthermore, we cannot rule out the possibility that OAB was secondary to outflow obstruction. Future studies should therefore either involve participants with a broad range of OAB symptoms in order to allow the identification of further OAB phenotypes, and should be extended to include more participants and those with mixed urinary incontinence or, alternatively, include more rigorous recruitment criteria to exclude other possible overlapping conditions such as stress incontinence and polyuria. Furthermore, the clinical utility of biomarker combinations should be tested in large, longitudinal studies.

Conclusions

Given a paucity of knowledge of OAB pathogenesis, there is a need to develop tools (i) to allow early detection, (ii) to differentiate from other, similarly presenting symptom complexes/diseases, and (iii) as an objective means to monitor the effects of treatment. In this pilot study (n=113) sampling from the general population, we demonstrate positive correlations of [ATP/Cr], [ADP/Cr], [(ATP/NO)/Cr] and [ATP/Cr*ADP/Cr]/[NO/Cr]) with OAB severity, with the latter combination also correlating with the severity of urinary frequency

and urgency. This study adds to a growing literature that demonstrates the potential of urinary biomarkers and provides a foundation for a larger, longitudinal study.

Tables

Table 1. Correlations between the urinary nitric oxide (NO), nitrite (NO_2^-), nitrate (NO_3^-) and the ratio of urinary ATP and/or ADP to NO with overactive bladder characteristic symptoms severity and age.

Correlations		**Urinary [NO]**	**Urinary [NO_2^-]**	**Urinary [NO_3^-]**	**Urinary [ATP]/[NO]**	**Urinary [ADP]/[NO]**	**Urinary [ATP*ADP/NO]**
Frequency symptom score	*p*	0.290	**0.000**	0.095	**0.001**	0.402	**0.003**
	r	0.116	0.405	0.184	0.404	0.110	0.434
Nocturia symptom score	*p*	0.598	0.288	0.477	0.898	0.469	0.877
	r	-0.058	-0.118	-0.079	0.016	0.095	-0.024
Urgency symptom score	*p*	0.785	0.502	0.878	0.134	0.131	**0.015**
	r	0.030	0.075	0.017	0.187	0.197	0.364
Incontinence symptom score	*p*	0.711	0.708	0.521	0.189	0.207	0.293
	r	-0.041	-0.042	-0.072	0.164	0.165	0.162
Total ICIQ-OAB symptom score	*p*	0.637	0.303	0.760	**0.007**	**0.021**	**0.015**
	r	0.052	0.115	0.034	0.329	0.297	0.364
Total ICIQ-OAB symptom plus bothersome score	*p*	0.882	0.772	0.889	**0.022**	**0.038**	**0.032**
	r	-0.018	0.035	-0.017	0.309	0.294	0.363
Age	*p*	0.660	0.312	0.952	**0.022**	0.696	0.249
	r	0.048	-0.112	0.007	0.281	0.051	0.178

Urinary concentrations of NO, NO_2^-, NO_3^-, ATP and ADP were normalised to urinary creatinine concentration. *p*: p-value. *r*: Spearman/Pearson r value. P<0.05 are in bold.

Figures

Figure 1.

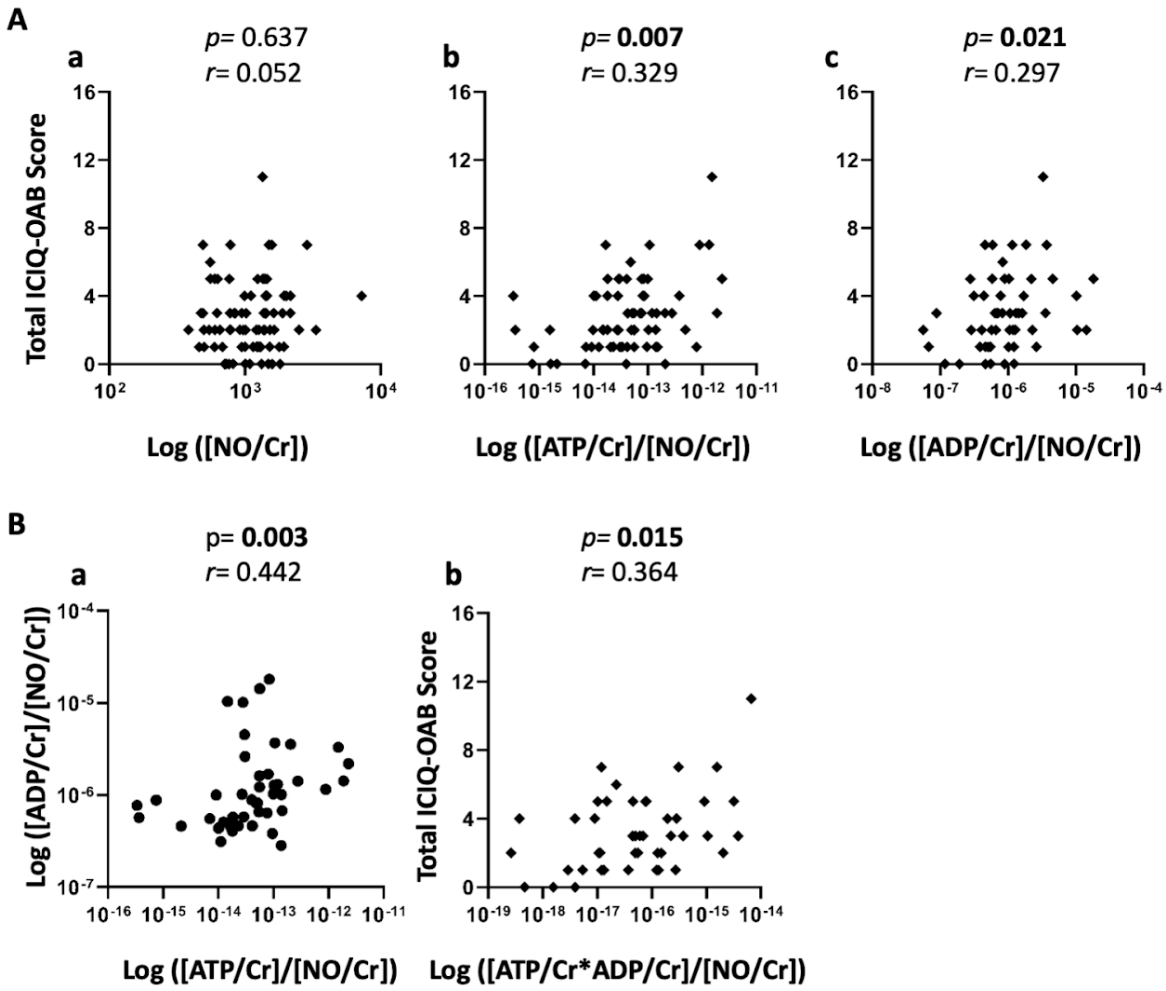


Figure Legends

Figure 1. Associations of nitric oxide (NO), nitrite (NO_2^-), nitrate (NO_3^-) and the ratio of urinary ATP and/or ADP to NO with overactive bladder (A) a. Correlation between the urinary levels of NO and participants' total ICIQ-OAB severity scores; b. Correlation between the ratio of urinary ATP to NO and participants' total ICIQ-OAB severity scores; c. Correlation between the ratio of urinary ADP to NO and participants' total ICIQ-OAB severity scores. (B) a. Correlation between participants' urinary ATP and ADP; b. Correlation between the ratio of the combination of urinary ATP and ADP to NO and participants' total ICIQ-OAB severity scores.

Urinary concentrations of NO, ATP and ADP were normalized to urinary creatinine (Cr) concentration. p: p-value. r: Spearman/Pearson r value. P<0.05 are in bold. All data presented on a lin-log plot except Ba, which is shown on a log-log plot.

Acknowledgements

We are thankful to all the participants involved in this study and to Rashedul Hoque and Nina Panesar who were involved in the early development of this study.

Funding

This work was supported by a PhD studentship awarded by the Institute of Biological and Biomedical Sciences (IBBS), University of Portsmouth, UK, to John S. Young.

CRediT Author Statement

Sepinoud Firouzmand: Methodology, Data curation, Formal analysis, Validation, Visualization, Roles/Writing - original draft, Writing - review & editing, Project administration. **John S. Young**: Methodology, Validation, Visualization, Roles/Writing - original draft, Writing - review & editing, Supervision, Project administration, Resources, Funding acquisition.

References

Abrams, P., Chapple, C. R., Jünemann, K.-P., & Sharpe, S. (2012). Urinary urgency: a review of its assessment as the key symptom of the overactive bladder syndrome. *World Journal of Urology, 30*, 385-392. https://doi.org/10.1007/s00345-011-0742-8.

Antunes-Lopes, T., & Cruz, F. (2019). Urinary biomarkers in overactive bladder: revisiting the evidence in 2019. *European Urology Focus, 5*, 329-336. https://doi.org/10.1016/j.euf.2019.06.006.

Birder, L. A., & de Groat, W. C. (2007). Mechanisms of disease: involvement of the urothelium in bladder dysfunction. *Nature Clinical Practice Urology, 4*, 46-54. https://doi.org/10.1038/ncpuro0672.

Birder, L., & Andersson, K.-E. (2013). Urothelial signaling. *Physiological Reviews, 93*, 653-680. https://doi.org/10.1152/physrev.00030.2012.

Drake, M. J. (2014). Do we need a new definition of the overactive bladder syndrome? ICI-RS 2013. *Neurourology and Urodynamics, 33*, 622-624. https://doi.org/10.1002/nau.22609.

Lightner, D.J., Gomelsky, A., Souter, L., Vasavada, S.P. (2019). Diagnosis and treatment of overactive bladder (non-neurogenic) in adults: AUA/SUFU guideline amendment. *Journal of Urology, 202*, 558-563. https://doi.org/10.1097/JU.0000000000000309.

Milsom, I., Kaplan, S. A., Coyne, K. S., Sexton, C. C., & Kopp, Z. S. (2012). Effect of bothersome overactive bladder symptoms on health-related quality of life, anxiety, depression, and treatment seeking in the united states: results From EpiLUTS. *Urology*, *80*, 90-96. https://doi.org/10.1016/j.urology.2012.04.004.

Munoz, A., Smith, C. P., Boone, T. B., & Somogyi, G. T. (2011). Overactive and underactive bladder dysfunction is reflected by alterations in urothelial ATP and NO release. *Neurochemistry International, 58*, 295-300. https://doi.org/10.1016/j.neuint.2010.12.002.

Ozawa, H., Chancellor, M. B., Jung, S. Y., Yokoyama, T., Fraser, M. O., Yu, Y., … Yoshimura, N. (1999). Effect of intravesical nitric oxide therapy on cyclophosphamide-induced cystitis. *Journal of Urology, 162*, 2211-2216. https://doi.org/10.1016/s0022-5347(05)68161-x.

Pandita, R. K., Mizusawa, H., & Andersson, K. E. (2000). Intravesical oxyhemoglobin initiates bladder overactivity in conscious, normal rats. *Journal of Urology*, *164*, 545-550. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10893641.

Peyronnet, B., Mironska, E., Chapple, C., Cardozo, L., Oelke, M., Dmochowski, R., … Cornu, J.-N. (2019). A comprehensive review of overactive bladder pathophysiology: on the way to tailored treatment. *European Urology*, *75*, 988-1000. https://doi.org/10.1016/j.eururo.2019.02.038.

Rovner, E. S., & Goudelocke, C. M. (2010). Urodynamics in the evaluation of overactive bladder. *Current Urology Reports*, *11*, 343-347. https://doi.org/10.1007/s11934-010-0130-8.

Sun, Y., Keay, S., De Deyne, P.G., Chai, T.C. (2001). Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis.

Journal of Urology, *166*, 1951-1956. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11586266.

Silva-Ramos, M., Silva, I., Oliveira, O., Ferreira, S., Reis, M. J., Oliveira, J. C., & Correia-de-Sá, P. (2013). Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome. PloS one, 8(5), e64696. https://doi.org/10.1371/journal.pone.0064696

Silva-Ramos, M., Silva, I., Oliveira, J.C., Correia-de-Sá, P. (2016). Increased urinary adenosine triphosphate in patients with bladder outlet obstruction due to benign prostate hyperplasia. *Prostate, 76*, 1353-1363. https://doi.org/10.1002/pros.23207.

Yoshida, M., Inadome, A., Maeda, Y., Satoji, Y., Masunaga, K., Sugiyama, Y., & Murakami, S. (2006). Non-neuronal cholinergic system in human bladder urothelium. *Urology, 67*, 425-430. https://doi.org/10.1016/j.urology.2005.08.014.

Appendices

Figure A1. Flow diagram of participants' recruitment, selection and subsequent tests.

a: See Methods and Materials section for inclusion and exclusion criteria; ICIQ-OAB: International consultation on incontinence questionnaire - overactive bladder; UTI: Urinary tract infection; NO: Nitric oxide; NO_2^-: Nitrite; NO_3^-: Nitrate. b: Urinary concentrations of NO, NO_2^-, NO_3^-, ATP and ADP were normalised to urinary creatinine concentration.

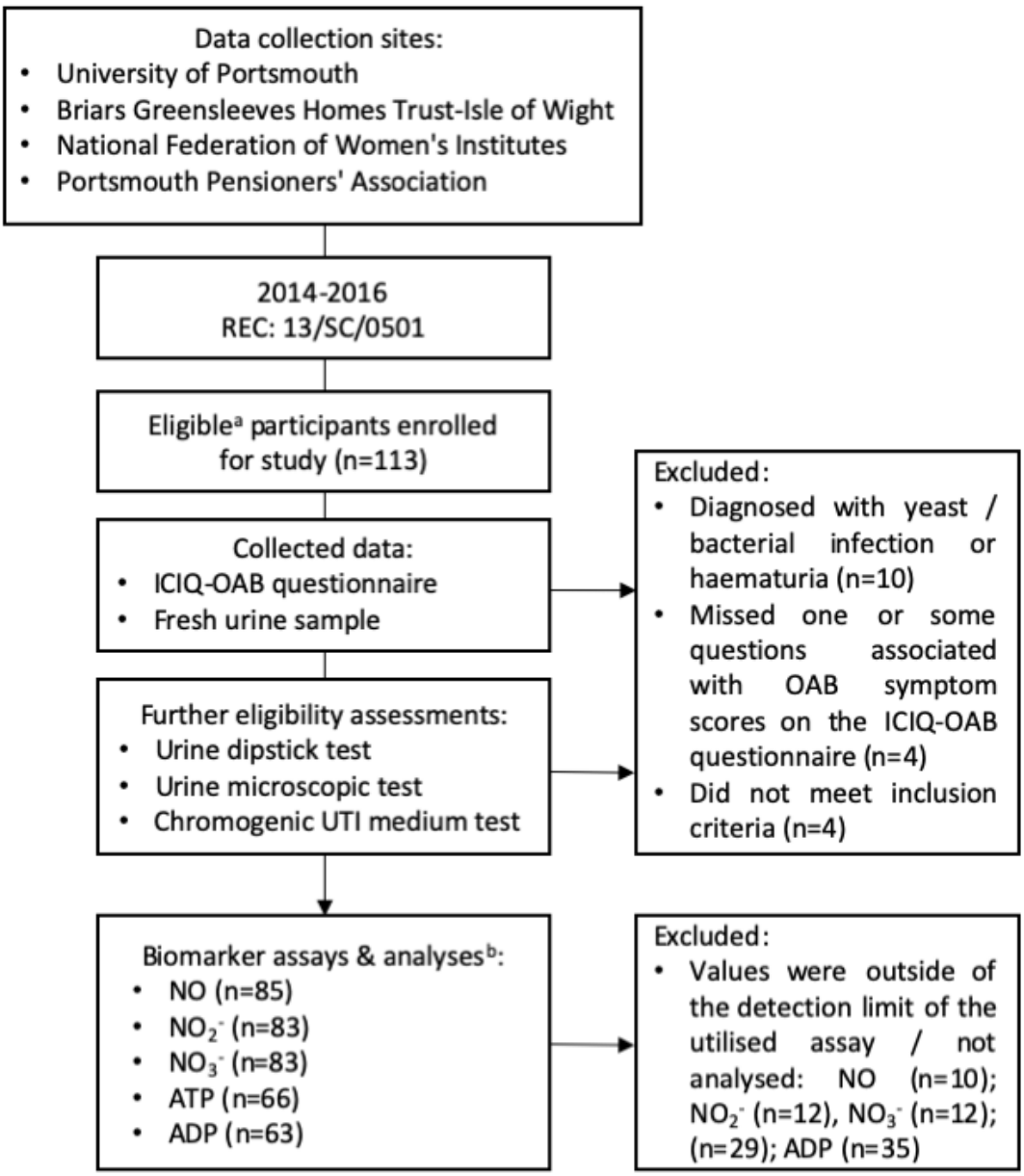


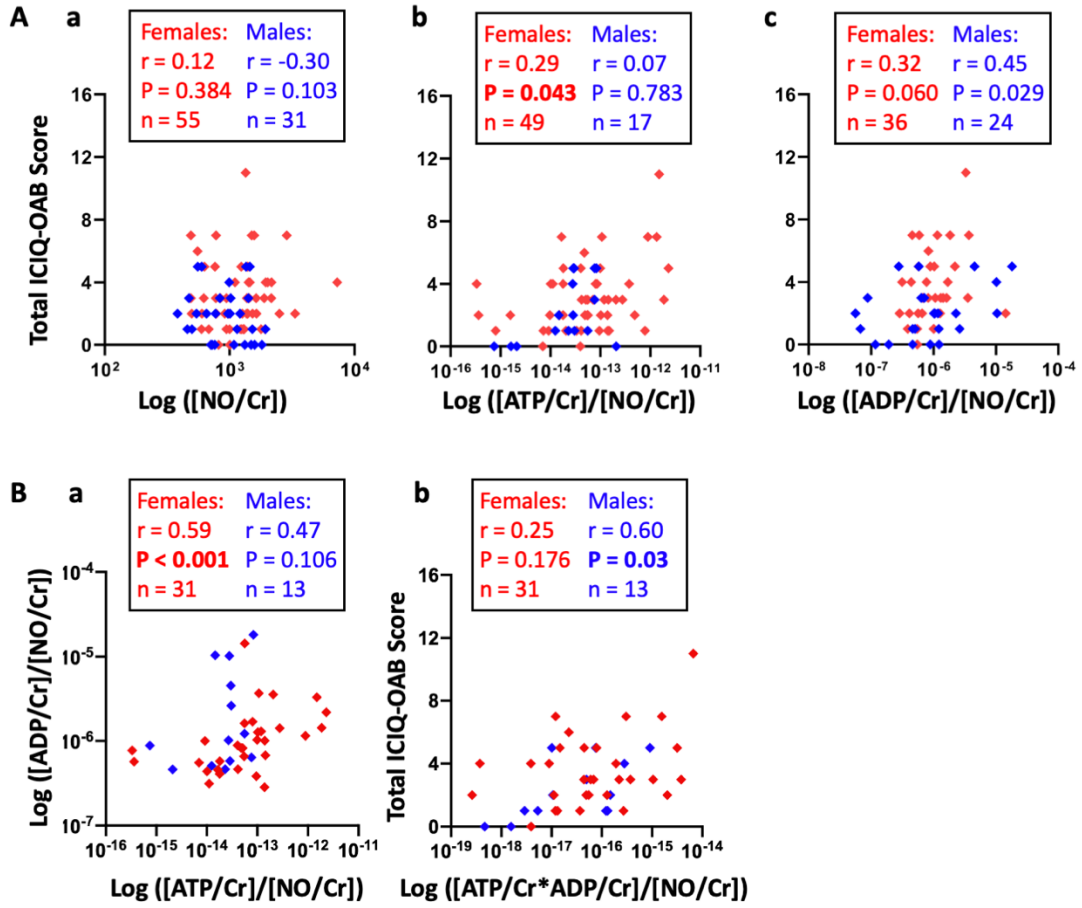
Table A1. Characteristics of study participants.

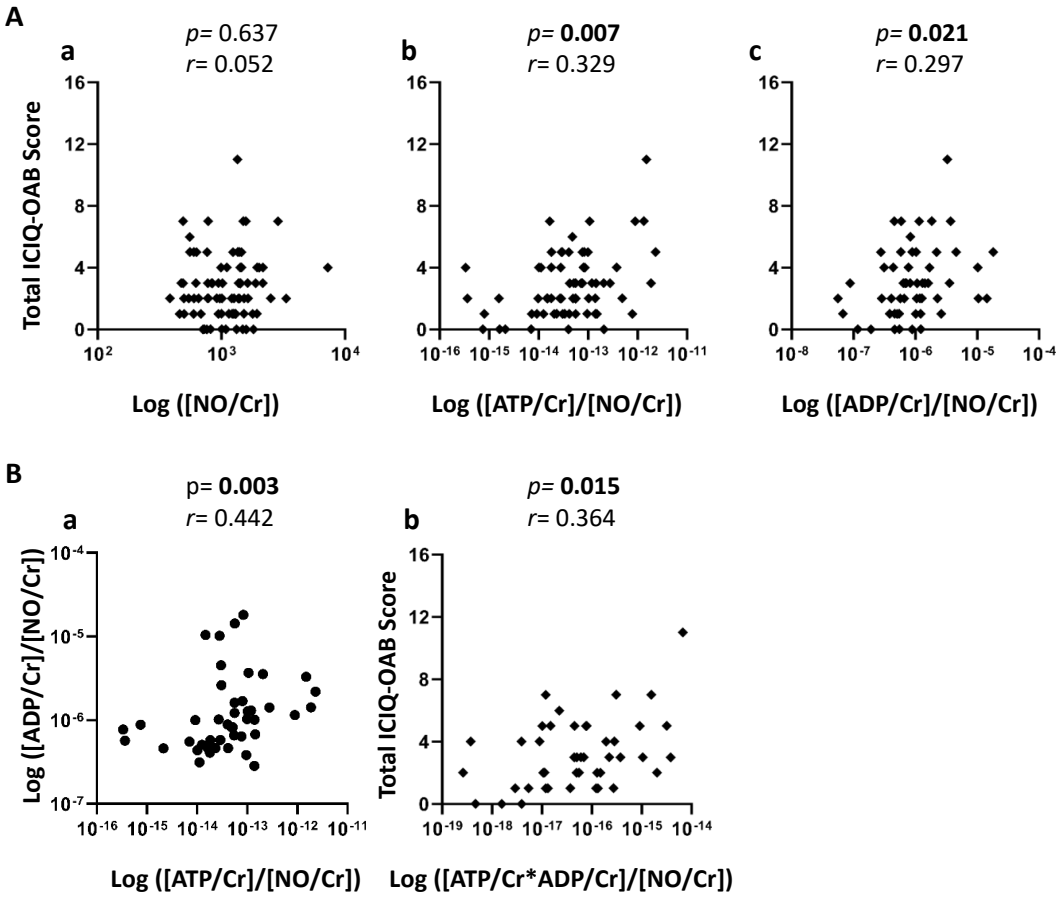
Participant characteristics	Urinary [NO] analysis	Urinary [NO_2^-] analysis	Urinary [NO_3^-] analysis	Urinary [ATP]/[NO] analysis	Urinary [ADP]/[NO] analysis	Urinary [ATP*ADP/NO] analysis
n	85	83	83	66	60	44
Age, mean (range) (yrs)	53 (21-93)	52 (21-93)	52 (21-93)	52 (21-93)	54 (21-93)	55 (21-93)
Gender						
Female	55	54	54	49	36	31
Male	30	29	29	17	24	13
ICIQ-OAB characteristics						
Frequency[a], mean (SD)	0.34 (±0.30)	0.33 (±0.30)	0.33 (±0.30)	0.37 (±0.31)	0.36 (±0.32)	0.39 (±0.32)
Nocturia[a], mean (SD)	0.14 (±0.17)	0.14 (±0.18)	0.14 (±0.18)	0.15 (±0.18)	0.17 (±0.19)	0.18 (±0.20)
Urgency[a], mean (SD)	0.18 (±0.18)	0.18 (±0.19)	0.18 (±0.19)	0.17 (±0.18)	0.20 (±0.20)	0.19 (±0.19)
Incontinence[a], mean (SD)	0.09 (±0.16)	0.09 (±0.16)	0.09 (±0.16)	0.09 (±0.17)	0.10 (±0.18)	0.11 (±0.19)
Total ICIQ-OAB symptom score[a], mean (SD)	0.17 (±0.13)	0.17 (±0.13)	0.17 (±0.13)	0.18 (±0.13)	0.19 (±0.14)	0.20 (±0.14)

NO: Nitric oxide; NO_2^-: Nitrite; NO_3^-: Nitrate; n: Number of participants included in the analysis; SD: Standard Deviation; [a] Symptom scores were range standardised on a 0 to 1 scale. Urinary concentrations of NO, NO_2^-, NO_3^-, ATP and ADP were normalised to urinary creatinine concentration.

Figure A2. Associations of nitric oxide (NO), nitrite (NO_2^-), nitrate (NO_3^-) and the ratio of urinary ATP and/or ADP to NO with overactive bladder for male and females. (A) a. Correlation between the urinary levels of NO and participants' total ICIQ-OAB severity scores; b. Correlation between the ratio of urinary ATP to NO and participants' total ICIQ-OAB severity scores; c. Correlation between the ratio of urinary ADP to NO and participants' total ICIQ-OAB severity scores. (B) a. Correlation between participants' urinary ATP and ADP; b. Correlation between the ratio of the combination of urinary ATP and ADP to NO and participants' total ICIQ-OAB severity scores.

Urinary concentrations of NO, ATP and ADP were normalized to urinary creatinine (Cr) concentration. p: p-value. r: Spearman/Pearson r value. P<0.05 are in bold. All data presented on a lin-log plot except Ba, which is shown on a log-log plot.




A
a
p= 0.637
r= 0.052
Total ICIQ-OAB Score
Log ([NO/Cr])
b
p= 0.007
r= 0.329
Log ([ATP/Cr]/[NO/Cr])
c
p= 0.021
r= 0.297
Log ([ADP/Cr]/[NO/Cr])
B
a
p= 0.003
r= 0.442
Log ([ADP/Cr]/[NO/Cr])
Log ([ATP/Cr]/[NO/Cr])
b
p= 0.015
r= 0.364
Total ICIQ-OAB Score
Log ([ATP/Cr*ADP/Cr]/[NO/Cr])