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TITLE: The Urinary Fungal Mycobiome and Host Responses in Patients with Interstitial Cystitis

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CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center

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14. ABSTRACT

The overall hypothesis of the original project was that uncultivated commensal microbial communities and their interactions with the host are associated with the development of IC/BPS and that the resultant inflammatory host responses, assessed in the urine and blood, create a diagnostic signature specific to IC/BPS. We used two complementary approaches to test this hypothesis: (1) state-of-the-art next-generation sequencing (NGS) resources in microbial community profiling to define fungal and bacterial microbiota of IC/BPS patients in comparison to normal subjects, and (2) proteomic analysis of urine and blood from IC/BPS subjects to identify a specific signature correlating with disease phenotype and severity.
We have made significant progress in each of our aims, identifying clinically relevant microbial biomarkers that 1) correlate with IC/BPS, 2) distinguish bladder pain from alternate patterns of pelvic pain refractory to interstitial cystitis treatments and 3) associate with specific inflammatory profiles. We have identified symptom-specific urinary microbial profiles, urotypes, associated with bladder and pelvic pain that are useful as diagnostic biomarkers. In addition, proteomic profiling of IC/BPS subgroups revealed distinct inflammatory associations. These results indicate promising pathways for further research into the mechanisms behind IC/BPS and potential treatment modalities.

15. SUBJECT TERMS

Interstitial Cystitis, Microbiome, Mycobiome, Genomics, catherization, Proteomics, biomarkers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Using blood samples and catheterized samples of urine from both healthy controls and IC/BPS patients, we studied the relationship of changes in the fungal and bacterial communities (urotypes) in the bladder to dysbiosis. We have developed symptom-based classifications of bladder and pelvic pain that correspond to significant associations with characteristic microbial communities to aid diagnosis and treatment. In addition, we examined changes in protein expression patterns in subjects' blood that may serve as biomarkers of disease. This work identifies promising pathways for further research into the mechanisms underlying IC/BPS.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Interstitial Cystitis, Pelvic Pain, Microbiome, Mycobiome, Genomics, Proteomics, Biomarkers

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The overall hypothesis of the original project was that uncultivated commensal microbial communities and their interactions with the host are associated with the development of IC/BPS and that the resultant inflammatory host responses, assessed in the urine and blood, create a diagnostic signature specific to IC/BPS. We additionally proposed that the perturbations in microbial communities associated with IC/BPS would only become evident by careful, objective clinical phenotyping. The original Aims of our PRMRP Discovery Award used two complementary approaches to test this hypothesis: (1) state-of-the-art next-generation sequencing (NGS) resources in microbial community profiling to define fungal and bacterial microbiota of IC/BPS patients in comparison to normal subjects, and (2) proteomic analysis of urine and blood from IC/BPS subjects to identify a specific signature correlating with disease phenotype and severity.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Using a novel approach to pelvic and bladder pain assessment based on objective, symptomatic measures, we have identified clinically relevant microbial biomarkers that 1) correlate with IC/BPS, 2) distinguish bladder pain from alternate patterns of pelvic pain refractory to interstitial cystitis treatments (Appendix Figure 1) and 3) associate with specific inflammatory profiles. We have defined distinctive microbial profiles that define microbial communities associated with specific subtypes of bladder and pelvic pain; unique urotypes are associated with each pain subtype (Appendix Figure 2). This work identifies particular species, *Lactobacillus iners* and *Escherichia coli*, that are significantly associated with distinctive patient/symptomatic groups and types of pain (Appendix Figure 3). Our proteomic analysis of urine and serum markers in cases and controls found clear differences in the patterns of inflammatory markers

associated with individual bacterial species. *E.coli* exhibited large elevations in urinary pro-inflammatory cytokines classically found in bacterial infections. In contrast, *Lactobacillus iners* had minimal urinary inflammation, instead exhibiting elevations in specific plasma cytokines. The distinctive inflammatory profiles for each urotype suggest unique patho-physiologies for the pain manifesting in each patient subgroup. We developed and validated a rapid and inexpensive qPCR method using these bacterial associations that can be developed to aid physicians in diagnosis and treatment of IC/BPS.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The Principal Investigator continues to participate in regular, professional conferences of the American Urology Association (AUA) and the Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction (SUFU). In addition, she has attended the meetings of the Western Section of the AUA. She completed an intensive course in Biostatistical Analysis of Microbial Populations at the Marine Biology Laboratory (Woods Hole, MA) in the summer of 2018. The PI is developing knowledge of statistical analyses and has gained proficiency with analytical software for microbial analysis, such as the R Studio integrated development environment and QIIME. The PI has also completed the Introduction to Data Science course at UCLA extension and is using her knowledge of several programming language, such as PYTHON, shell scripting, and SQL in data analysis for this study. The PI has also continued to develop these skills independently through the online learning platform, DataCamp, to expand familiarity and flexibility with these programming languages for health science statistical applications. The PI has continued to meet regularly with the mentors, Drs. Freeman and Anger, one-on-one on a regular bi-weekly schedule throughout the award period, to discuss project progress, future directions, study design, career support and progression and laboratory management. The PI also regularly participates in weekly immunology and urology conferences and seminars, including the research-in-progress series, journal clubs, microbiome club, urology grand rounds, Female Pelvic Medicine and Reconstructive Surgery rounds and didactics, and women's health center seminars.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Components of this work have been presented at the Annual Meetings of the American Urological Association (AUA), Society of Urodynamics, Female Pelvic Medicine and Reconstructive Surgery (SUFU), Association of Clinical and Translational Science (ACTS), Mediterranean Incontinence and Pelvic Floor Society (MIPS), Urobiome Annual Meeting, and AUA Western Section as well as in invited lectures at Duke University, University of California-Los Angeles, Beth Israel Deaconess Medical Center, Yale University, Virginia Commonwealth University, University of California-San Diego, and Cedars-Sinai Medical Center.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have developed testing for a useful, non-invasive biomarker that could revolutionize diagnosis and management of patients with pelvic pain, which can be an elusive and debilitating condition. The process potentially provides a rapid, inexpensive and, most importantly, definitive indicator that appears to correlate with disease, which will require further validation in a multi-institutional clinical population. As preliminary data indicates that the phenotypes identified by biomarker classification can also predict treatment responses, future studies will explore the utility of this biomarker in determining the optimal individual treatments for each patient population. The novel approach to pelvic and bladder pain assessment based on objective, symptomatic measures in combination with microbial biomarkers has the capability to separate patients into distinct sub-groups that will assist both physicians and researchers and has the potential to reduce the confounders often limiting research into IC/BPS.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The development of this technique dispels significant confusion in the diagnosis of patients with pelvic pain symptoms which will strongly impact a wide range of specialists and general practitioners. Patients with pelvic pain often seek multiple consultations from a wide range of specialists including urology, neurology, gynecology, and gastroenterology, because the nature of the pain is vague and frequently misattributed to a variety of pelvic origins. This work develops a test to identify subsets within the pelvic pain population that can more easily be directed to the appropriate specialist for care. For one group of these patients with significant gynecological symptoms, we can now direct patients into a more effective pathway for treatment, while patients with bladder-centric pain will be able to find appropriate care within urologic offices offering bladder-directed therapies.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*

- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

This research opens a path to a simple, point-of-care assay to guide the treatment of pelvic pain. We hope that future research will help to develop this moving forward to allow for clinical use and the incorporation of this assay into clinical evaluation of pelvic pain to better guide treatment decisions.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

The identification of a biologic foundation for a condition that has been considered a diagnosis of exclusion that many MD's consider a functional or even psychiatric, not physiologic, disease provides validation to a distressed population which has been historically marginalized.

Having a better understanding of the physiology behind this condition will help both prevention and care by non-specialty physicians, providing them with a tool to direct treatment, and will help researchers narrow study parameters to more homogeneous populations, facilitating a better understanding of the underlying etiologies of pelvic pain that could lead to new treatments or even prevent its development in the first place.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We have noted previously issues connected to the reliability of species level identification by next-generation sequencing (NGS). We therefore decided to confirm the accuracy of NGS data by external deep sequencing of an independent ribosomal locus. It was also necessary to overcome the potential variations resulting from any particular analytic pipeline variations to NGS. Dr. Ackerman's additional training in microbial bioinformatics and data science obtained with the assistance of this grant allowed her to understand these limitations and develop a more sophisticated and reliable microbial analysis. For example, we have replaced the use of Operational Taxonomic Units (OTUs) with Amplicon Sequence Variants (ASVs) to resolve variations in sequences that have allowed more reliable and specific microbial assignments. We also developed quantitative PCR approaches to identify and confirm species that could not be well defined by NGS analysis. This method was used alongside the next-generation sequencing (NGS) analysis originally proposed. In the last six months, we have confirmed the reliability of these techniques to duplicate/confirm the NGS findings and provide a greater specificity confirming the species-level identities of critical microbes altered in pelvic pain.

We had not originally anticipated the divergent microbiota between pre- and post-menopausal women, which has necessitated their analysis as separate populations. We have responded by focusing on the recruitment of pre-menopausal women, who are more homogeneous and have fewer confounding health conditions. We hope that future studies will be able to examine the applicability of this study's findings to other populations, such as post-menopausal women and men. Determination of the appropriate power to determine differences in this more narrow population indicated that 160 subjects would adequately power this study to identify changes of approximately 20% abundance between cases and controls. Therefore, we revised our recruitment goal to 200.

Our strict exclusion criteria excluding antibiotic usage prior to study enrollment, while necessary, initially disqualified a substantial proportion of eligible patients. We have engaged in a health system-wide didactic program for referring providers to encourage more selective use of antibiotics in patients with chronic pelvic symptoms. In addition, with the addition of two new specialty providers to our practice, we have further increased our patient volume, which has allowed us to establish a specialized pelvic pain clinic streamlined for the standardized care and research recruitment of IC/BPS patients. As a result, patient recruitment has increased substantially. We recruited 200 patients for our original study; since completing that analysis, we have recruited 65 additional patients, which is a dramatic improvement in recruitment rate.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Not Applicable

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Ackerman AL, Lai HH, Parameshwar PS, et al. Symptomatic Overlap in Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome - development of a new algorithm. BJU Int. 2019 Apr;123(4):682-693
2. Ackerman AL, Anger JT, Khalique MU, et al. Optimization of DNA extraction from human urinary samples for mycobiome community profiling. PLoS One. 2019 Apr 25;14(4).
3. Ackerman AL, Underhill DM. The mycobiome of the human urinary tract: potential roles for fungi in urology. Ann Transl Med. 2017 Jan;5(2):31.
4. Ackerman AL and TC Chai. The Bladder is Not Sterile: an Update on the Urinary Microbiome. Curr Bladder Dysfunct Rep. 2019 Nov; 14(2):1-11.
5. Ackerman AL, Khalique MU, Caron AT, et al. Microbiological phenotyping in bladder and pelvic pain reveals distinct clinical profiles with implications for prognosis and treatment. *Manuscript in preparation.*
6. Ackerman AL, Cheng Z, Lagree K, et al. Identification of a diverse mycobiome within the urinary tract of females that is altered in interstitial cystitis/bladder pain syndrome. *Manuscript in preparation.*

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Kuhlmann PK, Ackerman JE, Khalique MU, Caron AS, Kanji F, Anger JT, Eilber KS, Underhill DM, **Ackerman AL**. Urinary Aerococcus Defines a Severe, Treatment-refractory Phenotype of Urge Incontinence in Older Women. American Urological Association Annual Meeting, May 2020.
2. Dallas K, Caron AS, Anger JT, Eilber KS, Ackerman AL. Application of Machine Learning Algorithms to Classify Storage Lower Urinary Tract Symptoms. American Urological Association Annual Meeting, May 2020.
3. Ackerman AL, Khalique MU, Ackerman JE, Cheng Z, Eilber KS, Anger JT, Underhill DM. Microbial Composition Defines Pelvic Pain Phenotypes in Reproductive-age Women. American Urological Association Annual Meeting, May 2020.
4. Ackerman AL, Khalique M, Ackerman J, Cheng Z, Eilber K, Anger J, Underhill D. Microbial Composition Defines Pelvic Pain Phenotypes in Reproductive-Age Women. SUFU Annual Meeting; 2020 February; Scottsdale, AZ, USA.
5. Dallas K, Anger J, Caron A, Eilber K, Ackerman AL. Validation of the Diagnostic Accuracy of Diagnostic Groupings of Patients with Storage Lower Urinary Tract Symptoms Generated by Machine Learning Algorithms. SUFU Annual Meeting; 2020 February; Scottsdale, AZ, USA.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

We described an improved method for the purification of microbial DNA from clinical urine samples for deep sequencing analysis/microbial profiling of communities. This was published in a peer-reviewed article (Ackerman AL, Anger JT, Khalique MU, et al. Optimization of DNA extraction from human urinary samples for mycobiome community profiling. PLoS One. 2019 Apr 25;14(4)) as well as on the open access website, protocols.io (https://www.protocols.io/view/protocol-for-dna-extraction-from-urine-wgsfbwe).

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

We are currently preparing a patent application for the rapid qPCR-based assay for urinary microbial profiling. (Patent application in preparation).

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Dr. A. Lenore Ackerman no change

Dr. Michael Freeman no change

Dr. Jennifer Anger no change

Dr. Wei Yang no change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

The PI was recently awarded a Mentored Clinical Scientist Research Award from the NIH NIDDK (1K08DK118176-01A1).

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Not Applicable

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix A: Figures (manuscript in preparation)

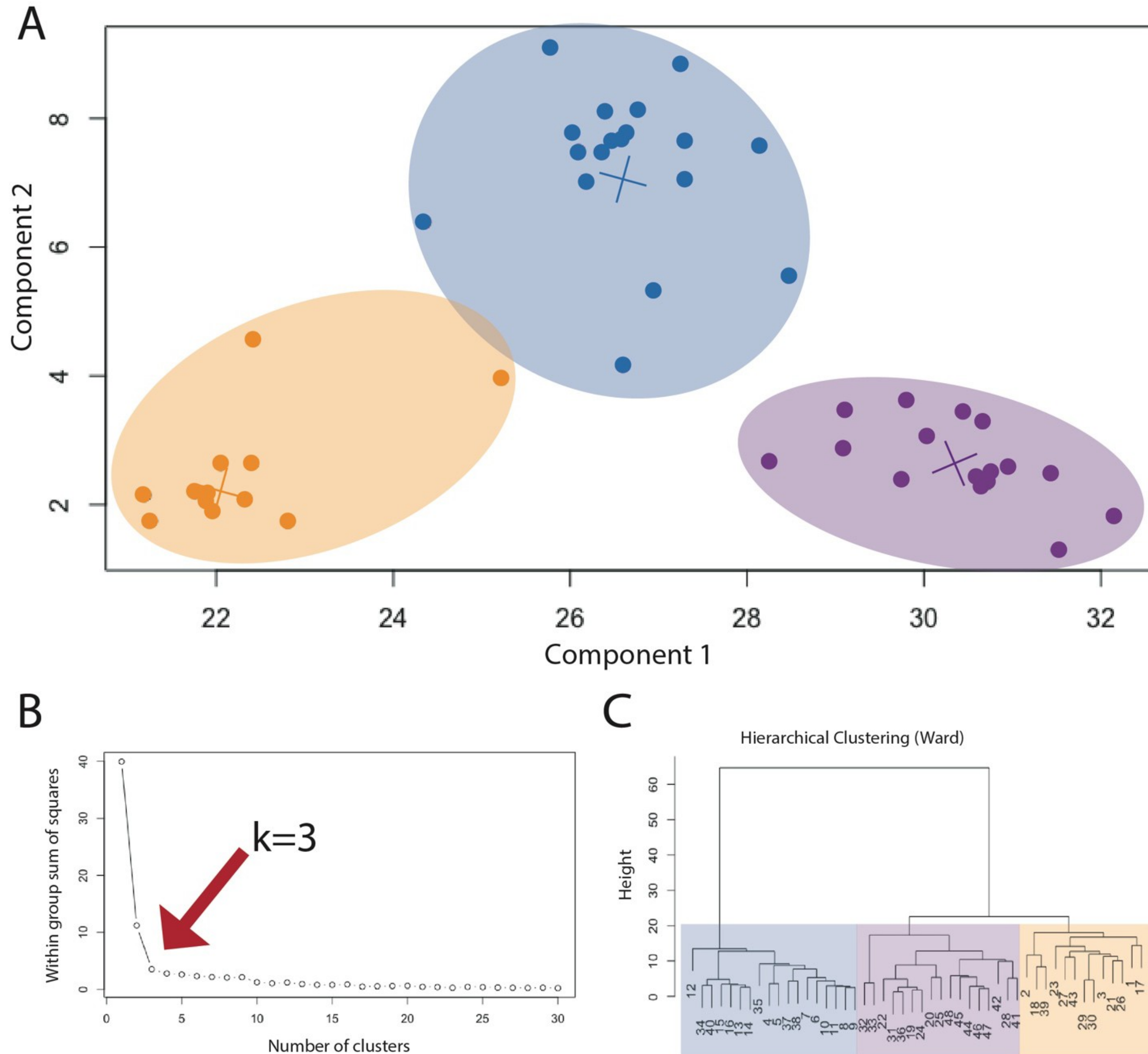

Figure 1. *K means clustering to categorize bacterial communities in the validation subset of pre-menopausal female subjects.* To classify bacterial communities into categories, with each category containing similar community patterns or "states" which differ from the individuals in other categories, we utilized a k-means algorithm of unsupervised clustering for an independent group of subjects. No demographics or clinical information was included in the clustering. (A) Centroid plot for the three-cluster solution demonstrating clear clustering of bacterial communities into three groups, corresponding to the L. iners-containing (purple), other Lactobacilli (orange) and Escherichia-containing (blue) groups. (B) The value of the number of clusters, k, was determined through successive solutions that increment the value of k by 1, with the chosen solution being the minimum number of groups that yields the most meaningful cluster profiles, indicated by the red arrow for k=3. (C) Dendrogram visualizing the order and distances of subjects for merges during the hierarchical clustering by Ward's method.

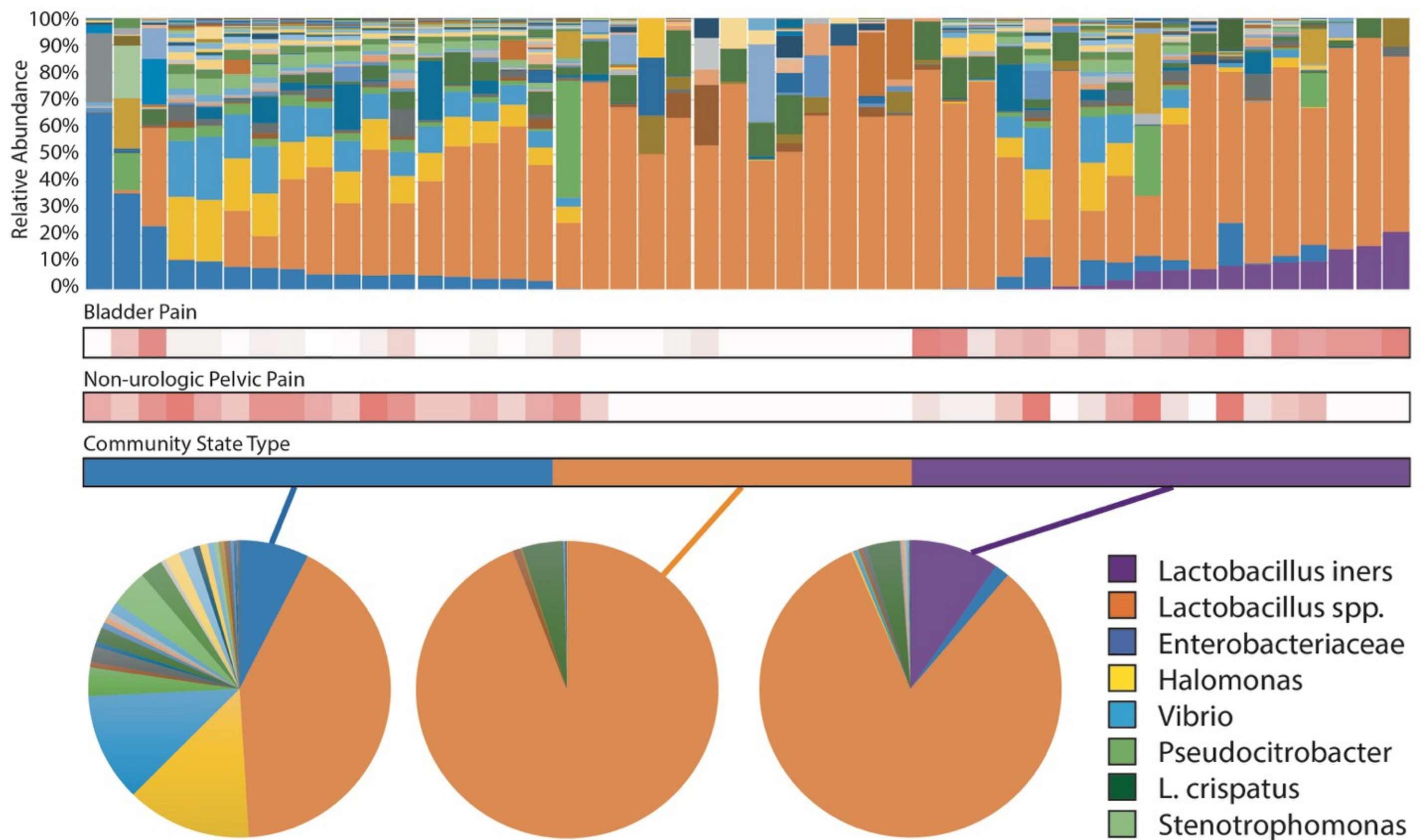

Figure 2. *Correlation of microbial patterns with pain phenotypes.* Individual urinary bacterial compositions of 49 pre-menopausal subjects with pelvic pain are shown as stacked box plots and classified into three unique "urotypes" (see color bar). *Lactobacillus iners* (in purple) was present in patients with bladder-specific pain and *Enterobacteriaceae* (blue) in subjects with non-urologic pelvic pain. In the heat bars below the plot, increasing red indicates higher pain levels. The remaining group, displaying predominantly mixed Lactobacilli (orange), lacked either clinical feature. The pie charts below indicate the median microbial composition for each phenotype.

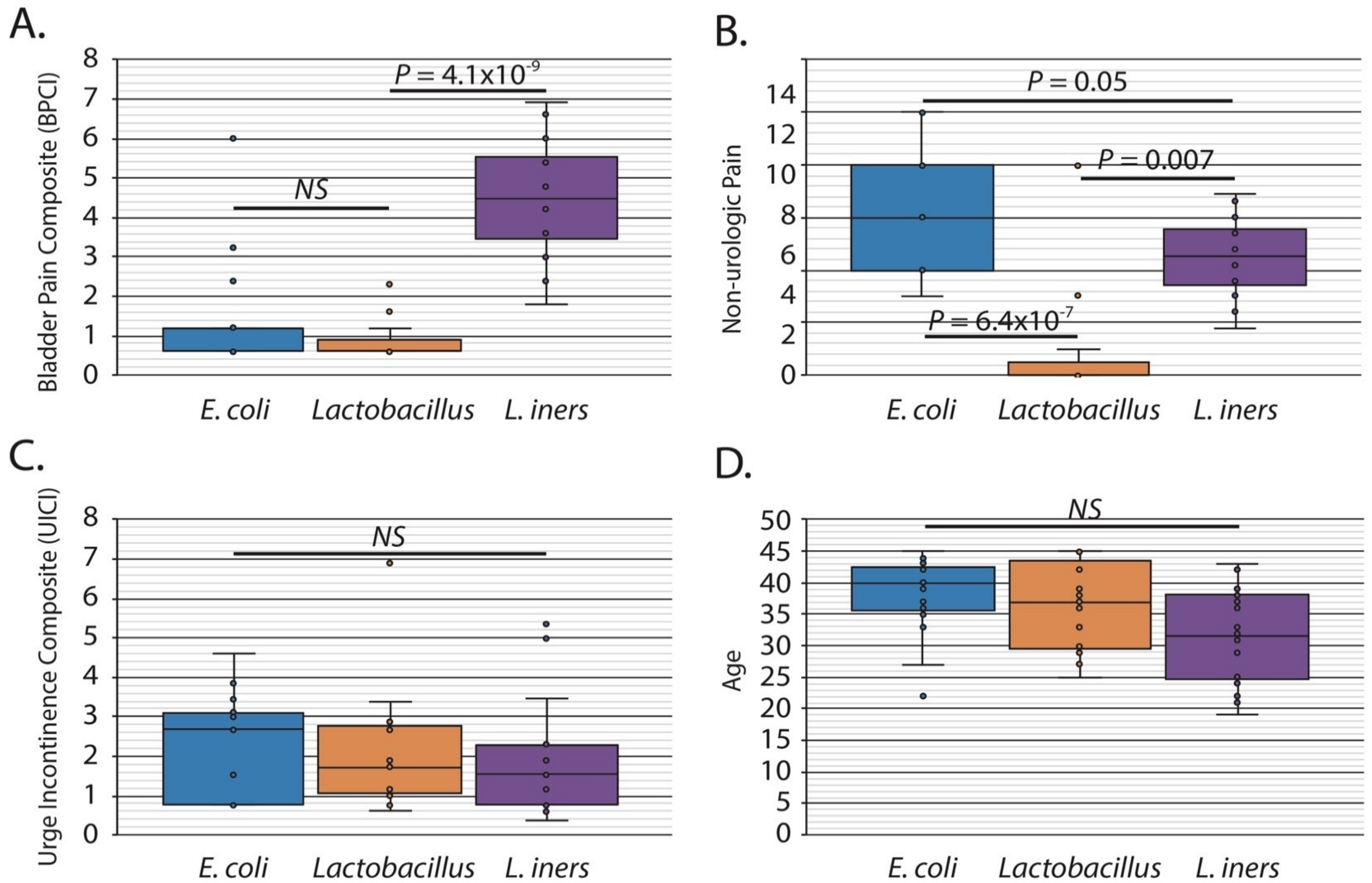

Figure 3. *Clinical features of pelvic pain patients by bacterial profiling.* Individual clinical features for presumptive IC/BPS patients are plotted as box and whisker plots. While patients were indistinguishable by pain scores on the Genitourinary Pain Index (A), subjects with different urinary bacterial profiles exhibited distinct pain patterns. The *L. iners* group (BPS; purple) was homogenously high for isolated bladder pain (A), while the *Escherichia* group (NUPP; blue) demonstrated pelvic pain unrelated to urination (B). The other Lactobacilli group (MFP; orange) had features of myofascial pain, recognized only on exam. None of these groups exhibited clinically relevant urgency incontinence (C). NS: not significant.

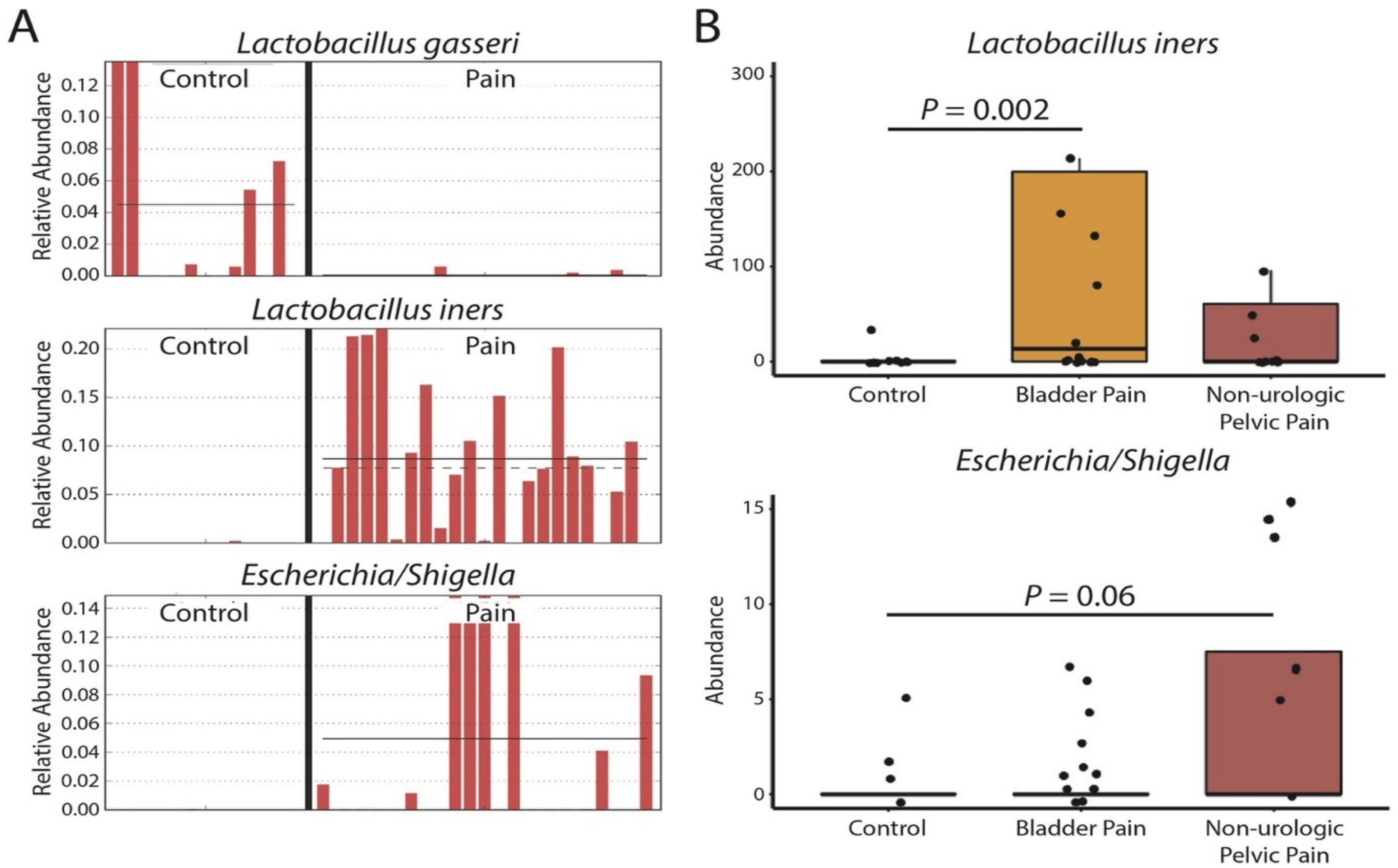

Figure 4. EC *and* LI *are discriminatory for pain.* A) Each panel indicates relative abundance of designated taxa for each subject. All patients with non-MFP pelvic pain exhibited detectable LI, EC, or both, while little of either could be detected in asymptomatic subjects. B) Subjects were separated into BPS, NUPP, and asymptomatic groups. Box and whisker plots of relative abundances for each symptom subgroup confirms the association of *LI* with BPS and *EC* with NUPP, respectively.

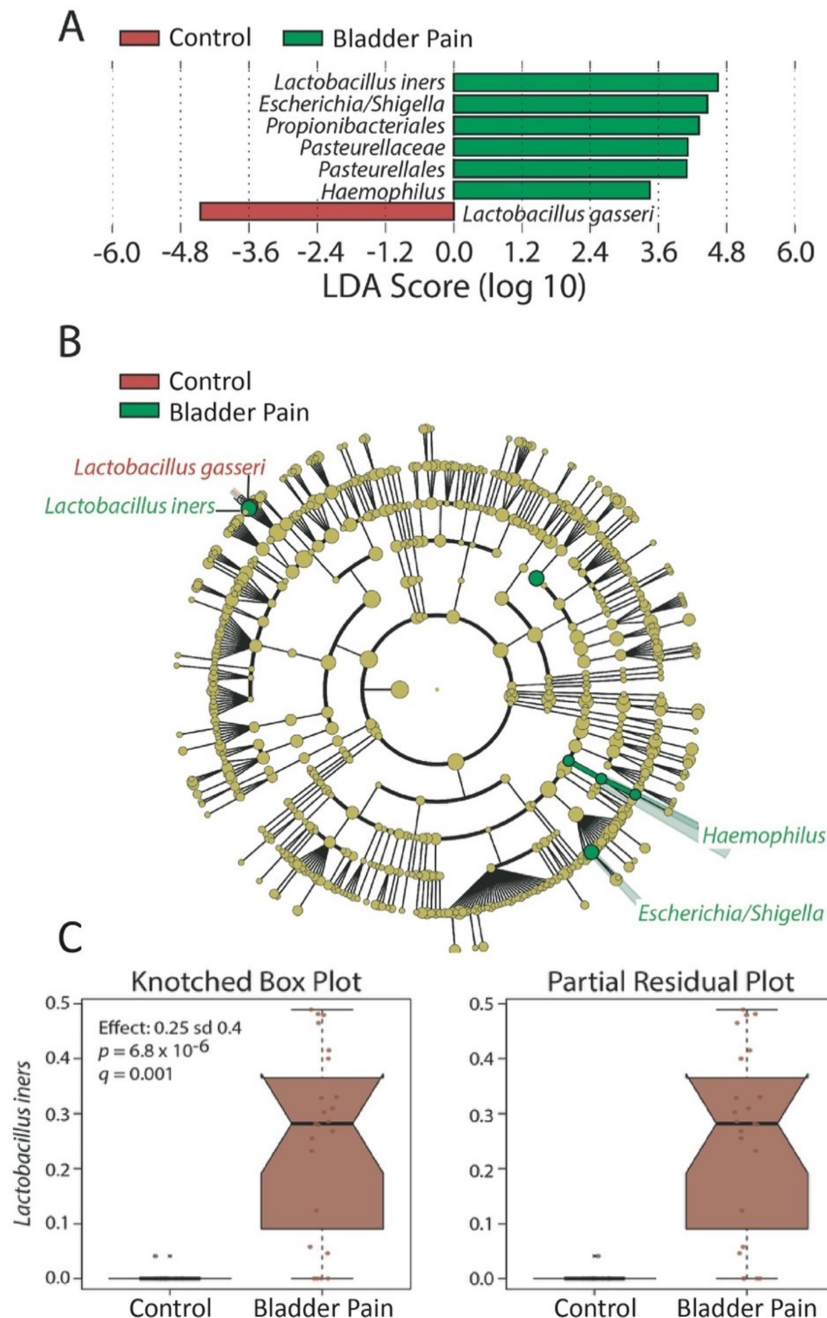

Figure 5. *Microbial associations persist even after correction for confounders and for multiple comparisons.* (A) Linear discriminant analysis (LDA) assesses the potential of specific taxa as biomarkers for bladder/pelvic pain. Differential bacterial features are ranked by linear discriminant analysis effect size (LEfSe), with green and red indicating associations with pain or the asymptomatic state, respectively. (B) The LDA is alternatively represented on a phylogenetic tree as a cladogram. (C) Multivariate Association with Linear Models (MaAsLin) Analysis confirmed the association of *Lactobacillus iners* with bladder pain. Notched box plots demonstrate the difference in *Lactobacillus iners* abundance in controls and the bloadder pain subgroup after controlling for age, BMI, and hormonal supplementation. Neither *Haemophilus* nor *Escherichia/Shigella* was associated with bladder-specific pain.

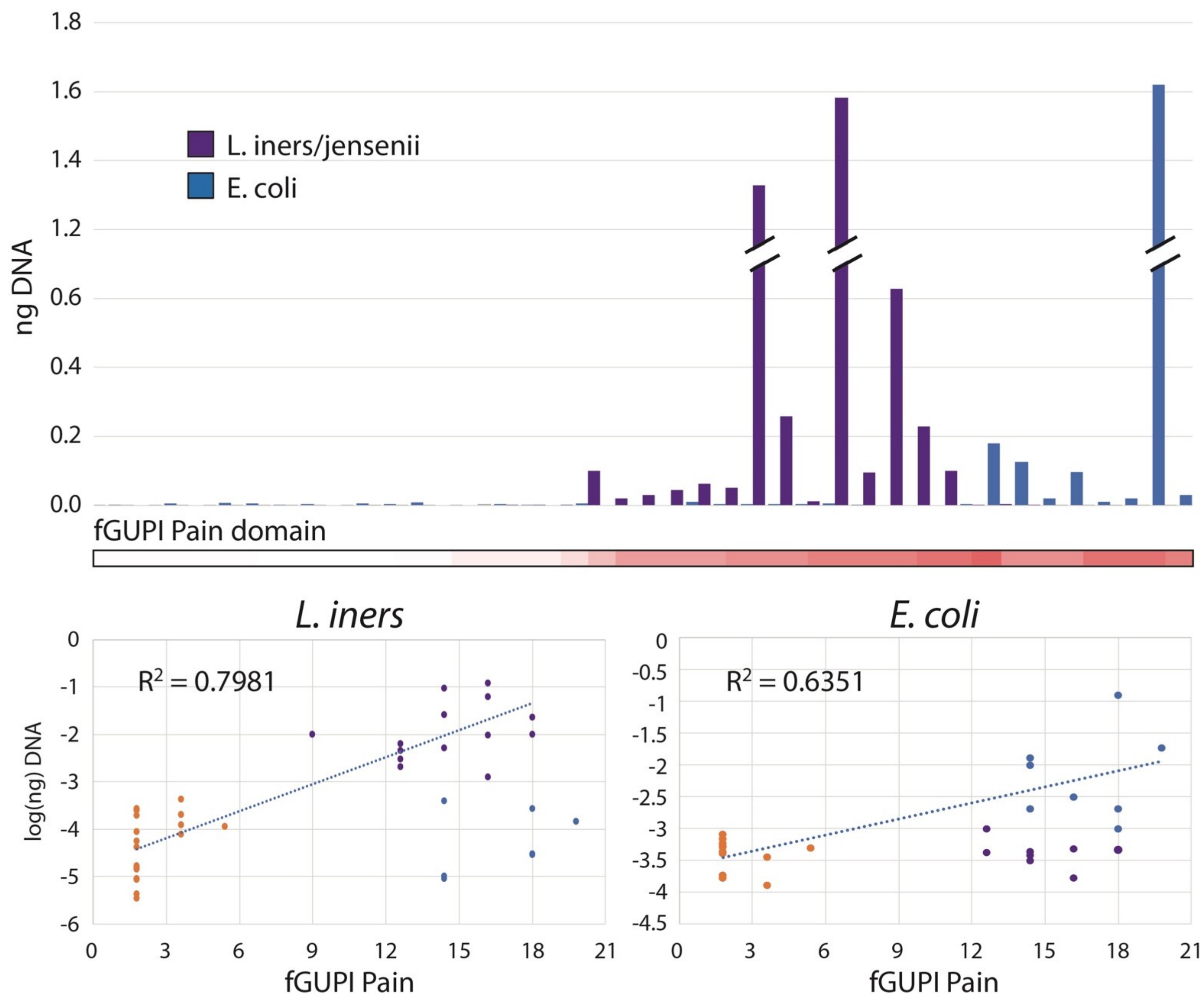

Figure 6. *Quantitative PCR provides rapid, scalable testing with objective thresholds to facilitate patient diagnosis and sub-classification.* Using the female Genitourinary Pain Index (fGUPI) as an indicator of patients presenting with and without pain, quantitative PCR detecting *Lactobacillus iners* and *Escherichia coli* can accurately separate patients with genitourinary pain syndromes from asymptomatic controls. In addition, these two microbial markers alone could subclassify unselected patients into bladder pain and non-urologic pelvic pain groups with diagnostically useful thresholds. As shown in the scatter plots (bottom), thresholds of absolute DNA quantities for each species in each specimen could clearly discriminate between symptomatic and asymptomatic subjects.

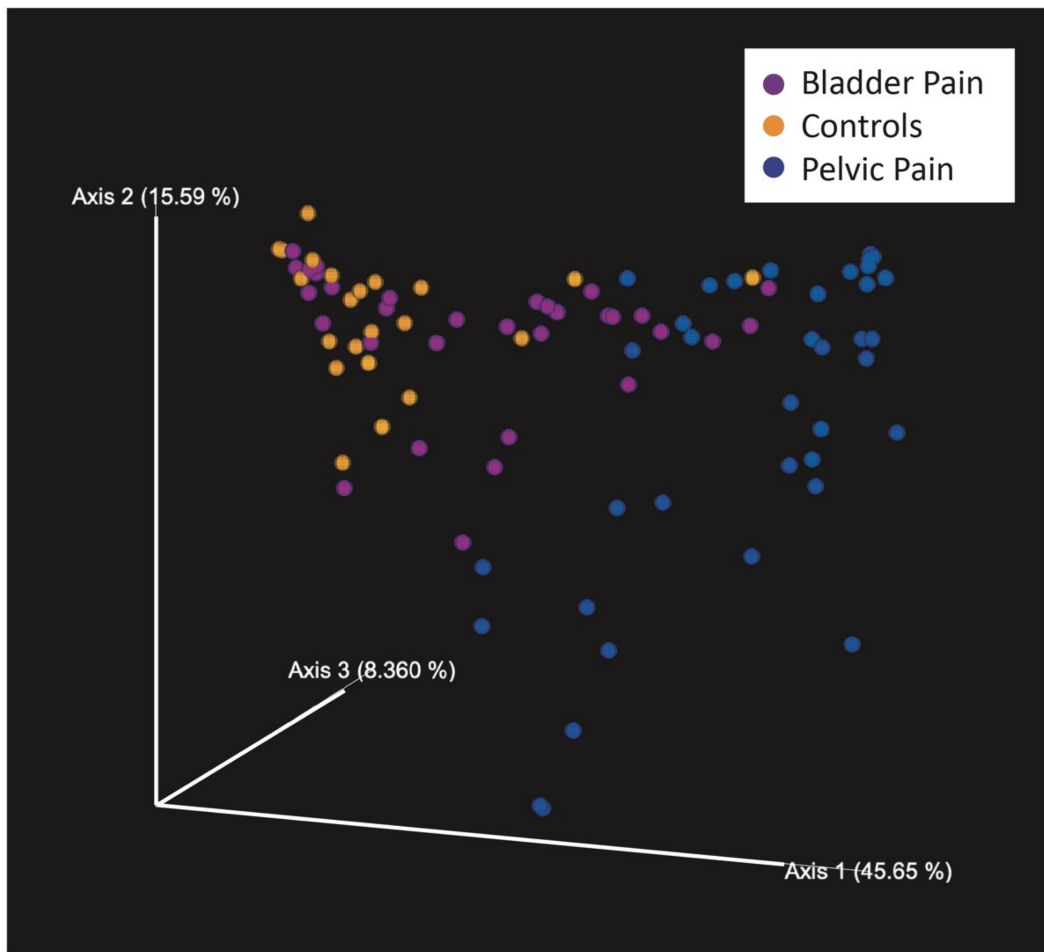

Figure 7. *Principle component analysis by Unifrac Distance for the entire population.* Bacterial communities in bladder pain (purple and asymptomatic controls (orange) are similar overall, differing primarily at the species level. Both of these groups, however, differed substantially from communities in patients with non-urologic pelvic pain (blue). This difference suggest pelvic pain may occur in the context of a local environmental shift that results in this bacterial divergence.

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Symptomatic overlap in overactive bladder and interstitial cystitis/bladder pain syndrome: development of a new algorithm

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Objectives

To address challenges in the diagnosis and classification of storage lower urinary tract symptoms (LUTS), we sought to define the fundamental features of overactive bladder (OAB) and interstitial cystitis/bladder pain syndrome (IC/BPS), two conditions with considerable symptomatic overlap. Through retrospective comparison of self-reported symptoms in women with a range of clinical presentations and symptom severities, we have attempted to refine the diagnostic features of OAB and IC/BPS and to develop a novel clinical nomogram to improve patient screening and classification.

Materials and Methods

We performed a univariate analysis comparing responses to the female Genitourinary Pain Index (fGUPI), the OAB Questionnaire and O'Leary–Sant Indices (the Interstitial Cystitis Symptom Index and Interstitial Cystitis Problem Index) in an initial cohort of 50 patients with OAB, patients with IC/BPS and control subjects. Only eight questions differed significantly between the IC/BPS and OAB groups; we used five unique questions and three measuring bother to generate a novel composite scoring system and nomogram that included urgency incontinence, bladder pain and symptomatic bother domains to differentiate these populations, which was validated in a second cohort of 150 patients. The addition of a self-reported bother index resulted in the creation of a diagnostic algorithm to identify and classify LUTS clusters across the total population.

Results

While all validated questionnaires could distinguish between controls and patients with storage LUTS, no combined symptom scores differed significantly between the IC/BPS and OAB groups. These results are reflective of the prevalence of significant bladder pain (35%) in patients with OAB and the presence of urge incontinence (25%) in patients with IC/BPS. Only the fGUPI pain domain scores differed between patients in the OAB and IC/BPS groups, but it was not accurate enough for diagnostic evaluation (68% accuracy). Our composite scores and nomogram gave a much-improved diagnostic accuracy (94%) and demonstrated utility as a screening tool to identify storage LUTS in patients presenting for unrelated complaints, e.g. microhaematuria.

Conclusions

There is significant overlap of urinary tract symptoms between OAB and IC/BPS. We present a novel algorithm that provides a binary output capable of guiding clinical diagnosis. Future studies aimed at assessing the diagnostic value of novel classification schemes that address symptoms rather than specific diagnoses may improve patient prognosis.

Keywords

interstitial cystitis, overactive bladder, diagnosis, questionnaires, bladder hypersensitivity, #InterstitialCystitis, #OAB

Introduction

Storage LUTS, which include urinary urgency, frequency, nocturia and bladder pressure/discomfort, refer to patient experiences when the bladder is unable to function normally as a urine reservoir. More than 50% of people experience at least one of these symptoms [1], which tend to be chronic and debilitating, and degrade physical and social activity and quality of life [2]. These symptoms are responsible for an economic burden of > $80 bn/year in the USA alone [3]. Appropriate treatment continues to be limited by challenges in identifying and classifying these conditions [4].

Interstitial cystitis/bladder pain syndrome (IC/BPS) and overactive bladder (OAB) are two prevalent conditions presenting with storage LUTS. These two syndromes lack any definitive diagnostic tests or markers and are defined by the collection of symptoms experienced and reported by patients

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[5,6], making diagnosis subjective and clinician-dependent. IC/BPS is characterized predominantly by bladder pain, including the subjective sensations of 'pressure' and 'discomfort', whereas the hallmark symptom of OAB is urinary urgency [5]. The presence of bladder pain in OAB or urinary urgency/urgency incontinence in IC/BPS had been considered rare [7], but recent evidence suggests considerable symptom overlap between IC/BPS and OAB [8]. Patients with IC/BPS commonly present with urgency, frequency, and nocturia in addition to pain [5,9], and many will also describe urgency incontinence. In fact, patients with IC/BPS can initially present with urgency or frequency symptoms alone, and may not develop pain for years [10]. Some investigators suggest that clinicians should suspect IC/BPS in patients diagnosed with OAB who are refractory to treatment with anticholinergics [11,12]. Patients with OAB are also heterogeneous in nature, with some exhibiting urgency and frequency only (OAB-dry) and others displaying urgency and frequency with urgency incontinence (OAB-wet) [13,14]. A third of patients with OAB complain of some pain with bladder filling, complicating simplistic divisions between the two conditions [8].

Conventional theory is that these two symptom clusters represent different disease pathologies. OAB is a disorder of detrusor and/or urothelial function, in which urgency is the result of abnormal muscular contractions and/or an afferent sensory disorder, while in IC/BPS, urgency results from visceral allodynia, in which lowered pain thresholds lead to bladder hypersensitivity [5,7,15–17]. The ways in which patients with IC/BPS or OAB describe urgency, however, do not seem to differ significantly between the two populations [15]. Urgency symptoms in particular have been a confounding feature because of their incidence in both conditions [5,18] and the variety of words patients use to describe bladder sensations. The terms bladder pain, urgency and frequency are language of the urologist, and are poorly understood by the average patient. Unprompted, patients tend not to use the term 'urgency' to describe a need to void, but describe a variety of other feelings, such as tingling or pressure [19].

This subjective terminology, substantial symptom overlap, and lack of objective diagnostic testing make differentiating IC/BPS from OAB challenging in some patients. Hence, some patients are under-treated or misdiagnosed [20], which is problematic as treatment strategies differ between the conditions [5,6]. While several validated questionnaires are routinely used to assess symptom severity in both IC/BPS and OAB, few studies have evaluated the extent to which these questionnaires can differentiate between patients exhibiting similar symptoms. These tools have limited utility, therefore, for the screening or categorization of patients with storage LUTS.

To address these limitations, we sought to quantitate the overlap between conditions to define the fundamental and unique features of each syndrome through a retrospective comparison of self-reported symptoms in women with a range of clinical presentations and symptom severities. We then used these distinctions to define a novel diagnostic nomogram for classification of storage LUTS and to provide a perspective on how these results may lead to innovative clinical approaches to patients with these symptoms.

Materials and Methods

Participants and Procedures

Under Cedars-Sinai IRBPro00040261, 200 consecutive women, including patients diagnosed with IC/BPS and OAB as well as asymptomatic controls, seen in a tertiary Female Pelvic Medicine and Reconstructive Surgery (FPMRS) practice between June 2015 and June 2016 were retrospectively enrolled in the study. Patients with IC/BPS and OAB were diagnosed by a board-certified FPMRS specialist based on the AUA and ICS definitions, respectively. Patients with diagnoses of both OAB and IC/BPS, only two of which were identified, were excluded to avoid confounding. Control subjects included those evaluated for conditions without urinary symptoms, such as microscopic haematuria. Patients with UTI, prior third-line therapies (e.g. onabotulinum injection or sacral neuromodulation), an active smoking habit, current pregnancy, cyclic pain at menses, diabetes, and neurogenic bladder were excluded from the study. Patients with other comorbid functional pain syndromes, such as irritable bowel syndrome or fibromyalgia, were included. The 200 patients were divided into an experimental cohort of the first 50 patients and a validation cohort of the remaining 150 patients. *Post hoc* confirmatory chart review excluded five additional patients, leaving a total of 48 and 147 patients in the experimental and validation cohorts, respectively (Fig. 1).

The patients included in the study completed three validated, written questionnaires, the female Genitourinary Pain Index (fGUPI) [21], the OAB Questionnaire (OAB-q) [22], and O'Leary-Sant Indices, which include the Interstitial Cystitis Symptom Index (ICSI) and Interstitial Cystitis Problem Index (ICPI) [23]. The fGUPI measures severity of genitourinary pain, containing subscales assessing pain, urinary symptoms, and quality of life [21]. The OAB-q measures both continent and incontinent OAB symptoms, containing subscales for symptom bother, coping behaviours, concern/worry, social interaction, sleep, and health-related quality of life [22]. The ICSI and ICPI are commonly used together to measure the severity of and bother associated with urinary frequency, urgency, nocturia, and bladder pain [23].

After a complete history, physical and diagnostic evaluation, patients were categorized as controls, IC/BPS, or OAB according to physicians board-certified in both Urology and FPMRS. Retrospective chart review also identified

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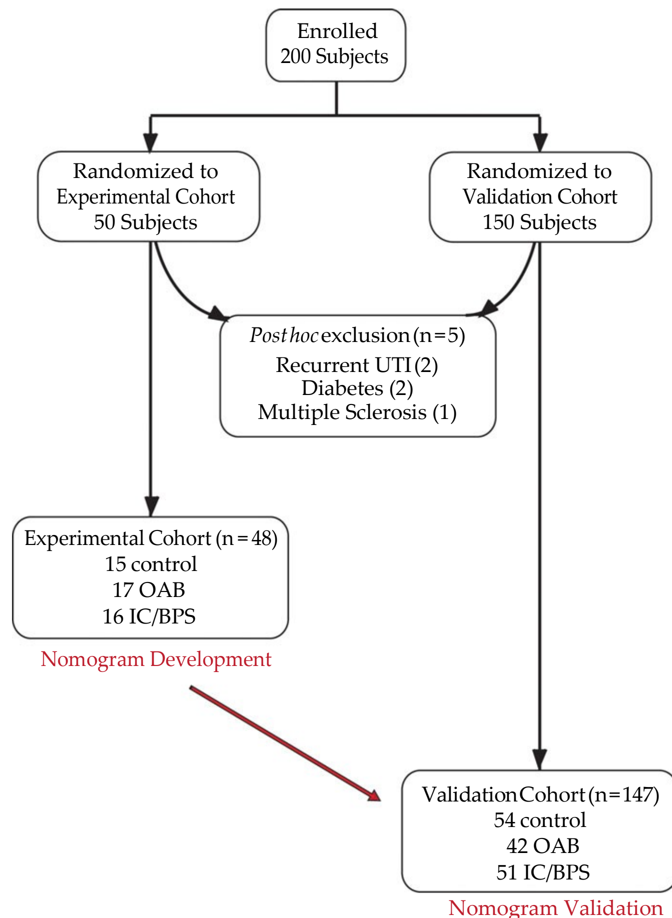

Fig. 1 Flow diagram of patients enrolled in this study. The first 50 of 200 consecutive patients with overactive bladder (OAB) and interstitial cystitis/bladder pain syndrome (IC/BPS) as well as asymptomatic controls were designated as the experimental cohort, the remaining 150 became the validation cohort. *Post hoc* chart review revealed exclusion criteria in five of the total patient population.

demographic data, including age, parity, menopausal status, self-described race, body mass index (BMI), and comorbidities.

Derivation of Novel Composite Symptom Indices and Nomogram

Statistical analysis was performed using SPSS version 22. Initial evaluation of the experimental group was performed using ANOVA and Student's *t*-test with Bonferroni correction for multiple comparisons. Of the eight questions identified as differing significantly between the IC/BPS and OAB groups, numerical responses to these questions were classified into either the bladder pain ($n = 5$) or urgency categories ($n = 3$).

Fisher's exact test was used to determine the interdependency of the responses to questions related to pain. From these values, we chose the two questions (ICSI 4 and fGUPI 2C; Fig. 2) that demonstrated the least interdependency to generate the Bladder Pain Composite Index (BPCI). All three urgency questions were used to generate the Urgency Incontinence Composite Index (UICI). Combinations of these variables were tested in both their native format and with a variety of weighting in an iterative fashion, using area under the receiver-operating characteristic curve to quantify the predictive accuracy of each index independently. To confirm the validity of exclusion of the three interdependent questions, we subjected the optimally weighted BPCI to a second set of iterations including all five questions, without any improvement in predictive accuracy performance. We then identified the most informative thresholds for each index using the *P* value approach [24].

Using the two novel composite indices, backward selection of an appropriate model relied on the Akaike Information Criterion [25] to identify the most accurate multivariate predictor for both conditions concurrently. The most informative model was depicted in a nomogram format to illustrate the distribution of patient phenotypes relative to the bladder pain and urgency incontinence symptom domains (Fig. 3). For internal validation, we determined the positive and negative predictive values and predictive accuracy for each patient group as well as the overall population within the validation cohort. This model, however, provided poor discrimination of the asymptomatic controls from the OAB cases, requiring addition of a third variable assessing symptomatic bother, the fGUPI quality-of-life subdomain, which is the sum of fGUPI questions 7–9. Beginning with the experimental cohort and after determination of the most informative threshold for this variable using the *P* value approach, the nomogram was combined with the binary output from this quality-of-life variable to create a final algorithm to classify patients as asymptomatic, as having OAB, or as having IC/BPS (Fig. S1). Finally, internal validation was repeated. Calibration plots were used to explore graphically the correlation between predicted and observed diagnoses.

An a value of 5% was considered as the threshold for significance. Data are typically presented in box and whisker plots to visualize the median (line inside the box), mean (X), interquartile range (box) and outliers (whiskers).

Results

An experimental cohort of 48 patients served as a pilot population to determine potential features distinguishing OAB and IC/BPS, while the validation cohort of 147 patients facilitated confirmation of the findings seen with the experimental group.

Patient demographics of the final cohorts (Table 1) demonstrated significant differences in these aggregate populations in terms of age and BMI. The OAB population

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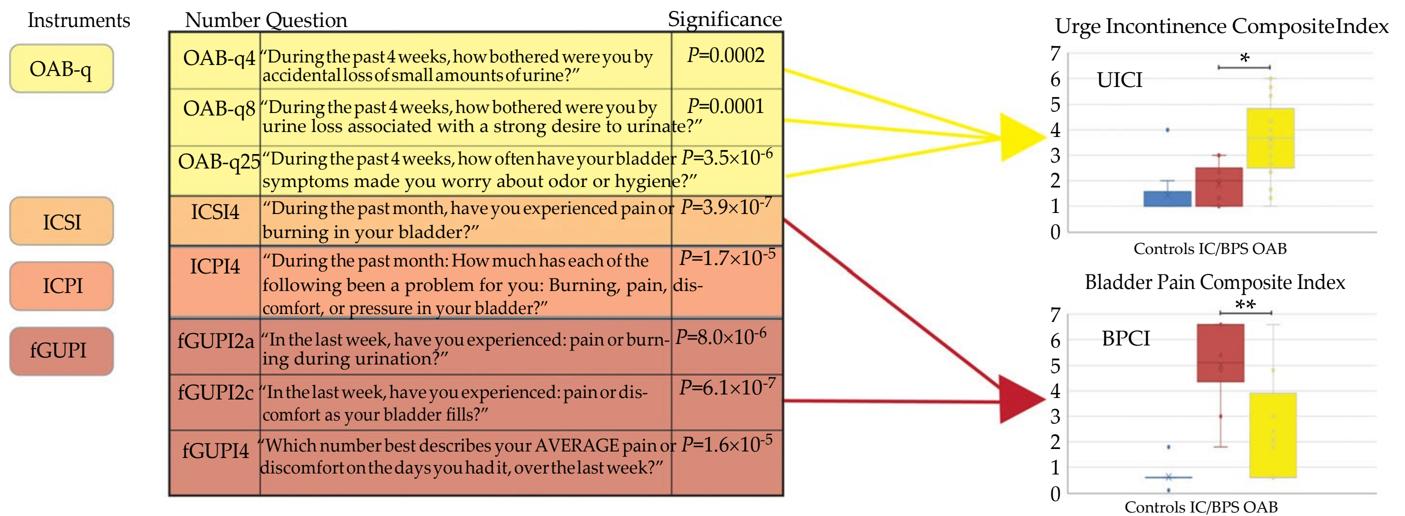
Fig. 2 Urge incontinence and bladder pain composite scores describe the unique features of overactive bladder (OAB) and interstitial cystitis/bladder pain syndrome (IC/BPS). We compared the results of 56 individual questions and nine subtotal and total scores on the OAB Questionnaire (OAB-q), Interstitial Cystitis Symptom Index (ICSI), Interstitial Cystitis Problem Index (ICPI) and female Genitourinary Pain Index (fGUPI) questionnaires for patients with IC/BPS and OAB. Only eight questions differed significantly between these populations after adjustment for multiple corrections (left), generally assessing two unique symptoms, urge incontinence and bladder pain, which we formulated into a novel composite scoring system. The three urge incontinence questions (yellow arrows) formed the Urgency Incontinence Composite Index (UICI), which exhibited elevated scores for the OAB patients alone, while two of the non-redundant questions addressing bladder pain (red arrows) were formulated into the Bladder Pain Composite Index (BPCI) that demonstrated elevated values in the IC/BPS patients. Box and whisker plots (right) demonstrate the means, medians, and ranges of each index for control patients, and patients with OAB and IC/BPS in the total population, demonstrating good differentiation of the patient groups. **P* = 0.00027, ***P* = 1 9 10^{-8}.



was significantly older and the IC/BPS group had a significantly lower BMI. Self-described ethnic identity distributions were similar between groups except for fewer Hispanic patients in the IC/BPS group. Review of prevalent comorbid conditions indicated that our study populations reflected the profiles for controls and cases with these symptomatic complexes that were seen in previous studies [26,27]. There were no significant differences in several common unrelated comorbidities, such as headaches, hyperlipidaemia, degenerative disc disease or glaucoma. Consistent with its older age, the OAB group had a significantly higher prevalence of arthritis and a trend toward greater hypertension. Consistent with previous reports [26,28–30], there was a significantly increased prevalence of multiple allergies and anxiety and a trend toward thyroid disease in patients with IC/BPS. Overall, the present study populations reflected the profiles for controls and cases with these symptomatic complexes seen previously [26,27].

Qualitative comparison of the scores for the fGUPI, OAB-q, ICSI and ICPI subdomains and totals revealed good distinction of controls from cases, but a high degree of overlap between scores on all questionnaires for OAB and IC/BPS populations (Fig. 4A). If IC/BPS were purely pain-predominant and OAB urgency-predominant, plots of individual patient scores on pain-focused instruments as opposed to those focused on urinary symptoms would be expected to separate these populations into unique quadrants. In our cohort, regardless of the scores used, patient populations demonstrated significant overlap, with increasing symptom severity on one measure correlating directly with all other measures (Fig. 4B). Thus, current validated tools do little to distinguish conditions associated with storage LUTS and serve primarily as measures of severity only.

Only the fGUPI pain subdomain identified a significant difference between the OAB and IC/BPS groups ($P = 0.005$). Attempts to use this subscore as a diagnostic classification measure, however, were unsuccessful; the optimal threshold for this subdomain provided an overall accuracy of 68% for symptomatic patients (sensitivity 60%, specificity 53% for IC/BPS). This inability to distinguish these conditions reflects the prevalence of significant bladder pain (35%) in patients with OAB and of urgency with fear of incontinence (25%) in patients with IC/BPS.

As all utilized questionnaires address the aggregate of multiple urinary/pelvic symptoms, we sought to determine which symptomatic features were associated with specific diagnoses. Out of 56 total questions across four questionnaires, only eight questions differed significantly between patients with OAB and IC/BPS when adjusted for multiple comparisons (Fig. 2). These questions were redundant, covering two symptom features: bladder pain and

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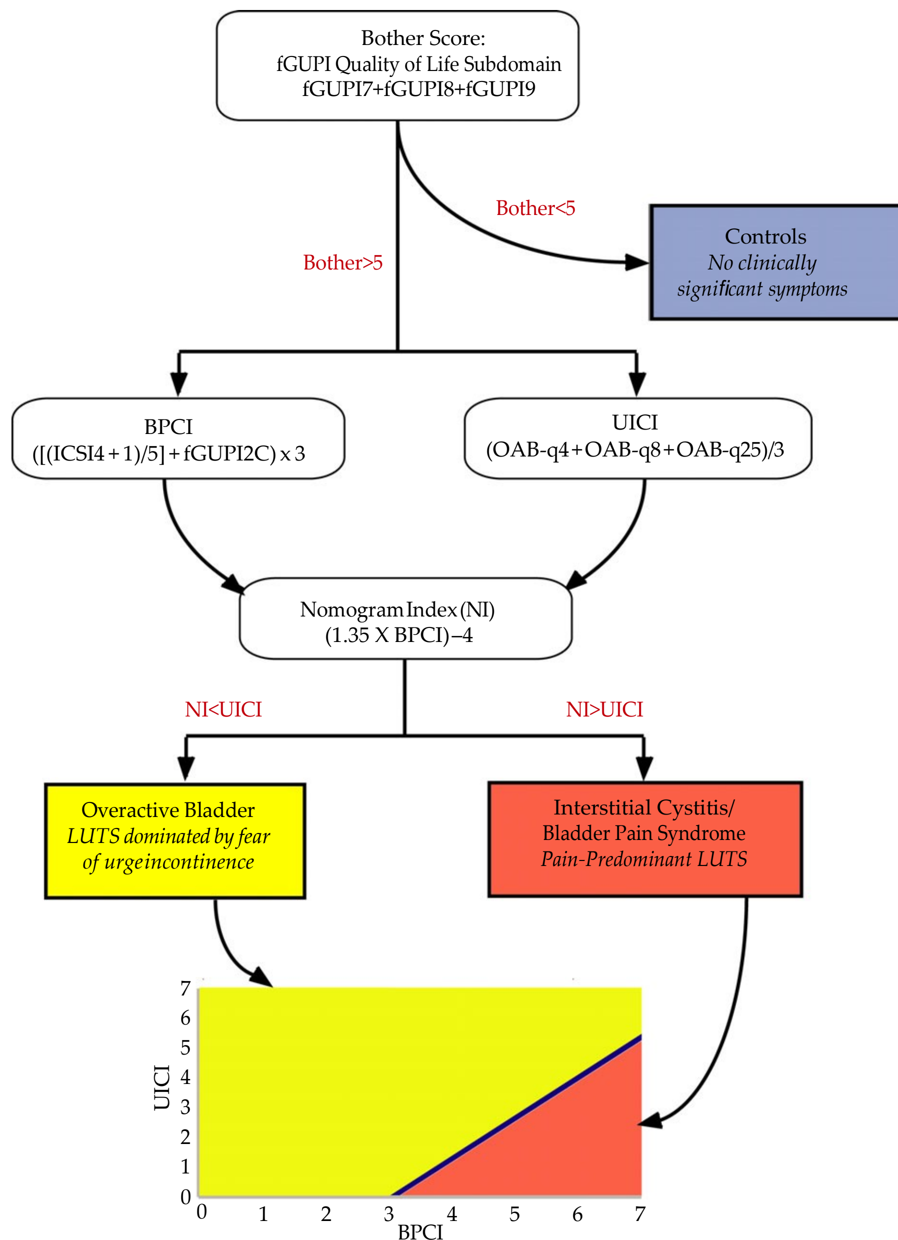
Fig. 3 Nomogram flow chart. Beginning with the determination of the patient's symptomatic bother, sequential calculations using eight symptomatic questions allow the categorization of patients into three symptomatic groups, overactive bladder, interstitial cystitis, and asymptomatic controls. BPCI, Bladder Pain Composite Index; UICI, Urge Incontinence Composite Index; NI, Nomogram Index; GUPI, Genitourinary Pain Index; ICSI, Interstitial Cystitis Symptom Index; OAB-q, OAB Questionnaire.



urgency incontinence. The responses to none of the questions addressing nocturia, frequency, urgency, incomplete emptying, lifestyle limitations, anxiety, frustration, or symptomatic bother revealed observable differences between the OAB and IC/BPS groups.

We used the questions with the greatest discriminatory power and least interdependency to generate a composite scoring system with urgency incontinence (UICI) and bladder pain (BPCI) composite indices. For pain assessment, we selected ICSI question 4 and fGUPI question 2c, while for urgency incontinence, we chose OAB-q questions 4, 8 and 25 (Fig. 2). As the scales for these questions differed, we used weighting of individual question scores with iterative assessment of the predictive accuracies of different weighting schemes to provide a more balanced composite (Fig. 3). Inclusion of additional variables did not result in improved performance.

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Table 1 Patient demographics.

	Control	OAB	IC/BPS
Median (range) age, years	46	*72**	46
Median (range) BMI, kg/m^2	24.3	27.5	*21.0***
Ethnicity, *n* (%)			
African-American	4 (5.8)	4 (6.0)	4 (6.8)
Asian/Pacific Islander	2 (2.9)	2 (3.0)	0 (0)
White	41 (59.4)	36 (53.7)	38 (64.4)
Hispanic	6 (8.7)	9 (13.4)	*1 (1.7)*[†]
Native American/Alaskan native	0 (0)	0 (0)	1 (1.7)
Other/Mixed	16 (23.2)	16 (23.9)	15 (25.4)
Comorbidities, %			
Allergies (multiple)	0	7.1	*23.1*[§]
Anxiety	8.8	18.8	*28.6*[§]
Arthritis	16.3	*35.7*[‡]	23.1
Hypertension	31.3	42.9	27.7
Migraine headaches	6.25	7.1	7.7
Thyroid disease	25.0	28.6	38.4

*BMI, body mass index; IC/BPS, interstitial cystitis/bladder pain syndrome; OAB, overactive bladder. Unless indicated, there were no significant differences between the experimental groups and controls. *P = 3* **9** *10^{-8}. **P = 0.007. †P = 0.002. ‡P = 0.05. §P = 0.04.*

Unlike the validated symptom questionnaires, these novel composite scores were capable of distinguishing OAB from IC/BPS with high significance (Fig. 2).

A two-dimensional graph of these indices provided good separation of the populations (Fig. 5A), in contrast to Fig. 3B. Patients in the control group clustered in the lower left-hand corner and patients in the IC/BPS in the lower right, with high pain and low urgency composite scores. Patients with OAB were seen throughout the remaining three quadrants. From this result, we derived a nomogram to divide these patient groups, indicated by the blue line, with a sensitivity of 100% for both OAB and IC/BPS overall and a specificity of 88% for IC/BPS and 100% for OAB in the validation cohort.

Composite scoring and the diagnostic nomogram were tested in the independent validation cohort, demonstrating a similar distribution of scoring on standardized questionnaires to the experimental cohort (data not shown), with significant differences between the OAB and IC/BPS populations only in the fGUPI pain subdomain ($P = 0.004$). Application of the nomogram again revealed good separation of the OAB and IC/BPS patients (Fig. 5B).

When the control subjects from the experimental cohort were classified according to this nomogram, their distribution overlapped significantly with patients in the OAB group. This overlap appeared to be attributable to the confounding factor of ~25% of controls exhibiting clinically bothersome urge incontinence (Fig. 6A). The severity of incontinence correlated positively with the degree of symptomatic bother (Fig. 6B). Division of the control population into two subgroups based on a bother threshold (fGUPI quality-of-life domain score > 5) was sufficient to identify patients with increased symptomatic scores on all questionnaires (Fig. 6C), confirming that these patients represent a confounding, symptomatic OAB population.

The addition of symptomatic bother (Fig. 7A) as a standard to separate the control group (patients not requiring treatment) from groups needing intervention, resulted in a diagnostic algorithm that distinguished patients with IC/BPS from those with OAB well, with an overall accuracy of diagnostic classification of 94%. In addition, the identification of the subset of control patients with high bother and significant LUTS (predominantly UI) displays the potential utility of this algorithm as a screening tool to identify bothersome OAB symptoms in the primary care setting.

In contrast to the overlap of symptoms seen when using standardized questionnaires (Fig. 3B), the use of these three measures, BPCI, UICI and the bother score, as visualized graphically in a three-dimensional plot (Fig. 8A), provides good separation of the IC/BPS and OAB populations. The overlap of a subset of the control subjects with the OAB population re-emphasizes the high prevalence of unrecognized, bothersome storage LUTS in this group. To assess the performance of our algorithm, we compared scoring on validated questionnaires of the populations classified by clinical diagnosis with that classified by algorithm designation. When separated by clinical diagnosis, the populations exhibited significant overlap (Fig. 8B), in contrast to the improved clustering of symptom scores seen when patients were classified by the algorithm (Fig. 8C).

Discussion

Although regarded as two separate clinical entities, there is significant symptomatic overlap between IC/BPS and OAB. Recent data from the Multidisciplinary Approach to the Study of Chronic Pelvic Pain Research Network recommended assessing pain and urinary symptoms separately, as these two symptom clusters segregated differentially even within the IC/BPS population [31]. Our results indicate that patients with OAB and IC/BPS are difficult to distinguish based on aggregated, self-reported symptomatic questionnaire scoring alone. In the present study, composite scores from the fGUPI, OAB-q and ICSI/ICPI questionnaires could not differentiate between patients with IC/BPS and those with OAB, revealing significant bladder pain in patients with OAB and urgency incontinence in patients with IC/BPS.

Questions addressing nocturia, urgency without fear of incontinence, frequency, incomplete emptying, bother, sleep disturbances, frustration, anxiety, and lifestyle limitations were similar in both groups; only two symptom clusters, bladder pain and urgency incontinence, differed significantly between the patient groups. It has been reported that one

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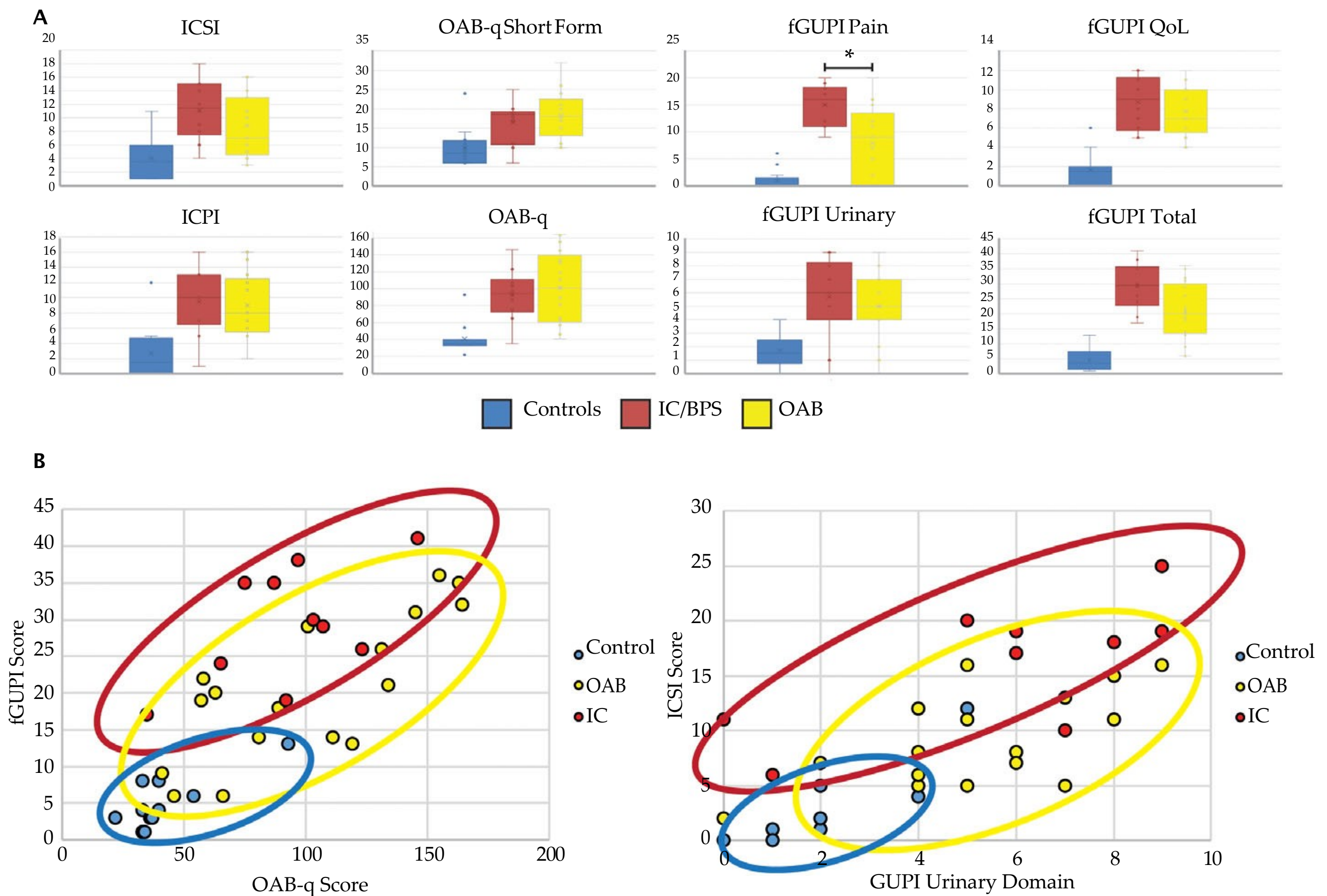
Fig. 4 Comparison of patient-reported symptom scores between patients with overactive bladder (OAB) and those with interstitial cystitis/bladder pain syndrome (IC/BPS) patients. (A) Box and whisker plots show the means, medians and ranges of individual questionnaire scores for patients in the control, OAB, and IC/BPS groups in the initial experimental cohort of 50 patients. $^*P = 0.005$. (B) Principal component analysis of symptomatic questionnaire scores. Scores from pain-focused questionnaires (left: female Genitourinary Pain Index [fGUPI] total score; right: Interstitial Cystitis Symptom Index [ICSI] total score) were plotted against scores for urinary urgency-focused scores (right: OAB Questionnaire [OAB-q] total score; left: fGUPI urinary score). The patterns of symptom scores for patients with either diagnosis (OAB and IC/BPS) exhibited elevated scores on all measures, with similar spreads of symptom severity, resulting in near-complete overlap of the two populations.



highly distinctive characteristic of IC/BPS is bladder pain associated with filling. This is the sensation captured by question 2c of the fGUPI, one of our three discriminating pain questions [32–35]. Using these features, we created and validated a novel composite scoring system with bladder pain and urgency incontinence domains to differentiate these populations. These scores, compiled into a new storage LUTS nomogram, help distinguish OAB from IC/BPS. The most commonly used, validated measures are good for assessing symptom severity, but provide little guidance when diagnosis is in doubt. Adjustment of the diagnostic nomogram for self-reported bother allowed the generation of a diagnostic algorithm identifying those with clinically relevant syndromes.

The presence of bothersome symptoms in many of the patients in the control group underscores the need for novel ways to identify significant LUTS. The control patients presented for conditions without LUTS and did not volunteer complaints of urinary symptoms. Despite our best screening, a quarter of these controls had clinically significant symptoms. Even after multiple negative screens at the primary and specialist levels, 22% (15/69) of controls reported bothersome LUTS on a written survey that they did not endorse orally, even when prompted. Patients frequently do not seek care for urinary symptoms for many reasons, such as shame, embarrassment or a feeling that symptoms are unavoidable [20]. While there may be some selection bias in the present study as these patients were evaluated in a urology office, the prevalence of LUTS among controls is consistent with previous population-based surveys [1], stressing the need for better screening.

Previous studies have demonstrated that the prevalence of IC symptoms in the primary care setting increased >20-fold

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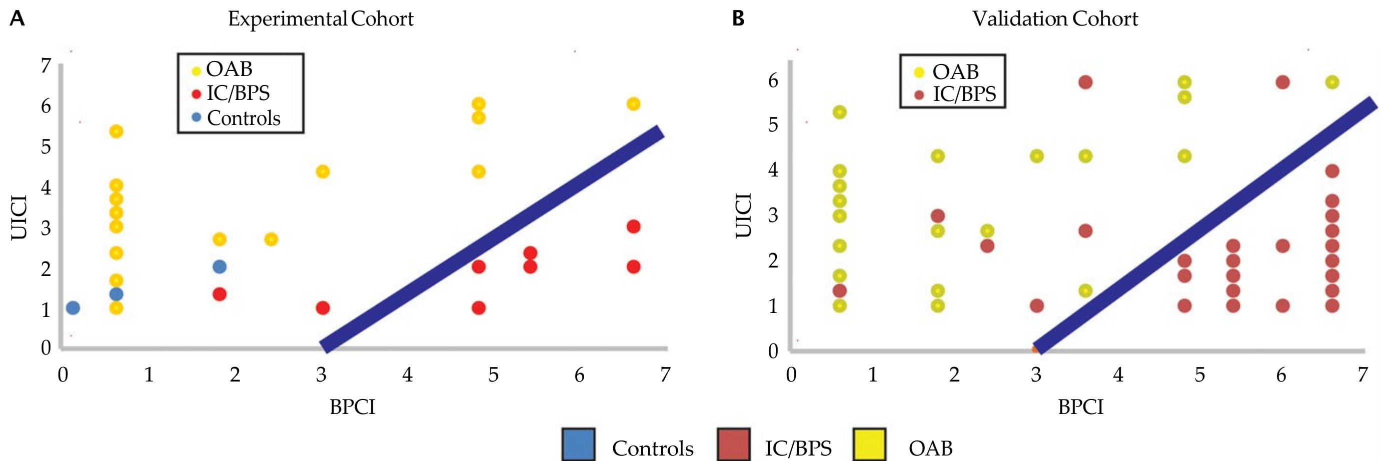
Fig. 5 Novel storage LUTS nomogram provides accurate classification of patients with irritative urinary symptoms. Our initial nomogram for the classification of urinary symptoms was initially defined using the experimental cohort from a plot of the urge incontinence (Urgency Incontinence Composite Index [UICI]) and bladder pain (Bladder Pain Composite Index [BPCI]) composite indices, with the blue line serving as the dividing point for these diagnoses (A). The validation population was then examined using this algorithm to confirm the representational accuracy of this approach, which confirmed a distribution similar to the experimental population (B).



(0.57-12.6%) when the Pelvic Pain and Urgency/Frequency patient symptom scale was administered in addition to the ICSI/ICPI [36]. This suggests that recognizing IC/BPS in the primary care setting relies largely on which methods and cut-off criteria are used to assess symptoms. Thus, it may be germane to implement algorithms such as this to provide a binary output capable of guiding discussion, initial treatment, and the need for referral without requiring subspecialty knowledge.

All control subjects with self-reported urge incontinence at levels greater than the threshold designated in our algorithm also self-described their symptoms as clinically bothersome, indicating that our composite indices differentiate meaningful urgency incontinence from symptoms that are not bothersome. Approximately one-quarter of patients seen by a physician for an unrelated complaint have clinically relevant LUTS that would benefit from intervention [1]. Our novel algorithm provides a simple, rapid and minimally burdensome screening tool, capable of identifying and even classifying patients with storage LUTS and offering busy providers a starting point for conversations and education about bladder issues.

Validated questionnaires are meant to establish thresholds and reliably classify symptom severity. For storage LUTS, however, evaluating aggregate symptoms with such questionnaires can mask critical individual symptoms [21]. Algorithmic systems may be more useful in profiling patients. Results from our algorithm show that assessment of three symptom features in eight questions can provide an appropriate diagnosis for storage LUTS, distinguishing OAB from IC/BPS with high accuracy and reliability. Additional research will be necessary to confirm these results prospectively and determine if this algorithm provides improved prognostic assessment. We believe this simple measure is at least as accurate as clinical evaluation by specialty clinicians. Future prospective studies will aim to determine if our diagnostic classification provides improved prediction of responses to specific treatment methods, particularly in patients reclassified from one condition to another. For example, a patient with IC/BPS reclassified as OAB by the algorithm may be more likely to demonstrate positive responses to anticholinergics or chemodenervation.

It is well established that IC/BPS and OAB are highly heterogeneous conditions, both in their presentation and in their responses to therapy. The two symptom clusters differ widely in definition, diagnosis and management by clinicians. Efforts have been made to develop clinical phenotyping systems for IC/BPS, such as the UPOINT system [37]; a need for similar stratification systems to be established has been suggested for OAB as well [14]. The difficulty in distinguishing patients with OAB from controls has been noted previously [38]. Classifying patients according to phenotypes can better inform more effective, targeted treatment plans, but many of these systems are unwieldy and unfamiliar to most providers. Diagnostic algorithms such as ours can provide a simpler and more useful starting point in the identification of clinically relevant storage LUTS.

It was rare for patients clinically categorized as having OAB to exhibit a high degree of bladder pain in the absence of high urgency, but approximately one in eight patients with storage LUTS (35% of OAB cases) exhibited both elevated bladder pain and urge incontinence, making this dually-afflicted phenotype a common disease subtype within the storage LUTS population. It is unclear if this patient group represents a

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Fig. 6 High prevalence of bothersome symptoms in control subjects. (A) Subgroup analysis of the control population using the nomogram demonstrated that ~25% of controls exhibited clinically significant scores on the urge incontinence composite index. (B) The severity of incontinence correlated positively with the degree of symptomatic bother, as measured by female Genitourinary Pain Index (fGUPI) question 9. (C) When the controls were separated into two subgroups on the basis of symptomatic bother, the true controls (low bother) were asymptomatic on all scores, while the patients with high bother exhibited a symptomatic profile indistinguishable from patients with overactive bladder (OAB), with elevated scores on all questionnaires. fGUPI QoL, fGUPI quality of life domain.

A
UICI
BPCI

B
UICI
Bother Score

C
ICSI
OAB-q Short Form
fGUPI Pain
fGUPI QoL
ICPI
OAB-q
fGUPI Urinary
fGUPI Total
Low bother
High bother

Fig. 7 Development of diagnostic algorithm for classification of storage LUTS based on novel nomogram. (A) A single question measurement of symptomatic bother discriminates true asymptomatic controls from patients with clinically relevant storage LUTS. (B) To correct for the overlap of unrecognized symptomatic controls with the overactive bladder (OAB) group, a third measure of symptomatic bother was incorporated to create a diagnostic algorithm distinguishing patients with clinically relevant conditions from truly asymptomatic controls. This formula provides an integer diagnostic code giving good separation and accurate classification of patients and controls.

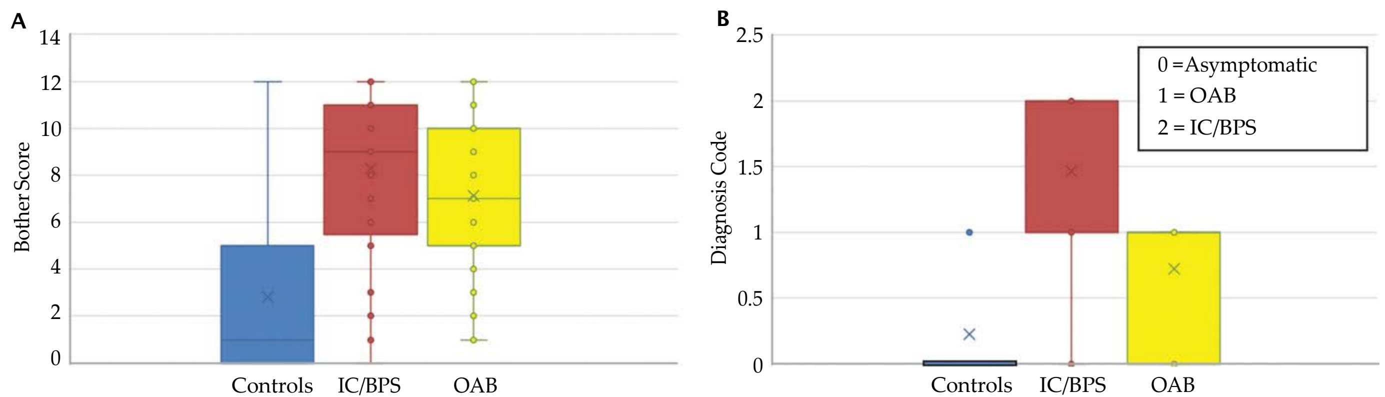

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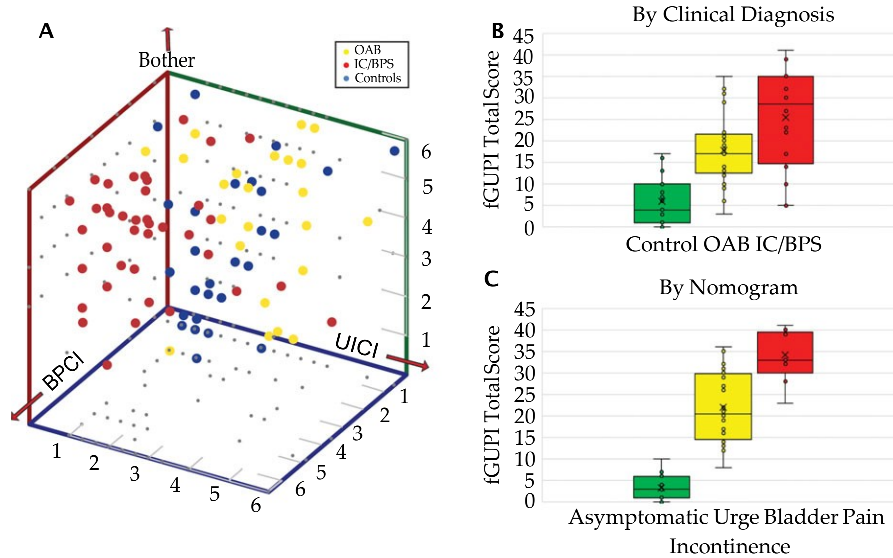
Fig. 8 Novel diagnostic algorithm accurately classifies patients with bothersome storage LUTS. (A) Three-dimensional plot of novel composite indices against symptomatic bother scores provides graphical representation of the nomogram classification, demonstrating good separation of patient populations (yellow: overactive bladder [OAB]; red: interstitial cystitis/bladder pain syndrome [IC/BPS]) by diagnosis. A subset of the control subjects (blue) overlap primarily with the OAB population as a result of the high prevalence of unrecognized bothersome storage LUTS. (B, C) The female Genitourinary Pain Index (fGUPI) total score is shown for each population as determined by either the clinical diagnosis given by a subspecialty physician (B) or diagnosis designated by our novel algorithm (C). The nomogram provides a better separation of these populations than clinician diagnosis.



unique population with both IC/BPS and OAB or a more severe phenotype in which greater urgency presents as bladder discomfort and pain. Hopefully, future studies can distinguish the natural history of these patients from those with more isolated urgency and begin to answer these questions.

Urgency symptoms may fundamentally differ between patients with IC/BPS and patients with OAB [16], with each group having unique underlying motivations to void. No clinical measurements of urgency/need to urinate could discern such differences; only questions addressing accidental urine loss varied between IC/BPS and OAB. This distinction is consistent with the medical definition of urgency by the International Continence Society (ICS) as 'the sudden and compelling desire to void that is difficult to defer' [39]. It is unclear, however, if patients internalize the definition in the same way as physicians, particularly urologists. The lack of a well-defined, commonly used language to describe the experiences of patients with storage LUTS outside of the clinical setting may be the foundation of the considerable challenges facing the identification, classification and management of these conditions. Instead of using medical terminology that is poorly understood by patients, the use of straightforward questions in which the symptoms of interest are clearly defined may be one reason why this algorithm is effective at categorizing patients.

The symptomatic overlap observed in the present study reveals the limitations of current measurement systems and our understanding of these two conditions. It may be fitting to conceptualize IC/BPS and at least a subset of OAB (OAB-dry) on a spectrum of bladder hypersensitivity syndromes, as opposed to disjointed conditions. Increasing evidence suggests IC/BPS and OAB-dry share a common underlying pathology [40], while IC/BPS with isolated bladder pain and OAB-wet with isolated urgency incontinence may lie on opposite ends of a spectrum [8]. Many patients, however, exist somewhere in the middle, with a complex mixture and overlap of frequency, urgency and bladder discomfort. Indeed, there are patients diagnosed with IC/BPS who do not report pain [41,42], and patients with OAB who do not demonstrate detrusor overactivity. As we excluded patients with both diagnoses, additional studies will be needed to determine how this classification system performs for patients meeting both clinical criteria. Although our algorithm does not directly address where such patients would be on such a spectrum, it does allow better discernment of the most salient symptoms. Shifting the focus from these diagnoses to symptoms may better inform management strategies. Physicians will find Table S1 in the Supporting Information supplies a simple template for using our nomogram. Ideally, an online tool will be available in the future to guide treatment plans.

While these early results are promising for the applicability of this algorithm, studies are needed to confirm its utility in clinical decision-making, particularly in men, who may manifest symptoms differently. We hope to investigate further

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the utility of this algorithm in the primary care setting as a screening tool to identify patients requiring urological assessment. This algorithm can be used in the initial evaluation of complex patients presenting with urinary tract complaints (Table S1); future prospective studies must examine its clinical accuracy, particularly in complex or treatment-refractory patients. In addition, this classification schema could be used as a prognostic indicator for patients with complex LUTS to predict the best therapeutic strategies, particularly in OAB and IC/BPS for which suboptimal patient responses are the norm and multiple trials of therapies are often needed for durable improvement.

In conclusion, there is significant overlap of urinary tract symptoms between OAB and IC/BPS, suggesting that there may be common pathological elements between the two conditions. Future studies assessing the diagnostic value of novel classification schemes that address symptoms rather than specific diagnoses may improve patient prognosis. A proposed novel diagnostic algorithm for the screening and classification of storage LUTS presents a new paradigm for how we approach storage urinary symptoms.

Conflict of Interest

Dr A. Lenore Ackerman is an investigator for Urigen and a consultant for Aquinox. Dr Henry Lai is an investigator for Medtronic and Allergan and a consultant for Aquinox. Dr Jennifer Anger is an expert witness for Boston Scientific. Dr Karyn Eilber is an investigator and expert witness for Boston Scientific, an investigator for Aquinox, and a consultant for Boston Scientific and Allergan. Ms. Pooja Parameshwar reports no conflicts of interest.

References

1 Coyne KS, Sexton CC, Thompson CL et al. The prevalence of lower urinary tract symptoms (LUTS) in the USA, the UK and Sweden: results from the Epidemiology of LUTS (EpiLUTS) study. *BJU Int* 2009; 104: 352–60

2 Milsom I, Kaplan SA, Coyne KS, Sexton CC, Kopp ZS. 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* 2012; 80: 90–6

3 Coyne KS, Wein A, Nicholson S, Kvasz M, Chen CI, Milsom I. Economic burden of urgency urinary incontinence in the United States: a systematic review. *J Manag Care Pharm* 2014; 20: 130–40

4 NIH. Meeeting on Measurement of Urinary Symptoms. Available at: http://www.niddk.nih.gov/news/events-calendar/Pages/meeting-on-measure ment-of-urinary-symptoms-momus.aspx. Accessed November 2017

5 Hanno PM, Burks DA, Clemens JQ et al. AUA guideline for the diagnosis and treatment of interstitial cystitis/bladder pain syndrome. *J Urol* 2011; 185: 2162–70

6 Gormley EA, Lightner DJ, Burgio KL et al. Diagnosis and treatment of overactive bladder (non-neurogenic) in adults: AUA/SUFU guideline. *J Urol* 2012; 188: 2455–63

7 Abrams P, Hanno P, Wein A. Overactive bladder and painful bladder syndrome: there need not be confusion. *Neurourol Urodyn* 2005; 24: 149–50

8 Lai HH, Vetter J, Jain S, Gereau RW, Andriole GL. The overlap and distinction of self-reported symptoms between interstitial cystitis/bladder pain syndrome and overactive bladder: a questionnaire based analysis. *J Urol* 2014; 192: 1679–86

9 Tincello DG, Walker AC. Interstitial cystitis in the UK: results of a questionnaire survey of members of the Interstitial Cystitis Support Group. *Eur J Obstet Gynecol Reprod Biol* 2005; 118: 91–5

10 Driscoll A, Teichman JM. How do patients with interstitial cystitis present? *J Urol* 2001; 166: 2118–20

11 MacDiarmid SA, Sand PK. Diagnosis of interstitial cystitis/painful bladder syndrome in patients with overactive bladder symptoms. *Rev Urol* 2007; 9: 9

12 Minaglia S, Özel B, Bizhang R, Mishell DR. Increased prevalence of interstitial cystitis in women with detrusor overactivity refractory to anticholinergic therapy. *Urology* 2005; 66: 702–6

13 Wein AJ, Rackley RR. Overactive bladder: a better understanding of pathophysiology, diagnosis and management. *J Urol* 2006; 175: S5–10

14 Lee UJ, Scott VC, Rashid R et al. Defining and managing overactive bladder: disagreement among the experts. *Urology* 2013; 81: 257–62

15 Clemens JQ, Bogart LM, Liu K, Pham C, Suttorp M, Berry SH. Perceptions of "urgency" in women with interstitial cystitis/bladder pain syndrome or overactive bladder. *Neurourol Urodyn* 2011; 30: 402–5

16 Hanno PM, Chapple CR, Cardozo LD. Bladder pain syndrome/interstitial cystitis: a sense of urgency. *World J Urol* 2009; 27: 717

17 Lai HH, Krieger JN, Pontari MA et al. Painful bladder filling and painful urgency are distinct characteristics in men and women with urological chronic pelvic pain syndromes: a MAPP research network study. *J Urol* 2015; 194: 1634–41

18 Abrams P. Urgency: the key to defining the overactive bladder. *BJU Int* 2005; 96: 1–3

19 Heeringa R, van Koeveringe GA, Winkens B, van Kerrebroeck PE, de Wachter SG. Do patients with OAB experience bladder sensations in the same way as healthy volunteers? A focus group investigation *Neurourol Urodyn* 2012; 31: 521–5

20 Filipetto FA, Fulda KG, Holthusen AE, McKeithen TM, McFadden P. The patient perspective on overactive bladder: a mixed-methods needs assessment. *BMC Fam Pract* 2014; 15: 96

21 Clemens JQ, Calhoun EA, Litwin MS et al. Validation of a modified National Institutes of Health chronic prostatitis symptom index to assess genitourinary pain in both men and women. *Urology* 2009; 74: 983–7 e3

22 Coyne K, Revicki D, Hunt T et al. Psychometric validation of an overactive bladder symptom and health-related quality of life questionnaire: the OAB-q. *Qual Life Res* 2002; 11: 563–74

23 O'Leary MP, Sant GR, Fowler FJ, Whitmore KE, Spolarich-Kroll J. The interstitial cystitis symptom index and problem index. *Urology* 1997; 49: 58–63

24 Mazumdar M, Glassman JR. Categorizing a prognostic variable: review of methods, code for easy implementation and applications to decision-making about cancer treatments. *Stat Med* 2000; 19: 113–32

25 Hens N, Aerts M, Molenberghs G. Model selection for incomplete and design-based samples. *Stat Med* 2006; 25: 2502–20

26 Fuoco MB, Irvine-Bird K, Curtis Nickel J. Multiple sensitivity phenotype in interstitial cystitis/bladder pain syndrome. *Can Urol Assoc J* 2014; 8: E758–61

27 Coyne KS, Wein A, Nicholson S, Kvasz M, Chen CI, Milsom I. Comorbidities and personal burden of urgency urinary incontinence: a systematic review. *Int J Clin Pract* 2013; 67: 1015–33

28 Alagiri M, Chottiner S, Ratner V, Slade D, Hanno PM. Interstitial cystitis: unexplained associations with other chronic disease and pain syndromes. *Urology* 1997; 49(5A Suppl): 52–7

© 2018 The Authors

29 Chung KH, Liu SP, Lin HC, Chung SD. Bladder pain syndrome/interstitial cystitis is associated with anxiety disorder. *Neurourol Urodyn* 2014; 33: 101–5
30 Chung SD, Liu SP, Lin CC, Li HC, Lin HC. Bladder pain syndrome/interstitial cystitis is associated with hyperthyroidism. *PLoS One* 2013; 8: e72284
31 Griffith JW, Stephens-Shields AJ, Hou XL et al. Pain and urinary symptoms should not be combined into a single score: psychometric findings from the MAPP research network. *J Urol* 2016; 195: 949–54
32 Warren JW, Diggs C, Horne L, Greenberg P. Interstitial cystitis/painful bladder syndrome: what do patients mean by "perceived" bladder pain? *Urology* 2011; 77: 309–12
33 Warren JW, Brown J, Tracy JK, Langenberg P, Wesselmann U, Greenberg P. Evidence-based criteria for pain of interstitial cystitis/painful bladder syndrome in women. *Urology* 2008; 71: 444–8
34 Birder LA. Urinary bladder, cystitis and nerve/urothelial interactions. *Auton Neurosci* 2014; 182: 89–94
35 Castro-Diaz D, Cardozo L, Chapple CR et al. Urgency and pain in patients with overactive bladder and bladder pain syndrome. What are the differences? *Int J Clin Pract* 2014; 68: 356–62
36 Rosenberg MT, Hazzard M. Prevalence of interstitial cystitis symptoms in women: a population based study in the primary care office. *J Urol* 2005; 174: 2231–4
37 Nickel JC, Shoskes D, Irvine-Bird K. Clinical phenotyping of women with interstitial cystitis/painful bladder syndrome: a key to classification and potentially improved management. *J Urol* 2009; 182: 155–60
38 Staskin DR, Dmochowski RR. Future studies of overactive bladder: the need for standardization. *Urology* 2002; 60(5 Suppl 1): 90–3
39 Abrams P, Cardozo L, Fall M et al. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Am J Obstet Gynecol* 2002; 187: 116–26
40 Parsons CL. The role of a leaky epithelium and potassium in the generation of bladder symptoms in interstitial cystitis/overactive bladder, urethral syndrome, prostatitis and gynaecological chronic pelvic pain. *BJU Int* 2011; 107: 370–5
41 Bogart LM, Berry SH, Clemens JQ. Symptoms of interstitial cystitis, painful bladder syndrome and similar diseases in women: a systematic review. *J Urol* 2007; 177: 450–6
42 Parsons CL. Interstitial cystitis: epidemiology and clinical presentation. *Clin Obstet Gynecol* 2002; 45: 242–9

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Abbreviations: OAB, overactive bladder; IC/BPS, interstitial cystitis/bladder pain syndrome; fGUPI, female Genitourinary Pain Index; OAB-q, OAB Questionnaire; ICSI, Interstitial Cystitis Symptom Index; ICPI, Interstitial Cystitis Problem Index; FPMRS, Female Pelvic Medicine and Reconstructive Surgery; BMI, body mass index; BPCI, Bladder Pain Composite Index; UICI, Urgency Incontinence Composite Index.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Nomogram worksheet.
Table S1. Survey questions used in nomogram with scoring range.

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RESEARCH ARTICLE

Optimization of DNA extraction from human urinary samples for mycobiome community profiling

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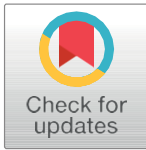

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Abstract

Introduction

Recent data suggest the urinary tract hosts a microbial community of varying composition, even in the absence of infection. Culture-independent methodologies, such as next-generation sequencing of conserved ribosomal DNA sequences, provide an expansive look at these communities, identifying both common commensals and fastidious organisms. A fundamental challenge has been the isolation of DNA representative of the entire resident microbial community, including fungi.

Materials and methods

We evaluated multiple modifications of commonly-used DNA extraction procedures using standardized male and female urine samples, comparing resulting overall, fungal and bacterial DNA yields by quantitative PCR. After identifying protocol modifications that increased DNA yields (lyticase/lysozyme digestion, bead beating, boil/freeze cycles, proteinase K treatment, and carrier DNA use), all modifications were combined for systematic confirmation of optimal protocol conditions. This optimized protocol was tested against commercially available methodologies to compare overall and microbial DNA yields, community representation and diversity by next-generation sequencing (NGS).

Results

Overall and fungal-specific DNA yields from standardized urine samples demonstrated that microbial abundances differed significantly among the eight methods used. Methodologies that included multiple disruption steps, including enzymatic, mechanical, and thermal disruption and proteinase digestion, particularly in combination with small volume processing and pooling steps, provided more comprehensive representation of the range of bacterial

mappnetwork.org. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: Dr. Anger is an expert witness for Boston Scientific, Inc. Dr. Eilber is an investigator and expert witness for Boston Scientific, an investigator for Aquinox, and a consultant for Boston Scientific and Allergan. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

and fungal species. Concentration of larger volume urine specimens at low speed centrifugation proved highly effective, increasing resulting DNA levels and providing greater microbial representation and diversity.

Conclusions

Alterations in the methodology of urine storage, preparation, and DNA processing improve microbial community profiling using culture-independent sequencing methods. Our optimized protocol for DNA extraction from urine samples provided improved fungal community representation. Use of this technique resulted in equivalent representation of the bacterial populations as well, making this a useful technique for the concurrent evaluation of bacterial and fungal populations by NGS.

Introduction

Multiple organs, such as the gut, oral cavity, and vagina, have long been known to harbor communities of microbes that can protect against or contribute to disease under different circumstances. The urinary tract, however, was widely thought to be sterile until only recently, when extended culture techniques and the detection of microbial DNA definitively demonstrated microbial communities of great diversity within this site.[1–3] Currently, culture-independent microbial characterization using the sequencing of highly conserved DNA regions, such as the ribosomal RNA gene locus (rDNA), is widely-accepted as a useful, sensitive tool to explore microbial populations. These next-generation sequencing (NGS) technologies are particularly useful in characterizing microbes that may be difficult to culture or that are present in low abundance (the "rare biosphere").[4] Therefore, the composition and diversity of the urinary microbiome has likely been drastically understated, in part, due to dependence on culture methods to identify resident species.

With the development of affordable, rapid, and scalable culture-independent methods for the study of bacterial communities, the last decade has seen a massive expansion in studies aimed at profiling commensal communities in a multitude of organs not included in the large-scale Human Microbiome Project (HMP), such as the urinary tract. Using NGS methods, multiple studies have demonstrated that perturbations in the urinary microbiota appear to correlate with Lower Urinary Tract Symptoms (LUTS).[5–13] The clinical significance and utility of these alterations, however, remain unclear, primarily due to challenges that persist for the characterization of microbes from low biomass specimens, such as urine.

Due to these limitations, we still lack vital information about the content of normal urine and its relationship to dysbiosis and/or disease. Studies examining the urinary microbiome thus far demonstrate wide variation in their ability to consistently detect microbial species. In many studies, approximately half of patient samples do not have bacterial sequences of sufficient quality for analysis[2, 6, 14]; in other studies, this efficiency could be improved with the use of multiple amplification steps[11], but this may introduce new biases that could skew results. This low sequencing efficiency is likely due to the combination of low biomass and the unique qualities of urine, which include high variability in osmolality/salt content, high abundance of PCR inhibitors, and fluctuating levels of cellular material, all in all making urine a challenging biological fluid to study. The question remains as to whether these sequence-negative samples are truly negative for microbes or whether our detection methods are inadequate

to fully characterize these specimens. Until this question can be answered, it remains a very real possibility that the subset of samples analyzed, the "sequence-positive" group, may represent a unique subgroup within the analyzed population with higher microbial loads, whose findings cannot be generalized to the larger sample population.

Even less is known about the composition of non-bacterial populations, such as fungi, viruses, archaea, and protozoa, in the genitourinary tract and other human organs, primarily from a lack of well-researched tools for their analysis. Despite these challenges, alterations in the fungal microbiota (the "mycobiome") in the absence of frank infection have been demonstrated in multiple human diseases, such as hepatitis [15], atopic dermatitis [16], inflammatory bowel disease [17–19], cystic fibrosis [20], allergy/atopy [21], asthma [22], and psoriasis [23, 24]. As yet, only a few analyses have examined aspects of the urinary mycobiome. *Candida spp*. have been detectable in urinary samples by culture,[5–8] demonstrating their viability. Fungi were also detectable in urine from patients with urological chronic pelvic pain syndromes (UCPPS) using the targeted Ibis T-5000 Universal Biosensor system.[25] Interestingly, fungi were detected more frequently in UCPPS patients during symptomatic flares, while no significant differences in the bacterial microbiota could be identified, implicating fungi as important players in lower urinary tract symptomatology. Even in this culture-independent study, however, fungi were detected in less than 10% of patients overall. Again, it is unclear if this low number is representative of the absence of fungi in the majority of subjects or represents severe limitations in our current technologies.

Further progress in identifying consistent microbial markers or understanding the pathophysiology of microbial interactions in the urinary tract requires methodologies that adequately and reliably characterize these populations, and which include fungi and other microbes in addition to bacteria. In this study, we sought to identify the most effective strategies for extracting and identifying microbial DNA from urine, with a focus on enhancing the detection of fungi. Using an iterative approach, we optimized urine sample processing at multiple steps to increase DNA yields and population representation to generate more consistent data from sequencing-based microbial population analyses.

Materials and methods

This study was approved by the Cedars-Sinai Institutional Review Board (Pro00033267) and written consent was obtained from all subjects.

DNA yield assessment

Overall DNA yields and quality (assessed by OD_{260}/OD_{280} ratios) were measured on the NanoDrop 2000 Spectrophotometer (Thermo Scientific). Fungal DNA levels were assessed in duplicate by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analyses on a Mastercycler Realplex2 (Eppendorf) using the SYBR Green PCR kit as instructed by the manufacturer (Applied Biosystems). Fungal levels were assessed using the Fungiquant primers (forward: 5′-GGRAAACTCACCAGGTCCAG-3′; reverse: 5′-GSWCTATCCCCAKCACGA-3′)[26] that recognize a highly-conserved segment of the fungal 18S rDNA region, while bacterial levels were assessed using 16S rDNA primers (forward: 5′-ACTCCTACGGGAGGCAGCAGT-3′; reverse: 5′-ATTACCGCGGCTGCTGGC-3′), a universal primer with broad specificity for bacteria. The qRT-PCR protocol employed an initial denaturation at 94˚C for 10 min, followed by 35 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s, and elongation at 72˚C for 2 min, followed by an elongation step at 72˚C for 30 min. Relative quantity of bacterial and fungal DNA yields, consistent from experiment to experiment, was calculated by the comparative CT method ($2^{-\Delta\Delta CT}$ method)[27] and normalized to a DNA standard curve

derived from a mixed bacterial and fungal culture that remained constant over all tests. Samples with greater than 3% variance between duplicates were reanalyzed in duplicate. An aliquot of 1 μl of the PCR product was evaluated by 2% agarose gel electrophoresis.

Evaluation of individual protocol enhancements

For the initial, iterative analyses, specimens were obtained from mid-stream urine collections from multiple male and female subjects, all of whom denied any urinary symptoms, after preparation of the external urethral meatus with chlorhexidine gluconate wipes. Urine specimens were mixed well, then divided into 1 ml samples and centrifuged at 5000 relative centrifugal force (rcf) to pellet cellular material prior to parallel processing to test the individual protocol variations described below.

Enzymatic disruption. Sample pellets were initially resuspended in 500 μl enzyme buffer containing a reducing agent (0.5 M Tris, 1mM EDTA, and 0.2% 2-mercaptoethanol, pH 7.5). We added 200 U/ml Lyticase (Sigma Aldrich), 20 mg/ml Lysozyme (Thermo Scientific), both enzymes, or buffer alone without enzyme. Samples were then incubated for 30 min. at 30˚C, with inversion of the tubes every 5–10 min. Subsequently, samples were centrifuged at 1500 rcf for 5 min, the supernatant removed, and the pellet resuspended in 800 μl of Stool DNA Stabilizer (Stratec Biomedical).

Mechanical disruption. Physical disruption of cell walls was accomplished with bead beating. The 800 μl post-enzymatic digestion cell suspension was transferred to a 2 ml centrifuge tube containing 100 μl 0.1 mm and 300 ul 0.5 mm silica beads (Biospec Products, Inc.). Samples were agitated twice for 1 min each on a standard Vortex mixer using a Vortex Adapter for bead beating (MO BIO Laboratories Inc.). Samples were centrifuged for 15 s at 17000 rcf between bead beating periods.

Thermal disruption. Samples were heated to 95˚C for 10 min, with a brief vortex to ensure adequate mixing 5 min into the incubation. After a second, brief vortexing step, samples were incubated on ice (0˚C) for 5 min, then centrifuged for one min. at 17000 rcf after each boil/freeze cycle.

Proteinase digestion. After cell wall disruption, cell lysates were transferred to new tubes containing an equal volume of buffer AL (Qiagen) containing varying concentrations of Proteinase K (0, 12, 24, 48, 72, 96, 120, or 144 mAU/ml)(Qiagen), then incubated at 70˚C for 10 min.

Addition of carrier DNA. When specified, polyadenylic acid carrier DNA (PolyA) (Roche Diagnostics) was added to the cell lysates at the time of proteinase K digestion.

Column DNA extraction. Following the specified disruption and digestion steps, 250 μl 100% Ethanol was added and briefly mixed by vortexing, prior to applying the cell lysates to Qiagen Mini DNA Spin columns (Qiagen). The columns were washed twice with a column volume of buffer AW (Qiagen) by centrifugation at 17000 rcf for 1 min and residual alcohol removed with a third spin without wash buffer. DNA was then eluted from the column in 60 μl warm Tris-EDTA (TE) buffer.

Confirmation of protocol components in aggregate analysis

For 4 subjects (2 male and 2 female), >60 ml of urine were obtained and divided into 1 ml samples within 1 h of sample collection. Twenty unique conditions were analyzed following centrifugation at one of three centrifugation conditions: 1) 1500 rcf for 15 min., 2) 5000 rcf for 20 min., or 3) 16000 rcf for 10 min. The resulting pellets were frozen and stored at -80˚C. 20 unique combinations of the conditions explored in initial, iterative analysis were performed, with inclusion or exclusion of the individual enzymatic treatments, mechanical and thermal

disruption steps, and proteinase digestion in almost all combinations. For this panel of conditions, carrier DNA was included in all samples to provide better discrimination of differences in these low volume samples.

Relative DNA yields for each condition were determined by fungal-specific qPCR as specified above; for each sample, yields were scaled to equal variance for all samples to allow plotting of the median yields for each condition as a heat map.

Determination of optimal sample volume

Large volume urine samples (>100 ml) from 3 male and 3 female subjects were mixed well and subdivided into 1, 2.5, 5, 10, 25, and 50 ml aliquots. Each sample was centrifuged at 1500 rcf and the supernatant decanted. After pelleting, all samples were identically processed using the optimized protocol detailed above. All aliquots for an individual subject were processed in batches to minimize batch-to-batch variation. The resulting fungal DNA concentrations were then quantitated by qRT-PCR. Taxal diversity was also examined by 2% agarose gel electrophoresis.

Sample subdivision and pooling

To evaluate if processing lysates in smaller volumes provided increased DNA yields, samples were subdivided into smaller aliquots after mechanical disruption. Seven identical urine specimens were pelleted, digested with lysozyme and lyticase, then subjected to bead beating with a mixture of silica beads as detailed above. Sample quantities ranging from 100 μl to 400 μl (of an approximately 500 μl total lysate volume) at 50 μl intervals were aspirated off of the beads and subjected to thermal disruption, proteinase K digestion and DNA-column binding and elution.

To examine if total DNA yields could be increased by pooling these smaller aliquots, sample lysates were subdivided into two 250 μl aliquots after mechanical disruption, then subjected to thermal disruption and proteinase digestion separately. These two samples were then applied to either a single DNA-binding column in succession or to two separate columns, eluted and pooled after elution. Overall and fungal-specific DNA yields were then measured using NanoDrop DNA quantitation and fungiquant qRT-PCR.

Light microscopy

After centrifugation, cellular pellets from urine were resuspended in 5 ml PBS and mixed well with a pipette. A 10 μl aliquot was transferred to a 75 × 26-mm glass slide and covered with an 18 × 18-mm coverslip, ensuring that the sediment was uniformly distributed but not escaping from the edges of the coverslip. Using an inverted IX51 microscope (Olympus), images without staining were captured at ×400 (objective lens 40× in combination with wide field 10x eyepiece) to generate a field area of 0.196 mm^2.

Comparison with commercial methods

We compared our optimized approach to three, commonly used commercial kits for DNA extraction: PSP Spin Stool DNA Plus Kit (Stratec Biomedical), PureLink Microbiome DNA Purification Kit (ThermoFisher Scientific), and QIAamp DNA Stool Mini Kit (Qiagen). Large volume urine specimens (>120 ml) from 9 subjects were divided into four 30 ml specimens and pelleted by centrifugation at 1500 rcf. Mid-vaginal swabs were obtained from female subjects using FloQSwabs (Copan Diagnostics). Swabs were gently agitated for 30 min in 500 μl enzyme buffer (0.5 M Tris, 1mM EDTA, and 0.2% 2-mercaptoethanol, pH 7.5), before

removing the swab; the resulting cell suspension was then processed as for urine specimens. The identical urine samples and vaginal swabs were processed according to the manufacturers' protocols for each kit or using our optimized protocol. Fungal and bacterial DNA yields in the eluents were then assessed by qPCR as specified above.

Microbial sequencing analysis

Library generation. DNA was isolated from urine using the specified protocols as described above. Fungal ITS1 and bacterial 16S regions amplicons were generated by PCR using the primers below (Table 1) modified to include Nextera XT v2 barcoded primers (Illumina) to uniquely index each sample. Mock samples run in parallel with urine samples lacking any starting cellular pellet as well as individual aliquots of all reagents and buffers were analyzed to ensure validity and rule out any systemic contamination.

PCR reactions utilized Platinum SuperFi DNA Polymerase (Invitrogen) according to the following protocol: initial denaturation at 94˚C for 10 min, followed by 40 cycles of denaturation at 94˚C for 30 s, annealing at 48˚C for 30 s, and elongation at 72˚C for 2 min., followed by an elongation step at 72˚C for 30 min.

Next-generation sequencing. Amplicons generated above were sequenced at 2x300 paired-end sequencing on the Illumina MiSeq sequencer, according to manufacturer's instructions. Raw data processing and de-multiplexing was performed using on-instrument MiSeq Reporter Software v2.6 as per manufacture recommendations. Demultiplexed 16S sequence data were processed and analyzed as previously described including OTU assignment by alignment to the GreenGenes reference database (May 2013 release) at 97% identity.[28] For analysis of ITS1 sequence data, raw FASTQ data were filtered to enrich for high quality reads including removing the adapter sequence by Cutadapt v1.4.1,[29] truncating reads with average quality scores less than 20 over a 3-base pair sliding window and removing reads that do not contain the proximal primer sequence or that contain a single unknown base. Filtered pair-end reads were then merged with overlap into single reads using SeqPrep v1.1 wrapped by QIIME v1.9.1.[30] Processed high-quality reads were then aligned to previously observed host sequences (including rRNA and uncharacterized genes in human) to deplete potential contamination. Operational taxonomic units (OTU) were identified by alignment of filtered reads to the Targeted Host Fungi (THF) custom fungal ITS database (version 1.6), [31] using BLAST v2.2.22 in the QIIME v1.9.1 wrapper with an identity percentage 2:97%.

Diversity analysis

We performed rarefaction analysis. The original OTU table was randomly subsampled (rarefied) to create a series of subsampled OTU tables. Alpha diversity was calculated on each sample using the OTU table and a variety of metrics (chao1, observed species, etc.). The results of the alpha diversity were collated into a single file and the number of species identified for each sample versus the depth of subsampling was plotted. Shannon diversity indices were selected to show composite readout of microbial population evenness and richness.

Table 1. Next-generation sequencing primers.

Amplicon	Forward	Reverse
ITS1	5′-CTTGGTCATTTAGAGGAAGTAA-3′	5′- GCTGCGTTCTTCATCGATGC-3′
16S (8F&R357)	5′-AGAGTTTGATCMTGGCTCAG-3′	5′-CTGCTGCCTYCCGTA-3′

https://doi.org/10.1371/journal.pone.0210306.t001

Statistical analysis

Differences in DNA yields between groups were compared using a two-tailed, paired Student's *t* test with a 95% confidence interval. Data are presented as means ± SEM, unless otherwise stated. Statistical analyses were performed using Microsoft Excel 2016 (version 1803) or RStudio version 3 as appropriate.

Results

Sequential optimization of fungal DNA extraction

To optimize the procedure of isolating urinary microbial DNA, we began with a protocol described in the initial isolation of bacteria from urine specimens,[5, 32]. Small volumes of urine were initially centrifuged to concentrate cells and microorganisms, then subjected to DNA extraction using a standardized kit involving DNA binding and elution from an affinity column.

To concentrate the rare cellular material present in urine, samples were centrifuged under three conditions previously described for the isolation of fungi from low biomass fluids.[32–34] Samples prepared with an initial centrifugation speed of 1500 relative centrifugal force (rcf) for 20 min. yielded fungal DNA levels at least 1.5-fold higher than those prepared at 5000 rcf for 10 min., while yields from those centrifuged at 16000 rcf for 10 min. were substantially lower (Fig 1A). While a subset of individual samples demonstrated similar yields following centrifugation at 5000 rcf as that seen after centrifugation at 1500 rcf, the lower speed was never associated with a decrease in overall or microbial DNA yields (S1 Fig).

Fungi and some bacteria have cell walls, which can be resistant to digestion, leading to their absence or underrepresentation in culture-independent analyses. To optimize the isolation of organisms with robust cell walls, we examined the utility of an initial enzymatic digestion step to aid in cell wall dissolution.[35] Lysozyme, a glycolytic hydrolase that catalyzes the breakdown of peptidoglycan in gram-positive bacterial cell walls, is known to enhance gram-positive bacterial detection.[36] Lyticase, which hydrolyzes the poly-β(1 ❢ 3)-glucose present in yeast cell wall glycans, has been widely used in yeast DNA extraction, including PCR-based clinical assays.[34, 37] These enzymes were tested alone and in combination in comparison to omission of this step. Consistently, the combination of the two enzymes resulted in improved yields of both total DNA (data not shown) and relative fungal DNA levels calculated by qPCR (Fig 1B).

Particularly for fungi, physical disruption techniques, such as the thermal and mechanical steps described above, significantly improve fungal DNA purification,[38–40] again by further breaking down tough cell walls. Bead beating, which we performed using multiple sizes of silica beads, can be particularly useful in isolation of fungi such as Aspergillus, which is known to play a role in multiple human diseases.[41, 42] An additional thermal disruption step, with two freeze-boil (0˚C/95˚C) cycles, was also evaluated. Both methods used in isolation enhanced DNA extraction efficiency 2-3-fold over baseline (Fig 1A).

These disruption steps were followed by an additional digestion step with Proteinase K, a broad-spectrum serine protease, to remove any protein contamination and inactivate any remaining DNAase activity prior to cell and nuclear lysis. We tested a range of proteinase concentrations; while inclusion of the enzyme was important in enhancing DNA extraction efficiency, varying the proteinase concentration had much less effect. While a concentration of 24 mAU/ml (0.8 μg/ml) tended to provide the best results, a range of concentrations from 12–144 mAU/ml (4.8 μg/ml) did not differ significantly in their enhancement of DNA recovery (data not shown).

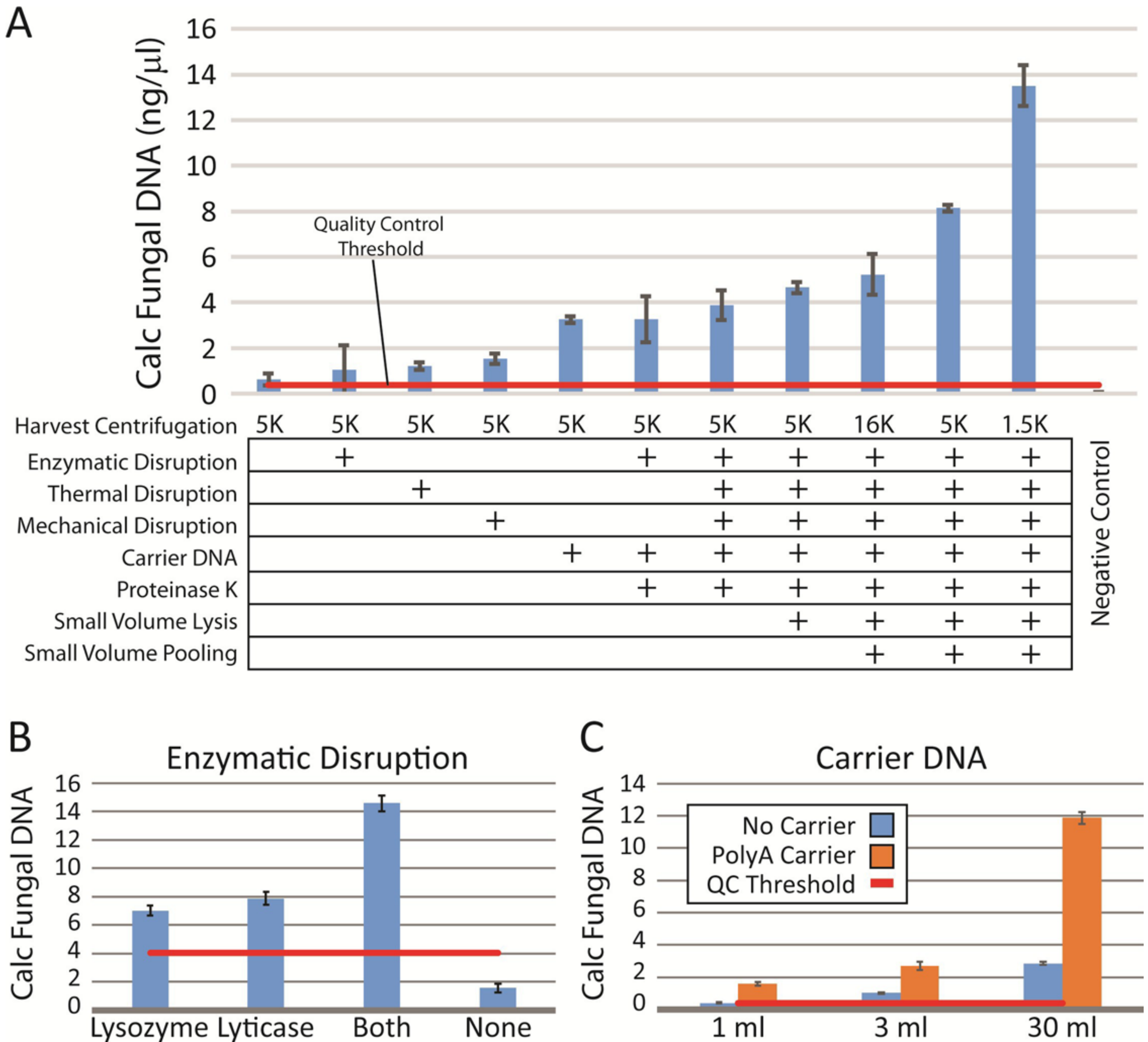

Fig 1. Optimization of microbial dna extraction requires multiple disruption steps. (A) Eight variations in the protocol (at left) were noted to increase yields as determined by quantitative PCR. Relative fungal DNA yields were calculated from quantitative PCR using the Fungiquant pan-fungal PCR primer pair and normalized to a mixed fungal DNA standard. The negative control samples were processed in parallel, but did not have any input cellular material. Multiple protocol variations, such as enzymatic pre-digestion (B) or carrier DNA use during DNA column binding (C), were tested individually in triplicate for multiple subjects (minimum n = 4), both male and female, before incorporating.

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To maximize DNA recovery, we also evaluated the addition of carrier DNA. Because naturally occurring carriers, such as salmon sperm DNA, contain rDNA sequences with partial homology to other eukaryotic DNA, we chose a synthetic carrier, polyadenylic acid, which has shown efficacy in enhancing recovery of low abundance DNA from human biological samples. [43] Supplementation of carrier DNA increased both overall (data not shown) and fungal-

specific DNA yields 2–4 fold across all samples (Fig 1C). The best combination of all techniques tested resulted in an almost 14-fold increase in fungal DNA yields, comprising an optimal protocol utilizing low-speed centrifugation, enzymatic, mechanical, and thermal cell wall disruption, inclusion of carrier DNA, and proteinase K digestion in combination.

Confirmation of protocol on standardized samples

Each of the individual conditions noted to increase DNA yields was tested in aggregate on a panel of urine specimens from both male and female patients. Large volume (>75 ml) urine specimens from 4 subjects (2 male and 2 female) were divided into small equal aliquots (1 ml), and then processed in parallel to confirm the enhancement of DNA purification with the modifications observed in the individual experiments detailed above. This larger-scale optimization panel assessed the variations in cell wall disruption methods (thermal and mechanical), enzymatic pre-treatment methods (lysozyme and lyticase), proteinase K digestion, and centrifugation speed in almost all combinations (Fig 2). Calculation of the relative fungal DNA yields from these 60 variations in isolation methodology revealed a clear pattern, with improved yields resulting from the optimized protocol defined above with multiple disruption methods, combined enzymatic digestion, and lower centrifugation speeds.

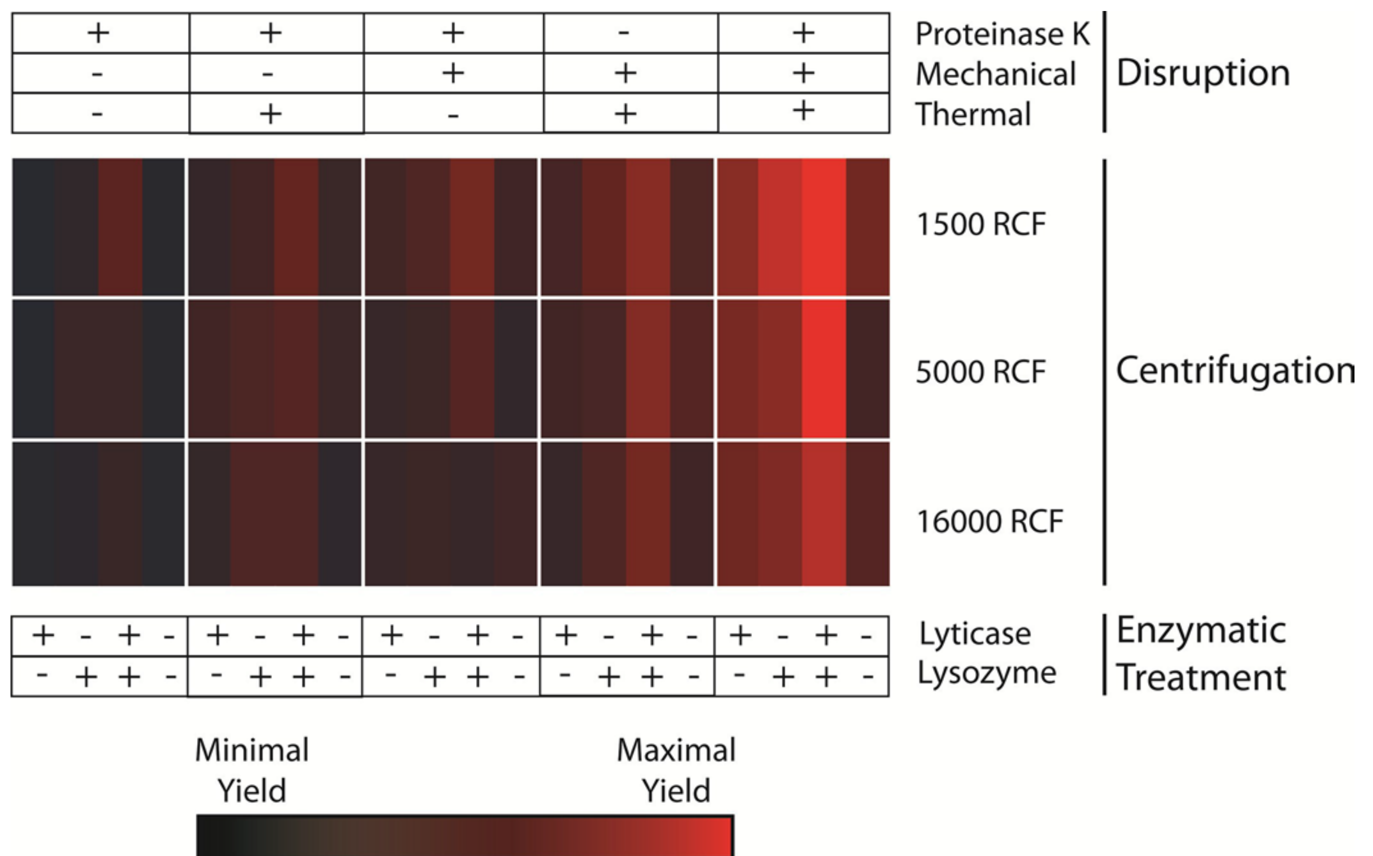

Fig 2. Large-scale confirmation of optimization of microbial DNA purification. The individual conditions noted to increase yields were tested in aggregate in a larger-scale optimization panel. DNA was concurrently isolated from 60 identical 1 ml urine samples from each of 4 subjects with variations in cell wall disruption methods (as indicated at the top), enzymatic pre-treatment methods (bottom), and centrifugation speeds (rows indicated at right adjacent to heat map). Fungal DNA yields from these 60 variations in isolation methodology were calculated from Fungiquant qPCR as described in Fig 1, then scaled across all samples. Values are expressed as a heat map, with bright red signifying the highest yields and black the lowest yields across all samples.

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Effect of sample volume on community profiling

Specimen volume is thought to influence the representation of microbial complexity determined by NGS, particularly in low biomass specimens, such as urine[44], which was also suggested by our preliminary results (Fig 1C). To determine the magnitude of the effect of sample volume on microbial yields and community depth and diversity, we examined microbial profiles across a range of urine sample volumes. Large volume urine specimens from individual subjects were divided into 1, 2.5, 5, 10, 25, and 50 ml aliquots and processed in parallel according to our optimized protocol described above. Fungal yields (Fig 3A) were greatest with larger urine volumes. However, the optimal volume of initial urine was 25 ml, with 10 and 25 ml samples yielding substantially greater DNA concentrations than smaller or larger amounts. Average yields across all specimens *decreased* in 50 ml samples.

While increasing the quantity of urine processed may increase the cellular material, it also increases urinary salts. We hypothesized that these increased salt loads interfered with DNA isolation when utilizing a DNA-binding column. We emulated this situation by adding increasing urinary phosphate salts to standardized urine specimens to emulate the salt burden of larger samples. As typical urinary phosphate salt concentrations are 1–30 mg/dl, an additional 20 ml of urine would provide an additional 2–60 mg of salt per sample. Addition of this amount of sodium phosphate salts was clearly associated with lower microbial DNA yields, particularly for bacteria (S2 Fig).

We also assessed community complexity by gel electrophoresis following PCR-based amplification of the fungal ITS1 rDNA region in which different sized products represent unique fungal taxa (Fig 3B). In comparison to sample sizes of 5 ml or less, 25 ml provided a more comprehensive representation of the range of fungal species with an increased number of bands of varying sizes representing unique taxa for larger initial sample sizes. Across all volumes, urine from male subjects consistently demonstrated lower yields. Only at the 25 ml volume were fungal DNA yields consistently above quality control thresholds.

Effect of urine storage and centrifugation conditions on DNA extraction efficiency

In handling urine, we sporadically observed after centrifugation a substantial, sand-like pellet of varying colors. The appearance of this non-cellular pellet material was observed with refrigeration (>2 hours) of urine samples prior to processing and with high-speed centrifugation (16,000 rcf). Post-centrifugation pellets from larger urine volumes (>50 ml) also would frequently contain this material, even when pelleted at lower speeds (1500–5000 rcf) and processed at room temperature. Microscopic examination of these samples revealed a range of crystalline forms, typically amorphous urates or phosphates, depending on urinary pH. When these microcrystal salts appeared, DNA quality, as assessed by OD_{260}/OD_{280} ratios, was significantly lower. Relative microbial DNA yields were also consistently lower, suggesting that larger crystal burden interfered with DNA purification (S1 Fig). One such post-centrifugation specimen (shown in Fig 4A) demonstrates a red-orange, sandy pellet, the "brick-layer's dust" characteristic of amorphous urates. Confirmation of crystal composition was supported by microscopic analysis (Fig 4B) as well as chemical properties; these pellets could be dissolved by either heating to a temperature >60˚C or adding sodium hydroxide. In a smaller subset of alkaline urine specimens, refrigeration or high-speed prolonged centrifugation resulted in a light-colored sandy pellet, which could be identified as amorphous phosphates by microscopy (Fig 4C). Chemical composition was confirmed by solubility in glacial acetic acid and resistance to dissolution with heating[45]. Other crystal forms were occasionally noted, such as the "envelope"-type crystals characteristic of calcium oxalate (Fig 4D inset), but these did not

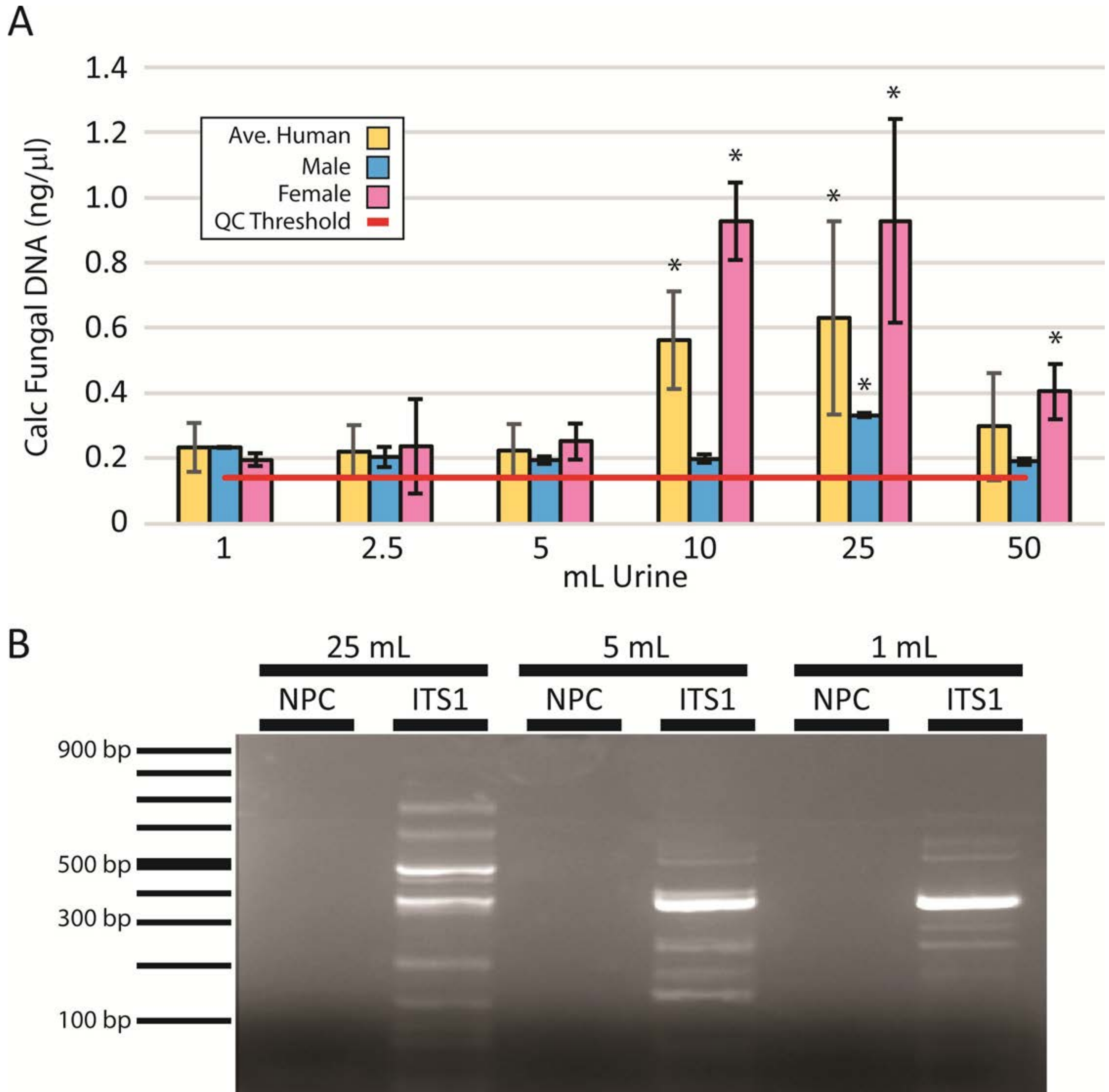

Fig 3. Fungal community representation is influenced by specimen volume. (A) DNA was isolated from a range of urinary volumes in male and female subjects (n = 3 each) and assessed by qPCR for fungal DNA. Calculated fungal DNA concentrations were calculated by normalization to a fungal standard. The optimal concentrations were achieved using 25 ml urine specimens. *: $P<0.05$ in comparison to 1 mL yields. (B) Following fungal DNA amplification by qPCR using broad-spectrum fungal primers, products from 25, 5 and 1 ml samples were assessed by 2% agarose gel electrophoresis. Standards indicating the PCR product size are shown on the left. Each band represents unique taxa within the urinary fungal population. NPC: no primer control.

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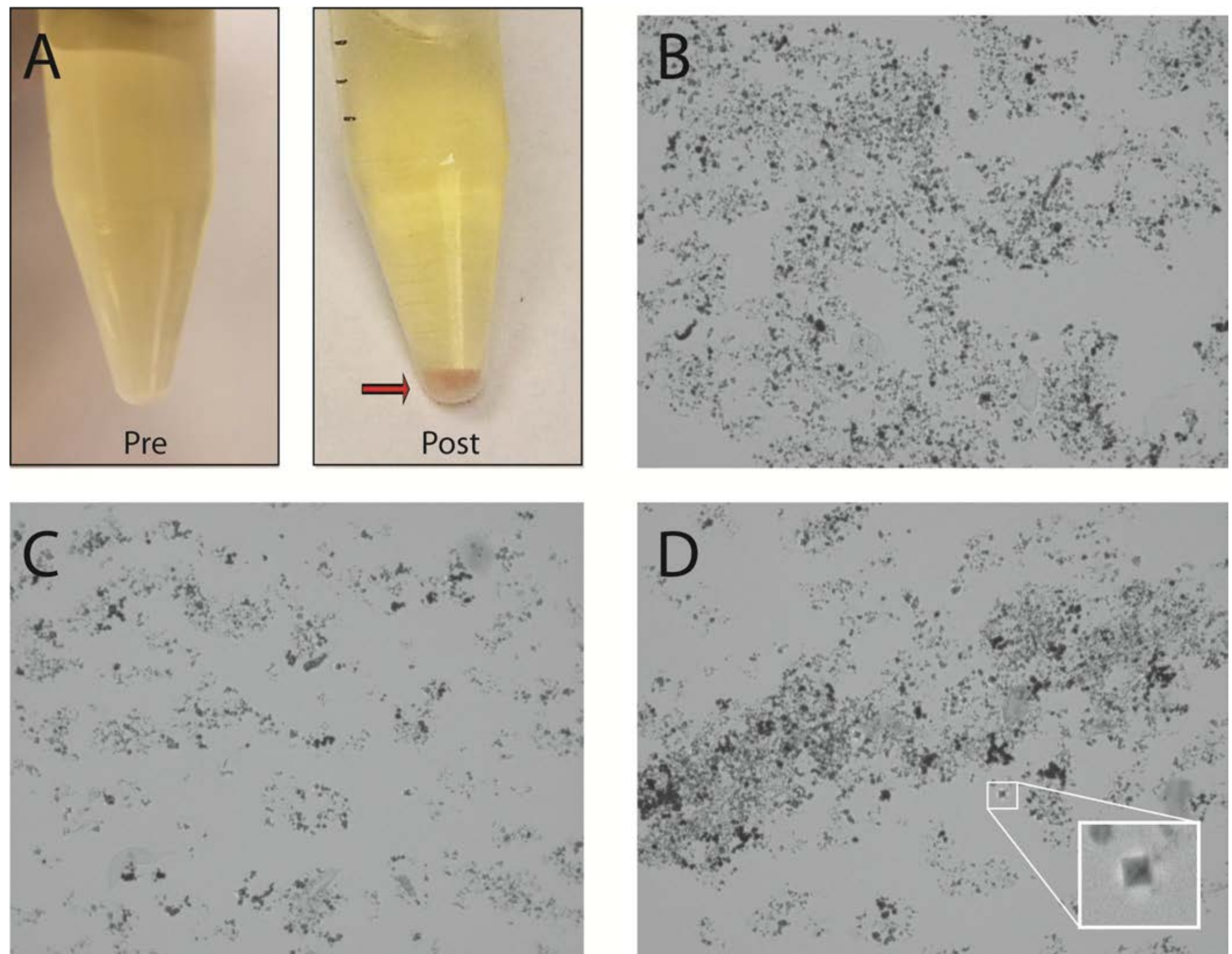

Fig 4. Urine storage and centrifugation conditions impact DNA extraction efficiency. In a subset of urine samples, both refrigeration and high-speed centrifugation were associated with precipitation of varying crystals that interfered with DNA purification. (A) A single urine specimen before and after refrigeration and centrifugation at 5000 rcf. In the post-centrifugation specimen, a red-orange, sandy pellet was observed after centrifugation consistent with the "brick-layer's dust" characteristic of amorphous urates. (B) The pellet seen in A was examined by light microscopy (x400 magnification), revealing disorganized amorphous urate crystals. (C) Amorphous phosphates from alkaline urine. (D) The "envelope"-type crystals characteristic of calcium oxalate could also be identified in urine (magnified in the inset picture), but did not constitute the majority of the crystalline material.

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typically constitute any sizable portion of the crystalline material. We were able to minimize the appearance of crystalline salts through a combination of expedient processing (within 4 hours of sample acquisition), the avoidance of refrigeration, and optimization of sample size and centrifugation speed.

As amorphous urates and phosphates can inhibit individual steps in DNA purification and PCR amplification, we next sought to determine if varying processing volumes could minimize any impact of these salt contaminants on DNA purification and subsequent PCR amplification. In addition, we hypothesized that smaller sample volumes might be more effectively heated for thermal disruption. After combined enzymatic treatment and mechanical cell wall disruption, we subdivided samples into varying aliquot sizes for the two boil/freeze cycles, proteinase K digestion, and DNA isolation using a DNA-binding column. Volumes ranging from

25% to 80% (100–400 μl in 50 μl increments) of the total sample lysate were applied to the spin columns before washing and DNA elution. Small sequential increases in DNA yields were seen up to 250 μl, but then plateaued, without additional increase in DNA yields with larger volumes (S3A Fig).

These data suggested that a portion of the DNA in our samples was not either effectively digested or binding to the extraction column. We therefore attempted pooling of subdivided samples; sample lysates were divided into equal halves (~250 μl) and processed in parallel before column binding. Lysates were then either pooled onto a single column in two subsequent binding steps and eluted in a single elution or bound and eluted from separate columns and pooled after elution. Pooling of two 200–250 μl aliquots on a single DNA column provided the best DNA yields (S3B Fig).

Our optimized method outperforms previously described and commercial DNA isolation methods

We then evaluated our method in comparison to several commercial DNA kits commonly used for microbial analysis. This optimized protocol yielded higher concentrations of DNA and greater species diversity for fungal DNA than identical samples processed with the PSP Spin Stool DNA Plus Kit, PureLink Microbiome DNA Purification Kit, and QIAamp DNA Stool Mini Kit (Fig 4). As the ideal goal of this method would be the simultaneous examination of both fungal and bacterial populations, we also assessed the utility of the optimized protocol in the isolation of bacterial DNA. Our protocol consistently outperformed commercial methods for the purification of fungal as well as bacterial DNA (Fig 5A). To assess the applicability of this protocol to other human commensal microbial communities, we analyzed a panel of vaginal swabs as well. Our protocol enhanced fungal and bacterial recovery from vaginal swabs significantly. While the method previously described for urine samples[5, 32] using Qiagen DNA isolation kits (Qiagen) was already better than the commercial kits tested, the optimized protocol increased the yield of fungal DNA approximately 200% ($p<0.001$) for vaginal swabs and 130% ($p<0.005$) for 30 ml urine samples. Bacterial yields differed even more profoundly, increasing yields approximately 240% for vaginal swabs and 200% for urine over levels seen with the best of previously described methods ($p<0.001$).

The improved yields translated to an improved representation of urinary microbial community diversity as assessed by NGS. Qualitatively, a community of greater richness and evenness, as measured using the Shannon Diversity Index (Fig 5C), was seen; multiple taxa were absent or underrepresented in other purification methods (Fig 5B). The optimized method consistently resulted in the highest diversity of all methods. While these differences were not statistically significant, our optimized technique provides equivalent or improved bacterial and fungal community representation across multiple biological sample types.

Discussion

Extraction of DNA from fungal cells in urine has proven challenging for multiple reasons. Fungi are thought to be low abundance in most body sites and are structurally more robust and difficult to lyse. Multiple challenges in the identification and characterization of fungal species, such as incomplete annotation in common databases, inconsistent taxonomic classification, and variable conservation of the ribosomal locus across divisions of the fungal kingdom[46], complicate studies of fungi in any biologic niche. The combination of these problems with the technical difficulties of working with urine specimens has left previous explorations of the urinary fungal microbiota inadequate to examine anything more than a few, well-characterized species.[25] Given the experiences of others attempting fungal isolation

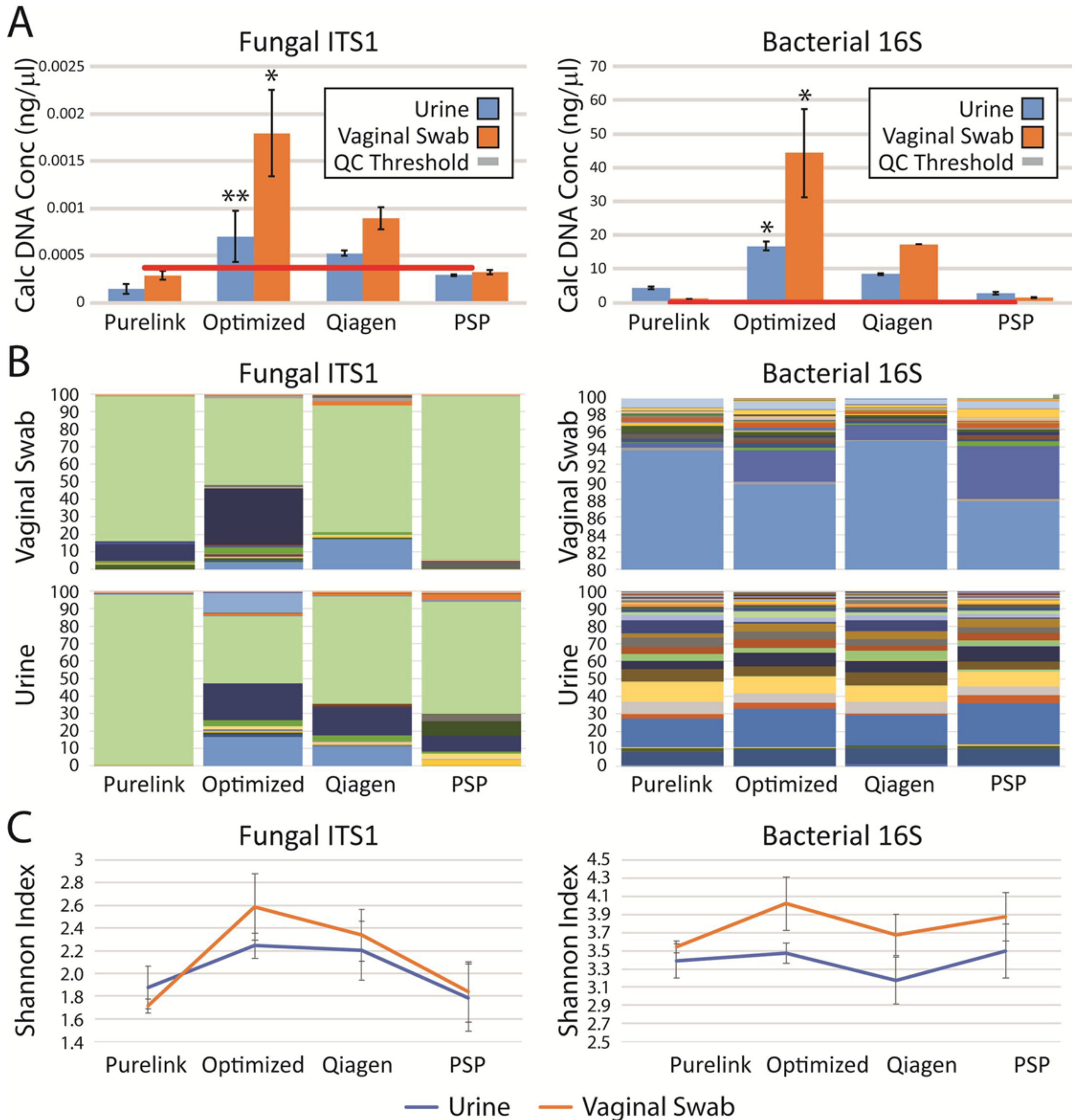

Fig 5. Our optimized DNA extraction method outperforms commercial methods. We compared fungal (left) and bacterial (right) extraction and characterization after our optimized protocol in comparison to three commercial DNA preparation kits using standardized urine and vaginal swab samples. (A) Individual urine specimens were divided into equal aliquots of 30 ml each. DNA was isolated from each aliquot using the specified methods; this process was repeated in quadruplicate. Samples were assessed by qPCR for fungal (left) and bacterial (right) DNA. Calculated DNA concentrations were determined by normalization to a mixed fungal and bacterial standard with a known DNA concentration. *: $P<0.001$, **: $P<0.005$. (B, C) Samples were sequenced in quadruplicate by next generation sequencing for the ITS1 (left) and 16S (right) primers. (B) The stacked bar plots represent the mean relative abundances for the fungal (left) and bacterial (right) populations in individual sequencing runs. (C) Shannon diversity indices were calculated from the microbial populations resulting from NGS for each purification method.

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in low biomass specimens, such as blood, we anticipated that substantial modifications of typical protocols used for the isolation of bacteria from urine would be necessary to assess adequately the fungal populations present. As optimal depth of sequencing requires the highest concentrations of DNA possible, we hypothesized that successful fungal DNA extraction for sequencing would require concentration of cellular material from larger volumes of urine, multiple disruption steps to break down fungal cell walls, and inactivation of the abundant PCR inhibitors present in urine. An iterative approach to the optimization of fungal DNA extraction confirmed these suspicions, with multiple modifications from commonly-used standard DNA extraction methods needed to provide consistent, good quality fungal DNA for sequencing-based assessments.

Sample size was very important. Approximately 40% of low volume specimens (e.g. 1 ml urine) did not provide adequate sequencing depth for analysis (<1000 reads per sample), while samples >10 ml consistently provided excellent depth of coverage in ~95% of samples. While this volume threshold had previously been suggested[44], our data provides objective confirmation that such a threshold is important for microbial analyses. Unexpected was the discovery that the best results were not associated with the largest, initial sample size, with an optimal sample size of 25–30 ml. We believe that this is due to an increase in urinary salts with larger sample volumes beyond a critical point that begins to interfere with DNA isolation. In this study, we utilized urine from asymptomatic subjects; patients with lower urinary tract symptoms tend to restrict fluids as a method to control their symptoms, resulting in urinary salt concentrations that are typically higher. Use of larger sample volumes of 50 ml or greater could unintentionally bias results, artifactually decreasing yields and diversity in patients with urinary symptoms. We selected the 25–30 ml volume to avoid this bias for routine use across study populations in the exploration of urinary symptom pathogenesis.

Interestingly, centrifugation speed made a substantial difference, with slower speeds yielding better results. While this result may seem counter-intuitive, our data suggest that this decrease in fungal DNA seen with higher centrifugation speeds and larger sample volumes is due to the accumulation of amorphous crystals common in urine that interfere with DNA extraction and amplification by PCR. While centrifugation at higher speeds may provide better pelleting of cellular matter, the lowest speed appears sufficient for this purpose; this is the speed routinely used to pellet eukaryotic cells. While lower concentration samples tended to demonstrate similar yields with centrifugation at 5000 rcf, the 1500 rcf centrifugation speed was more reliable across the population and was not associated with any significant loss of material in any samples tested. In addition, the lower speed reduces pelleting of microscopic crystals and *de novo* crystal formation seen at ultracentrifugation speeds. As we wanted to create a standardized protocol for all samples, we chose this lower speed. In addition, centrifugation at these speeds is readably achievable with most clinical centrifuges that can be found in outpatient centers, which may make rapid processing of samples in the clinical setting feasible. While it is possible that other methodologic variations, such as filter-based concentration methods or magnetic bead separations, could provide improved results, our initial attempts using these methods did not appearpromising.

As anticipated, multiple cell wall disruption methods (thermal, mechanical, and enzymatic) provided much improved fungal DNA yields. An additional digestion with proteinase K was helpful at improving DNA quality as well, although the precise amount of enzyme was less important. The use of carrier DNA to enhance DNA column binding efficiency was crucial. Parallel processing of cell lysates in smaller batches with serial application of these samples to a single DNA binding column also improved yields. The individual improvements in fungal DNA extraction for each of these steps justified their inclusion into the optimized protocol.

The final optimized protocol (available at protocols.io) includes limited storage at room temperature prior to centrifugation of a 25–30 ml urine sample at 1500 rcf to pellet cellular material, followed by an initial enzymatic digestion in lyticase and lysozyme. Subsequent mechanical disruption using silica beads and thermal digestion at 95˚C was followed by division of the sample into 2 smaller batches to allow an effective secondary digestion with proteinase K to inactivate any contaminating DNases. After addition of synthetic carrier DNA to each batch, sample DNA was subsequently isolated by binding to a commercial DNA-binding column. We attempted multiple other variations on our protocol that are not described in this paper, such as preheating the DNA elution buffer to 37˚C or performing a second elution from the DNA-binding column in a small volume, as none of these possibilities made significant differences in the resulting DNA concentrations. Our results without these additional steps were sufficient for genomic sequencing.

One drawback to these additional steps is that this protocol takes significantly more time than the available commercial kits, 150–180 min in contrast to 75–90 min. The substantial improvement in the quality and quantity of isolated microbial DNA, however, is clear, consistently providing reliable DNA for NGS analyses of microbial populations.

The samples utilized as test specimens throughout this paper were voided. Contamination from nearby sites, such as skin, urethra, and vagina (in women), can contribute heavily to the microbial content of voided samples[44]. When compared directly (Fig 3), fungal levels in samples from women were 2-3- fold higher than those seen for men. While this could reflect a difference in the urinary fungal content between genders that may be a product of the differing anatomy of the lower urinary tract between genders, it may also merely reflect differences in contamination from nearby urogenital sites. As a result, this paper does not seek to make conclusions about the composition of the urinary mycobiome, but instead sought to explore the solutions needed to characterize microbial content from urine specimens. Larger scale studies, which are currently underway, using a multitude of samples will be needed to explore the urinary mycobiome. However, while the samples used in this study were voided in origin, we have since confirmed that this enhanced protocol is successful at producing sufficient quality fungal DNA to obtain good depth of sequencing from a limited number of catheterized urine samples and those obtained by suprapubic aspirate.

For microbial populations of low abundance, as presumed for the urinary tract, maximizing the quantity of template DNA for analysis is extremely important. When DNA quantities are barely in the range of detection, small variations in sample quantity or quality or even minor fluctuations in physiologic conditions may result in large misleading population shifts. If certain benign urologic conditions are associated with changes in the overall abundance of fungi in urine, as has been suggested for UCPPS,[25] then methods that fail to adequately represent the population at the lower, baseline levels will underrepresent the populations present in these circumstances. It is likely in that situation that culture-independent microbial analyses will incorrectly identify the upregulation or novel appearance of particular taxa, providing misleading conclusions about disease pathophysiology. These problems are compounded by the fact that urine composition and concentration is highly variable, even within a single individual. Certain disease conditions are associated with systematically smaller void volumes, which might also significantly bias such results. The increased DNA concentration and quality achieved using this optimized approach seek to minimize these biases and provide the most accurate results in the use of sequencing-based methods to define the urinary mycobiome.

It has been widely recognized for bacterial DNA extraction that different sample preparation and DNA extraction protocols can produce dramatically different results.[47–50] Protocols utilizing mechanical and enzymatic disruption steps have consistently given the best representations of bacterial community structure, but in no case have the obtained results

provided completely accurate representations of standardized samples.[50] In fungal studies, [51] optimal conditions vary for individual fungal species; therefore, while standardized methods are generally useful for fungal and bacterial DNA extraction from biologic specimens, every method will have some bias in extraction efficiency. No single extraction method is reliable and optimal for all species in all specimens. While our results from a range of subjects and specimens confirmed the efficacy of this optimized protocol in aggregate, there were individual variations in fungal community patterns. Our optimized protocol as defined was not always the most effective for every subject assessed. The greatest variations occurred with centrifugation conditions; it is likely that for subjects for whom there is a lower urinary salt content there would be improved results with higher centrifugation speeds. Such biases are inevitable for all stages in the process of culture-independent sequencing-based identification of microorganisms. It remains important to keep these biases in mind when interpreting results, as well as to confirm results through multiple methodologies.

In conclusion, we present a method for microbial DNA isolation that results in a better representation of the overall fungal and bacterial populations, both in terms of the population diversity as well as identification of low abundance taxa that are lost with less sensitive methods. All of these benefits appear to occur without a significant loss in bacterial community representation, making this the best available method for microbial analyses of urine samples.

Conclusion

Studies examining urinary fungal populations have been limited by the inability to consistently isolate the microbial DNA from low biomass urinary samples. This report describes an optimized protocol for the analysis of urinary fungi that is also highly effective for the concurrent analysis of urinary bacterial populations. The simultaneous and efficient extraction of fungal and bacterial DNA from urine for use in culture-independent microbial analyses is thus possible with this refined technique, providing more reliable methods for the detection and exploration of multiple microbial kingdoms from a single specimen.

Supporting information

S1 Fig. Lower Centrifugation is optimal across intra-individual variation. In individual subjects, lower centrifugation speeds are associated with increased total and fungal DNA yields from urine samples. Urine samples from eight subjects, four male (in blue) and four female (in pink), were divided into three 30 ml samples and subjected to centrifugation at the indicated speeds. (A) Total DNA yields from each condition were measured using a Nanodrop Spectrophotometer and expressed at a fold increase over the levels seen in the 16000 rcf sample. (B) Fungal DNA yields from these variations in centrifugation speed were calculated by quantitative PCR using primers specific for the 18S ribosomal DNA locus, then scaled across all samples. Values are expressed as a heat map, with bright red signifying the highest yields and black the lowest yields across all samples.
(TIF)

S2 Fig. Phosphates inhibit urinary microbial DNA isolation. Large volume urine samples were divided into eight 30 ml samples. After adding the indicated amount of sodium phosphate to each sample, each specimen was processed according to the optimal DNA isolation protocol. (A) Fungal and (B) bacterial DNA yields from these samples were calculated by quantitative PCR in triplicate. Profound decreases in microbial DNA yields, despite equal starting DNA quantities, occur with increasing phosphate concentrations. This decrease is directly dependent on phosphate concentration, as seen in plots of the log of the phosphate

concentration against the log of the fungal (C) and bacterial (D) DNA yields.
(TIF)

S3 Fig. Smaller volume sample processing and pooling increases DNA purification yields. Standardized urine samples from 4 subjects were pelleted and processed using the optimized purification protocol for enzymatic treatment and cell wall disruption. (A) Prior to the addition of proteinase K, varying quantities of the total sample lysate were transferred to new tubes for digestion and DNA column binding. Lysate quantities 2:250 μl provided equivalent yields. (B) Prior to the addition of proteinase K, sample lysates were divided into 250 μl aliquots. Processing of a single 250 μl aliquot (No pooling) was compared to the results if the lysate was split into two aliquots and processed in parallel, then later pooled on either a single DNA-binding column and eluted as a single sample (1 column) or purified separately on two columns, eluted independently and then pooled (2 columns). Control samples were processed in parallel and did not have any input cellular material.
(TIF)

S1 File. Ackerman_PLOS_NGS.
(XLSX)

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References

1. Siddiqui H, Nederbragt AJ, Lagesen K, Jeansson SL, Jakobsen KS. Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA amplicons. BMC Microbiol. 2011; 11:244. https://doi.org/10.1186/1471-2180-11-244 PMID: 22047020; PubMed Central PMCID: PMCPMC3228714.
2. Pearce MM, Zilliox MJ, Rosenfeld AB, Thomas-White KJ, Richter HE, Nager CW, et al. The female urinary microbiome in urgency urinary incontinence. American journal of obstetrics and gynecology. 2015; 213(3):347 e1-11. Epub 2015/07/27. https://doi.org/10.1016/j.ajog.2015.07.009 PMID: 26210757; PubMed Central PMCID: PMCPMC4556587.
3. Wolfe AJ, Brubaker L. "Sterile Urine" and the Presence of Bacteria. Eur Urol. 2015; 68(2):173–4. Epub 2015/03/17. https://doi.org/10.1016/j.eururo.2015.02.041 PMID: 25774008; PubMed Central PMCID: PMCPMC4659483.
4. Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, Neal PR, et al. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci U S A. 2006; 103(32):12115–20. Epub 2006/08/02. https://doi.org/10.1073/pnas.0605127103 PubMed Central PMCID: PMCPMC1524930. PMID: 16880384
5. Pearce MM, Hilt EE, Rosenfeld AB, Zilliox MJ, Thomas-White K, Fok C, et al. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. MBio. 2014; 5(4): e01283–14. Epub 2014/07/10. https://doi.org/10.1128/mBio.01283-14 PubMed Central PMCID: PMCPMC4161260. PMID: 25006228
6. Thomas-White KJ, Hilt EE, Fok C, Pearce MM, Mueller ER, Kliethermes S, et al. Incontinence medication response relates to the female urinary microbiota. Int Urogynecol J. 2016; 27(5):723–33. Epub 2015/10/02. https://doi.org/10.1007/s00192-015-2847-x PubMed Central PMCID: PMCPMC5119460. PMID: 26423260
7. Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, et al. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. J Clin Microbiol. 2014; 52(3):871–6. Epub 2013/12/29. https://doi.org/10.1128/JCM.02876-13 PubMed Central PMCID: PMCPMC3957746. PMID: 24371246
8. Khasriya R, Sathiananthamoorthy S, Ismail S, Kelsey M, Wilson M, Rohn JL, et al. Spectrum of bacterial colonization associated with urothelial cells from patients with chronic lower urinary tract symptoms. J Clin Microbiol. 2013; 51(7):2054–62. Epub 2013/04/19. https://doi.org/10.1128/JCM.03314-12 PubMed Central PMCID: PMCPMC3697662. PMID: 23596238
9. Fouts DE, Pieper R, Szpakowski S, Pohl H, Knoblach S, Suh MJ, et al. Integrated next-generation sequencing of 16S rDNA and metaproteomics differentiate the healthy urine microbiome from asymptomatic bacteriuria in neuropathic bladder associated with spinal cord injury. J Transl Med. 2012; 10:174. Epub 2012/08/30. https://doi.org/10.1186/1479-5876-10-174 PMID: 22929533; PubMed Central PMCID: PMCPMC3511201.
10. Groah SL, Perez-Losada M, Caldovic L, Ljungberg IH, Sprague BM, Castro-Nallar E, et al. Redefining Healthy Urine: A Cross-Sectional Exploratory Metagenomic Study of People With and Without Bladder Dysfunction. J Urol. 2016; 196(2):579–87. Epub 2016/01/26. https://doi.org/10.1016/j.juro.2016.01.088 PMID: 26807926.
11. Thomas-White KJ, Kliethermes S, Rickey L, Lukacz ES, Richter HE, Moalli P, et al. Evaluation of the urinary microbiota of women with uncomplicated stress urinary incontinence. American journal of obstetrics and gynecology. 2017; 216(1):55 e1- e16. Epub 2016/08/09. https://doi.org/10.1016/j.ajog.2016.07.049 PMID: 27498309; PubMed Central PMCID: PMCPMC5182144.
12. Siddiqui H, Lagesen K, Nederbragt AJ, Jeansson SL, Jakobsen KS. Alterations of microbiota in urine from women with interstitial cystitis. BMC Microbiol. 2012; 12:205. Epub 2012/09/15. https://doi.org/10.1186/1471-2180-12-205 PMID: 22974186; PubMed Central PMCID: PMCPMC3538702.
13. Nelson DE, Van Der Pol B, Dong Q, Revanna KV, Fan B, Easwaran S, et al. Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. PloS one. 2010; 5(11):

e14116. Epub 2010/12/03. https://doi.org/10.1371/journal.pone.0014116 PMID: 21124791; PubMed Central PMCID: PMCPMC2991352.

14. Fok CS, Gao X, Lin H, Thomas-White KJ, Mueller ER, Wolfe AJ, et al. Urinary symptoms are associated with certain urinary microbes in urogynecologic surgical patients. Int Urogynecol J. 2018. Epub 2018/08/18. https://doi.org/10.1007/s00192-018-3732-1 PMID: 30116843.
15. Chen Y, Chen Z, Guo R, Chen N, Lu H, Huang S, et al. Correlation between gastrointestinal fungi and varying degrees of chronic hepatitis B virus infection. Diagn Microbiol Infect Dis. 2011; 70(4):492–8. https://doi.org/10.1016/j.diagmicrobio.2010.04.005 PMID: 20846815.
16. Zhang E, Tanaka T, Tajima M, Tsuboi R, Nishikawa A, Sugita T. Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects. Microbiol Immunol. 2011; 55 (9):625–32. https://doi.org/10.1111/j.1348-0421.2011.00364.x PMID: 21699559.
17. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, et al. Fungal microbiota dysbiosis in IBD. Gut. 2016. https://doi.org/10.1136/gutjnl-2015-310746 PMID: 26843508.
18. Hoarau G, Mukherjee PK, Gower-Rousseau C, Hager C, Chandra J, Retuerto MA, et al. Bacteriome and Mycobiome Interactions Underscore Microbial Dysbiosis in Familial Crohn's Disease. MBio. 2016; 7(5). https://doi.org/10.1128/mBio.01250-16 PMID: 27651359; PubMed Central PMCID: PMCPMC5030358.
19. Chehoud C, Albenberg LG, Judge C, Hoffmann C, Grunberg S, Bittinger K, et al. Fungal Signature in the Gut Microbiota of Pediatric Patients With Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015; 21(8):1948–56. https://doi.org/10.1097/MIB.0000000000000454 PMID: 26083617; PubMed Central PMCID: PMCPMC4509842.
20. Delhaes L, Monchy S, Frealle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community—implications for therapeutic management. PloS one. 2012; 7(4):e36313. https://doi.org/10.1371/journal.pone.0036313 PMID: 22558432; PubMed Central PMCID: PMCPMC3338676.
21. Sellart-Altisent M, Torres-Rodriguez JM, Gomez de Ana S, Alvarado-Ramirez E. [Nasal fungal microbiota in allergic and healthy subjects]. Rev Iberoam Micol. 2007; 24(2):125–30. PMID: 17604431.
22. Knutsen AP, Bush RK, Demain JG, Denning DW, Dixit A, Fairs A, et al. Fungi and allergic lower respiratory tract diseases. J Allergy Clin Immunol. 2012; 129(2):280–91; quiz 92–3. https://doi.org/10.1016/j.jaci.2011.12.970 PMID: 22284927.
23. Paulino LC, Tseng CH, Blaser MJ. Analysis of Malassezia microbiota in healthy superficial human skin and in psoriatic lesions by multiplex real-time PCR. FEMS Yeast Res. 2008; 8(3):460–71. https://doi.org/10.1111/j.1567-1364.2008.00359.x PMID: 18294199.
24. de Koning HD, Rodijk-Olthuis D, van Vlijmen-Willems IM, Joosten LA, Netea MG, Schalkwijk J, et al. A comprehensive analysis of pattern recognition receptors in normal and inflamed human epidermis: upregulation of dectin-1 in psoriasis. J Invest Dermatol. 2010; 130(11):2611–20. https://doi.org/10.1038/jid.2010.196 PMID: 20631729.
25. Nickel JC, Stephens A, Landis JR, Mullins C, van Bokhoven A, Lucia MS, et al. Assessment of the Lower Urinary Tract Microbiota during Symptom Flare in Women with Urologic Chronic Pelvic Pain Syndrome: A MAPP Network Study. J Urol. 2016; 195(2):356–62. Epub 2015/09/28. https://doi.org/10.1016/j.juro.2015.09.075 PMID: 26410734; PubMed Central PMCID: PMCPMC4770794.
26. Liu CM, Kachur S, Dwan MG, Abraham AG, Aziz M, Hsueh PR, et al. FungiQuant: a broad-coverage fungal quantitative real-time PCR assay. BMC Microbiol. 2012; 12:255. Epub 2012/11/10. https://doi.org/10.1186/1471-2180-12-255 PMID: 23136846; PubMed Central PMCID: PMCPMC3565980.
27. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008; 3(6):1101–8. Epub 2008/06/13. PMID: 18546601.
28. McHardy IH, Goudarzi M, Tong M, Ruegger PM, Schwager E, Weger JR, et al. Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. Microbiome. 2013; 1(1):17. Epub 2014/01/24. https://doi.org/10.1186/2049-2618-1-17 PMID: 24450808; PubMed Central PMCID: PMCPMC3971612.
29. Martin M. Cutadapt Removes Adapter Sequences from High-throughput Sequencing Reads. EMBnet-journal. 2011; 17(1):10–2.
30. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010; 7(5):335–6. Epub 2010/04/13. https://doi.org/10.1038/nmeth.f.303 PMID: 20383131; PubMed Central PMCID: PMCPMC3156573.
31. Tang J, Iliev ID, Brown J, Underhill DM, Funari VA. Mycobiome: Approaches to analysis of intestinal fungi. J Immunol Methods. 2015; 421:112–21. Epub 2015/04/22. https://doi.org/10.1016/j.jim.2015.04.004 PMID: 25891793; PubMed Central PMCID: PMCPMC4451377.

32. Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, Fitzgerald M, et al. Evidence of uncultivated bacteria in the adult female bladder. J Clin Microbiol. 2012; 50(4):1376–83. Epub 2012/01/27. https://doi.org/10.1128/JCM.05852-11 PMID: 22278835; PubMed Central PMCID: PMCPMC3318548.
33. Kinsey GC, Paterson RR, Kelley J. Methods for the determination of filamentous fungi in treated and untreated waters. J Appl Microbiol. 1998; 85 Suppl 1:214S–24S. Epub 1998/12/01. https://doi.org/10.1111/j.1365-2672.1998.tb05301.x PMID: 21182711.
34. van Deventer AJ, Goessens WH, van Belkum A, van Vliet HJ, van Etten EW, Verbrugh HA. Improved detection of Candida albicans by PCR in blood of neutropenic mice with systemic candidiasis. J Clin Microbiol. 1995; 33(3):625–8. Epub 1995/03/01. PMID: 7751367; PubMed Central PMCID: PMCPMC228002.
35. Goldschmidt P, Degorge S, Merabet L, Chaumeil C. Enzymatic treatment of specimens before DNA extraction directly influences molecular detection of infectious agents. PloS one. 2014; 9(6):e94886. Epub 2014/06/18. https://doi.org/10.1371/journal.pone.0094886 PMID: 24936792; PubMed Central PMCID: PMCPMC4061000.
36. Gill C, van de Wijgert JH, Blow F, Darby AC. Evaluation of Lysis Methods for the Extraction of Bacterial DNA for Analysis of the Vaginal Microbiota. PloS one. 2016; 11(9):e0163148. Epub 2016/09/20. https://doi.org/10.1371/journal.pone.0163148 PMID: 27643503; PubMed Central PMCID: PMCPMC5028042.
37. Williamson EC, Leeming JP, Palmer HM, Steward CG, Warnock D, Marks DI, et al. Diagnosis of invasive aspergillosis in bone marrow transplant recipients by polymerase chain reaction. Br J Haematol. 2000; 108(1):132–9. Epub 2000/01/29. PMID: 10651736.
38. Haugland RA, Heckman JL, Wymer LJ. Evaluation of different methods for the extraction of DNA from fungal conidia by quantitative competitive PCR analysis. J Microbiol Methods. 1999; 37(2):165–76. Epub 1999/08/13. PMID: 10445315.
39. Lech T, Sygula-Cholewinska J, Szostak-Kot J. An economical and combined method for rapid and efficient isolation of fungal DNA. Genet Mol Res. 2014; 13(4):10779–86. Epub 2014/12/20. https://doi.org/10.4238/2014.December.18.19 PMID: 25526198.
40. van Burik JA, Schreckhise RW, White TC, Bowden RA, Myerson D. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med Mycol. 1998; 36(5):299–303. Epub 1999/03/13. PMID: 10075499.
41. Griffiths LJ, Anyim M, Doffman SR, Wilks M, Millar MR, Agrawal SG. Comparison of DNA extraction methods for Aspergillus fumigatus using real-time PCR. J Med Microbiol. 2006; 55(Pt 9):1187–91. Epub 2006/08/18. https://doi.org/10.1099/jmm.0.46510-0 PMID: 16914647.
42. Fredricks DN, Smith C, Meier A. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J Clin Microbiol. 2005; 43(10):5122–8. Epub 2005/10/07. https://doi.org/10.1128/JCM.43.10.5122-5128.2005 PMID: 16207973; PubMed Central PMCID: PMCPMC1248488.
43. Beranek M, Sirak I, Vosmik M, Petera J, Drastikova M, Palicka V. Carrier molecules and extraction of circulating tumor DNA for next generation sequencing in colorectal cancer. Acta Medica (Hradec Kralove). 2016; 59(2):54–8. Epub 2016/08/16. https://doi.org/10.14712/18059694.2016.54 PMID: 27526306.
44. Bao Y, Al KF, Chanyi RM, Whiteside S, Dewar M, Razvi H, et al. Questions and challenges associated with studying the microbiome of the urinary tract. Ann Transl Med. 2017; 5(2):33. Epub 2017/02/22. https://doi.org/10.21037/atm.2016.12.14 PubMed Central PMCID: PMCPMC5300849. PMID: 28217698
45. Hallson PC, Rose GA. Measurement of calcium phosphate crystalluria: influence of pH and osmolality and invariable presence of oxalate. Br J Urol. 1989; 64(5):458–62. Epub 1989/11/01. PMID: 2611613.
46. Ackerman AL, Underhill DM. The mycobiome of the human urinary tract: potential roles for fungi in urology. Ann Transl Med. 2017; 5(2):31. Epub 2017/02/22. https://doi.org/10.21037/atm.2016.12.69 PubMed Central PMCID: PMCPMC5300854. PMID: 28217696
47. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 2010; 6(1):e1000713. Epub 2010/01/15. https://doi.org/10.1371/journal.ppat.1000713 PubMed Central PMCID: PMCPMC2795202. PMID: 20072605
48. Eisner ER. Transcoronal approach to the palatal root of the maxillary fourth premolar in the dog. J Vet Dent. 1990; 7(2):14–5. Epub 1990/04/01. PMID: 2073359.
49. Dollive S, Peterfreund GL, Sherrill-Mix S, Bittinger K, Sinha R, Hoffmann C, et al. A tool kit for quantifying eukaryotic rRNA gene sequences from human microbiome samples. Genome Biol. 2012; 13(7): R60. Epub 2012/07/05. https://doi.org/10.1186/gb-2012-13-7-r60 PMID: 22759449; PubMed Central PMCID: PMCPMC4053730.

50. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PloS one. 2012; 7(3):e33865. Epub 2012/03/30. https://doi.org/10.1371/journal.pone.0033865 PMID: 22457796; PubMed Central PMCID: PMCPMC3311548.

51. Rittenour WR, Park JH, Cox-Ganser JM, Beezhold DH, Green BJ. Comparison of DNA extraction methodologies used for assessing fungal diversity via ITS sequencing. J Environ Monit. 2012; 14(3):766–74. Epub 2012/01/11. https://doi.org/10.1039/c2em10779a PMID: 22230933; PubMed Central PMCID: PMCPMC4685454.

Review Article

The mycobiome of the human urinary tract: potential roles for fungi in urology

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Abstract: The mycobiome, defined as the fungal microbiota within a host environment, is an important but understudied component of the human microbial ecosystem. New culture-independent approaches to determine microbial diversity, such as next-generation sequencing methods, have discovered specific, characteristic, commensal fungal populations present in different body sites. These studies have also identified diverse patterns in fungal communities associated with various diseases. While alterations in urinary bacterial communities have been noted in disease states, a comprehensive description of the urinary mycobiome has been lacking. Early evidence suggests the urinary mycobiome is a diverse community with high intraindividual variability. In other disease systems, the mycobiome is thought to interact with other biomes and the host to play a role in organ homeostasis and pathology; further study will be needed to elucidate the role fungi play in bladder health and disease.

Keywords: Mycobiome; fungi; fungal microbiome; urology; urinary tract; lower urinary tract symptom (LUTS)

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Introduction

The microbiome, the aggregate collection of microorganisms within a physiologic niche, modulates human physiology in ways that we are just beginning to comprehend, influencing organ permeability and barrier function, vitamin synthesis, metabolism, neurological activity, and inflammation and immunity (1,2). Studies of these microbial communities were previously hindered by limitations of culture methods, but the development of culture-independent discovery techniques aimed at the detection of microbial DNA has revealed complex populations in sites previously thought to be sterile, such as the lung, brain, and breast. This insight prompted multiple comprehensive studies looking at bacterial ecosystems and their role in health and disease over the past decade, exemplified by the large-scale National Institutes of Health (NIH) Human Microbiome Project (HMP) in the United States and the Metagenomics of the Human Intestinal Tract (MetaHIT) Project in Europe.

Before the advent of culture-independent analyses, the uninfected urinary tract had been assumed to be a sterile environment and was therefore not included in the HMP (3). It has since become clear that bacterial communities exist within the urinary tract, even in healthy, asymptomatic subjects (4-8). Large shifts in urinary bacterial communities are found in a variety of pathologies, such as neurogenic bladder, stress and urgency incontinence, interstitial cystitis/painful bladder syndrome (IC/PBS), and sexually transmitted infections (4-7,9-13), suggesting the resident microbial communities change in disease states [reviewed in (14)].

With the development of affordable, rapid, and scalable culture-independent methods for the study of bacterial communities, the last decade has seen a massive expansion in studies aimed at profiling commensal bacterial communities. Despite this new interest in and understanding of the

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importance of microbial communities in human physiology, non-bacterial populations, such as fungi, viruses, archae, and protozoa, have remained understudied. As the term "microbiome" is frequently used to refer to the study of bacteria alone, the term "mycobiome", a combination of microbiome and mycology, emerged in 2010 (15) to designate the endogenous community of fungi within a specific host anatomic location. This field is still in its infancy; the term "mycobiome" or "fungal microbiome" only appears in PubMed-indexed articles 105 times. Of these articles, only 37 provide original data regarding communities in human hosts, several of which utilize only culture-based methods. None of these examines the urinary tract.

There are several explanations for the inattention to the role of fungi in host physiology, including a lack of standardized information regarding the vast numbers of species, a lack of reagents and tools for the study of fungal populations, and a (potentially) erroneous belief that these organisms are not as critical as bacteria in human disease. Only a few fungal species are known to cause invasive infections in humans despite hundreds of thousands of fungal species throughout the environment (16). The pathogenic potential of these organisms is clear; while rare, fungal infections of the urinary tract can be life-threatening due to the limitations in culture methodologies, frequent low suspicion for fungal involvement, and the lack of preventative and therapeutic options (17). *Candida* spp. are overwhelmingly the most prevalent urinary pathogen, but all of the common invasive fungal species, such as *Cryptococcus*, *Aspergillus*, *Mucoraceae*, *Histoplasma*, *Blastomyces*, and *Coccidioides*, can infect the urinary tract. Recent epidemiological studies have noted increases in both the populations infected and in the development of more virulent organisms resistant to common antifungals (18,19). This shift may be due to increases in the use of antineoplastic drugs, systemic immunosuppressants, prosthetic grafts and implants, as well as the wide-spread use of broad-spectrum antibiotics (20).

It is becoming clear that there are associations between changes in the mycobiome and common, significant diseases in the immunocompetent host such as hepatitis, cystic fibrosis, and inflammatory bowel disease (IBD), which can be seen in the absence of frank, fungal infections. But is this the case for fungi in the urinary tract? As early as the 1850s, clinicians were able to demonstrate viable fungi in the urine of asymptomatic subjects and note alterations in fungal composition in association with diabetes and renal disease, again, in the absence of overt infection (21). But more than 150 years have gone by with little increase in our knowledge or understanding of urinary fungi. A substantive portion of this lack of progress may be due to the lack of tools available to identify and study this kingdom and its interactions with the human host.

Tools to study the human mycobiome

Culture techniques

The majority of fungal culture techniques date back to the 1920s with few changes in the past 100 years. Identifications are made by examining growth structures and evaluating growth on different media (22). Even for cultivatable species, low abundance organisms and organisms that require microbe-microbe interactions cannot be cultivated optimally (23). More importantly, unculturable fungi comprise the largest part of the human mycobiome. In studies of the pulmonary fungal and bacterial microbiome in cystitc fibrosis, 60% of genera were missed by culture (24). In one study of the oral mycobiome, 11 of 85 fungal genera identified by culture-independent methods could not be cultured at all (15). In 30% of the participants, non-cultivatable fungi were 50% or more of the fungi identified. In a direct comparison, extended culture conditions identified five species, while culture-independent analyses identified 37 unique fungal genera within the gut mycobiome (25). Although culture will remain an important tool in our armamentarium, culture-based methods are a poor methodology to examine the complexity and depth of commensal fungal communities.

Culture-independent methods

At the heart of culture-independent approaches to the detection of microorganisms is an assumption that DNA isolated from an anatomic site will contain genomic sequences from resident microbes which provide a snapshot of the ecosystem at that moment in time. Several methodologies allow the rapid assessment of the relative diversities of samples without a need for specialized expertise and expensive equipment, such as restriction fragment length polymorphism (RFLP) analysis, oligonucleotide fingerprinting of *rRNA* genes (OFRG), denaturing gradient gel electrophoresis (DGGE) and *in situ* hybridization. While these methods can be informative, they lack the specificity to identify fungal differences in

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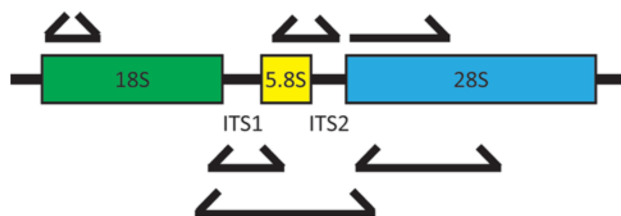

Figure 1 Schematic representation of the fungal ribosomal gene cluster. The 18S, 5.8S, and 28S genes are separated within the locus by two ITS. These ITS sequences are noncoding regions, and therefore have the highest diversity within the locus. Binding locations for several of the commonly used fungal primers for NGS are shown above and below the schematic. ITS, internal transcribed spacer; NGS, next generation sequencing.

larger populations at the species level.

In general, direct sequencing methods provide the best representation of diversity and complexity in microbial community profiling. Shotgun metagenomics and metatranscriptomics, which involve the sequencing and analysis of total genomic DNA or RNA transcripts respectively (26,27), theoretically provide the most comprehensive and unbiased data, but have proven challenging in the analysis of fungi from human biological samples given their low abundance. Previous attempts in other organ systems have been unable to produce data sets with enough sequence data to reconstruct the genomes of fungi from complex human samples such as stool (26).

The majority of mycobiome studies utilize next generation sequencing (NGS) of pools of specific DNA targets. After isolation of DNA from a biological sample, small variable regions of the fungal genome are amplified by polymerase chain reaction (PCR), followed by large-scale pyrosequencing of thousands or even millions of these fragments. The taxon corresponding to each fragment is identified by structured comparison to a database. In comparison to traditional Sanger sequencing, NGS has made community-level analyses more efficient and cost-effective, making it the effective gold standard for microbiome profiling. There are, however, significant limitations to this technology and unique challenges for fungal identification at every step of sample processing and analysis.

Challenges in fungal community profiling

DNA extraction

Previous studies examining the optimization of DNA isolation and purification for analysis of bacterial species ("microbiome") in samples with a standardized, diverse bacterial community demonstrated that different sample preparation and DNA extraction protocols have dramatically different results (1,15,28,29). While more extensive protocols utilizing mechanical disruption by bead beating and multiple enzymatic treatments consistently gave the best representations of bacterial community structure, in no case did the obtained results provide a completely accurate picture of standardized samples (29). Isolation of DNA from fungi can be even more challenging than from bacterial cells, as fungi are often more structurally robust and difficult to lyse effectively. As no extraction method will disrupt all species uniformly, hardier fungi may be underrepresented in analyses. In addition, the optimal cellular disruption/lysis and subsequent DNA extraction and purification conditions for fungal samples have not been clearly defined (30). These conditions may differ significantly from those used for bacterial isolation and may influence both overall DNA yields and relative species representation.

Amplification methodology

In contrast to metagenomics, methods based on sequencing pools of specific DNA targets typically rely on the selection of a relatively conserved genomic region to serve as a proxy for the entire genome. As in bacterial profiling, these sequences are amplified using primers designed to hybridize to regions conserved between fungi but containing between them areas of sufficient variability to allow taxonomic assignment. For profiling of bacteria, the 16S rRNA locus is targeted. The fungal rDNA cluster of genes encodes the 18S, 5.8S, and 28S ribosomal subunits in a single locus separated by two internal transcribed spacers (ITS1 and ITS2) (*Figure 1*). Multiple regions within this locus have been utilized for community profiling, most commonly the 28S large subunit (LSU), 18S small subunit (SSU) and the ITS. The LSU is highly conserved but lacks discriminatory power for many species. The SSU is less conserved than the LSU, providing better phylogenetic distinction, and allows the detection of a number of non-fungal eukaryotes, such as parasitic protozoa (28,31). The ITS regions are most commonly used as the higher diversity at these loci enable greater genus-level phylogenetic assignment (32). This diversity, however, can lead to underamplification of certain families, inevitably biasing the results towards certain species. For example, commonly-used ITS1

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primer combinations can be biased toward amplification of Basidiomycetes, while ITS2 primer sets can be biased towards the Ascomycetes (33,34).

Sample processing and analysis

The sequencing platform utilized can also impact the quality and character of information obtained by NGS (30,35). Pyrosequencing using the 454 GS-FLX (Roche Diagnostics Corporation) technology is the most expensive, but produces the longest sequence reads (>500 bp) (36). The Illumina© HiSeq and MiSeq platforms (Illumina, Inc.) dominate the NGS market, providing a good balance of convenience and quality. HiSeq gives the highest data output at lowest cost, while MiSeq is better balanced for quick turnaround time and longer read lengths (37,38). The Ion Torrent systems (Thermo Fisher Scientific, Inc.) offer low cost, scalable, high-throughput sequencing competitive with the Illumina technology (39), although we have noted that it has more trouble handling the diverse amplicon lengths generated when amplifying ITS1 or ITS2 regions (30).

Once sequencing is complete, pipelines for the analysis of sequence data compile bioinformatics tools that allow the trimming, screening, and alignment of sequences for the assignment of operational taxonomic units (OTUs), phylogenetic analyses, and determinations of fungal diversity within and across groups (α and a diversity). The QIIME (40) and Mothur (41) analytical methods are widely used for all varieties of microbial profiling. More recent tools, such as CloVR-ITS (32) and BROCC (28), were uniquely developed for mycobiome studies, using customized automated analysis pipelines containing elements from both QIIME and Mothur to optimize taxonomic assignment of ITS and 18S amplicons, respectively.

Taxonomic classification

The most limiting step in mycobiome analyses is the choice of sequence database. There does not exist for fungi a database as rich as that available for bacterial 16S rDNA. For ITS sequences, the UNITE database (42) is most commonly used and contains the greatest number of overall annotated fungal sequences, but may not be best for the analysis of human biological samples. The targeted host-associated fungi (THF) database, designed specifically for biologic, not environmental, fungal discovery, consistently outperforms UNITE and other large ITS1 databases (30) on comprehensiveness, taxonomy assignment accuracy, and computational efficiency in analyzing sequencing data from mammalian sources. There exist a multitude of additional sequence collections, such as the SILVA database of 18S and 28S sequences (43) and the PHYMYCO-DB collection of SSU and EF1-α gene sequences (44).

While the curation of public databases has significantly improved in the past decade, a 2006 estimate suggested that only 1% of fungal species were represented in GenBank and that approximately 20% of entries may be misclassified to the species level (45). Also lacking from these databases is the capability to categorize fungi at the subspecies level. Additional problems arise for fungi which have not been addressed or resolved in the literature given the lack of systematic nomenclature for fungi; e.g., a single species may have been given multiple names. In addition, sexual and asexual forms of the same fungal species are often classified as different taxa (28). These resources are themselves evolving and improving, but require continued annotation and curation to move forward.

Relative abundance readouts

The ability of NGS technology to amplify and detect very low abundance species provides tremendous analytic power, but this sensitivity is also a source of significant consternation; contamination and context can influence results, particularly in low density samples. Quantification of absolute fungal levels is not possible with NGS alone; these analyses typically only provide a relative abundance of detection of specific sequences within the overall population. Trace environmental contamination, carryover of populations from nearby sites, variations in sample quantity or quality, and even minor fluctuations in physiologic conditions may result in large, misleading population shifts. The predominance of a fungal species may represent robust expansion of that population, selective survival of that species under selective pressure, or both. While the significance of these situations is different, as are the absolute numbers of live fungi, there is no way to discriminate these possibilities based on NGS data alone. Further, rDNA loci in bacteria and fungi are generally present in genomes in multiple (and often variable) copy-numbers. Thus, it is critical to combine NGS-based determinations of microbial changes with alternative molecular and microbiological techniques to draw conclusions about the roles of particular population shifts in disease conditions.

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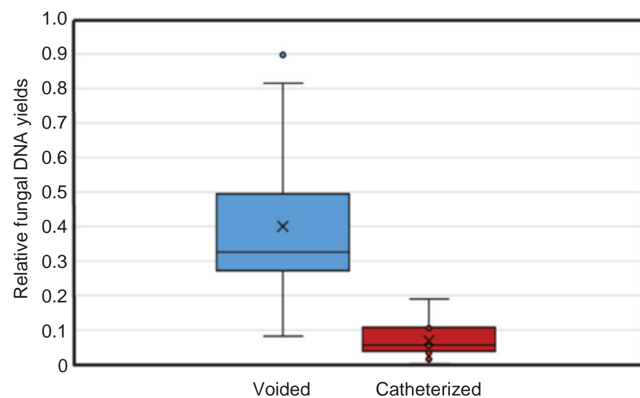

Figure 2 Relative fungal DNA quantities in urine samples. Quantitative PCR for fungal ribosomal DNA was used to determine the median levels of fungal DNA in urine samples from asymptomatic female patients. The median levels in voided urinary samples were nearly an order of magnitude higher (8.5-fold) than those seen in catheterized specimens. These data imply the presence of substantial contamination from vaginal and possibly skin sources, suggesting that the analysis of voided specimens may be dominated by non-urinary microbes. PCR, polymerase chain reaction.

Challenges in evaluating the urinary mycobiome in particular

Host contamination

As fungi are eukaryotes, the presence of non-fungal eukaryotic DNA (e.g., from host cells) can compete with fungal DNA for reagents during extraction and amplification. The more human material, the worse this interference becomes. Thus, contaminating human cells may interfere with the specific amplification and sequencing of fungal ribosomal sequences. In urinary samples, the ratio of fungi to sloughed human urothelial (and contaminating epithelial) cells is unclear, but presumed to be extremely low, reducing the sensitivity of mycobiome analyses in lower urinary tract disease.

Low abundance of source material

In the gut, metagenomics studies demonstrated that only ~0.1% of detectable sequences in the total microbiota were attributed to fungi (26), in contrast to the 99% that were bacterial. There are no data about what this ratio might be in the bladder or any reason to believe that this ratio would be similar. Even when considering these estimates, there is cause to doubt this conclusion, as this assessment was based on sequences identified from available annotated reference databases, in which fungi are highly underrepresented (46). Regardless, the prevailing belief is that the total abundance of microorganisms in urine is orders of magnitude less than the gut, with fungi comprising only a small percentage.

Thus, for populations of low abundance, as presumed for the urinary tract, the difficulties detailed above with appropriate sensitivity are amplified. Low numbers of fungi would result in the omission of large numbers of species from community profiling as a result of abundances below detection levels. As urine samples vary considerably from void to void even in the same individual, small volume samples may not be representative of the overall population. For example, analysis of 1 mL urine samples without controlling for the overall voided volume, urine concentration, and circumstances of collection is likely to introduce a multitude of additional biases that may influence results. We have noted significant vaginal and skin contamination in voided specimens that cannot be entirely eradicated by catheterization. Given the microbial load in the vagina, both fungal and bacterial, any vaginal contamination is likely to obscure the urinary microbiota almost completely (*Figure 2*). Suprapubic aspiration offers the purest examination of urine with minimal urethral, vaginal, or skin contamination (47), but due to its invasive nature, is not practical for the study of urinary fungi in larger human populations.

Bladder vs. urine

Urinary samples may not reflect the bladder microbiota. In the gut, direct comparison of mucosal biopsies to stool samples or brush biopsies demonstrate that mucosal-associated bacteria are not equally distributed between these two locations (48,49). Fungal interactions with the urothelium are entirely uncharacterized; it is possible that fungi may attach directly to the urothelium to form mixed biofilms with bacterial species or grow in soluble microcolonies in the urine, never making direct contact with the urothelium. It is even possible that fungi could remain quiescent within cells of the bladder wall, as can be seen with *E. coli* intracellular bacterial colonies (50). Similar mechanisms of intracellular sequestration have been observed for other fungal species in human hosts (51-54). If all of these mechanisms are in play, then the urinary microbiota would be expected to vary with urine concentration, the amount of urothelial sloughing,

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and intrinsic host and environmental factors, such as psychosocial stress, that alter growth conditions for these organisms.

Temporal fluctuations in urine microbial composition

As yet, no one has analyzed the stability of urinary microbial communities over time, leading to uncertainty as to whether observed differences between subjects represent stable communities with high interindividual variability, highly fluctuant intraindividual variation over time, or differences in collection methods unrelated to microbial content. Even using similar collection and processing methodologies, two studies of the oral mycobiome exhibited significant differences in the most abundant taxa (15,55), likely reflective of both the high variability between the subjects in each study as well as the different techniques utilized. Thus, a comparison of species abundance across samples using data from NGS can be misleading. With variations in collection and analytic methods, each of which introduces its own unique biases, comparison of fungal data across studies becomes challenging. All of these caveats again stress the point that these methods do not truly provide evidence of viable, stable fungal communities without confirmatory studies. While NGS studies are useful at generating an overall sense of microbial changes in disease, the limitations of these studies are many and require validation and mechanistic investigations to place the results into the context of bladder physiology.

The composition of the human mycobiome

The initial characterizations of the human mycobiome were dependent on culture-based methods, which limited these analyses to a select few species known to be pathogenic to humans, such as *Candida albicans, Candida glabrata, Crytococcus neoformans, Aspergillus fumigatus, Coccidioides* spp., *Histoplasma* spp., and *Blastomyces* spp. With extended culture conditions and culture-independent methods, however, we now recognize that sites thought to be sterile, such as the lung, harbor fungi (24) of astounding diversity. The composition of these communities is influenced by a multitude of factors, including age, gender, environmental variations (56), and hygiene (57). The limited fungal community profiling data available so far suggest that fungal communities may be more fluctuant than bacterial ones (58) and exhibit a greater interindividual variation than seen for bacteria (59-62). One plausible hypothesis suggests that because bacteria are numerically more abundant than fungi, bacterial communities are more stable and robust and are less influenced by environmental fluctuations.

In general, different predominant organisms are seen in different anatomic niches, but related, neighboring sites often have similar patterns (63). For example, *Cladosporium*, *Aspergillus,* and *Penicillium* spp. dominate in both the oral and nasal cavities (15,64), but differ from those seen in lung bronchoalveolar lavage specimens (65). The regions surrounding the urethra, however, are highly divergent in their composition; the inguinal crease skin is dominated by *Malassezia* spp. (31), while the vagina contains predominantly *Candida* spp. (66). The local environment is important as a shift in the populations can be pathogenic; a predominance of *Candida* in the inguinal crease is associated with dermatitis (67).

In the vagina, *Candida* spp. have long been recognized as colonizers of the genital epithelium without causing disease (under most circumstances). In a culture-independent analysis of the vaginal mycobiome in healthy, asymptomatic women (66), ITS1 NGS confirmed this predominance of the Ascomycota, the largest proportion of which were *Candida* spp. *Candida* was not, however, the only fungal species detected or even the predominant one; 30% of patients did not have detectable Candida at all in vagina swab specimens. As seen in other organ systems, alterations in vaginal fungal diversity were associated with pathologic conditions, such as in diabetes (68) and allergic rhinitis (69). In all of these studies, however, there are a large proportion of sequences that cannot be matched to specific taxa, which again reflects the nascent status of this field.

The urinary mycobiome

As yet, there have not been any comprehensive attempts at characterizing the urinary mycobiome reported. Low levels of Candida spp. have been detectable in urinary samples from a variety of patients, including healthy controls, using expanded quantitative urine culture (EQUC) (4-7), demonstrating their viability within urine. Using the Ibis T-5000 universal biosensor system to identify microbes, fungi were detectable in urine from patients with urological chronic pelvic pain syndromes (70). This approach recognized an increase in the number of patients with detectable fungi (3.9% *vs.* 15.7%) during symptomatic flares, while no significant differences in the bacterial microbiota could be identified. Of note, this detection method is only able to quantitate the *Saccharomycetales*, which includes

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Figure 3 The urinary mycobiome of asymptomatic control subjects. Clean-catch mid-stream urine samples were obtained from a panel of women without urinary complaints, as confirmed by validated urinary symptom questionnaires, then subjected to NGS using the ITS1 fungal ribosomal primer set. (A) The pie chart represents the overall prevalence of individual classes of fungi across the population as determined by taxonomic assignment using the THF database; (B) stacked bar-plot representation of the relative abundances at the class level of the urinary mycobiota of healthy subjects demonstrates significant interindividual variability as well as intraindividual diversity. NGS, next generation sequencing.

Candida and *Saccharomyces*, but excludes many of the species implicated in other organ system pathologies. Regardless, these transformative results implicate a role for fungi in lower urinary tract symptomatology that was not previously recognized.

We have performed a preliminary, culture-independent characterization of urinary fungi from a range of asymptomatic subjects (manuscript in preparation). Utilizing NGS with amplification of the ITS1 region, we have identified a diverse population of fungi in urinary samples. As detailed in *Figure 3*, our results indicate that each individual exhibited a diverse fungal population without single predominant taxa. Fungi belonging to the *Saccharomycetes* class, which includes the *Saccharomyces* and *Candida* spp., are the only fungi documented to be detected in urine as yet (4,70). These were commonly present in our analysis but were not the most common, demonstrating the limitations of culture-based analyses at characterizing the breadth of fungal populations in urine. Similar to studies of the vagina, a proportion of the OTUs (approximately 40%) observed could not be matched to known fungal species despite comprehensive searches of multiple fungal-specific databases and the NIH GenBank sequence database of all publicly available DNA sequences.

Interestingly, one patient in the analysis (#V002 in *Figure 3*) had a dramatically different profile of urinary fungi than the other subjects; approximately 32% of the detected OTUs were identified as belonging to the glomeromycetes, a fungal division completely absent from all other patients. In addition, the relative levels of fungal DNA found in this subject were nearly three orders of magnitude greater than the population median. After performing the analysis, it was noted that this patient was taking steroids for an unrelated skin condition at the time of sampling. While this result only details a single aberrant patient, the dramatic diversion of this subject's resident urinary fungi from the patterns seen in the majority of other subjects suggests that the composition of the urinary mycobiome is linked to changes in systemic physiology. In this particular case, modulation of host immune status is closely tied to dramatic changes in both fungal overall quantity and community composition.

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The mycobiome in health and disease

Despite the limitations in community profiling data, patterns in fungal populations begin to emerge as do their associations with organ physiology and pathology. Alterations in the mycobiome occurring in the absence of classical infections have been implicated in a wide range of diseases, such as chronic hepatitis B virus (HBV) hepatitis (25), atopic dermatitis (71), dandruff (72), IBD (73-75), cystic fibrosis (24), allergy/atopy (64), asthma (76), and psoriasis (77,78). There is no obvious pattern of fungal alterations in these diseases; pathology can be associated with either a decreased or increased diversity from that seen in the healthy state. The particular species alterations also differ in each pathology. Unlike the case of *Helicobacter pylori* in gastritis, the lack of simple associations of specific diseases with particular organisms underlies the complex interdependency between the mycobiome, host immune status, and other members of microbiota (including the equally neglected protozoa, viruses, and archaebacteria).

A major caveat for studies identifying associations of disease states with microbial alterations is the questionable cause logical fallacy; that is, the correlation of alterations in the microbiota with disease states does not provide evidence that these changes are causative or even impact disease progression. Changes in the mycobiome may directly lead to the generation or progression of disease, but they may also be merely a sign of altered tissue microenvironments, perhaps as a result of altered metabolism or inflammatory dysfunction. It is equally plausible that the disease state results in local environmental changes that lead to the development of an altered microbial community better adapted to the new conditions. Even if microbial changes are not involved in pathogenesis, however, specific microbial signatures may be useful as diagnostic or prognostic markers of disease.

It is unlikely that the simple introduction of a specific species or even the expansion of a class of organisms results in disease without alterations in the host that facilitate the pathology. In other diseases linked to mycobiome alterations, fungal colonization is typically coincident with an immunomodulatory state that results in dysregulated inflammation presenting without a clear etiology (79). While it is well understood that immunosuppressed patients (such as HIV+, transplant, or chemotherapy patients) are more likely to contract opportunistic fungal (and protozoal) infections than immunocompetent patients, the specific mechanisms by which immunomodulatory states lead to increased fungal pathogenesis are not well understood.

In addition to the influence of host immune status on fungal community composition, the transition to virulence of commensal fungi may also be complicated by the ability of individual fungal species to exist in both a commensal and pathogenic relationship with the host (80). The mechanisms controlling such modifications are unclear. The switch from commensal to pathogen within a single ecosystem may be associated with gene expression changes triggered by as-yet poorly understood environmental or host factors. This evidence suggests that the host response to fungi is highly complex with constant integration of complex messages from both local environments and systemic host health. Overall fungal burden, community composition, and unique fungal virulence states likely interact continually with and respond to host metabolic state and immune status as well as perturbations in other microbial communities, such that minor fluctuations in any of these factors can bias the host from symbiosis towards disease.

Future directions

Myriad challenges await in the field of urologic mycology. Most fundamental is an understanding of how the fungal mycobiota of the bladder contribute to the regulation of bladder health, function, and inflammation. While much is assumed from studies of other organ systems, nothing is known about the development/inoculation and maturation of the urinary mycobiome in early life, how the urinary mycobiota interact with neighboring communities, and the susceptibility of this ecosystem to perturbations from nutritional changes, metabolic stresses, and other host inflammatory disorders. While multiple lessons from other organ systems have demonstrated the close interplay between different kingdoms, virtually nothing is known of how urinary fungi influence urinary bacterial community composition and stability.

Hopefully, with a better understanding of the role of fungi in urinary tract disease, will come the ability to use targeted manipulation of the mycobiome for therapeutic purpose, as is becoming a reality in other organ systems. *Saccharomyces boulardii* is being trialed as a probiotic used in the treatment of diarrheal diseases (81,82). With recognition of the role of fungi in graft-versus-host disease, antifungals are being explored as agents to ameliorate disease development and severity (83). As we learn more about the role of fungi in urinary tract disease, novel potential therapeutic approaches, such as fungus-specific

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vaccinations, fungal probiotics, or targeted antifungal drugs, may become plausible adjunctive treatments.

Conclusions

While long ignored, fungi are an essential part of the human microbiota. While this field remains underdeveloped and in need of improved analytic tools and approaches, novel sequencing-based approaches to the study of fungal mycobiomes have revealed diverse communities of fungi throughout the human body, including the urinary tract. Accumulating data indicate a role for fungi in normal human physiology as well as the progression and prevention of human disease. In general, an understanding of the role of fungi in health and disease requires a systems-level, integrated approach, as opposed to a focus on specific disease-causing taxa; unlike the case of *H. pylori* in gastritis, microbial alterations affiliated with disease rarely are due to the presence of a single causative microbe. While fungi likely play an important role in the maintenance of microbial community structure, modulating immune function and influencing metabolism, the mechanisms by which fungi interact with other components of the microbiome and the host remain poorly characterized. These variations in fungal communities are likely closely tied to host physiology in ways we have only begun to characterize. Almost nothing is known about these interactions in the bladder; the field is ripe for future studies.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Wu GD, Lewis JD, Hoffmann C, et al. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol 2010;10:206.
2. Bäckhed F, Ley RE, Sonnenburg JL, et al. Host-bacterial mutualism in the human intestine. Science 2005;307:1915-20.
3. Proctor LM. The Human Microbiome Project in 2011 and beyond. Cell Host Microbe 2011;10:287-91.
4. Pearce MM, Hilt EE, Rosenfeld AB, et al. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. MBio 2014;5:e01283-14.
5. Thomas-White KJ, Hilt EE, Fok C, et al. Incontinence medication response relates to the female urinary microbiota. Int Urogynecol J 2016;27:723-33.
6. Hilt EE, McKinley K, Pearce MM, et al. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. J Clin Microbiol 2014;52:871-6.
7. Khasriya R, Sathiananthamoorthy S, Ismail S, et al. Spectrum of bacterial colonization associated with urothelial cells from patients with chronic lower urinary tract symptoms. J Clin Microbiol 2013;51:2054-62.
8. Siddiqui H, Nederbragt AJ, Lagesen K, et al. Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA amplicons. BMC Microbiol 2011;11:244.
9. Fouts DE, Pieper R, Szpakowski S, et al. Integrated next-generation sequencing of 16S rDNA and metaproteomics differentiate the healthy urine microbiome from asymptomatic bacteriuria in neuropathic bladder associated with spinal cord injury. J Transl Med 2012;10:174.
10. Groah SL, Pérez-Losada M, Caldovic L, et al. Redefining Healthy Urine: A Cross-Sectional Exploratory Metagenomic Study of People With and Without Bladder Dysfunction. J Urol 2016;196:579-87.
11. Thomas-White KJ, Kliethermes S, Rickey L, et al. Evaluation of the urinary microbiota of women with uncomplicated stress urinary incontinence. Am J Obstet Gynecol 2017;216:55.e1-16.
12. Siddiqui H, Lagesen K, Nederbragt AJ, et al. Alterations of microbiota in urine from women with interstitial cystitis. BMC Microbiol 2012;12:205.
13. Nelson DE, Van Der Pol B, Dong Q, et al. Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. PLoS One 2010;5:e14116.
14. Whiteside SA, Razvi H, Dave S, et al. The microbiome of the urinary tract--a role beyond infection. Nat Rev Urol 2015;12:81-90.
15. Ghannoum MA, Jurevic RJ, Mukherjee PK, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog 2010;6:e1000713.
16. Iliev ID, Underhill DM. Striking a balance: fungal

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commensalism versus pathogenesis. Curr Opin Microbiol 2013;16:366-73.
17. Brown GD, Denning DW, Gow NA, et al. Hidden killers: human fungal infections. Sci Transl Med 2012;4:165rv13.
18. Sanglard D. Emerging Threats in Antifungal-Resistant Fungal Pathogens. Front Med (Lausanne) 2016;3:11.
19. Enoch DA, Ludlam HA, Brown NM. Invasive fungal infections: a review of epidemiology and management options. J Med Microbiol 2006;55:809-18.
20. Brandt ME, Park BJ. Think fungus–prevention and control of fungal infections. Emerg Infect Dis 2013;19:1688-9.
21. Hassall A. On the Development of Torulæ in the Urine, and on the relation of these Fungi to Albuminous and Saccharine Urine. Med Chir Trans 1853;36:23-78.9.
22. Marloth RH. An apparatus for the study of matforming fungi in culture solutions. Science 1929;69:524-5.
23. Beck JM, Young VB, Huffnagle GB. The microbiome of the lung. Transl Res 2012;160:258-66.
24. Delhaes L, Monchy S, Fréalle E, et al. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community--implications for therapeutic management. PLoS One 2012;7:e36313.
25. Chen Y, Chen Z, Guo R, et al. Correlation between gastrointestinal fungi and varying degrees of chronic hepatitis B virus infection. Diagn Microbiol Infect Dis 2011;70:492-8.
26. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-65.
27. Gilbert JA, Hughes M. Gene expression profiling: metatranscriptomics. Methods Mol Biol 2011;733:195-205.
28. Dollive S, Peterfreund GL, Sherrill-Mix S, et al. A tool kit for quantifying eukaryotic rRNA gene sequences from human microbiome samples. Genome Biol 2012;13:R60.
29. Yuan S, Cohen DB, Ravel J, et al. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One 2012;7:e33865.
30. Tang J, Iliev ID, Brown J, et al. Mycobiome: Approaches to analysis of intestinal fungi. J Immunol Methods 2015;421:112-21.
31. Findley K, Oh J, Yang J, et al. Topographic diversity of fungal and bacterial communities in human skin. Nature 2013;498:367-70.
32. White JR, Maddox C, White O, et al. CloVR-ITS: Automated internal transcribed spacer amplicon sequence analysis pipeline for the characterization of fungal microbiota. Microbiome 2013;1:6.
33. Bellemain E, Carlsen T, Brochmann C, et al. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiol 2010;10:189.
34. Větrovský T, Kolařík M, Žifčáková L, et al. The rpb2 gene represents a viable alternative molecular marker for the analysis of environmental fungal communities. Mol Ecol Resour 2016;16:388-401.
35. Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet 2010;11:31-46.
36. Gilles A, Meglécz E, Pech N, et al. Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequencing. BMC Genomics 2011;12:245.
37. Bartram AK, Lynch MD, Stearns JC, et al. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. Appl Environ Microbiol 2011;77:3846-52.
38. Caporaso JG, Lauber CL, Walters WA, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 2012;6:1621-4.
39. Whiteley AS, Jenkins S, Waite I, et al. Microbial 16S rRNA Ion Tag and community metagenome sequencing using the Ion Torrent (PGM) Platform. J Microbiol Methods 2012;91:80-8.
40. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335-6.
41. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537-41.
42. Abarenkov K, Henrik Nilsson R, Larsson KH, et al. The UNITE database for molecular identification of fungi--recent updates and future perspectives. New Phytol 2010;186:281-5.
43. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590-6.
44. Mahé S, Duhamel M, Le Calvez T, et al. PHYMYCO-DB: a curated database for analyses of fungal diversity and evolution. PLoS One 2012;7:e43117.
45. Nilsson RH, Ryberg M, Kristiansson E, et al. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS One 2006;1:e59.
46. Underhill DM, Iliev ID. The mycobiota: interactions between commensal fungi and the host immune system. Nat Rev Immunol 2014;14:405-16.
47. Wolfe AJ, Toh E, Shibata N, et al. Evidence of uncultivated bacteria in the adult female bladder. J Clin

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Microbiol 2012;50:1376-83.
48. Araújo-Pérez F, McCoy AN, Okechukwu C, et al. Differences in microbial signatures between rectal mucosal biopsies and rectal swabs. Gut Microbes 2012;3:530-5.
49. Huse SM, Young VB, Morrison HG, et al. Comparison of brush and biopsy sampling methods of the ileal pouch for assessment of mucosa-associated microbiota of human subjects. Microbiome 2014;2:5.
50. Rosen DA, Hooton TM, Stamm WE, et al. Detection of intracellular bacterial communities in human urinary tract infection. PLoS Med 2007;4:e329.
51. Alvarez M, Casadevall A. Phagosome extrusion and host-cell survival after Cryptococcus neoformans phagocytosis by macrophages. Curr Biol 2006;16:2161-5.
52. Nicola AM, Robertson EJ, Albuquerque P, et al. Nonlytic exocytosis of Cryptococcus neoformans from macrophages occurs in vivo and is influenced by phagosomal pH. MBio 2011;2. pii: e00167-11.
53. Charlier C, Nielsen K, Daou S, et al. Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans. Infect Immun 2009;77:120-7.
54. Faro-Trindade I, Willment JA, Kerrigan AM, et al. Characterisation of innate fungal recognition in the lung. PLoS One 2012;7:e35675.
55. Dupuy AK, David MS, Li L, et al. Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of malassezia as a prominent commensal. PLoS One 2014;9:e90899.
56. Xu J, Mitchell TG. Geographical differences in human oral yeast flora. Clin Infect Dis 2003;36:221-4.
57. Avila M, Ojcius DM, Yilmaz O. The oral microbiota: living with a permanent guest. DNA Cell Biol 2009;28:405-11.
58. Dollive S, Chen YY, Grunberg S, et al. Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. PLoS One 2013;8:e71806.
59. Ott SJ, Kühbacher T, Musfeldt M, et al. Fungi and inflammatory bowel diseases: Alterations of composition and diversity. Scand J Gastroenterol 2008;43:831-41.
60. Scupham AJ, Presley LL, Wei B, et al. Abundant and diverse fungal microbiota in the murine intestine. Appl Environ Microbiol 2006;72:793-801.
61. Scanlan PD, Marchesi JR. Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces. ISME J 2008;2:1183-93.
62. Pandey PK, Siddharth J, Verma P, et al. Molecular typing of fecal eukaryotic microbiota of human infants and their respective mothers. J Biosci 2012;37:221-6.
63. Cui L, Morris A, Ghedin E. The human mycobiome in health and disease. Genome Med 2013;5:63.
64. Sellart-Altisent M, Torres-Rodríguez JM, Gómez de Ana S, et al. Nasal fungal microbiota in allergic and healthy subjects. Rev Iberoam Micol 2007;24:125-30.
65. Cui L, Lucht L, Tipton L, et al. Topographic diversity of the respiratory tract mycobiome and alteration in HIV and lung disease. Am J Respir Crit Care Med 2015;191:932-42.
66. Drell T, Lillsaar T, Tummeleht L, et al. Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. PLoS One 2013;8:e54379.
67. Gray M, Beeckman D, Bliss DZ, et al. Incontinence-associated dermatitis: a comprehensive review and update. J Wound Ostomy Continence Nurs 2012;39:61-74.
68. Zheng NN, Guo XC, Lv W, et al. Characterization of the vaginal fungal flora in pregnant diabetic women by 18S rRNA sequencing. Eur J Clin Microbiol Infect Dis 2013;32:1031-40.
69. Guo R, Zheng N, Lu H, et al. Increased diversity of fungal flora in the vagina of patients with recurrent vaginal candidiasis and allergic rhinitis. Microb Ecol 2012;64:918-27.
70. Nickel JC, Stephens A, Landis JR, et al. Assessment of the Lower Urinary Tract Microbiota during Symptom Flare in Women with Urologic Chronic Pelvic Pain Syndrome: A MAPP Network Study. J Urol 2016;195:356-62.
71. Zhang E, Tanaka T, Tajima M, et al. Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects. Microbiol Immunol 2011;55:625-32.
72. Park HK, Ha MH, Park SG, et al. Characterization of the fungal microbiota (mycobiome) in healthy and dandruff-afflicted human scalps. PLoS One 2012;7:e32847.
73. Sokol H, Leducq V, Aschard H, et al. Fungal microbiota dysbiosis in IBD. Gut 2016. pii: gutjnl-2015-310746.
74. Hoarau G, Mukherjee PK, Gower-Rousseau C, et al. Bacteriome and Mycobiome Interactions Underscore Microbial Dysbiosis in Familial Crohn's Disease. MBio 2016;7. pii: e01250-16.
75. Chehoud C, Albenberg LG, Judge C, et al. Fungal Signature in the Gut Microbiota of Pediatric Patients With Inflammatory Bowel Disease. Inflamm Bowel Dis 2015;21:1948-56.
76. Knutsen AP, Bush RK, Demain JG, et al. Fungi and allergic lower respiratory tract diseases. J Allergy Clin Immunol 2012;129:280-91; quiz 292-3.
77. Paulino LC, Tseng CH, Blaser MJ. Analysis of Malassezia

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microbiota in healthy superficial human skin and in psoriatic lesions by multiplex real-time PCR. FEMS Yeast Res 2008;8:460-71.
78. de Koning HD, Rodijk-Olthuis D, van Vlijmen-Willems IM, et al. A comprehensive analysis of pattern recognition receptors in normal and inflamed human epidermis: upregulation of dectin-1 in psoriasis. J Invest Dermatol 2010;130:2611-20.
79. Iliev ID, Funari VA, Taylor KD, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 2012;336:1314-7.
80. Romani L. Immunity to fungal infections. Nat Rev Immunol 2011;11:275-88.
81. Sharif MR, Kashani HH, Ardakani AT, et al. The Effect of a Yeast Probiotic on Acute Diarrhea in Children. Probiotics Antimicrob Proteins 2016;8:211-4.
82. Das S, Gupta PK, Das RR. Efficacy and Safety of Saccharomyces boulardii in Acute Rotavirus Diarrhea: Double Blind Randomized Controlled Trial from a Developing Country. J Trop Pediatr 2016;62:464-70.
83. van der Velden WJ, Netea MG, de Haan AF, et al. Role of the mycobiome in human acute graft-versus-host disease. Biol Blood Marrow Transplant 2013;19:329-32.

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The Bladder is Not Sterile: an Update on the Urinary Microbiome

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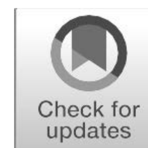

The Bladder is Not Sterile: an Update on the Urinary Microbiome

A. Lenore Ackerman[1] & Toby C. Chai[2]

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Abstract

Purpose of Review The article discusses (1) techniques used to study bacterial urinary microbiota; (2) existence of non-bacterial urinary microbiota; (3) associations between changes in urinary microbiota and various benign lower urinary tract disorders.

Recent Findings Urine harbors a diverse microbial community that resides within it. A multitude of studies have identified differences in these communities associated with urologic conditions, suggesting that microbial communities may maintain normal bladder homeostasis. Technological advances in analytic approaches have improved our understanding of the urinary microbiome. The choice of urine sampling method (voided, catheterized, or aspirated) will significantly influence microbiome findings. Sex and age highly influence urinary microbiota; in addition to rigorous inclusion criteria, microbial studies must be sufficiently powered to overcome the substantial interindividual variability of urinary microbiota. Regardless of these complicating factors, studies have identified microbial patterns correlating with both urologic diagnoses and treatment responses.

Summary Without a clear understanding of the variability of and exogenous influences on the urinary microbiota in the absence of disease, it has been challenging to reveal the microbial patterns responsible for disease pathophysiology. Host mechanisms in response to the urinary microbiome are also poorly understood. Additional research can address whether the manipulation of urinary microbiota will benefit lower urinary tract health.

Keywords Urinary microbiome · Benign lower urinary tract disorders

Introduction

The healthy bladder is not sterile. Urine harbors a complex microbial community even in healthy, asymptomatic individuals. This microbial community is thought to perform critical functions in bladder homeostasis, with potential roles in the maintenance of urothelial integrity, protection against infection, regulation of neurotransmission, and promotion of normal immune function [1]. Shifts in resident urinary microbiota towards different communities that do not perform these beneficial functions are termed dysbiosis, such imbalances have been implicated in dysfunction of nearly every organ system, from the central nervous system to the genitourinary tract (Fig. 1). Highly sensitive, culture-based, and state-of-the-art culture-independent techniques have opened a window into understanding these communities (reviewed in [2]). Detection of a microbial community in urine specimens, however, cannot presume an identical microbial community within or on tissue of either the lower (bladder, prostate, urethra) or upper (ureter, renal pelvis) urinary tract. In comparison with other body sites, there remains a substantial gap between curiosity about urinary microbes and our understanding of their role in urologic symptomatology. Of the literature identified preparing this review, approximately half of PubMed-indexed articles concerning the urinary microbiota were reviews lacking primary data. While a decade of study has revealed fundamental roles for the microbiota in health and disease in other systems, multiple technical and operational challenges specific to the urinary tract have challenged progress to understanding the urinary microbiome's specific functions.

This article is part of the Topical Collection on *Overactive Bladder*

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Technical Challenges

Deep sequencing of variable regions within the 16S rRNA locus (commonly referred to as next-generation sequencing

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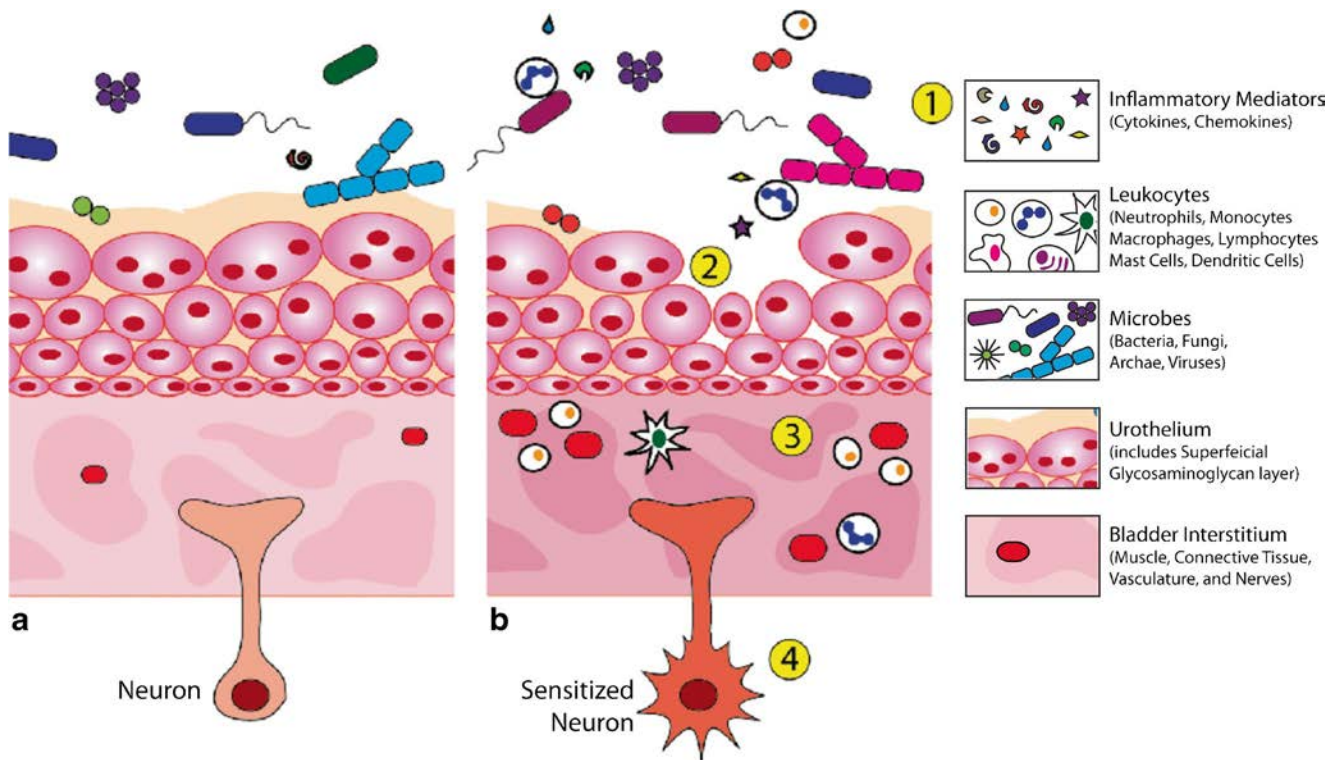

Fig. 1. The proposed role of urinary dysbiosis in the development of urinary pathology. (A) In a healthy bladder, a symbiotic microbiota maintains the urothelial barrier, prevents pathologic infection, and preserves normal neurotransmission. (B) In patients with bladder dysfunction, a dysbiotic urinary microflora alters bladder function and results in pain. (1) Pathobiont microbes, enriched in urinary dysbiosis, interact with a susceptible host immune system to induce inflammation. (2) Infiltration of immune cells and inflammatory mediator production (cytokines and chemokines) in concert with loss of the pro-homeostatic, microbially associated factors leads to a breakdown of the urothelial barrier and increased tissue permeability. (3) Host inflammation leads to increased vascularity, tissue edema, and leukocyte recruitment. (4) These fundamental changes in the bladder microenvironment lead to changes in both sensory and motor neurotransmission, leading to sequelae such as altered pain thresholds or detrusor muscle overactivity

(NGS)) remains the most common methodology to examine bacterial populations. Several important limitations to this technology are particularly relevant to the analysis of urinary populations, and urine presents unique technical challenges requiring special consideration before applying standard techniques and pipelines used for other biological samples. In contrast to stool, which contains ample bacteria, urine contains low numbers of microbes and significant host material in shed urothelial cells. To date, numerous studies have demonstrated a significant influence on the results of population analyses of collection technique, sample volume, DNA extraction methodologies, choice of consensus sequencing region and database for taxonomic assignment, and computational analysis for data interpretation [3 • •, 4 •, 5, 6]. Even in higher biomass samples, such as stool, such choices influence results, producing disparate findings for similar studies with small variations in analytic approaches [7–10].

Standardized collection methods and analytic approaches may improve the relatability and scalability of independent studies, but as technology develops, continual refinement will be needed to address the limitations of culture-independent microbial profiling. Given the low biomass of urine samples, the intrinsic error rates of standard NGS can produce significant inaccuracy. In addition, contaminants, such as environmental, skin or vaginal microbes, and urothelial cells, can be amplified to levels similar to those of the community under analysis, creating considerable confounders [6]. The use of larger volume samples and more stringent lysis conditions improves microbial community representation [5, 11]. There have also been intriguing advances, such as PacBio circular consensus sequencing (CCS); sequencing and error-correcting of full-length bacterial 16S rRNA genes provides high-fidelity species-level microbiome data unobtainable with standard NGS [12]. Regardless of method, recognition of the biases in multiple decisions inherent for any individual study prompts caution in relying on any single study as conclusive in our exploration of the urinary microbiome and reinforces the need for confirmatory analyses using alternative approaches.

Defining Pathologic Changes in the Urinary Microbiota

To an extent, early studies of urinary microbes bypassed the iterative nature of scientific exploration, attempting to identify

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pathognomonic bacteria for urologic diseases without first establishing the techniques needed for the study of urinary populations, reaching consensus on what defines normal or even that urinary microbiota represent the bladder environment.

Problematic is the proximity of the lower urinary tract (LUT) to higher biomass sites, such as the vagina, through which urine is typically sampled. Only one study looked at the microbiome of urinary samples collected by suprapubic aspirates [4 •]. Large differences were found between urinary microbial communities collected by suprapubic aspiration from those seen in voided samples from matched patients, with voided samples exhibiting more similarity to matched vaginal samples. Catheterized samples were far more similar to aspirates, but these catheterizations were performed after sterile, surgical preparation of the urethra. Even under these conditions, catheterized samples contained more vaginal commensals (e.g., *Lactobacillus*, *Prevotella*, *Atopobium*) than aspirated samples. Operating room–based catheterization represents a different scenario than clinic-based catheterization, which will likely contain more genital flora. Suprapubic aspiration is impractical for routine studies, voided samples contain substantial contaminant microorganisms from neighboring urogenital sites, and catheterization reduces but does not eliminate that sampling contamination [13].

Also, there is no guarantee that the urinary microbiome, even when sampled by aspiration, reflects the bladder microbiome. In the gut, the microbial content of stool does not mirror that of the colonic mucosa [14, 15]. Microbes present within bladder tissue or at the bladder-urine interface are presumably selected for different environmental pressures than those in urine; the bladder-associated microbiome likely differs from urinary microbiota. This debate returns us to the question of why we study the urinary microbiome. Most studies seek biomarkers of disease in the hopes that recognizable alterations in urinary flora can provide clinically useful information about diagnosis, prognosis, or causative pathology. The appropriate urine specimen to study may therefore differ for the question being asked; for this reason, the study of voided urine, while generally less specific for the LUT, may serve a more valuable purpose if the goal is identifying diagnostic or prognostic, non-invasive biomarkers. Suprapubic aspirates, while likely to be most representative of the true LUT urinary microbiome, may still be insufficient to understand bladder pathophysiology if the disease is mediated at the microbe-urothelial interface.

A recent comparison of bacteria isolated from gastrointestinal, vaginal, and urinary samples revealed strong similarities between the vaginal and urinary microbiota, which were distinct from the gastrointestinal microbiota. Whole-genome phylogenetic analyses of bacterial strains isolated from the vagina and bladder within single subjects were similar and differed from isolates catalogued from stool. The authors suggested the possibility of interlinked urogenital microbiota, at least in women, which may reframe how we understand microbiota-related pathologies and potential interventions [16 • •].

Defining urotypes

In addition to technical challenges, difficulties defining experimental and control populations confound many current studies. Some of the best evidence linking the urinary microbiome exist for urgency incontinence, a condition that is more objectively defined than a subjectively defined symptom cluster lacking objective diagnostic criteria, such as interstitial cystitis/bladder pain syndrome (IC/BPS) or chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS). Given substantial interindividual variability and intraindividual variation over time, large sample groups and longitudinal samples, particularly with monitoring of symptom fluctuations, may be necessary to overcome these limitations.

Instead of attempting to examine hundreds of taxa independently, several research groups have separated patients into low and high diversity groups, observing associations of these categories with urologic conditions from bladder cancer to IC/BPS [2, 17–19, 20 • , 21, 22 • , 23, 24]. Most studies, however, did not utilize an age-matched control cohort; the higher diversity group tended to reflect an older patient population for which age and menopausal status, not disease, may be the dominant influence. At least in women, age and menopausal status are associated with urinary microbial differences [17, 25–28], the magnitudes of which can exceed those associated with disease states. Rigorous matching is needed to avoid artifactual findings, particularly in age-associated conditions such as overactive bladder (OAB). Consensus on appropriate ways to structure both subject and control populations will allow faster progress in understanding types of dysbiosis.

The role of microbial diversity as a measure of disease must take these baseline differences into account. For younger women, in whom the microbiome is less diverse, increasing diversity may be the pathologic state; for older women, who are more diverse at baseline, decreased diversity may represent dysbiosis. Thus, while diversity measures can be helpful descriptors, their meaning is often confused or overstated. Many indices factor in both evenness and richness, which can lead to false equivalency between bacterial communities. The need for diversity measures to describe differences in urinary communities reflects a lack of simple associations of diseases with single organisms and emphasizes the complex interdependency between disease, host health and immune status, and the complex resident microbial community (including bacteria and the frequently neglected fungi, protozoa, viruses, and archaebacteria).

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Non-bacterial organisms

While bacteria are the best-studied population within the urinary tract, we are beginning to see early explorations of other organism classes there, such as fungi and viruses.

Fungi Viable fungi in urine were identified as early as the 1850s, with alterations in fungal composition noted in diabetes and renal disease [29]. Yet 150 years later, there has been little improvement in our understanding of urinary fungi. Several reports examining urinary microbiota using expanded culture conditions identified viable Candida species [30, 31•, 32], reiterating the viability of fungi from urinary samples obtained by catheterization. Targeted analysis of fungi using multi-locus PCR coupled with electrospray ionization/mass spectrometry revealed increased fungal DNA during symptomatic flares in patients with IC/BPS [33, 34•]. Fungal community profiling by NGS using the ITS1 region of fungal rRNA, however, revealed that the taxa detected in previous studies are only a small component of the complement of genera present in urinary specimens [35, 36].

Multiple issues are responsible for the lack of progress in fungal characterization, including inadequate fungal reference databases, challenges in the isolation and processing of these more robust organisms, and an erroneous belief that these organisms are not critical in human disease. An increasing number of diseases are being linked to fungal dysbiosis, implicating a profound impact on health and disease [37]. In addition, microbiota are a collective community; the importance of synergistic and antagonistic associations with other microbial components has likely been underestimated.

Viruses Several studies have attempted to identify viral DNA from urine and link these to urinary symptomatology [38]. Viruses, however, lack conserved sequences to permit high-throughput amplification of conserved regions; it is necessary to perform either targeted detection of known viruses or mass DNA sequencing with subsequent viral identification after genomic reconstruction (metagenomic sequencing). Given the low urinary biomass and contaminating DNA, viral DNA may be a needle in the host DNA haystack. One study examining human papilloma viruses could not identify any associations with LUT symptomatology [39]. Another examined the correlation of JC polyoma viruses (JCPyV) with glomerulosclerosis, suggesting a protective effect for JCPyV [40]. Several recent studies were able to distinguish viral sequences from JCPyV and BK and Torque teno virus from urine after whole genome sequencing [41, 42]. As these reports examine urine, not tissue, such approaches may miss important viral reservoirs. Viral peptide mapping using mass spectroscopy–mediated proteomics avoids the pitfalls of genomic sequencing and has identified multiple novel viral proteins in urine, which occur at different frequencies in healthy subjects and patients with several renal diseases [43].

Bacteriophages In the urine, genetic sequences of numerous bacteriophages, viruses that kill bacteria, were found within the genetic sequences of urinary bacteria, suggesting their presence within the urinary microbiome. Bacteriophages play a fundamental role in modulating bacterial communities [44]. A few recent studies have examined this population more comprehensively; analysis of genomic sequences obtained from prior studies revealed a diverse abundance of phages, many of which had little homology to previously described phages [45, 46•]. In addition, abundance of bacteriophage gene sequences differed between asymptomatic subjects and individuals with urinary symptoms, implicating an impact of phages on bladder health [46]. This influence on bacterial microbiota suggests obvious therapeutic potential; bacteriophages have already been proposed as a treatment for complicated urinary tract infection (UTI) [47] as well as biofilm encrustation of indwelling urinary devices [48].

The Urinary Microbiome in Disease

The preponderance of evidence implicates urinary microbes as at least a bystander in or a modifier or promoter of LUT disease. As with all studies of non-malignant LUT conditions, a strict definition of the patient population under study with rigorous inclusion and exclusion criteria is essential for success. A substantial proportion of women will objectively have urinary symptoms, but not complain of these when asked [49, 50]. Given the high prevalence of LUT symptoms, contamination of control groups with symptomatic patients may result in inconclusive or false negative results. For urologic research in general, better and more careful specification of study populations and careful screening of asymptomatic patients using multiple modalities will improve future research.

Urinary Incontinence Most studies have focused on urgency urinary incontinence (UUI). Only one has primarily addressed stress urinary incontinence (SUI) and did not find any associations of microbiota with SUI symptoms, but did not compare these women to an age-matched control group [51].

Microbiota of UUI are by far the best studied, with multiple studies observing associations of the urinary microbes with symptoms [19, 20•, 24, 26, 30, 52, 53]. The specific differences, however, are not consistent between studies, with little overlap or conflicting results between different research groups. One interesting study examined baseline differences in urinary microbiota prior to treatment with solifenacin, an anticholinergic given for UUI, linking increased *Actinomyces*, *Corynebacterium*, and *Streptococcus* abundances with improved responses to medication [20]. These data provide an intriguing potential prognostic factor for individualized UUI treatment decisions and suggest medications, such as beta3-agonists, may be able to modify the urinary microbiome.

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Studies from multiple groups implicate variations in *Lactobacillus* species identity and abundance in UUI. *Lactobacillus* predominance appears to associate with healthy controls, while Lactobacilli within a more diverse population are associated with disease, despite no significant differences in lactobacillus abundance across populations [26]. *Lactobacillus* species also segregate with UUI, particularly *Lactobacillus gasseri* with UUI and *Lactobacillus crispatus* with healthy controls [30]. Control and UUI populations differed significantly in age, exogenous sex hormone usage, and body mass index, however, which likely impact these associations. While further larger-scale studies will be necessary to clarify UUI-associated microbial changes, a role for lactobacilli in health and disease appears likely.

Genitourinary Pain (IC/BPS and CP/CPPS) For genitourinary pain syndromes, a single pathogen or microbial pattern is unlikely to cause all types of genitourinary pain. In a study by the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network, bacterial composition of voided urine from men with CPPS differed significantly from healthy controls, with *Burkholderia cenocepacia* overrepresented in affected subjects [54]. A second study of voided samples reiterated differences between CPPS and controls, with overrepresentation of *Clostridia* and *Bacteroides* and underrepresentation of *Bacilli* in affected patients [21].

In women, a single, small study of catheterized urine samples revealed decreased bacterial diversity, with reduced *Lactobacillus* levels and increased proinflammatory cytokines in IC/BPS compared with controls [22 •]. Three studies examining voided urine samples in women with IC/BPS were unable to identify differences in individual taxa between control subjects and IC/BPS participants [55–57], but did observe trends towards differential abundances of several species, such as increased *L. gasseri* and lower *Corynebacterium* prevalence in IC/BPS patients. Earlier studies using both voided and catheterized specimens are similar, visualizing overall changes in diversity without unique disease-associated species. In all these studies, quantity and species variations in the individual *Lactobacillus* spp. were common although typically not significant and frequently contradictory between studies. In studies of the vaginal microbiota, *Lactobacillus* species and strain variations can highly influence disease [58, 59], which may explain this ambiguity and confusion. Collectively, these studies implicate a critical role for *Lactobacilli* in IC/BPS, but the nature of this role remains undefined.

An additional MAPP study of women with IC/BPS revealed increased levels of detectable fungi, particularly *Candida* spp., without changes in bacterial community patterns [34]. A follow-up study examining fungal taxa in detail revealed greater fungal diversity and increased fungal burden in IC/BPS subjects with increased urinary symptom severity [33]. Unfortunately, neither study compared IC/BPS with controls. Altered levels of *Lactobacilli* may implicate a possible connection; selective *Lactobacillus* species will inhibit Candida growth and hyphae formation [60, 61]. Another potential confounder is that IC/BPS is often treated with antibiotics empirically, especially during painful flares; antibiotics can result in fungal overrepresentation within microbial communities.

These contradictory and inconclusive studies of genitourinary pain only promote confusion regarding any role for or correlation with urinary microbial communities. Direct comparisons are challenging due to a lack of consistency in study structure and subject differentiation, making strict, homogeneous definitions of study populations necessary to differentiate pathologic correlates. We cannot expect conclusive findings from microbiome or any field of study until we meet this standard.

Urinary Tract Infection (UTI) Our established diagnostic and management paradigm for UTI has come under fire with the revelation of a complex, symbiotic microbiome within the healthy genitourinary tract [62]. No consensus exists for a strict definition of UTI [63]; significant debate still surrounds the bacterial colony-forming unit-based thresholds that define infection by standard clinical urine culture. This uncertainty is compounded by the discovery that urinary bacteria can be detected in the urine of nearly all subjects, including those without urinary symptoms [64]. Thus, according to the strict definition, all individuals have "bacteriuria."

Asymptomatic bacteriuria has been implicated as protective against recurrent UTI [65]; better understanding of the urinary microbiome suggests a mechanism for this clinical observation. Multiple asymptomatic bacteriuria strains exhibit reduced virulence and effective inhibition of subsequent colonization with uropathogenic *Escherichia coli* strains [66, 67]. Interspecies bacterial antagonism is an emerging theme in UTI prevention, particularly with growing antibiotic resistance. Certain *Enterobacteriaceae*, including some asymptomatic bacteriuria *E. coli* strains, secrete a siderophore, escherichelin, that inhibits *Pseudomonas aeruginosa* from causing symptomatic infection [68]. In a comparative genomic study of *E. coli* isolates from women with recurrent UTI, the virulence potential of diverse bacterial strains significantly impacted UTI risk and outcome, stressing the importance of strain-level differences in bacterial uropathogenicity [69 • •]. Differences in virulence potential of *E. coli* strains similarly influence infection outcomes in prostatitis. Certain strains are associated with acute infection with subsequent clearance while others lead to persistence and pain [70]. Similar studies have noted significant strain effects for other species, which must reframe our concept of uropathogens and bacteriuria.

Novel diagnostic methods have capitalized on culture-independent DNA amplification–based molecular techniques for the improved detection of bacterial pathogens in urine from symptomatic patients. Such methods have the potential

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to rapidly identify causative organisms. While there is evidence that these methods may be able to recognize antibiotic susceptibility [71], avoiding inappropriate or delayed treatment, it is unclear how more sensitive detection methods will impact the accuracy of UTI diagnosis. Current understanding stresses that urinary bacteria are present in the healthy state, and that certain bacteria, even some historically considered uropathogens, may be beneficial to bladder health. Thus, more sensitive bacterial detection in the absence of clinical context may be associated with diagnostic confusion and unnecessary overtreatment. As urinary frequency and urgency are associated with multiple urologic conditions, dysuria remains a more accurate diagnostic marker than any bacterial, threshold-based measure [72]. Context and judgement are important, especially in light of evidence refuting the concept that any strict detection threshold or particular species is pathognomonic for UTI.

Early data also suggest a role for the vaginal microbiota in recurrent UTI [73]. In an animal model of recurrent UTI in which animals are chronically colonized with uropathogenic *E. coli*, urethral inoculation with *Gardnerella* will prompt healthy mice to redevelop an *E. coli* UTI [74 •]. These data support the concept that endogenous microbes affecting the local, visceral environment modulate disease independent of frank infection.

Effects of Antibiotics on the Urinary Microbiome No studies have directly examined the longitudinal effects of antibiotics on the healthy microbiome. While most studies of the urinary microbiome attempt to control for antibiotic effects by excluding patients with recent use, it is unclear what period free from treatment is needed to prevent confounding. The stable gut microbiome can be permanently perturbed by just two courses of ciprofloxacin, resulting in a stable alternative composition [75]. While causality is unclear, repeated antibiotic use has been linked to a variety of localized and systemic conditions such as depression, schizophrenia, and allergy/atopy [76–78].

With regard to the urinary microbiome, a few studies have implicated systemic antibiotic effects in this biological niche. In a small study of 27 subjects, prior antibiotic use was associated with decreased *Lactobacillus* and *Finegoldia* and increased *E. coli* and *Parabacteroides* [79 •]. In women given oral metronidazole for bacterial vaginosis, the relative abundances of BV-associated pathogens such as *Gardnerella vaginalis*, *Atopobium vaginae*, and *Sneathia amnii* decreased, but did not disappear, and remained at levels not significantly different from those seen in asymptomatic controls [80]. After metronidazole treatment, increases in *Lactobacillus iners* were seen in vaginal and urinary specimens, and *Lactobacillus crispatus*, which commonly predominates in healthy, pre-menopausal women, became undetectable in urinary specimens after treatment despite persistence in vaginal samples.

In renal transplant recipients, urine samples taken 1 month after surgery demonstrate increased bacterial diversity as well as increased relative abundances of *Enterococcus*, *Escherichia coli*, *Gardnerella*, and *Prevotella* [18, 81]. As antibiotic suppression is standard for patients post-transplant, these changes were suspected to be due to daily antibiotic usage, but one cannot rule out effects of the metabolic and inflammatory dysregulation of chronic renal failure, transplantation surgery stress, and immunosuppression.

While it is unclear if such genitourinary dysbiosis is the result of antimicrobial drug use or a risk factor for urinary tract infections, data suggest that antimicrobials modify the urinary microbiome, possibly selecting for more pathogenic bacteria with increased antibiotic resistance. The long-term consequences of these shifts are unclear but urge caution when considering therapeutic antimicrobial interventions. Furthermore, antibiotics for non-urologic indications may have unintended alterations to urinary microbiota.

Genitourinary Malignancies Intravesical instillation of *Bacille Calmette-Guerin* (BCG), a live mycobacterium strain, has long been used for therapeutic manipulation of urinary microbiota as treatment of non-muscle invasive bladder urothelial carcinoma, demonstrating the potential effect of bacteria on cancer progression. Several studies identified pre-treatment differences in urinary communities between patients with bladder cancer and healthy controls [82, 83]. Increased levels of Fusobacterium, which is pro-tumorigenic in colonic malignancies [84, 85], were identified in patients with bladder cancer in comparison with controls [82]. While correlative at this point, the tumor promotional potential of the urinary microbiome promotes an interesting hypothesis for the gender differences observed in bladder cancer incidence [86]. Healthy women at baseline have a higher abundance of *Mycobacteria* and other *Actinomycetes* [87], which have been hypothesized to have an inhibitory effect on cancer promotion or progression. While immature, hints of evidence suggest certain urinary microbial profiles are associated with risk of cancer recurrence, progression, and treatment responses [83].

Urolithiasis Urinary stone disease is highly prevalent, affecting almost 10% of the US population, and linked to metabolic disorders, such as atherosclerosis, obesity, and diabetes mellitus [88]. The relationship between the gut microbiota, oxalate metabolism, and stone formation has been well described in animal models and human populations [89–92]. The role of urease-producing bacteria in the generation and promotion of struvite stones has long been accepted, but only in the past few years, however, have culture-independent techniques been applied to further our understanding of the role of urinary bacteria in urolithiasis.

Several studies have identified an enriched, altered group of bacteria in association with urinary stones, suggesting specific genera contribute to the urolithiasis pathophysiology [93]. Stone cultures yielded bacterial and even fungal growth, even

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after surgical removal and perioperative antibiotics, indicating that microbes are viable within the stone fragments. While these bacteria are not urease-producing, many of the isolated genera promote crystal aggregation in vitro [94–96] which promotes stone formation [97]. Bacteria may also decrease citrate levels in urine via bacterial production of citrate lyase, which also promotes stone formation [98]. While urinary microbiota likely function in the promotion of urolithiasis, additional research is needed to understand its role in nidus formation and stone promotion as well as any possible protective role for alternative genera in the larger urinary milieu.

Conclusions and Future Directions

Advances in microbiome science promote a vision of the future in which tailored adjustments in an individual's resident communities can be exploited to improve health and combat disease. The substantial knowledge gap between this potential future and our present, however, requires a deeper dive into the basic host-microbe and microbe-microbe interactions within the LUT, including a characterization of microbial variability over time; the impact of environmental stressors, dietary intake, and medications on urinary microbial composition; and the consequences of microbial shifts on LUT function, all of which are unstudied. Also, increased granular understanding of host response mechanisms to the urinary microbiome, both in healthy and disease states, is lagging behind the burgeoning numbers of descriptive urinary microbiome studies. While cataloguing studies describe the constituents, from bacteria to bacteriophages, of the urinary microbiome in healthy and diseased hosts, it is "mission critical" to frame these taxonomical studies as to why and how the host responds (or does not respond) to these microbial communities.

Compliance with Ethical Standards

Conflict of Interest A. Lenore Ackerman has no conflict of interest. Toby C. Chai has no conflict of interest.

Human and Animal Rights and Informed Consent The authors did not perform any studies with human or animal subjects in this review article.

References

Papers of particular interest, published recently, have been highlighted as:
· Of importance
· · Of major importance

1. Whiteside SA, Razvi H, Dave S, Reid G, Burton JP. The microbiome of the urinary tract–a role beyond infection. Nat Rev Urol. 2015;12(2):81–90. https://doi.org/10.1038/nrurol.2014.361.
2. Thomas-White K, Brady M, Wolfe AJ, Mueller ER. The bladder is not sterile: history and current discoveries on the urinary microbiome. Curr Bladder Dysfunct Rep. 2016;11(1):18–24. https://doi.org/10.1007/s11884-016-0345-8.
3. · · Karstens L, Asquith M, Caruso V, Rosenbaum JT, Fair DA, Braun J, et al. Community profiling of the urinary microbiota: considerations for low-biomass samples. Nat Rev Urol. 2018;15(12):735–49. https://doi.org/10.1038/s41585-018-0104-z This is an in-depth exploration of the detailed decisions in the analysis of microbial sequence data that can influence the results and findings of microbiome studies of the urinary tract.
4. · Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, Fitzgerald M, et al. Evidence of uncultivated bacteria in the adult female bladder. J Clin Microbiol. 2012;50(4):1376–83. https://doi.org/10.1128/JCM.05852-11 One of the first descriptions of the presence of bacteria within the urinary tract; it is also the only study to detail the profound differences in microbial communities detected with different methods of sampling the urinary tract.
5. Ackerman AL, Khalique MU, Ackerman JE, Tang J, Kim J, Underhill DM et al. Optimization of DNA extraction from human urinary samples for mycobiome community profiling. PLoS One. 2019;14(4).
6. Caruso V, Song X, Asquith M, Karstens L. Performance of microbiome sequence inference methods in environments with varying biomass. mSystems. 2019;4(1). doi:https://doi.org/10.1128/mSystems.00163-18.
7. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 2010;6(1):e1000713. https://doi.org/10.1371/journal.ppat.1000713.
8. Dollive S, Peterfreund GL, Sherrill-Mix S, Bittinger K, Sinha R, Hoffmann C, et al. A tool kit for quantifying eukaryotic rRNA gene sequences from human microbiome samples. Genome Biol. 2012;13(7):R60. https://doi.org/10.1186/gb-2012-13-7-r60.
9. Wu GD, Lewis JD, Hoffmann C, Chen YY, Knight R, Bittinger K, et al. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol. 2010;10:206. https://doi.org/10.1186/1471-2180-10-206.
10. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One. 2012;7(3):e33865. https://doi.org/10.1371/journal.pone.0033865.
11. Bao Y, Al KF, Chanyi RM, Whiteside S, Dewar M, Razvi H, et al. Questions and challenges associated with studying the microbiome of the urinary tract. Ann Transl Med. 2017;5(2):33. https://doi.org/10.21037/atm.2016.12.14.
12. Earl JP, Adappa ND, Krol J, Bhat AS, Balashov S, Ehrlich RL, et al. Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes. Microbiome. 2018;6(1):190. https://doi.org/10.1186/s40168-018-0569-2.
13. Sianou A, Galyfos G, Kaparos G. Re: Alan J. Wolfe, Linda Brubaker. "Sterile urine" and the presence of bacteria. Eur Urol 2015;68:173-4. Eur Urol. 2016;69(1):e7. doi:https://doi.org/10.1016/j.eururo.2015.06.052.
14. Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, de Vos WM. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol. 2002;68(7):3401–7. https://doi.org/10.1128/aem.68.7.3401-3407.2002.
15. Momozawa Y, Deffontaine V, Louis E, Medrano JF. Characterization of bacteria in biopsies of colon and stools by high throughput sequencing of the V2 region of bacterial 16S rRNA

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gene in human. PLoS One. 2011;6(2):e16952. https://doi.org/10.1371/journal.pone.0016952.

16. • • Thomas-White K, Forster SC, Kumar N, Van Kuiken M, Putonti C, Stares MD, et al. Culturing of female bladder bacteria reveals an interconnected urogenital microbiota. Nat Commun. 2018;9(1): 1557. https://doi.org/10.1038/s41467-018-03968-5 Cross- sectional sampling of the urogenital tract in a cohort of female patients reveals strong interrelationship between the vaginal and urinary microbiome that may suggest an interconnected urogenital microbiome, at least in women.
17. Liu F, Ling Z, Xiao Y, Lv L, Yang Q, Wang B, et al. Dysbiosis of urinary microbiota is positively correlated with type 2 diabetes mellitus. Oncotarget. 2017;8(3):3798–810. https://doi.org/10.18632/oncotarget.14028.
18. Rani A, Ranjan R, McGee HS, Andropolis KE, Panchal DV, Hajjiri Z, et al. Urinary microbiome of kidney transplant patients reveals dysbiosis with potential for antibiotic resistance. Transl Res. 2017;181:59–70. https://doi.org/10.1016/j.trsl.2016.08.008.
19. Karstens L, Asquith M, Davin S, Stauffer P, Fair D, Gregory WT, et al. Does the urinary microbiome play a role in urgency urinary incontinence and its severity? Front Cell Infect Microbiol. 2016;6:78. https://doi.org/10.3389/fcimb.2016.00078.
20. • Thomas-White KJ, Hilt EE, Fok C, Pearce MM, Mueller ER, Kliethermes S, et al. Incontinence medication response relates to the female urinary microbiota. Int Urogynecol J. 2016;27(5):723–33. https://doi.org/10.1007/s00192-015-2847-x The baseline urinary microbiome prior to treatment in a population of women with UUI was related to medication responses, providing the first evidence that the urinary microbiome may be a useful prognostic biomarker in the classification of patients with benign urologic conditions.
21. Shoskes DA, Altemus J, Polackwich AS, Tucky B, Wang H, Eng C. The urinary microbiome differs significantly between patients with chronic prostatitis/chronic pelvic pain syndrome and controls as well as between patients with different clinical phenotypes. Urology. 2016;92:26–32. https://doi.org/10.1016/j.urology.2016.02.043.
22. • Abernethy MG, Rosenfeld A, White JR, Mueller MG, Lewicky- Gaupp C, Kenton K. Urinary microbiome and cytokine levels in women with interstitial cystitis. Obstet Gynecol. 2017;129(3):500–6. https://doi.org/10.1097/AOG.0000000000001892 This small study of a cohort of women with interstitial cystitis and age-matched controls identified alterations in the microbiome that also correlated with both increased inflammatory cytokine levels and worsening symptom scores, suggesting a functional consequence associated with shifts in urogenital microbial communities.
23. Kramer H, Kuffel G, Thomas-White K, Wolfe AJ, Vellanki K, Leehey DJ, et al. Diversity of the midstream urine microbiome in adults with chronic kidney disease. Int Urol Nephrol. 2018;50(6):1123–30. https://doi.org/10.1007/s11255-018-1860-7.
24. Wu P, Chen Y, Zhao J, Zhang G, Chen J, Wang J, et al. Urinary microbiome and psychological factors in women with overactive bladder. Front Cell Infect Microbiol. 2017;7:488. https://doi.org/10.3389/fcimb.2017.00488.
25. Curtiss N, Balachandran A, Krska L, Peppiatt-Wildman C, Wildman S, Duckett J. Age, menopausal status and the bladder microbiome. Eur J Obstet Gynecol Reprod Biol. 2018;228:126–9. https://doi.org/10.1016/j.ejogrb.2018.06.011.
26. Komesu YM, Richter HE, Carper B, Dinwiddie DL, Lukacz ES, Siddiqui NY, et al. The urinary microbiome in women with mixed urinary incontinence compared to similarly aged controls. Int Urogynecol J. 2018;29(12):1785–95. https://doi.org/10.1007/s00192-018-3683-6.
27. Komesu YM, Richter HE, Dinwiddie DL, Siddiqui NY, Sung VW, Lukacz ES, et al. Methodology for a vaginal and urinary microbiome study in women with mixed urinary incontinence. Int Urogynecol J. 2017;28(5):711–20. https://doi.org/10.1007/s00192-016-3165-7.
28. Liu F, Ling Z, Xiao Y, Yang Q, Zheng L, Jiang P, et al. Characterization of the urinary microbiota of elderly women and the effects of type 2 diabetes and urinary tract infections on the microbiota. Oncotarget. 2017;8(59):100678–90. https://doi.org/10.18632/oncotarget.21126.
29. Hassall A. On the development of torulae in the urine, and on the relation of these fungi to albuminous and saccharine urine. Med Chir Trans. 1853;36:23–78 9. https://doi.org/10.1177/095952875303600103.
30. Pearce MM, Hilt EE, Rosenfeld AB, Zilliox MJ, Thomas-White K, Fok C, et al. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. mBio. 2014;5(4):e01283–14. https://doi.org/10.1128/mBio.01283-14.
31. • Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, et al. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. J Clin Microbiol. 2014;52(3):871–6. https://doi.org/10.1128/JCM.02876-13 This paper describes the use of an enhanced culture technique to show that multiple genera of bacteria are culturable from urine obtained by catheterization from asymptomatic women.
32. Price TK, Dune T, Hilt EE, Thomas-White KJ, Kliethermes S, Brincat C, et al. The clinical urine culture: enhanced techniques improve detection of clinically relevant microorganisms. J Clin Microbiol. 2016;54(5):1216–22. https://doi.org/10.1128/JCM.00044-16.
33. Nickel JC, Stephens A, Landis JR, Mullins C, van Bokhoven A, Anger JT, et al. Urinary fungi associated with urinary symptom severity among women with interstitial cystitis/bladder pain syndrome (IC/BPS). World J Urol. 2019. https://doi.org/10.1007/s00345-019-02764-0.
34. • Nickel JC, Stephens A, Landis JR, Mullins C, van Bokhoven A, Lucia MS, et al. Assessment of the lower urinary tract microbiota during symptom flare in women with urologic chronic pelvic pain syndrome: a MAPP network study. J Urol. 2016;195(2):356–62. https://doi.org/10.1016/j.juro.2015.09.075 In a large cohort of patients with urologic chronic pelvic pain syndrome, an association of worsening symptoms, dubbed symptomatic "flares", was associated with increased detection of urinary fungi, revealing an importance for non-bacterial organisms in disease is is being increasingly recognized for other organ systems.
35. Ackerman AL, Underhill DM. The mycobiome of the human urinary tract: potential roles for fungi in urology. Ann Transl Med. 2017;5(2):31. https://doi.org/10.21037/atm.2016.12.69.
36. Wheeler ML, Limon JJ, Bar AS, Leal CA, Gargus M, Tang J, et al. Immunological consequences of intestinal fungal dysbiosis. Cell Host Microbe. 2016;19(6):865–73. https://doi.org/10.1016/j.chom.2016.05.003.
37. El-Jurdi N, Ghannoum MA. The mycobiome: impact on health and disease states. Microbiol Spectr. 2017;5(3). doi:https://doi.org/10.1128/microbiolspec.FUNK-0045-2016.
38. Santiago-Rodriguez TM. Identification and quantification of DNA viral populations in human urine using next-generation sequencing approaches. Methods Mol Biol. 1838;2018:191–200. https://doi.org/10.1007/978-1-4939-8682-8_14.
39. Santiago-Rodriguez TM, Ly M, Bonilla N, Pride DT. The human urine virome in association with urinary tract infections. Front Microbiol. 2015;6:14. https://doi.org/10.3389/fmicb.2015.00014.
40. Divers J, Langefeld CD, Lyles DS, Ma L, Freedman BI. Protective association between JC polyoma viruria and kidney disease. Curr Opin Nephrol Hypertens. 2019;28(1):65–9. https://doi.org/10.1097/MNH.0000000000000464.

Author's personal copy

41. Rani A, Ranjan R, McGee HS, Metwally A, Hajjiri Z, Brennan DC, et al. A diverse virome in kidney transplant patients contains multiple viral subtypes with distinct polymorphisms. Sci Rep. 2016;6: 33327. https://doi.org/10.1038/srep33327.
42. Garretto A, Thomas-White K, Wolfe AJ, Putonti C. Detecting viral genomes in the female urinary microbiome. J Gen Virol. 2018;99(8):1141–6. https://doi.org/10.1099/jgv.0.001097.
43. Sigdel TK, Mercer N, Nandoe S, Nicora CD, Burnum-Johnson K, Qian WJ, et al. Urinary virome perturbations in kidney transplantation. Front Med (Lausanne). 2018;5:72. https://doi.org/10.3389/fmed.2018.00072.
44. Garretto A, Miller-Ensminger T, Wolfe AJ, Putonti C. Bacteriophages of the lower urinary tract. Nat Rev Urol. 2019;16(7):422–32. https://doi.org/10.1038/s41585-019-0192-4.
45. Malki K, Shapiro JW, Price TK, Hilt EE, Thomas-White K, Sircar T, et al. Genomes of Gardnerella strains reveal an abundance of prophages within the bladder microbiome. PLoS One. 2016;11(11):e0166757. https://doi.org/10.1371/journal.pone.0166757.
46. • Miller-Ensminger T, Garretto A, Brenner J, Thomas-White K, Zambom A, Wolfe AJ et al. Bacteriophages of the urinary microbiome. J Bacteriol. 2018;200(7). doi:https://doi.org/10.1128/JB.00738-17. This study provides early evidence of the existence of abundant and novel bacteriophages within the urinary tract that likely influence both the composition of the urinary microbiome as well as urologic disease.
47. Ujmajuridze A, Chanishvili N, Goderdzishvili M, Leitner L, Mehnert U, Chkhotua A, et al. Adapted bacteriophages for treating urinary tract infections. Front Microbiol. 2018;9:1832. https://doi.org/10.3389/fmicb.2018.01832.
48. Nzakizwanayo J, Hanin A, Alves DR, McCutcheon B, Dedi C, Salvage J, et al. Bacteriophage can prevent encrustation and blockage of urinary catheters by Proteus mirabilis. Antimicrob Agents Chemother. 2015;60(3):1530–6. https://doi.org/10.1128/AAC.02685-15.
49. Coyne KS, Sexton CC, Thompson CL, Milsom I, Irwin D, Kopp ZS, et al. The prevalence of lower urinary tract symptoms (LUTS) in the USA, the UK and Sweden: results from the Epidemiology of LUTS (EpiLUTS) study. BJU Int. 2009;104(3):352–60. https://doi.org/10.1111/j.1464-410X.2009.08427.x.
50. Ackerman AL, Lai HH, Parameshwar PS, Eilber KS, Anger JT. Symptomatic overlap in overactive bladder and interstitial cystitis/painful bladder syndrome - development of a new algorithm. BJU Int. 2018. https://doi.org/10.1111/bju.14568.
51. Thomas-White KJ, Kliethermes S, Rickey L, Lukacz ES, Richter HE, Moalli P, et al. Evaluation of the urinary microbiota of women with uncomplicated stress urinary incontinence. Am J Obstet Gynecol. 2017;216(1):55 e1–e16. https://doi.org/10.1016/j.ajog.2016.07.049.
52. Fok CS, Gao X, Lin H, Thomas-White KJ, Mueller ER, Wolfe AJ, et al. Urinary symptoms are associated with certain urinary microbes in urogynecologic surgical patients. Int Urogynecol J. 2018;29(12):1765–71. https://doi.org/10.1007/s00192-018-3732-1.
53. Pearce MM, Zilliox MJ, Rosenfeld AB, Thomas-White KJ, Richter HE, Nager CW, et al. The female urinary microbiome in urgency urinary incontinence. Am J Obstet Gynecol. 2015;213(3):347 e1–11. https://doi.org/10.1016/j.ajog.2015.07.009.
54. Nickel JC, Stephens A, Landis JR, Chen J, Mullins C, van Bokhoven A, et al. Search for microorganisms in men with urologic chronic pelvic pain syndrome: a culture-independent analysis in the MAPP Research Network. J Urol. 2015;194(1):127–35. https://doi.org/10.1016/j.juro.2015.01.037.
55. Bresler L, Price TK, Hilt EE, Joyce C, Fitzgerald CM, Wolfe AJ. Female lower urinary tract microbiota do not associate with IC/PBS symptoms: a case-controlled study. Int Urogynecol J. 2019. https://doi.org/10.1007/s00192-019-03942-9.
56. Nickel JC, Stephens-Shields AJ, Landis JR, Mullins C, van Bokhoven A, Lucia MS et al. A culture-independent analysis of the microbiota of female interstitial cystitis/bladder pain syndrome participants in the MAPP Research Network. J Clin Med. 2019;8(3). doi:https://doi.org/10.3390/jcm8030415.
57. Meriwether KV, Lei Z, Singh R, Gaskins J, DTG H. Jala V. The vaginal and urinary microbiomes in premenopausal women with interstitial cystitis/bladder pain syndrome as compared to unaffected controls: a pilot cross-sectional study. Front Cell Infect Microbiol. 2019;9:92. https://doi.org/10.3389/fcimb.2019.00092.
58. Greenbaum S, Greenbaum G, Moran-Gilad J, Weintraub AY. Ecological dynamics of the vaginal microbiome in relation to health and disease. Am J Obstet Gynecol. 2019;220(4):324–35. https://doi.org/10.1016/j.ajog.2018.11.1089.
59. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011;108(Suppl 1):4680–7. https://doi.org/10.1073/pnas.1002611107.
60. De Gregorio PR, Silva JA, Marchesi A, Nader-Macias MEF. Anti-Candida activity of beneficial vaginal lactobacilli in in vitro assays and in a murine experimental model. FEMS Yeast Res. 2019;19(2). doi:https://doi.org/10.1093/femsyr/foz008.
61. Jang SJ, Lee K, Kwon B, You HJ, Ko G. Vaginal lactobacilli inhibit growth and hyphae formation of Candida albicans. Sci Rep. 2019;9(1):8121. https://doi.org/10.1038/s41598-019-44579-4.
62. Finucane TE. 'Urinary tract infection' and the microbiome. Am J Med. 2017;130(3):e97–e8. https://doi.org/10.1016/j.amjmed.2016.08.018.
63. Anger J, Lee U, Ackerman AL, Chou R, Chughtai B, Clemens JQ et al. Recurrent uncomplicated urinary tract infections in women: AUA/CUA/SUFU Guideline. J Urol. 2019: 101097JU0000000000000296. doi:https://doi.org/10.1097/JU.0000000000000296.
64. Price TK, Hilt EE, Dune TJ, Mueller ER, Wolfe AJ, Brubaker L. Urine trouble: should we think differently about UTI? Int Urogynecol J. 2018;29(2):205–10. https://doi.org/10.1007/s00192-017-3528-8.
65. Cai T, Mazzoli S, Mondaini N, Meacci F, Nesi G, D'Elia C, et al. The role of asymptomatic bacteriuria in young women with recurrent urinary tract infections: to treat or not to treat? Clin Infect Dis. 2012;55(6):771–7. https://doi.org/10.1093/cid/cis534.
66. Roos V, Ulett GC, Schembri MA, Klemm P. The asymptomatic bacteriuria Escherichia coli strain 83972 outcompetes uropathogenic E. coli strains in human urine. Infect Immun. 2006;74(1):615–24. https://doi.org/10.1128/IAI.74.1.615-624.2006.
67. Stork C, Kovacs B, Rozsai B, Putze J, Kiel M, Dorn A, et al. Characterization of asymptomatic bacteriuria Escherichia coli isolates in search of alternative strains for efficient bacterial interference against uropathogens. Front Microbiol. 2018;9:214. https://doi.org/10.3389/fmicb.2018.00214.
68. Ohlemacher SI, Giblin DE, d'Avignon DA, Stapleton AE, Trautner BW, Henderson JP. Enterobacteria secrete an inhibitor of Pseudomonas virulence during clinical bacteriuria. J Clin Invest. 2017;127(11):4018–30. https://doi.org/10.1172/JCI92464.
69. •• Schreiber HLt, Conover MS, Chou WC, Hibbing ME, Manson AL, Dodson KW et al. Bacterial virulence phenotypes of Escherichia coli and host susceptibility determine risk for urinary tract infec- tions. Sci Transl Med. 2017;9(382). doi:https://doi.org/10.1126/ scitranslmed.aaf1283. This comparative genomic study examining a panel of E. coli isolates from women with urinary tract infections revealed conserved functional, but not genomic, virulence patterns at the bacterial strain level, suggesting

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important host-pathogen interactions at the root of infection susceptibility.

70. Krieger JN, Thumbikat P. Bacterial Prostatitis: Bacterial Virulence, Clinical outcomes, and new directions. Microbiol Spectr. 2016;4(1). doi:https://doi.org/10.1128/microbiolspec.UTI-0004-2012.
71. Tchesnokova V, Avagyan H, Rechkina E, Chan D, Muradova M, Haile HG, et al. Bacterial clonal diagnostics as a tool for evidence-based empiric antibiotic selection. PLoS One. 2017;12(3): e0174132. https://doi.org/10.1371/journal.pone.0174132.
72. Dune TJ, Price TK, Hilt EE, Thomas-White KJ, Kliethermes S, Brincat C, et al. Urinary symptoms and their associations with urinary tract infections in urogynecologic patients. Obstet Gynecol. 2017;130(4):718–25. https://doi.org/10.1097/AOG.0000000000002239.
73. Stapleton AE. The vaginal microbiota and urinary tract infection. Microbiol Spectr. 2016;4(6). doi:https://doi.org/10.1128/microbiolspec.UTI-0025-2016.
74. · Gilbert NM, O'Brien VP, Lewis AL. Transient microbiota expo- sures activate dormant Escherichia coli infection in the bladder and drive severe outcomes of recurrent disease. PLoS Pathog. 2017;13(3):e1006238. https://doi.org/10.1371/journal.ppat. 1006238 In mice primed for recurrent urinary tract infections, intraurethral innoculation of Gardnerella, a bacterium associated in humans with bacterial vaginosis, can promote the development of E. coli urinary tract infection, suggesting that infection risk is a complex interplay not only between host and pathogen but that the microbial environment can also promote infection.
75. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011;108(Suppl 1):4554–61. https://doi.org/10.1073/pnas.1000087107.
76. Kohler O, Petersen L, Mors O, Mortensen PB, Yolken RH, Gasse C, et al. Infections and exposure to anti-infective agents and the risk of severe mental disorders: a nationwide study. Acta Psychiatr Scand. 2017;135(2):97–105. https://doi.org/10.1111/acps.12671.
77. Kohler-Forsberg O, Petersen L, Gasse C, Mortensen PB, Dalsgaard S, Yolken RH, et al. A nationwide study in denmark of the association between treated infections and the subsequent risk of treated mental disorders in children and adolescents. JAMA Psychiatry. 2018. https://doi.org/10.1001/jamapsychiatry.2018.3428.
78. Noverr MC, Falkowski NR, McDonald RA, McKenzie AN, Huffnagle GB. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. Infect Immun. 2005;73(1):30–8. https://doi.org/10.1128/IAI.73.1.30-38.2005.
79. · Mulder M, Radjabzadeh D, Hassing RJ, Heeringa J, Uitterlinden AG, Kraaij R, et al. The effect of antimicrobial drug use on the composition of the genitourinary microbiota in an elderly popula- tion. BMC Microbiol. 2019;19(1):9. https://doi.org/10.1186/ s12866-018-1379-1 Despite widespread use of antibiotics to treat uropathogens such as E. coli, this examination of older adults after treatment with antibiotics revealed decreases in the classic anti-inflammatory commensals such as Lactobacillus and increases in E. coli after treatment, which should caution the use of antimicrobials for "Eradication" of uropathogens, particularly in asymptomatic patients.
80. Gottschick C, Deng ZL, Vital M, Masur C, Abels C, Pieper DH, et al. The urinary microbiota of men and women and its changes in women during bacterial vaginosis and antibiotic treatment. Microbiome. 2017;5(1):99. https://doi.org/10.1186/s40168-017-0305-3.
81. Modena BD, Milam R, Harrison F, Cheeseman JA, Abecassis MM, Friedewald JJ, et al. Changes in urinary microbiome populations correlate in kidney transplants with interstitial fibrosis and tubular atrophy documented in early surveillance biopsies. Am J Transplant Off J Am Soc Transplant Am Soc Transplant Surg. 2017;17(3): 712–23. https://doi.org/10.1111/ajt.14038.
82. Bucevic Popovic V, Situm M, Chow CT, Chan LS, Roje B, Terzic J. The urinary microbiome associated with bladder cancer. Sci Rep. 2018;8(1):12157. https://doi.org/10.1038/s41598-018-29054-w.
83. Wu P, Zhang G, Zhao J, Chen J, Chen Y, Huang W, et al. Profiling the urinary microbiota in male patients with bladder cancer in China. Front Cell Infect Microbiol. 2018;8:167. https://doi.org/10.3389/fcimb.2018.00167.
84. Viljoen KS, Dakshinamurthy A, Goldberg P, Blackburn JM. Quantitative profiling of colorectal cancer-associated bacteria reveals associations between fusobacterium spp., enterotoxigenic Bacteroides fragilis (ETBF) and clinicopathological features of colorectal cancer. PLoS One. 2015;10(3):e0119462. https://doi.org/10.1371/journal.pone.0119462.
85. Bullman S, Pedamallu CS, Sicinska E, Clancy TE, Zhang X, Cai D, et al. Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. Science. 2017;358(6369):1443–8. https://doi.org/10.1126/science.aal5240.
86. Raoult D. Is there a link between urinary microbiota and bladder cancer? Eur J Epidemiol. 2017;32(3):255. https://doi.org/10.1007/s10654-016-0213-z.
87. Lewis DA, Brown R, Williams J, White P, Jacobson SK, Marchesi JR, et al. The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults. Front Cell Infect Microbiol. 2013;3: 41. https://doi.org/10.3389/fcimb.2013.00041.
88. Lee JA, Stern JM. Understanding the link between gut microbiome and urinary stone disease. Curr Urol Rep. 2019;20(5):19. https://doi.org/10.1007/s11934-019-0882-8.
89. Tang R, Jiang Y, Tan A, Ye J, Xian X, Xie Y, et al. 16S rRNA gene sequencing reveals altered composition of gut microbiota in individuals with kidney stones. Urolithiasis. 2018;46(6):503–14. https://doi.org/10.1007/s00240-018-1037-y.
90. Stern JM, Moazami S, Qiu Y, Kurland I, Chen Z, Agalliu I, et al. Evidence for a distinct gut microbiome in kidney stone formers compared to non-stone formers. Urolithiasis. 2016;44(5):399–407. https://doi.org/10.1007/s00240-016-0882-9.
91. Suryavanshi MV, Bhute SS, Jadhav SD, Bhatia MS, Gune RP, Shouche YS. Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures. Sci Rep. 2016;6:34712. https://doi.org/10.1038/srep34712.
92. Ticinesi A, Milani C, Guerra A, Allegri F, Lauretani F, Nouvenne A, et al. Understanding the gut-kidney axis in nephrolithiasis: an analysis of the gut microbiota composition and functionality of stone formers. Gut. 2018;67(12):2097–106. https://doi.org/10.1136/gutjnl-2017-315734.
93. Dornbier RA, Bajic P, Van Kuiken M, Jardaneh A, Lin H, Gao X, et al. The microbiome of calcium-based urinary stones. Urolithiasis. 2019. https://doi.org/10.1007/s00240-019-01146-w.
94. Amimanan P, Tavichakorntrakool R, Fong-Ngern K, Sribenjalux P, Lulitanond A, Prasongwatana V, et al. Elongation factor Tu on Escherichia coli isolated from urine of kidney stone patients promotes calcium oxalate crystal growth and aggregation. Sci Rep. 2017;7(1):2953. https://doi.org/10.1038/s41598-017-03213-x.
95. Barr-Beare E, Saxena V, Hilt EE, Thomas-White K, Schober M, Li B, et al. The interaction between Enterobacteriaceae and calcium oxalate deposits. PLoS One. 2015;10(10):e0139575. https://doi.org/10.1371/journal.pone.0139575.
96. Chutipongtanate S, Sutthimethakorn S, Chiangjong W, Thongboonkerd V. Bacteria can promote calcium oxalate crystal growth and aggregation. J Biol Inorg Chem. 2013;18(3):299–308. https://doi.org/10.1007/s00775-012-0974-0.

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97. Schwaderer AL, Wolfe AJ. The association between bacteria and urinary stones. Annals of translational medicine. 2017;5(2):32. https://doi.org/10.21037/atm.2016.11.73.
98. De Ferrari ME, Macaluso M, Brunati C, Pozzoli R, Colussi G. Hypocitraturia and Ureaplasma urealyticum urinary tract infection in patients with idiopathic calcium nephrolithiasis. Nephrol Dial Transplant. 1996; 11(6): 1185. https://doi.org/10.1093/oxfordjournals.ndt.a027486.

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Appendix C: Curriculum Vitae (A. Lenore Ackerman MD PHD)

A. Lenore Ackerman

CURRICULUM VITAE

June 30, 2020

Professional Contact Information: Cedars-Sinai Medical Center
99 N. La Cienega Boulevard, Suite M-102
Beverly Hills, CA 90211

Education:

Bachelor of Science	Yale University	1998
Doctor of Philosophy	Yale University	2004
Doctor of Medicine	Yale University	2008
Internship	UCLA-David Geffen	2008-2009
Residency	UCLA-David Geffen	2009-2014
Fellowship	UCLA-Reagan Medical Center	2014-2016
Clinical Scholars Program	Cedars-Sinai Medical Center	2016-2018

Licensure: Medical Board of California, #A112910

Board Certification:

American Board of Urology 2018-
Female Pelvic Medicine and Reconstructive Surgery 2018-

Professional Experience:

Staff Physician	Cedars-Sinai Medical Center Dept. of Surgery, Division of Urology	2016-
Assistant Professor	Cedars-Sinai Medical Center Female Pelvic Medicine and Reconstructive Surgery	2016-
Adjunct Asst. Professor	UCLA-David Geffen Urology	2016-

Professional Activities: Professional Society Membership

Western Section – American Urologic Association	2018-
Society for Basic Urologic Research	2016 -
International Urogynecological Association	2016 -
Society for Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction	2015 -
American Urogynecological Society	2015 -
Federation of Clinical Immunology Societies	2015 -
Society for Women in Urology	2014 -
Los Angeles Urological Society	2008 -
American Urological Association	2008 -
American Medical Association	2004 -
American Association of Immunologists	2000 –

Committee/Professional Service

Women's Health Technology (WHT) Coordinated Registry Network (CRN), POP Working Group	2018-2019
Recurrent Urinary Tract Infections Panel (American Urological Association)	2018-
Basic Science Program Committee (Society of	2018-

Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction)

AUA Clinical Problem-Solving (CPS) Module: Recurrent Uncomplicated Urinary Tract Infections in Women	2019
Section Editor, Microbiome and Urologic Infections, Grand Rounds in Urology	2020-
Committee Member, Terminology on Female Bladder Pain (International Urogynecological Association/AUGS)	2020-
Chair, Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction Grant Research Committee	2020-

Community Service Activities

Volunteer – Urology Community Outreach, Leo Baeck	2019
Volunteer – Medicine for Humanity	2015
Volunteer – Yale-HAVEN Free Clinic	2005-2008
Coordinator – Medical Reserve Corps	2004-2008
Program Coordinator – Graduate Student Research Symposium, Yale University,	2000
Volunteer – Genetics Education Outreach Program, Yale University	1999-2002
Volunteer – Pediatric Emergency Department, Yale-New Haven Hospital	1998-2000

Editorial Services

European Urology	2018 -
Neurourology and Urodynamics	2016 -
Journal of Urology	2015 -
International Urogynecological Journal	2015 -
Urology	2015 -
PLOS One	2015 -

Professional Activities:

CSMC Clinical Competency Committee, Member	2017-2020
CSMG Clinical Care Optimization/Urology Member	2019-2020

Honors and Special Awards:

American Urologic Association: Best Poster Infections/Inflammation/Cystic Disease of the Genitourinary Tract: Kidney and Bladder II	2020
Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction- Basic Science Poster Award, Second Place	2019
American Urologic Association: Research Forum: First Place Early-Career Investigators Showcase	2018
American Urologic Association: Best Poster Basic Research and Pathophysiology II	2017
American Urologic Association: Best Poster Practice Patterns, Quality of Life and Shared	2017

	Decision Making V	
	Cedars-Sinai Patient Satisfaction Award	2016-2018
	Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction-Best Basic Science Poster	2016
	Pfizer/Urology Care Foundation Research Scholar	2015
	Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction Resident Travel Award	2012
	American Urogynecologic Society Resident Research Scholar	2011
	Yale Minority Science, Technology, and Research Fellow	1996
	Yale University Class of 1954 Fellow	1995-1997
	Yale University Hiram B. Manville Scholar	1995
	Yale Club of Atlanta Community Service Fellow	1995
	Coca-Cola National Scholar	1994

Research Grants and Fellowships Received:

Funder	Project / Award	Amount / Effort	Years
National Institute of Diabetes and Digestive And Kidney Diseases (NIH)	The Urinary Microbiota and Host Inflammation in Lower Urinary Tract Symptoms Mentored Clinical Scientist Research Career Development Award (K08) Principal Investigator	$850,000 75% effort	2019-2024
University of California Los Angeles, Clinical and Translational Science Institute	The Microbiome of Interstitial Cystitis Subjects with Hunner's Ulcers Technology Core Vouchers Co-Investigator	$10,000 1% effort	2019-2020
Society for Urodyn. Female Pelvic Med. and Urogenital Reconstruction	The Role of the GU Microbiota in predicting Responses to Sacral Neuromodulation for Overactive Bladder in Older Women Neuromodulation Award Principal Investigator	$25,000 1% effort	2019-2021
University of CA Los Angeles, Clinical and Translational Science Institute	The Role of the Vaginal Microbiota in Urgency Urinary Incontinence Iris Cantor-UCLA Executive Advisory Board/CTSI Pilot Award Principal Investigator	$30,000 1% effort	2019-2020
University of CA Los Angeles, Clinical and Translational Science Institute	The Role of the Vaginal Microbiota in Urgency Urinary Incontinence KL2 Mentored Clinical Scientist Training Award Principal Investigator	$125,000 75% effort	2019-2020
Shaffer Family Foundation	Vaginal Fractionated CO2 Laser Treatment – Effects on the Genitourinary Microbiome Co-Investigator	$235,000 1% effort	2018-2020

Department of Defense Congressionally-Directed Medical Research Program	The Urinary Fungal Mycobiome and Host Responses in Patients with Interstitial Cystitis PRMRP Discovery Award Principal Investigator	$350,000 5% effort	2017-2019
Society for Urodyn. Female Pelvic Med. and Urogenital Recon.	The Microbiome as a Predictor of Outcome of Intravesical Botox Chemodenervation Award Principal Investigator	$25,000 1% effort	2017-2019
University of California Los Angeles, Clinical and Translational Science Institute	Temporal Dynamics of the Female Genitourinary Microbiota Technology Core Vouchers Principal Investigator	$10,000 1% effort	2016-2017
Urology Care Foundation	Inflammatory Responses and the Microbiome in OAB Urologic Research Training Award Principal Investigator	$40,000 50% effort	2015-2016
National Institute of Allergy and Infectious Diseases (NIH)	Cellular Mechanisms of Cross-presentation in Dendritic Cells Individual National Research Service Award Minority Fellowship Principal Investigator	$200,000 100% effort	2000-2004
National Heart, Lung, and Blood Institute (NIH)	Generation of Soluble Antibody/T-cell Receptor Chimeras as Immunosuppressive Adjuncts in Xenotransplantation Under-represented Minority Summer Research Fellowship Principal Investigator	$2,500 100% effort	1998

Research Projects and Research Focus or Interest:

Clinical Trials — I have been involved in multiple trials of novel therapeutics in the treatment of benign uroloic diseases, serving as site coordinator for multiple industry-sponsored trials of oral and local therapies for interstitial cystitis and overctive bladder. My primary interest, however, is the application of machine learning to the diagnosis and phenotyping of benign urologic diseases to provide more objective diagnostic schemata and effective screening in the primary care setting as well as better prognostication in treatment decision-making.

Clinical Research — My primary interest is the application of machine learning to the diagnosis and phenotyping of benign urologic diseases to provide more objective diagnostic schemata and effective screening in the primary care setting as well as better prognostication in treatment decision-making.

Basic Research — I have a particular interest in the relationship between genitourinary microbial communities and host inflammatory responses and how the interactions between them influence lower urinary tract physiology and pathology. My laboratory is focused on identifying the associations of particular microbial communities with benign urologic disease phenotypes and modeling these phenotypes in in-vitro systems and animal models.

Invited Lectures and Presentations:

Microbiome: How is it used in urogynecology?	PFD Week 2020 (American Urogynecologic Society Annual	Online	2020

	Meeting		
Urinary Tract Infections	FPMRS Fellows Webinar American Urogynecologic Society	Online	2020
Fungal Infections in Urology, Past and Future	Society for Infection and Inflammation in Urology at the AUA	Washington, DC	2020
Take Home Messages: Infection/Inflammation	American Urological Assoc. Annual Meeting	Washington, DC	2020
Recurrent UTI in Women: 40 year-old with Recurrent UTIs Treated – Is there a Role for Cranberry or Other Non-Antibiotic Solutions?	SUFU at the American Urological Association Annual Meeting	Washington, DC	2020
Beyond Bacteria: Fungi In the Urinary Tract	UROBIOME 2020	San Diego, CA	2020
Recurrent Urinary Tract Infections: Imaging and Molecular Diagnostics	Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction	Scottsdale, AZ	2020
Recurrent Urinary Tract Infection: Lessons from the Guidelines	American Urologic Association Annual Meeting	Chicago, IL	2019
Female Pelvic Pain: Why Does It Hurt Down There?	Advanced Practice Provider Program at the AUA	Chicago, IL	2019
Vaginal Laser Therapy in Today's Urology Practice: What the Evidence Tells Us	SUFU at the American Urological Association Annual Meeting	Chicago, IL	2019
The Urinary Mycobiome and Lower Urinary Tract Symptoms	Duke University, Annual Multidisciplinary Benign Urology Research Day	Durham, NC	2019
Expert Panel-Recurrent UTI A Case-based Approach	Western Section, American Urological Association	Maui, HI	2018
The Mycobiome of the Urinary Tract and Its Potential Role in Urologic Conditions	University of California, San Diego	San Diego, CA	2018
The Urinary Microbiota and Host Inflammation in Lower Urinary Tract Symptoms	American Urological Association Annual Meeting Research Forum	San Francisco, CA	2018
Recent Advances in Urinary Microbiota and the Diagnosis of UTI	Mediterranean Incontinence and Pelvic Floor Society Annual Meeting	Rome, Italy	2018
Pathways to Independence in Urologic Research: It Takes a	Individualizing Treatment of Urinary Incontinence	Bethesda, MD	2018

Village	NIDDK/NIH		
Imaging in FPMRS: Ultrasound	Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction	Austin, TX	2018
The Urinary Microbiota: A New Paradigm in the Study of Lower Urinary Tract Symptoms	Yale University, Department of Urology	New Haven, CT	2017
Female Sexual Dysfunction in the Older Patient	Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction	Scottsdale, AZ	2017
Instructor, Female Pelvic Medicine and Reconstructive Surgery, re: AUA/SUFU Guidelines Curso de Actualizacion en Urologia	Sociedad Argentina de Urología/Ameican Urological Association	San Miguel de Tucuman, Argentina	2016
Genitourinary Fistulae: Approaches, Tips and Techniques Urological Association Joint Symposium	Congresso Argnetino de Urologia, Sociedad Argentina de Urología/Ameican	San Miguel de Tucuman, Argentina	2016
Overactive Bladder: A New Perspective on Bladder Hypersensitivity Syndromes Urological Association Joint Symposium	Congresso Argnetino de Urologia, Sociedad Argentina de Urología/American	San Miguel de Tucuman, Argentina	2016
Basic Sciences Symposium. Inflammatory Responses and the Microbiome in OAB.	American Urological Association Annual Meeting	San Diego, CA	2016

Teaching Activities and/or Other off-campus:

Cedars-Sinai Teaching Activities:

Urology Residents:

- Weekend Teaching Rounds
- Quarterly Journal Club
- Lectures bimonthly on the Urology Core Curriculum
- Individual teaching on consulting cases, operative cases, clinical supervision
- Participation/Teaching during weekly Case Conference, Morbidity and Mortality Conferences

Female Pelvic Medicine and Reconstructive Surgery Fellows:

- Weekly Didactic Conferences
- Individual teaching on consulting cases, operative cases, clinical supervision
- Participation/Teaching during weekly Case Conference, Morbidity and Mortality Conferences
- Quarterly FPMRS Los Angeles Area Journal Club
- Academy for Pelvic Surgery Regional Cadaver Lab for FPMRS Fellows

Graduate Students/Research Residents:

Weekly Laboratory Meetings
Weekly Host-Microbe Journal Club
Participation/Teaching during weekly Research In Progress Seminars
Yearly Guide to Funding for Fellow/Early Career Physician-Scientists

Research Mentees:
Ariel Moradzadeh, M.D. (2017-2018)
Victoria C.S. Scott, M.D. (2018-2019)
Kai Dallas, M.D. (2019-)
Colby P. Souders, M.D. (2019-2020)
Paige Kuhlmann, M.D. (2019-)
Ashley Caron, B.S. (2019-)
Julia Guo, B.S. (2019-)
Sarah B. Andebrhan, MPH (2020-)

CME Events:

Prolapse and Sexual Dysfunction after Menopause: Diagnostic Approaches and Treatments	Annual Cedars-Sinai Update In Obstetrics and Gynecology Cedars-Sinai Medical Center	2020
Does My Vagina Need a Makeover? Prolapse, Female Sexual Dysfunction, And Vaginal Rejuvenation	What's New in Urology: A Primer for the Primary Care Physician Cedars-Sinai Medical Center	2019
Obstructive Sleep Apnea and Personal Urological Health	Otolaryngology Symposium Cedars-Sinai Medical Center	2019
Urinary Incontinence and Pelvic Organ Prolapse: Common Complaints	Community Urology CME Event Cedars-Sinai Medical Center	2017
Why Does It Hurt Down There? Primary Care and Pelvic Pain	What's New in Urology: A Primer for the Primary Care Physician Cedars-Sinai Medical Center	2018
Management of Urinary Incontinence	Community Urology CME Event Cedars-Sinai Medical Center	2017
Urinary Complications of Prostate Cancer Treatment	Urologic Cancer Update Cedars-Sinai Medical Center	2017
Urinary Incontinence: Diagnosis And Management	Annual Cedars-Sinai Update In Obstetrics and Gynecology Cedars-Sinai Medical Center	2016

Grand Rounds/Noon Conferences Lectures:

Female Sexual Dysfunction in Older Patients	Obstetrics and Gynecology Grand Rounds, Cedars-Sinai Medical Center	2020
The Urinary Microbiota in Older Women: Influences of Sex Hormones on LUTS	Center for Research in Women's Health and Sex Differences, CSMC	2020
The Urinary Microbiome in Inflammation of the Urogenital Tract	Microbe Club, Cedars-Sinai Medical Center	2020

Urinary Microbiota and Host Inflammation In Lower Urinary Tract Symptoms	Yale University Department of Urology Grand Rounds, Yale-New Haven Hospital	2020
The Urinary Microbiome in LUTS: A New Paradigm	BIDMC Urology Grand Rounds, Beth Israel-Deaconess Medical Center	2019
Urinary Microbial Profiling in Lower Urinary Tract Symptoms	Urology Grand Rounds, Virginia Commonwealth University	2019
The Urinary Microbiome and Urinary Tract Infection	UCLA FPMRS Grand Rounds, UCLA Medical Center	2019
The Vaginal Microbiome in Health and Disease	Obstetrics and Gynecology Grand Rounds, Cedars-Sinai Medical Center	2019
Recent Advances in our Understanding Of the Diagnosis and Treatment of Urinary Tract Infection	Urgent Care Grand Rounds Cedars-Sinai Medical Center	2018
The Urinary Mycobiome in the Development of Lower Urinary Tract Symptoms	IBIRI Center Group Meeting Cedars-Sinai Medical Center	2017
Host Responses to the Urinary Microbiome in Lower Urinary Tract Symptoms	Immunology Research In Progress Cedars-Sinai Medical Center	2017
A New Perspective on Bladder Hypersensitivity Syndromes: The Urinary Microbiome	Urology Grand Rounds, Cedars-Sinai Medical Center	2017
The Urinary Microbiome and Bladder Hypersensitivity Syndromes	General Surgery Grand Rounds, Cedars-Sinai Medical Center	2017
Interstitial Cystitis: Etiology, Management and Mismanagement	Cedars-Sinai Medical Center Obstetrics and Gynecology Grand Rounds	2016

Undegraduate/Graduate Courses:

Medical Impact of Basic Science	Teaching Assistant, Yale University	2001-2003
Graduate Teaching Center at Yale	Science Teaching Consultant	2000-2002
Biological Mechanisms of Reaction to Injury	Teaching Assistant, Yale University	2000
Biology of AIDS	Adjunct Professor, Albertus Magnus College	2000
Cellular Basis of Behavior	Teaching Assistant, Yale University	1999

PUBLICATION/BIBLIOGRAPHY:

RESEARCH PAPERS

Research Papers (Peer Reviewed)

A. RESEARCH PAPERS – PEER REVIEWED

1. Fleming KG, **Ackerman AL**, Engelman DM. The effect of point mutations on the free energy of transmembrane alpha-helix dimerization. *J Mol Biol.* 1997 Sep 19;272(2):266-75.

2. **Ackerman AL**, Cresswell P. Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. *J Immunol.* 2003 Apr 15;170(8):4178-88.

3. **Ackerman AL**, Kyritsis C, Tampé R, Cresswell P. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc Natl Acad Sci U S A.* 2003 Oct 28;100(22):12889-94.

4. **Ackerman AL**, Cresswell P. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat Immunol*. 2004 Jul;5(7):678-84.

5. **Ackerman AL**, Kyritsis C, Tampé R, Cresswell P. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat Immunol*. 2005 Jan;6(1):107-13.

6. Shen H, **Ackerman AL***, Cody V, Giodini A, Hinson ER, Cresswell P, Edelson RL, Saltzman WM, Hanlon DJ. Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. *Immunology*. 2006 Jan;117(1):78-88. *Co-first author.

7. **Ackerman AL**, Giodini A, Cresswell P. A role for the endoplasmic reticulum protein retrotranslocation machinery during cross presentation by dendritic cells. *Immunity*. 2006 Oct;25(4):607-17.

8. Kiyosaki K, **Ackerman AL**, Histed S, Sevilla C, Eilber K, Maliski S, Rogers RG, Anger J. Patients' understanding of pelvic floor disorders: what women want to know. Female Pelvic Med Reconstr Surg. 2012 May-Jun;18(3):137-42.

9. Lee U, **Ackerman AL**, Wu A, Zhang R, Leung J, Bradesi S, Mayer E, Rodriguez LV. Chronic Psychological Stress in High Anxiety Rats Induces Sustained Bladder Hyperalgesia. *Physiol Behav*. 2015 Feb;139:541-548.

10. **Ackerman AL**, Lee UJ, Jellison F, Tan N, Patel M, Raman SS, Rodriguez LV. MRI suggests increased tonicity of the levator ani in women with Interstitial Cystitis/Bladder Pain Syndrome. *Int Urogyn J.* 2016 Jan;27(1):77-83.

11. **Ackerman AL**, Jellison FC, Lee UJ, Bradesi S, Rodriguez LV. Glt1 glutamate receptor mediates the establishment and perpetuation of chronic visceral pain in an animal model of stress-induced bladder hyperalgesia. *Am J Physiol Renal Physiol.* 2016 April;310(7):F628-F636.

12. Cohen SA, Chaudhry Z, Oliver JL, Kreydin EI, Nguyen MT, Mills SA, **Ackerman AL**, Kim JH, Tarnay CM, Raz S. Comparison of Times to Ureteral Efflux After Administration of Sodium Fluorescein and Phenazopyridine. *J Urol.* 2017 Feb;197(2):519-523.

13. Ramart P, **Ackerman AL***, Cohen SA, Kim JH, Raz S. The Frequency of Urinary Incontinence after Suburethral Mesh Removal Requiring Anti-Incontinence Procedure. *Urology*. 2017 May 2. (*co-first author)

14. Clemens JQ, Mullins C, **Ackerman AL**, Bavendam T, van Bokhoven A, Ellingson BM, Harte SE, Kutch JJ, Lai HH, Martucci KT, Moldwin R, Naliboff BD, Pontari MA, Sutcliffe S, Landis JR; MAPP Research Network Study Group. Urologic chronic pelvic pain syndrome: insights from the MAPP Research Network. Nat Rev Urol. 2018 Dec 18.

15. Weinberger JM, Houman J, Caron AT, Patel DN, Baskin AS, **Ackerman AL**, Eilber KS, Anger JT. Female Sexual Dysfunction and the Placebo Effect: A Meta-analysis. Obstet Gynecol. 2018 Jul 10.

16. Moradzadeh A, Jamnagerwalla J, Eilber KS, Anger JT, **Ackerman AL**. High catastrophizing in subjects with painful mesh complications have worse outcomes. Urology. 2018 Aug 1.

17. **Ackerman AL**, Lai HH, Parameshwar PS, Eilber KS, Anger JT. Symptomatic Overlap in Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome - development of a new algorithm. BJU Int. 2018 Sep 25.

18. Patel DN, Zhao HH, Houman J, **Ackerman AL**, Eilber KS, Anger JT. Comparative effectiveness of one versus two-stage sacral neurostimulation device placement. Neurourol Urodyn. 2019 Jan 8.

19. **Ackerman AL**, Anger JT, Khalique MU, Ackerman JE, Tang J, Kim J, Underhill DM, Freeman MR; NIH Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP). Optimization of DNA extraction from human urinary samples for mycobiome community profiling. PLoS One. 2019 Apr 25;14(4).

20. Nickel JC, Stephens A, Landis JR, Mullins C, van Bokhoven A, Anger JT, **Ackerman AL**, Kim J, Sutcliffe S, Krol JE, Sen B, Hammond J, Ehrlich GD; Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network. Urinary fungi associated with urinary symptom severity among women with interstitial cystitis/bladder pain syndrome (IC/BPS). World J Urol. 2019 Apr 26.

21. Anger J, Lee U, **Ackerman AL,** Chou R, Chughtai B, Clemens JQ, Hickling D, Kapoor A, Kenton KS, Kaufman MR, Rondanina MA, Stapleton A, Stothers L, Chai TC. Recurrent Uncomplicated Urinary Tract Infections in Women: AUA/CUA/SUFU Guideline. J Urol. 2019 Aug;202(2):282-289.

22. Samimi P, **Ackerman AL**, Handler S, Eilber KS, Anger J. Recurrent Urinary Tract Infection in Women: Primary Care Referral Patterns in a Tertiary Care Center.Female Pelvic Med Reconstr Surg. 2019 Jul 8.

23. Handler SJ, **Ackerman AL,** Samimi PA, Bresee C, Anger JT, Eilber KS. Referral Patterns for the Evaluation of Asymptomatic Microscopic Hematuria in Women in a Single Health Care System: Room for Improvement. Obstetrics & Gynecology: July 09, 2019.

24. Souders CP, Zhao H, **Ackerman AL**. Considerations for Bedside Urologic Procedures in Patients with Severe Acute Respiratory Syndrome Coronavirus-2. Urology. 2020 Apr 24.

25. Cohen TN, Cohen KA, Burton CS, Kanji F, Francis SE, Patel D, **Ackerman AL**, Eilber KS, Anger JT. Identifying opportunities to improve patient experience with sacral neuromodulation: A human factors approach. Urology. 2020 May 7.

26. Scott VCS, Lerner LB, Eilber KS, Anger JT, **Ackerman AL**. Re-evaluation of birth trends and pregnancy complications among female urologists: Have we made any progress?. Neurourol Urodyn. 2020 May 25;. doi: 10.1002/nau.24409. [Epub ahead of print] PubMed PMID: 32449995.

27. Souders CP, Lo EM, **Ackerman AL**. Underrepresentation of functional conditions of the lower urinary tract in adults in US federal research funding. Neurourol Urodyn. 2020 Jun 7;. doi: 10.1002/nau.24413. [Epub ahead of print] PubMed PMID: 32506674.

B. RESEARCH PAPERS – PEER REVIEWED (IN PRESS)

1. Parameshwar PS, Borok JF, Wood LN, **Ackerman AL**, Eilber KS, Anger JT. Writing in the Margins of Sexual Function Questionnaires: a Qualitative Analysis of Data from Women with Pelvic Floor Disorders. J Sex Med. 2020. *In press.*

2. Guo JZ, Souders CP, McClelland L, Anger JT, Scott VCS, Eilber KS, **Ackerman AL**. Vaginal Laser Treatment of Genitourinary Syndrome of Menopause: Does the Evidence Support the FDA Safety Communication? Menopause. 2020. *In press.*

C. RESEARCH PAPERS – PEER REVIEWED (SUBMITTED)

1. Scott VCS, Souders CP, Ackerman JE, Khalique MU, Eilber KS, Anger JT, Underhill DM, **Ackerman AL**. The Zombie Idea that Will Not Die: Clinical Mycoplasma and Ureaplasma Testing for Patients with Irritative Urinary Symptoms. *Submitted*

D. RESEARCH PAPERS – NON-PEER REVIEWED

1. Ackerman AL, Anger JT. The Role of the Urinary Microbiome in Urological Disease. AUA News, September, 2017.
2. Ackerman, AL Raz, S. Complete Mesh Removal is Appropriate for Chronic Mesh Related Pain. AUA News, October, 2017.
3. Ackerman, AL. Emergent Themes in Infection and Inflammation of the Genitourinary Tract. AUA News, August, 2020.

Chapters

1. The role of power morcellation and controversies
by **A. Lenore Ackerman**
in: Use of Robotic Technology in Female Pelvic Floor Reconstruction
Ed: Jennifer T. Anger, Karyn S. Eilber

2. Native Tissue Repair After Failed Synthetic Materials
by: **A. Lenore Ackerman**, Seth A. Cohen, Shlomo Raz
in: Native Tissue Repair For Incontinence and Prolapse
Ed: Phillipe Zimmern, Elise De

3. Bulbocavernosus Muscle and Fat Pad Supplement
by: **A. Lenore Ackerman**, Shlomo Raz
in: Hinman's Atlas of Urologic Surgery
Ed: Dr. Joseph A. Smith, Jr., Dr. Stuart S. Howards, Dr. Glenn M. Preminger and Dr. Roger M. Dmochowski

Letters to the Editor None

Reviews

1. Cresswell P, **Ackerman AL**, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev*. 2005 Oct;207:145-57.
2. **Ackerman AL**, Blavais J, Anger J. Female Urethral Reconstruction. *Curr Bladder Dysfunct Rep*. 2010 Oct; 5:225-232.
3. **Ackerman AL**, Rodriguez, LV. Evaluation of Primary Bladder Neck Obstruction in Men. *Curr Bladder Dysfunct Rep*. 2012 Sept; 7(3):235-241.
4. **Ackerman AL**, Underhill DM. The mycobiome of the human urinary tract: potential roles for fungi in urology. *Ann Transl Med.* 2017 Jan;5(2):31.
5. **Ackerman AL,** Parameshwar P, Anger JT. Diagnosis and Treatment of Patients with Prostatic Abscess in the Post-Antibiotic Era. *Int J Urol.* Int J Urol. 2017 Sep 24.
6. Nik-Ahd F, **Ackerman AL**, Anger J. Recurrent Urinary Tract Infections in Females and the Overlap with Overactive Bladder. Curr Urol Rep. 2018 Sep 13;19(11):94.
7. **Ackerman AL** and TC Chai. The Bladder is Not Sterile: an Update on the Urinary Microbiome. *Curr Bladder Dysfunct Rep*. 2019 Nov; 14(2):1-11.
8. Dallas KB, Stewart CS, **Ackerman AL,** Anger JT. OAB and IC/BPS: Two Conditions or a Continuum of One? Curr Bladder Dysfunct Rep. 2020; 15, 15–20.

Editorials

1. **Ackerman AL** and Raz S. Difference of Opinion – Are Synthetic Slings Safe? Opinion: No. Int Braz J Urol. 2016 Jul-Aug;42(4):640-4. PMID: 27564272.
2. **Ackerman AL**. Symptom and Quality of Life Improvements After Pelvic Floor Physical Therapy in a Clinical Population of Women With Pelvic Pain and Other Symptoms. PracticeUpdate. 2020 January.

https://www.practiceupdate.com/content/symptom-and-qol-improvements-after-pelvic-floor-physical-therapy/93850/65/3/1.

Papers in Preparation (Research Completed)

1. **Ackerman AL,** You S, Caron AT, Furie EA, Ackerman JE, Kaufman M. Persistency: A novel symptomatic domain correlating with pelvic floor dysfunction in lower urinary tract symptoms.
2. **Ackerman AL**, Khalique MU, Cheng Z, Ackerman JE, Anger JT, Eilber KS, Underhill DM. Microbiological phenotyping of bladder and pelvic pain reveals distinct clinical profiles with implications for prognosis and treatment.
3. Scott VCS, Thum LW, Khalique MU, Ackerman JE, Underhill DM, **Ackerman AL.** Temporal Dynamics of the Female Geintourinary Microbiome.
4. **Ackerman AL,** Liu G, Hu X, Scott VCS, Kreydin E, Shi W, Raz S**.** Bacterial persistence on Transvaginal Slings is Associated with Delayed-Onset Chronic Pain.
5. **Ackerman AL**, Lima BP, Mellano EM, Ramart P, Pizarro-Rojas M, Shi W, Lux R, Raz S. A Pro-Inflammatory Mesh-Associated Microbiota in Transvaginal Slings Explanted from Patients with Delayed-Onset Chronic Pain.

Abstracts

1. **Ackerman AL** and Cresswell P. Regulation of MHC Class I Transport in Human Dendritic Cells and the Dendritic-like Cell Line KG-1. International Congress of Immunology, Stockholm, Sweden, July 2001.
2. **Ackerman AL** and Cresswell P. Regulated MHC Class I Transport in Human Dendritic Cells Facilitates Proper Immune Activation and Limits Autoimmunity. Keystone Symposium on Dendritic Cells, Keystone, Colorado, March 2003.
3. Hanlon DJ, **Ackerman AL**, Shen H, Cresswell P, Saltzman M. Nanoparticle-Mediated Delivery of Antigens to Dendritic Cells: Increased Efficiency and Duration of Antigen Processing and Presentation. International Congress of Immunology, Montreal, Canada, July 2004.
4. **Ackerman AL**, Kyritsis C, Tampé R, Cresswell P. Early Phagosomes in Dendritic Cells Form a Cellular Compartment Sufficient for Cross Presentation of Exogenous Antigens. International Congress of Immunology, Montreal, Canada, July 2004.
5. **Ackerman AL**, Giodini A, Cresswell P. A Role for the Endoplasmic Reticulum Protein Retrotranslocation Machinery in Cross Presentation by Dendritic Cells. Annual MD/PhD Student Conference, Keystone, Colorado, July 2007. Recipient of Diversity Travel Award.
6. Le NB, Jellison F, **Ackerman AL**, Rogo-Gupta L, Chow D, Baxter ZC, Treat E, Hartshorn T, Kim JH, Rodriguez L, Raz S. Anterior Colporraphy Using Polypropylene Sutures: Our Two-year Experience. Society of Urodynamics and Female Urology Annual Meeting, New Orleans, LA, February 2012.
7. Chow D, Jellison F, Hartshorn T, Rogo-Gupta L, Le NB, **Ackerman AL**, Staack A, Rodriguez LV, Raz S. Translabial Ultrasound for Localization of Vaginal Mesh. Society of Urodynamics and Female Urology Annual Meeting, New Orleans, LA, February 2012.
8. **Ackerman AL**, Chestovich P, Dubina E, Tillou A, Anger JT. Patterns of Care for Urethral Trauma in Patients with Pelvic Fracture. Society of Urodynamics and Female Urology Annual Meeting, New Orleans, LA, February 2012.
9. **Ackerman, AL**, Lee U, Zhang R, Le N-B, Leung J, Bradesi S, Rodriguez LV. Alterations in Neurotransmitter Processing in Rodents Exposed to Chronic Water Avoidance Stress. Society of Urodynamics and Female Urology Annual Meeting, New Orleans, LA, February 2012.
10. **Ackerman AL**, Le N-B, Jellison F, Rogo-Gupta L, Chow D, Hartshorn TG, Rodriguez LV, Raz S. Anatomical, functional, and quality of life outcomes of transvaginal sacrouterine ligament

suspension for vaginal vault prolapse. Society of Urodynamics and Female Urology Annual Meeting, New Orleans, LA, February 2012. Recipient of Resident Travel Award.

11. Jellison F, Le NB, **Ackerman AL**, Rogo-Gupta L, Chow D, Chamie K, Raman S, Rodriguez LV, et. al. Relationship Between Physical Examination, Dynamic MRI, and Intra-operative Findings in the Treatment of Pelvic Organ Prolapse. American Urological Association Annual Meeting, Atlanta, GA, May 2012.
12. Rogo-Gupta L, Hartshorn TG, Chow D, Le NB, Jellison F, **Ackerman AL**, Rodriguez LV, Raz S. Complications of Mesh-augmented Pelvic Organ Prolapse and Incontinence Repairs: Case Series of 319 Procedures. American Urological Association Annual Meeting, Atlanta, GA, May 2012.
13. **Ackerman AL**, Lee UJ, Tan N, Raz S, Raman SS, Rodriguez LV. Alterations in the Pelvic Floor Musculature on Pelvic MRI in Patients with Interstitial Cystits. American Urological Association Annual Meeting, Atlanta, GA, May 2012.
14. Le N-B, **Ackerman AL**, Zhang R, Lee UJ, Leung J, Rodriguez LV. Mast Cell Activation, Mediator Levels, Tactile Allodynia, and Urothelial Topography in Wistar-Kyoto Rodents Exposed to Chronic Water Avoidance Stress. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Las Vegas, NV, February 2013.
15. Mellano EM, Lima B, Ramart P, **Ackerman AL**, Lux R, Shi W, Raz S. The Role of Bacterial Biofilms and Chronic Inflammation in the Delayed Development of Pain following Transvaginal Placement of Mesh Slings for Incontinence. AUGS Annual Meeting, October 2015, Seattle, WA.
16. Kang D, Hartshorn T, Pollard M, Choi J, Rodriguez L, Kim JH, **Ackerman AL**, Cohen S, Ramapart P, Raz S. Patient Quality of Life after Removal of Vaginal Mesh. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, February 2015.
17. Choi JM, Ramart P, Kang D, **Ackerman AL**, Cohen S, Raz S. Abdominal Sacrocolpopexy Mesh Complications: Presentation and Surgical Removal Techniques. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, February 2015.
18. **Ackerman AL**, Lima BP, Ramart P, Mellano E, Lux R, Shi W, Raz S. The Role of Bacterial Biofilms and Chronic Inflammation in the Delayed Development of Systemic Side Effects Following Transvaginal Placement of Mesh Slings for Incontinence. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, February 2015.
19. **Ackerman AL**, Lee UJ, Jellison FC, Margolis DC, Tan N, Patel M, Raz S, Raman SS, Rodriguez LV. MRI Imaging Suggests Increased Tonicity of the Levator Ani Muscle Complex in Women with Interstitial Cystitis/Bladder Pain Syndrome. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, February 2015.
20. Cohen SA, Mellano EM, Chaudhry Z, **Ackerman AL**, Ramart P, Scott VC, Kim J, Raz S. Cystocele Repair Using Autologous Iliotibial Band. Presentation: Video 15. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, February 2015.
21. **Ackerman AL**, Jellison FC, Lee UJ, Bradesi S, Rodríguez LV. Glt1 Glutamate Receptor Mediates The Establishment And Perpetuation Of Chronic Visceral Pain In An Animal Model Of Bladder Pain Syndrome/Interstitial Cystitis. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, February 2015.
22. Choi JM, Ramart P, Kang DC, Cohen SA, **Ackerman AL**, Raz S. Pubovaginal Sling with Tensor Fascia Lata. American Urological Association Annual Meeting, New Orleans, LA, May 2015.
23. Cohen SA, Viragh KA, Nakamura LY, **Ackerman AL**, Ramart P, Kang DC, Choi JM, Kim J-H, Raman SS, Raz S. Using Translabial Ultrasound as an Effective Tool to Visualize Mesh Erosion into the Urethra and Bladder. American Urological Association Annual Meeting, New Orleans, LA, May 2015.
24. Ramart P**, Ackerman AL**, Cohen SA, Kang DC, Choi JM, Kim J-H, Raz S. Urinary incontinence after suburethral mesh removal requiring anti-incontinence procedure. International Continence Society Annual Meeting, Montreal, Canada, October 2015.
25. **Ackerman AL**, Eilber KS, Caron AT, Pollard ME, Anger JT. Outcomes of Pregnancy Following Surgery for Pelvic Organ Prolapse: A Systematic Review. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016.

26. Scott VCS, Cohen SA, Greenberg SA, Kreydin EI, Oliver JL, **Ackerman AL**, Chaudhry Z, Nguyen MT, Kim J-H, Raz S. Use of Iliotibial Band (Fascia Lata) as a Salvage Continence Repair After Mesh Removal – At Least 6 Months Follow Up. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016.
27. Kreydin EI, Kim MM, Oliver JL, Cohen SA, **Ackerman AL**, Kim J-H, Raz S. Higher Urine Levels of Environmental Toxins Are Associated with Increased Incontinence and Nocturia in Men. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016.
28. Kreydin EI, Oliver JL, Kim MM, **Ackerman AL**, Cohen SA, Kim J-H, Raz S. 'Til Death Do Us Part: The Relationship Between Urinary Incontinence and Marital Status Among US Women and Men. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016.
29. Cohen SA, Oliver JL, Kreydin EI, Chaudhry Z, Nguyen MT, Mills SA, **Ackerman AL**, Kim J-H, Tarnay CM, Raz S. Comparison of Times to Ureteral Efflux After Administeration of Sodium Fluorescein and Phenazopyridine. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016.
30. Ramart P, **Ackerman AL**, Cohen SA, Kim J-H, Raz S. Urinary Incontinence After Suburethral Mesh Removal Requiring Anti-Incontinence Procedures. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016.
31. **Ackerman AL**, Anger JT, Eilber KS, Funari V, Tang J, Kim J, Freeman MR. Identification of a Diverse Fungal Community ("Mycobiome") in the Normal Female Human Lower Urinary Tract. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, New Orleans, LA, February 2016. Recipient of Best Basic Science Poster Award.
32. Kreydin E, Oliver J, Kim M, Cohen S, **Ackerman AL**, Raz S, Lerner L. Prostate Ablation and Enucleation: Comparison of Patient Characteristics and 30-day Surgical Outcomes Using a National Database. American Urological Association Annual Meeting, San Diego, CA, May 2016.
33. Kreydin E, Kim M, Oliver J, Cohen S, **Ackerman AL**, Chaudhry Z, Nguyen MT, Kim JH, Raz S. Til Death Do Us Part: The Relationship between Urinary Incontinence and Marital Status among US Women and Men. American Urological Association Annual Meeting, San Diego, CA, May 2016.
34. **Ackerman AL**, Anger JT, Eilber K, Funari V, Tang J, Kim J, Freeman M. Identification of a Diverse Fungal Community ("Mycobiome") in the Normal Female Human Lower Urinary Tract. American Urological Association Annual Meeting, San Diego, CA, May 2016.
35. **Ackerman AL**, Eilber KS, Tang J, J. Kim3 , Freeman MR, Anger JT. A Diverse, Viable Fungal Community ("Mycobiome") Exists in the Urine of Healthy Asymptomatic Females. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, September/October 2016, V22:5 p. S35. Oral Poster 1.
36. Samimi P, Handler SJ, **Ackerman AL**, Eilber KS, Anger JT. Recurrent UTI in Women: Patient Characteristics, Natural History, and Referral Practice Patterns. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale,AZ, September/October 2016, V22:5 p. S103. Poster 76.
37. Handler SJ, Samimi P, **Ackerman AL**, Anger JT, K. S. Eilber KS. Practice Patterns for the Evaluation of Asymptomatic Micoscopic Hematuria in Women in a Single Healthcare System: Room for Improvement? Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, September/October 2016, V22:5 p. S103. Poster 77.
38. Nguyen MT, Cohen SA, Mei JY, **Ackerman AL**, Oliver J, Kreydin KI. Preliminary Report on the Use of the Vastur Lateralis-Fascia Lata Graft for Repair of Anterior Vaginal Wall Prolapse. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, Scottsdale, AZ, September/October 2016, V22:5 p. S149. Poster 180.
39. **AL Ackerman**, KS Eilber, J Tang, J Kim, MR Freeman, JT Anger. A Diverse, Viable Fungal Community ("Mycobiome") Exists in the Urine of Healthy Asymptomatic Females. American Urogynecologic Society Annual Meeting/Pelvic Floor Disorders Week, September 2016, Denver, CO.

40. Van den Broek I, Fu Q, Ackerman AL, Anger JT, Kushon S, Chansky K, Millis K, Percy A, Agreste T, Van Eyk JE. Accuracy of Volumetric Absorptive Microsampling for Quantification of Protein Biomarkers. The Association for Mass Spectrometry: Applications to the Clinical Lab, January 2017, Palm Springs, CA.
41. Van den Broek I, Fu Q, Kushon S, Chansky K, Kowalski MP, Millis K, Percy A, Agreste T, **Ackerman AL**, Anger JT, Holewinski R, Venkatraman V, Van Eyk JE. A Precision Proteomics Pipeline for Remote Blood Monitoring: Integrating Volumetric Absorptive Microsampling with High-Throughput Mass Spectrometric Proteotyping. US HUPO 13th Annual Meeting: Precision Proteomics for Discovery and Health, March 2017, San Diego, CA.
42. Weinberger J, Houman J, Caron A, Baskin A, **Ackerman AL**, Eilber KS, Anger JT. Female Sexual Dysfunction Treatment: A Meta-Analysis of the Placebo Effect Across Randomized Controlled Trials. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
43. **Ackerman AL**, Lai HH, Eilber KS, Anger JT. Symptomatic Overlap in Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
44. **Ackerman AL**, Handler S, Samimi P, Anger JT, Eilber KS. Practice Patterns for the Evaluation of Asymptomatic Microscopic Hematuria in Women in a Single Healthcare System: Room for Improvement? Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
45. Parameshwar PS, Kayondo M, **Ackerman AL**, Anger JT, Tarnay C. Effects of Group Rehabilitation upon Women Undergoing Surgery for Obstetric Fistula. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
46. Thum DJ, Wood LM, Moradzadeh A, Hannemann A, Li A, **Ackerman AL**, Anger JT, Eilber KS. Concomitant Treatment of Stress Urinary Incontinence and Gynecologic Oncology Surgery: Are We Undertreating? Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
47. **Ackerman AL**, Eilber KS, Tang J, Kim J, Underhill DM, Anger JT, Freeman MR. Optimization of DNA Extraction from Human Urinary Samples for Microbial Community Profiling. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
48. **Ackerman AL**, Tang J, Eilber KS, Kim J, Anger JT, Underhill DM, Freeman MR. Decreased Urinary Fungal Burden and Diversity in Overactive Bladder. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
49. **Ackerman AL**, Anger JT, Tang J, Eilber KS, Kim J, Freeman MR, Underhill DM. Alterations in the Urinary Fungal Mycobiome in Patients with Bladder Pain and Urinary Urgency. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
50. **Ackerman AL**, Jie Tang J, Eilber KS, Kim J, Nickel JC, Ehrlich G, Underhill DM, Jennifer Anger JT. Shared Alterations in Urinary Bacterial Communities in Patients with Interstitial Cystitis and Overactive Bladder. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2017, Scottsdale, AZ.
51. Parameshwar PS, Borok JF, Wood LN, **Ackerman AL**, Eilber KS, Anger JT. Writing in the Margins of Sexual Function Questionnaires: A Qualitative Analysis From Women With Pelvic Floor Disorders. American Urological Association Annual Meeting, May 2017, Boston, MA.
52. **Ackerman AL**, Anger JT, Tang J, Eilber KS, Kim J, Freeman MR, Underhill DM. Alterations in the Urinary Fungal Mycobiome in Patients with Bladder Pain and Urinary Urgency. American Urological Association Annual Meeting, May 2017, Boston, MA.
53. **Ackerman AL**, Lai HH, Eilber KS, Anger JT. Symptomatic Overlap in Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome. American Urological Association Annual Meeting, May 2017, Boston, MA.

54. Weinberger J, Houman J, Caron A, Baskin A, **Ackerman AL**, Eilber KS, Anger JT. Female Sexual Dysfunction Treatment: A Meta-Analysis of the Placebo Effect Across Randomized Controlled Trials. American Urological Association Annual Meeting, May 2017, Boston, MA.
55. Juzar Jamnagerwalla J, Anger JT, Eilber KS, **Ackerman AL**. High Catastrophizing in Patients with Self-reported Painful Mesh Complications Have Poorer Outcomes. American Urological Association Annual Meeting, May 2017, Boston, MA.
56. **Ackerman AL**, Tang J, Eilber KS, Kim J, Anger JT, Underhill DM, Freeman MR. Decreased Urinary Fungal Burden and Diversity in Overactive Bladder. American Urological Association Annual Meeting, May 2017, Boston, MA.
57. **Ackerman AL**, Jie Tang J, Eilber KS, Kim J, Nickel JC, Ehrlich G, Underhill DM, Jennifer Anger JT. Shared Alterations in Urinary Bacterial Communities in Patients with Interstitial Cystitis and Overactive Bladder. American Urological Association Annual Meeting, May 2017, Boston, MA.
58. Parameshwar PS, Kayondo M, **Ackerman AL**, Eilber KS, Anger JT, Tarnay CM. Post-Operative Pain Management In Patients Undergoing Perineal And Vaginal Reconstructive Surgery: An Alternative To Narcotics. American Urogynecologic Society Annual Meeting/Pelvic Floor Disorders Week, October 2017, Providence, RI.
59. Kuhlmann P, Chen A, Johnson J, Hubbard L, **Ackerman AL**, Eilber KS, Anger JT. Concomitant Procedures Performed at the Time of Midurethral Sling Affect Post-operative Urinary Retention Rate. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2018, Austin, TX.
60. Moradzadeh A, Jamnagerwalla J, Eilber KS, Anger JT, **Ackerman AL**. High Catastrophizing in Subjects with Self-Reported Painful Mesh Complications Have Poorer Outcomes. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2018, Austin, TX.
61. Scott V, Tang J, Drell T, Simm J, Salumets A, Metsis M, Underhill D, **Ackerman AL**. Evaluation of the Vaginal Mycobiome in Asymptomatic Pre-menopausal Women. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2018, Austin, TX.
62. Scott V, **Ackerman AL**, Liu G, Shi W, Raz S. Immunofluorescence Localization of Bacterial Biofilms on Explanted Transvaginal Mesh Slings Removed for Chronic Pain. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2018, Austin, TX.
63. Anger JT, **Ackerman AL**, Spivia W, van den Broek I, Crear D, Eilber KS, Freeman M, Kim J, Fu Q, Van Eyk J. Differential Protein Expression in Patients with UCPPS: A MAPP Study. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2018, Austin, TX.
64. **Ackerman AL**, Scott V, Liu G, Shi W, Raz S. Characterization of Bacteria Identified on Explanted Mesh Slings Using Next-generation Sequencing Techniques. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2018, Austin, TX.
65. Scott V, **Ackerman AL**, Liu G, Shi W, Raz S. Immunofluorescence Localization of Bacterial Biofilms on Explanted Transvaginal Mesh Slings Removed for Chronic Pain. Annual Meeting. American Urological Association; May 2018; San Francisco, CA, USA.
66. **Ackerman AL**, Scott V, Liu G, Shi W, Raz S. Characterization of Bacteria Identified on Explanted Mesh Slings Using Next-generation Sequencing Techniques. Annual Meeting. American Urological Association; May 2018; San Francisco, CA, USA
67. Scott V, Tang J, Drell T, Simm J, Salumets A, Metsis M, Underhill D, **Ackerman AL**. Evaluation of the Vaginal Mycobiome in Asymptomatic Pre-menopausal Women. Annual Meeting. American Urological Association; May 2018; San Francisco, CA, USA.
68. Kuhlmann PK, Chen A, Johnson J, Hubbard L, **Ackerman AL**, et al. Concomitant Procedures Performed at the Time of Midurethral Sling Affect Post-operative Urinary Retention Rate. Annual Meeting. American Urological Association; May 2018; San Francisco, CA, USA.
69. Anger J, Spivia W, van den Broek I, Crear D, **Ackerman AL**, et al. Differential Protein Expression in Patients with UCPPS: A MAPP Study. American Urological Association Annual Meeting, May 2018, San Francisco, CA, 199:4 e506: J Urol; April, 2018.

70. van den Broek I, Mouapi KN, Mastali M, Holewinski R, Venkataraman V, Fu Q, **Ackerman AL**, Kim J, Freeman M, Anger JT, Millis K, Percy A, Van Eyk J. A stable-isotope labeled protein and peptide mixture for global normalization in targeted and data-independent quantitative bottom-up proteomics. ASMS Conference on Mass Spectrometry and Allied Topics, June 2018, San Diego, CA.
71. Kuhlmann PK, Chen A, **Ackerman AL**, Anger JT, Eilber KS. Higher BMI and Concomitant Pelvic Organ Prolapse Repair are Protective Against Intraoperative Bladder Perforation During Midurethral Sling Placement. American Urogynecologic Society Annual Meeting/Pelvic Floor Disorders Week, October 2018, Chicago, IL.
72. Patel DN, Gonzales-Alabastro C, Houman J, **Ackerman AL**, Eilber KS, Anger JT. Comparative Effectiveness of One- Versus Two-Stage Sacral Neuromodulation Device Placement. American Urogynecologic Society Annual Meeting/Pelvic Floor Disorders Week, October 2018, Chicago, IL
73. **Ackerman AL**, Khalique MU, Ackerman JE, Tang J, Eilber KS, Anger JT, Underhill DM. The Urinary Microbiota and Host Inflammation in Lower Urinary Tract Symptoms. Western Section American Urological Association Annual Meeting, Maui, HI, October 2018.
74. Scott VCS, Thum LW, Ackerman JE, Khalique MU, Eilber KS, Anger JT, Underhill DM, **Ackerman AL**. Temporal Dynamics of the Genitourinary Microbiome. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
75. Burton C, O'Brien R, Eilber KS, **Ackerman AL**, Anger JT. Identifying barriers to urinary incontinence care among primary care providers. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
76. Scott VCS, Lerner LB, Anger JT, Van Kuiken M, **Ackerman AL**. Satisfaction of women urologists with maternity leave, childbirth timining and work-family balanace ten years later. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
77. Souders C, Caron AT, Obi N, Clark K, McClelland L, **Ackerman AL,** Anger JT, Eilber KS. 60 minutes and vaginal mesh: did the media tell the whole story? Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
78. Cohen T, Burton C, Francis S, Patel D, Othman N, Lam P, **Ackerman AL,** Eilber KS, Anger JT. Improving patient experience with sacral neuromodulation: a human factors approach. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
79. Caron AT, Scott VCS, Eilber KS, Anger JT, **Ackerman AL.** Evaluation of recurrent uti-like symptoms should prompt consideration for alternative etiologies. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
80. Stewart C, Maniskas S, **Ackerman AL**, Eilber KS, Freedland S, Anger JT. Evidence linking comestibles to interstitial cystitis and bladder pain syndrome. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
81. Scott VCS, Lerner LB, Anger JT, Van Kuiken M, **Ackerman AL**. Re-evaluation of birth trends and pregnancy complications among female urologists: have we made any progress? Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
82. Burton C, Gonzalez-Alabastro C, Choi E, Parameshwar P, Gonzalez G, Bresee C, Eilber KS, **Ackerman AL,** Anger JT. Overuse of specialty care for women with urinary incontinence. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
83. Souders C, McClelland L, Caron AT, Alazzeh M, Zukotynski B, Ackerman AL, Eilber KS, Anger JT. Transvaginal mesh litigation has significant geographic variation. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
84. Caron AT, **Ackerman AL,** Parameshwar P, Eilber KS, Anger JT. Outcomes of pregnancy following surgery for pelvic organ prolapse: a systematic review. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.

85. Stewart CA, Zhao H, Gonzales G, Gonzales-Alabastro C, Zaghiyan K, Moore B, Magner D, **Ackerman AL,** Anger JT, Eilber KS. Outcomes of sacrocolpopexy with concurrent colorectal surgery for multicompartment prolapse repair. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
86. Zhao H, Stewart CA, Gonzalez G, Gonzales-Alabastro C, **Ackerman AL,** Eilber KS, Anger JT. De novo defecatory symptoms and posterior compartment prolapse as a complication of sacrocolpopexy. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction Annual Meeting, March 2019, Miami, FL.
87. Stewart CA, Zhao H, Gonzales G, Gonzales-Alabastro C, **Ackerman AL,** Eilber KS, Anger JT. Outcomes of prophylactic sling at the time of robotic assisted sacrocolpopexy. American Urological Association Annual Meeting, May 2019, Chicago, IL.
88. Kuhlmann P, Stewart CA, Souders C, Moradzadeh A, Houman J, **Ackerman AL,** Anger JT, Eilber KS. What is "vaginal rejuvenation"? A survey of medical professionals and lay persons. American Urological Association Annual Meeting, May 2019, Chicago, IL.
89. Cohen T, Burton CS, Francis S, **Ackerman AL**, Eilber KS, Anger JT. Improving patient experience with sacral neuromodulation: a human factors approach. American Urological Association Annual Meeting, May 2019, Chicago, IL.
90. Anger JT, Spivia W, **Ackerman AL,** Eilber KS, Kim J, Fu Q, Michael Freeman MR, Van Eyk J. The Serum Proteome Correlates with Clinical Phenotypes of UCPPS: a MAPP Study. American Urological Association Annual Meeting, May 2019, Chicago, IL.
91. Zhao H, Stewart CA, Souders CP, Gonzalez G, Gonzales-Alabastro C, **Ackerman AL,** Eilber KS, Anger JT. De novo Defecatory Symptoms and Posterior Compartment Prolapse as a complication of Sacrocolpopexy. American Urological Association Annual Meeting, May 2019, Chicago, IL.
92. Dallas K, **Ackerman AL**, Eilber K, Rogo-Gupta L, Scott V, Troung M, Cohen T, Anger J. Validated Surgical Steps of the Robotic Assisted Sacrocolpopexy Operation. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
93. Burton C, Markowitz M, Kanji F, Stewart C, Scott V, Eilber K, **Ackerman AL**, Anger J. Validation of the Pelvic Floor Awareness and Knowledge Survey (PFAKS). Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
94. Burton C, Gonzalez G, Bresee C, Scott V, Eilber K, **Ackerman AL**, Wieslander C, Anger J. Urinary Incontinence Referral Patterns in Academic and County Hospitals: The Impact of Econsult. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
95. Kuhlmann P, Ackerman J, Khalique M, Caron A, Kanji F, Anger J, Eilber K, Underhill D, **Ackerman AL**. Urinary Aerococcus Defines a Severe, Treatment-refractory Phenotype of Urgency Urinary Incontinence in Older Women. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
96. Scott V, Souders C, Khalique M, Ackerman J, **Ackerman AL**. Is Ureaplasma Truly a Urinary Tract Pathogen? Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
97. Guo J, Souders C, McClelland L, Anger J, Eilber K, **Ackerman AL**. Safety and Legal Environment for Vaginal Lasers: Uncovering the Evidence Behind the FDA Safety Communication. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
98. Kuhlmann P, Dallas K, Masterson J, Patel D, Casteneda P, Reddy A, Tsai K, **Ackerman AL**. Risk Factors for Intraoperative Bladder Perforation at the Time of Midurethral Sling Placement. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
99. Masterson J, Kuhlmann P, Dallas K, Reddy A, Tsai K, Castaneda P, Eilber K, Anger J, **Ackerman AL**. Overactive Bladder Following Midurethral Sling Placement: De Novo or Persistent? Society of

Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.

100. Burton C, Lo E, Kanji F, Caron A, Cohen T, Miller D, Wenger N, Scott V, **Ackerman AL**, Eilber K, Anger J. Implementation of a Primary Care Intervention to Improve Care for Women with Urinary Incontinence. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
101. **Ackerman AL**, Caron A, Ackerman J, Anger J, Eilber K, Kaufman M. 'Persistency': A Novel Urinary Symptom Measure Encompassing the Myofascial Component of LUTS. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA
102. Dallas K, Kuhlmann P, Masterson J, Reddy A, Tsai K, Casteneda P, **Ackerman AL**, Eilber K, Anger J. Urinary Tract Infection after Mid-urethral Sling: Rates and Risk Factors. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
103. Kuhlmann P, Masterson J, Dallas K, Tsai K, Reddy A, Casteneda P, **Ackerman AL**, Eilber K, Anger J. Development of Post-operative Urinary Retention after Midurethral Sling Placement: Can We Counsel Patients on Duration?. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
104. Dallas K, Caron A, Anger J, Eilber K, **Ackerman AL**. Application of Machine Learning Algorithms to Classify Storage Lower Urinary Tract Symptoms. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
105. Burton C, Gonzalez G, Kanji F, Caron A, Bresee C, Eilber K, **Ackerman AL**, Anger J. Rates of Tertiary Procedures among Women Referred for Urinary Incontinence. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
106. Souders C, **Ackerman AL** Underrepresentation of FPMRS in Federal Funding for Benign Urologic Conditions. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
107. **Ackerman AL**, Khalique M, Ackerman J, Cheng Z, Eilber K, Anger J, Underhill D. Microbial Composition Defines Pelvic Pain Phenotypes in Reproductive-Age Women. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
108. Dallas K, Anger J, Caron A, Eilber K, **Ackerman AL**. Validation of the Diagnostic Accuracy of Diagnostic Groupings of Patients with Storage Lower Urinary Tract Symptoms Generated by Machine Learning Algorithms. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
109. Cohen T, Burton C, Cohen K, **Ackerman AL**, Eilber K, Anger J. Using a Human Factors Approach to Develop Interventions Aimed at Improving Patient Experience with Sacral Neuromodulation. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
110. Burton C, Tran J, Gonzalez G, Bresee C, Choi E, Scott V, **Ackerman AL**, Eilber K, Anger J. Urinary Incontinence Care for Older Adults: Difference or Disparity. Society of Urodynamics, Female Pelvic Medicine, and Urogenital Reconstruction. Annual Meeting; 2020 February; Scottsdale, AZ, USA.
111. **Ackerman AL**, Caron AS, Ackerman JE, Anger JT, Eilber KS, Kaufman M. ‘Persistency’: A Novel Urinary Symptom Measure Encompassing the Myofascial Component of LUTS. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
112. **Ackerman AL**, Khalique MU, Ackerman JE, Cheng Z, Eilber KS, Anger JT, Underhill DM. Microbial Composition Defines Pelvic Pain Phenotypes in Reproductive-age Women. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
113. Scott VCS, Souders CP, Khalique MU, Ackerman JE, **Ackerman AL**. Is Ureaplasma a Urinary Tract Pathogen? American Urological Association Annual Meeting, May 2020, Virtual Meeting.

114. Dallas K, Caron AS, Anger JT, Eilber KS, **Ackerman AL**. Application of Machine Learning Algorithms to Classify Storage Lower Urinary Tract Symptoms. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
115. Dallas K, **Ackerman AL**, Eilber KS, Rogo-Gupta L, Scott VCS, Troung M, Cohen T, Anger JT. Validated Surgical Steps of the Robotic Assisted Sacrocolpopexy Operation. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
116. Burton CS, Markowitz MA, Kanji F, Scott VCS, **Ackerman AL**, Eilber KS, Anger JT. Validation of the Pelvic Floor Awareness and Knowledge Survey. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
117. Kanji F, Burton CS, Cohen T, Bresee C, Scott VCS, **Ackerman AL**, Eilber KS, Anger JT. Developing a Urinary Incontinence Screening Tool for Use in Primary Care: Trials and Tribulations. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
118. Burton CS, Tran J, Gonzalez G, Bresee C, Choi E, Scott VCS, **Ackerman AL**, Eilber KS, Anger JT. Urinary Incontinence Care for Older Adults: Difference or Disparity? American Urological Association Annual Meeting, May 2020, Virtual Meeting.
119. Kuhlmann PK, Ackerman JE, Khalique MU, Caron AS, Kanji F, Anger JT, Eilber KS, Underhill DM, **Ackerman AL**. Urinary Aerococcus Defines a Severe, Treatment-refractory Phenotype of Urge Incontinence in Older Women. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
120. Lai HH, Clemens JQ, Newcomb C, Appleby D, **Ackerman AL**, Anger JT, Nickel JC, Gupta P, Rodriguez L, Landis JR. Comparison of Deep Phenotyping Features of UCPPS Patients with and without Hunner Lesion – A MAPP Research Network Study. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
121. Burton CS, Lo EM, Kanji F, Caron A, Cohen T, Miller D, Wenger N, Scott VCS, **Ackerman AL**, Eilber KS, Anger JT. Implementation of a Primary Care Intervention to Improve Care for Women with Urinary Incontinence. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
122. Gupta P, Lai HH, Anger JT, **Ackerman AL**, Gallop R, Landis JR, Clemens JQ. Impact of History of Urinary Tract Infections on Patients with Urologic Chronic Pelvic Pain Syndromes: Findings from Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Study. American Urological Association Annual Meeting, May 2020, Virtual Meeting.
123. Hopp M, **Ackerman AL**, et al. Sexual and Urinary Health in OSA Patients. American Academy of Otolaryngology-Head and Neck Surgery Annual Meeting, September 2020, Boston, MA.
124. Dallas K, Anger JT, Elliott CS, **Ackerman AL**. Racial Disparities Exist in Outcomes in a Population Based Cohort of 35,000 Women Undergoing Myomectomy for Fibroid Disease. PFD Week 2020 – American Urogynecologic Society Annual Meeting, Vancouver, Canada, October, 2020.
125. Tholemeier LN, Choi E, Bresee CT, Kanji FF, **Ackerman AL**, Anger JT, Eilber KS. IMACTIV: A Pilot Study of the Impact of Unrestricted Activity Following Mid-Urethral Sling Surgery. PFD Week 2020 – American Urogynecologic Society Annual Meeting, Vancouver, Canada, October, 2020.

DIAGNOSTIC TESTING USING MICROBIAL BIOMARKERS TO CLASSIFY PELVIC AND BLADDER PAIN

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[01] This invention was made with Government support under award number W81XWH-17-1-0433 awarded by the Department of Defense. The Government has certain rights in the invention.

FIELD OF INVENTION

[02] This invention relates to the detection and treatment of pelvic and bladder pain.

BACKGROUND

[03] All publications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

[04] In reproductive-age women, there is significant symptomatic overlap between interstitial cystitis/bladder pain syndrome, chronic pelvic pain, overactive bladder syndrome (OAB), vulvodynia, and endometriosis leading to frequent misdiagnosis and delayed care. The epidemiology of pelvic pain suggests a microbial involvement in its etiology, but previous studies have failed to definitively identify specific bacteria associated with pain diagnoses.

[05] Interstitial cystitis/bladder pain syndrome and other chronic pelvic pain syndromes are highly refractory to treatment. Many patients are not properly diagnosed, and even those who do eventually get a diagnosis are frequently misdiagnosed and will often experience profound delays in diagnosis of as much as 9 years. Even after diagnosis, clinicians must employ a process of trial and error to eventually be able to find an effective treatment. These substantial barriers to appropriate care are the cause of a lack of testing to identify these conditions and any prognostic testing or information to help providers identify which therapies may be most beneficial for patients.

[06] As such, there is a need in the art to provide ways to distinguish each of these conditions and to determine treatments that would be appropriate.

BRIEF DESCRIPTION OF THE FIGURES

[07] Exemplary embodiments are illustrated in referenced figures. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive.

[08] Figure 1, panels A-B, show that Microbial Patterns are associated with pelvic pain subtypes in pre-menopausal women. In a pilot population of 35 pre-menopausal women, we observed a strong association of bladder pain with the presence of *Lactobacillus iners* and of vaginal/urethral pain unrelated to voiding (denoted as non-urologic pelvic pain[NUPP]) with *Enterobacteriaceae* (classified as *Escherichia/Shigella* in SILVA, *Enterobacteriaceae* in GreenGenes). A) Stacked bar plots display the relative composition of bacterial microbiota in each patient, the shades of red below each subject's column indicate the scaled pain in each of these two domains. B) The association of each pain domain was dependent on relative abundance, as demonstrated by the scatter plots of pain scores vs. the relative abundance of each taxa. Linear regression analysis demonstrated that these associations were statistically significant for both associations.

[09] Figure 2, panels A-B, show that Escherichia/Shigella *and* Lactobacillus iners *are discriminatory for pain.* A) Each panel indicates the relative abundance for each of the 35 pilot subjects. All patients with VAS pain scores >4 exhibited detectable *Lactobacillus iners*, *Escherichia/Shigella,* or both, while little of either could be detected in the asymptomatic subjects. B) Patients were separated into subgroups of patients with dominant bladder pain, dominant non-urologic pelvic pain and asymptomatic controls. Box and whisker plots of relative abundance in each subgroup relate the association of *Lactobacillus iners* and *Escherichia/Shigella* with each phenotype, respectively.

[10] Figure 3 shows the correlation of microbial patterns with pain phenotypes. The individual urinary microbial compositions of an independent verification population of 49 additional subjects are again exhibited in stacked box plots, ordered by urotype. This independent population reiterated the correlation of *Lactobacillus* iners with bladder pain and *Enterobacteriaceae* with non-urologic pelvic pain, which are quantitate in the heat bar below the plot with increasing red indicating the higher pain levels. The pie charts below indicate the median microbial composition for each phenotype. The orange non-iners Lactobacillus group and the purple bladder pain group differed very little except for the presence of Lactobacillus iners in the bladder pain group. The *Enterobacteriaceae* group was significantly more diverse, frequently featuring *Vibrio* and *Halomonas spp*.

[11] Figure 4, panels A-D, show clinical features of all patients by bacterial community state type. Urotypes were defined as *L. iners*, other *Lactobacilli*, and *Escherichia*. Individual clinical features were plotted as box and whisker plots. The L. iners urotype was homogenously high for isolated bladder pain (A), while the Escherichia group demonstrated non-urologic pelvic pain (B). There were no significant differences in the severity of urgency incontinence (C), which was low across all groups, or age (D) between groups

[12] Figure 5, panels A-C, show that microbial associations persist even after correction for confounders and for multiple comparisons. (A) Linear discriminant analysis (LDA) assesses the potential of specific taxa as biomarkers for bladder/pelvic pain. Differential bacterial features are ranked by linear discriminant analysis effect size (LEfSe), with green and red indicating associations with pain or the asymptomatic state, respectively. (B) The LDA is alternatively represented on a phylogenetic tree as a

cladogram. (C) Multivariate Association with Linear Models (MaAsLin) Analysis confirmed the association of *Lactobacillus iners* with bladder pain. Notched box plots demonstrate the difference in *Lactobacillus iners* abundance in controls and the bladder pain subgroup after controlling for age, BMI, and hormonal supplementation. Neither *Haemophilus* nor *Escherichia/Shigella* was associated with bladder-specific pain.

[13] Figure 6 shows that quantitative PCR provides rapid, scalable testing with objective thresholds to facilitate patient diagnosis and sub-classification. Using the female Genitourinary Pain Index (fGUPI) as an indicator of patients presenting with and without pain, quantitative PCR detecting Lactobacillus iners and Escherichia coli can accurately separate patients with genitourinary pain syndromes from asymptomatic controls. In addition, these two microbial markers alone could subclassify unselected patients into bladder pain and non-urologic pelvic pain groups with diagnostically useful thresholds. As shown in the scatter plots (bottom), thresholds of absolute DNA quantities for each species in each specimen could clearly discriminate between symptomatic and asymptomatic subjects.

[14] Figure 7 shows the principle component analysis by Unifrac Distance. Bacterial communities in bladder pain (purple and asymptomatic controls (orange) are similar overall, differing primarily at the species level. Both of these groups, however, differed substantially from communities in patients with non-urologic pelvic pain (blue). This difference suggest pelvic pain may occur in the context of a local environmental shift that results in this bacterial divergence.

DESCRIPTION OF THE INVENTION

[15] All references cited herein are incorporated by reference in their entirety as though fully set forth. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology 3rd ed.*, *Revised*, J. Wiley & Sons (New York, NY 2006); March, *Advanced Organic Chemistry Reactions, Mechanisms and Structure 7th ed.*, J. Wiley & Sons (New York, NY 2013); and Sambrook and Russel, *Molecular Cloning: A Laboratory Manual 4th ed.*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY 2012), provide one skilled in the art with a general guide to many of the terms used in the present application.

[16] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

[17] As used herein the term "about" when used in connection with a referenced numeric indication means the referenced numeric indication plus or minus up to 5% of that referenced numeric indication, unless otherwise specifically provided for herein. For example, the language "about 50%" covers the range of 45% to 55%. In various embodiments, the term "about" when used in connection with a referenced numeric indication

can mean the referenced numeric indication plus or minus up to 4%, 3%, 2%, 1%, 0.5%, or 0.25% of that referenced numeric indication, *if* specifically provided for in the claims.

[18] Given the substantial diagnostic confusion surrounding pelvic pain, we examined urinary bacterial associations with specific symptom clusters, not diagnoses. The present invention seeks to objectively identify subsets of bladder and pelvic pain as well as provide guidance as to the best possible treatment modalities for individuals.

[19] Genitourinary bacteria appear to play a role in the development of chronic pain and genitourinary symptoms in female patients. The present invention reveals subtypes of bacterial colonization associated with different pain phenotypes and different responses to therapy. We describe the identification of clinically-useful bacterial biomarkers for specific pelvic and bladder pain phenotypes. Objective, rapid, and inexpensive testing to identify and classify reproductive-age women with bladder and pelvic pain would allow more accurate diagnosis and improve treatment decisions. The direct association of pathologic bacterial species concentration with severity of specific pain symptoms implicates a microbial role in the pathogenesis of genitourinary pain.

[20] Various embodiments of the present invention provide for a method of detecting levels of one or more microorganisms in a subject in need thereof, comprising: assaying a biological sample obtained from the subject, wherein the subject has one or more symptoms of interstitial cystitis, pelvic pain or bladder pain; and detecting the levels of one or more microorganisms in the biological sample.

[21] In various embodiments, the method comprises detecting the levels of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more microorganisms in the biological sample. In various embodiments, the method comprises detecting the levels of 10, 15, 20, 25, 30, 35, 40, 45 or 50 or more microorganisms in the biological sample.

[22] In various embodiments, the one or more microorganisms are *Lactobacillus* and *Enterobacteriaceae* bacteria. In various embodiments, the one or more microorganisms is *Lactobacillus iners* (*L. iners*).

[23] In various embodiments, the biological sample is urine. Additional examples of biological samples include but are not limited to body fluids, whole blood, plasma, serum, vaginal fluids or aspirate, stool, intestinal fluids or aspirate, and stomach fluids or aspirate, cerebral spinal fluid (CSF), sweat, saliva, cervical scraping, and mucous.

[24] In various embodiments, the method further comprises comparing each detected level of the one or more microorganisms to each microorganism's reference level.

[25] In some embodiments, the reference level can be established from biological samples from a healthy subject. For example, if the biological sample is urine, then the reference value can be obtained from the urine of a healthy subject. In other embodiments, the reference value is the average bacteria count for the same type of biological sample from a population of healthy subjects. In other embodiments, the reference value is the average plus one or two standard deviations of average methanogen count for the same type of biological

sample from a population of healthy subjects. In some embodiments, the population of healthy subjects can range from at least three healthy individuals to 25 healthy individuals, more than 50 healthy individuals, and even more than 100 healthy individuals. A healthy individual is an individual who does not have interstitial cystitis, pelvic pain or bladder pain, or who does not have any symptoms of interstitial cystitis, pelvic pain or bladder pain.

[26] Symptoms of interstitial cystitis include but are not limited to pain in the pelvis or between the vagina and anus in women, chronic pelvic pain, a persistent, urgent need to urinate, frequent urination, often of small amounts, throughout the day and night (e.g., up to 60 times a day), pain or discomfort while the bladder fills and relief after urinating, and pain during sexual intercourse. Symptoms of pelvic pain include but are not limited to pain or cramps before or during a menstrual period, pain during or after sex, pain during ovulation, painful bowel movements, rectal bleeding during a menstrual period, pain during urination, lower back pain, infertility, spotting between periods, and bloating in the abdomen.

[27] In various embodiments, the subject does not have an active urinary tract infection as defined by a negative bacterial culture.

[28] In various embodiments, the assay comprising using a technique selected from the group consisting of qPCR, RT-PCT, and next generation sequencing.

[29] A method for treating interstitial cystitis, pelvic pain or bladder pain, comprising: administering a local bladder therapy to a subject who has been detected to have a level of *Lactobacillus iners* (*L. iners*) higher than a reference level.

[30] A method for treating interstitial cystitis, pelvic pain or bladder pain in a subject, comprising: obtaining or requesting the results of an analysis of levels of one or more microorganisms the subject; and administering a local bladder therapy to a subject who has been detected to have a level of *Lactobacillus iners* (*L. iners*) higher than a reference level.

[31] A method of selecting a treatment for a subject having interstitial cystitis, pelvic pain or bladder pain, comprising detecting levels of one or more microorganisms in a biological sample obtained from a subject in need thereof, selecting local bladder treatment for subjects with levels of *Lactobacillus iners (L. iners)* that are higher than a reference level indicates a local bladder treatment for the subject, and selecting a non-local bladder treatment for subjects with levels of *Enterobacteriaceae* bacteria that are higher than a reference level indicates based on the fact that the subject will be refractory to local bladder therapies.

[32] In various embodiments, the biological sample is urine. Additional examples of biological samples are as provided herein.

[33] In various embodiments, the local bladder therapy is a bladder instillation.

[34] In various embodiments, the bladder instillation comprises instilling an agent selected from the group consisting of alkalinized lidocaine and heparin, dimethyl sulfoxide (DMSO), sodium hyaluronate, heparin, a bladder cocktail, and an experimental solution.

[35] In various embodiments, the bladder cocktail comprises a combination selected from the group consisting of bupivacaine, heparin, hydrocortiosone and sodium bicarbonate; bupivacaine, lidocaine jelly, triamcinolone, heparin, and gentamicin; lidocaine, sodium bicarbonate, heparin, and gentamicin; DMSO, heparin, triamcinolone, sodium bicarbonate, and gentamicin; heparin and bupivacaine; DMSO, hydrocortisone, bupivacaine, sodium bicarbonate, and optionally heparin.

[36] In various embodiments, the experimental solution is selected from the group consisting of PSD597, URG101, URACYST; LIPELLA liposomes, and misoprostol.

[37] PSD597 (PLETHORA SOLUTIONS) is a formulation of alkalinized lidocaine. It includes a delivery system and a proprietary formulation said to protect the active drug in the bladder and ensure that the drug remains in the optimal chemical form for transport across the bladder wall to its site of action. URG101 (URIGEN) is a formulation of alkalinized lidocaine and heparin for instillation. URACYST (STELLAR PHARMACEUTICALS) is a formulation of sterile sodium chondroitin sulfate solution (2.0%). LIPOSOMES FOR INSTILLATION (LIPELLA) (see e.g., Chuang YC et al. J Urol. 2009;182:1393-1400) is a treatment wherein liposomes are instilled once a week for four weeks. MISOPROSTOL (CYTOTEC) FOR INSTILLATION is a prostaglandin E1 analog.

[38] In various embodiments, the subject does not have an active urinary tract infection as defined by a negative bacterial culture.

[39] In various embodiments, *Lactobacillus iners* (*L. iners*) is detected by using a technique selected from the group consisting of qPCR, RT-PCT, and next generation sequencing.

EXAMPLES

[40] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

Example 1

[41] **Methods:** Catheterized urinary samples were obtained from 78 pre-menopausal women (age 18-45) with a variety of urinary complaints, including bladder and pelvic pain. 16S next-generation sequencing (NGS) was used to characterize urinary microbial populations; validated questionnaires (female GenitoUrinary Pain Index, OAB questionnaire, O'Leary-Sant Indices) were used to quantify symptom type and severity. *K* means unsupervised clustering analysis of NGS data was used to assign subjects to urotypes, based on the urinary bacterial community state types. Quantitative PCR (qPCR) served to confirm the NGS results and

provide objective concentrations for taxa of interest. Linear regression analysis confirmed the associations of bacterial concentrations and specific symptoms.

[42] **Results:** In an exploratory population of 35 reproductive-age women with a variety of complaints, 16S NGS revealed four urotypes that strongly correlated with symptomatology. Isolated urgency incontinence was rare; the majority of subjects with symptoms complained of genitourinary pain. Bladder-specific pain (worse with filling, relieved by voiding) was strongly associated with *Lactobacillus iners,* a *Lactobacillus* spp. that does not produce lactic acid. Asymptomatic patients almost universally had a non-*iners*, *Lactobacillus*-predominant microbiota. Vaginal and urethral pain unrelated to voiding was positively correlated with increasing Enterobacteriaceae, confirmed on qPCR to be primarily *Escherichia coli.* Detection of these two pathobiont species by qPCR in a second validation population (n=43) was highly predictive of each phenotype ($P<0.00001$).

[43] Retrospective chart review indicated that only the *L. iners*-associated population responded to local bladder therapies, such as bladder instillations. Objective, rapid, and scalable testing to classify bladder and pelvic pain appropriately would allow more accurate diagnosis and improve treatment. The rising pain levels seen in association with increasing pathogenic bacterial abundance suggests a possible role for these taxa in pain physiology.

[44] Various embodiments of the invention are described above in the Detailed Description. While these descriptions directly describe the above embodiments, it is understood that those skilled in the art may conceive modifications and/or variations to the specific embodiments shown and described herein. Any such modifications or variations that fall within the purview of this description are intended to be included therein as well. Unless specifically noted, it is the intention of the inventors that the words and phrases in the specification and claims be given the ordinary and accustomed meanings to those of ordinary skill in the applicable art(s).

[45] The foregoing description of various embodiments of the invention known to the applicant at this time of filing the application has been presented and is intended for the purposes of illustration and description. The present description is not intended to be exhaustive nor limit the invention to the precise form disclosed and many modifications and variations are possible in the light of the above teachings. The embodiments described serve to explain the principles of the invention and its practical application and to enable others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. Therefore, it is intended that the invention not be limited to the particular embodiments disclosed for carrying out the invention.

[46] While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that, based upon the teachings herein, changes and modifications may be made without departing from this invention and its broader aspects and, therefore, the appended claims are to encompass within their scope all such changes and modifications as are within the true spirit and scope of this

invention. It will be understood by those within the art that, in general, terms used herein are generally intended as “open” terms (*e.g.*, the term “including” should be interpreted as “including but not limited to,” the term “having” should be interpreted as “having at least,” the term “includes” should be interpreted as “includes but is not limited to,” etc.).

[47] As used herein the term “comprising” or “comprises” is used in reference to compositions, methods, and respective component(s) thereof, that are useful to an embodiment, yet open to the inclusion of unspecified elements, whether useful or not. Although the open-ended term “comprising,” as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as “consisting of” or “consisting essentially of.”

WHAT IS CLAIMED IS:

1. A method of detecting levels of one or more microorganisms in a subject in need thereof, comprising:

assaying a biological sample obtained from the subject, wherein the subject has one or more symptoms of interstitial cystitis, pelvic pain or bladder pain; and

detecting the levels of one or more microorganisms in the biological sample.

2. The method of claim 1, wherein the one or more microorganisms is *Lactobacillus* and *Enterobacteriaceae* bacteria.

3. The method of claim 1, wherein the one or more microorganisms is *Lactobacillus iners* (*L. iners*).

4. The method of claim 1, wherein the biological sample is urine.

5. The method of claim 1, further comprising comparing each detected level of the one or more microorganisms to each microorganism's reference level.

6. The method of claim 1, wherein the subject does not have an active urinary tract infection as defined by a negative bacterial culture.

7. The method of claim 1, wherein the assay comprising using a technique selected from the group consisting of qPCR, RT-PCT, and next generation sequencing.

8. A method for treating interstitial cystitis, pelvic pain or bladder pain, comprising:

administering a local bladder therapy to a subject who has been detected to have a level of *Lactobacillus iners* (*L. iners*) higher than a reference level.

9. A method for treating interstitial cystitis, pelvic pain or bladder pain in a subject, comprising:

obtaining or requesting the results of an analysis of levels of one or more microorganisms the subject; and

administering a local bladder therapy to a subject who has been detected to have a level of *Lactobacillus iners* (*L. iners*) higher than a reference level.

10. A method of selecting a treatment for a subject having interstitial cystitis, pelvic pain or bladder pain, comprising

detecting levels of one or more microorganisms in a biological sample obtained from a subject in need thereof,

selecting local bladder treatment for subjects with levels of *Lactobacillus iners (L. iners)* that are higher than a reference level indicates a local bladder treatment for the subject, and

selecting a non-local bladder treatment for subjects with levels of *Enterobacteriaceae* bacteria that are higher than a reference level indicates based on the fact that the subject will be refractory to local bladder therapies.

11. The method of any one of claims 8-9, wherein the biological sample is urine.

12. The method of any one of claims 8-9, wherein the local bladder therapy is a bladder instillation.

13. The method of claim 12, wherein the bladder instillation comprises instilling an agent selected from the group consisting of alkalinized lidocaine and heparin, dimethyl sulfoxide (DMSO), sodium hyaluronate, heparin, a bladder cocktail, and an experimental solution.

14. The method of claim 13, wherein the bladder cocktail comprises a combination selected from the group consisting of bupivacaine, heparin, hydrocortiosone and sodium bicarbonate; bupivacaine, lidocaine jelly, triamcinolone, heparin, and gentamicin; lidocaine, sodium bicarbonate, heparin, and gentamicin; DMSO, heparin, triamcinolone, sodium bicarbonate, and gentamicin; heparin and bupivacaine; DMSO, hydrocortisone, bupivacaine, sodium bicarbonate, and optionally heparin.

15. The method of claim 13, wherein the experimental solution is selected from the group consisting of PSD597, URG101, URACYST; LIPELLA liposomes, and misoprostol.

16. The method of any one of claims 8-9, wherein the subject does not have an active urinary tract infection as defined by a negative bacterial culture.

17. The method of any one of claims 8-9, wherein *Lactobacillus iners* (*L. iners*) is detected by using a technique selected from the group consisting of qPCR, RT-PCT, and next generation sequencing.

ABSTRACT

The invention describes methods for selecting treatment and treating interstitial cystitis, pelvic pain and bladder pain.

FIG. 1

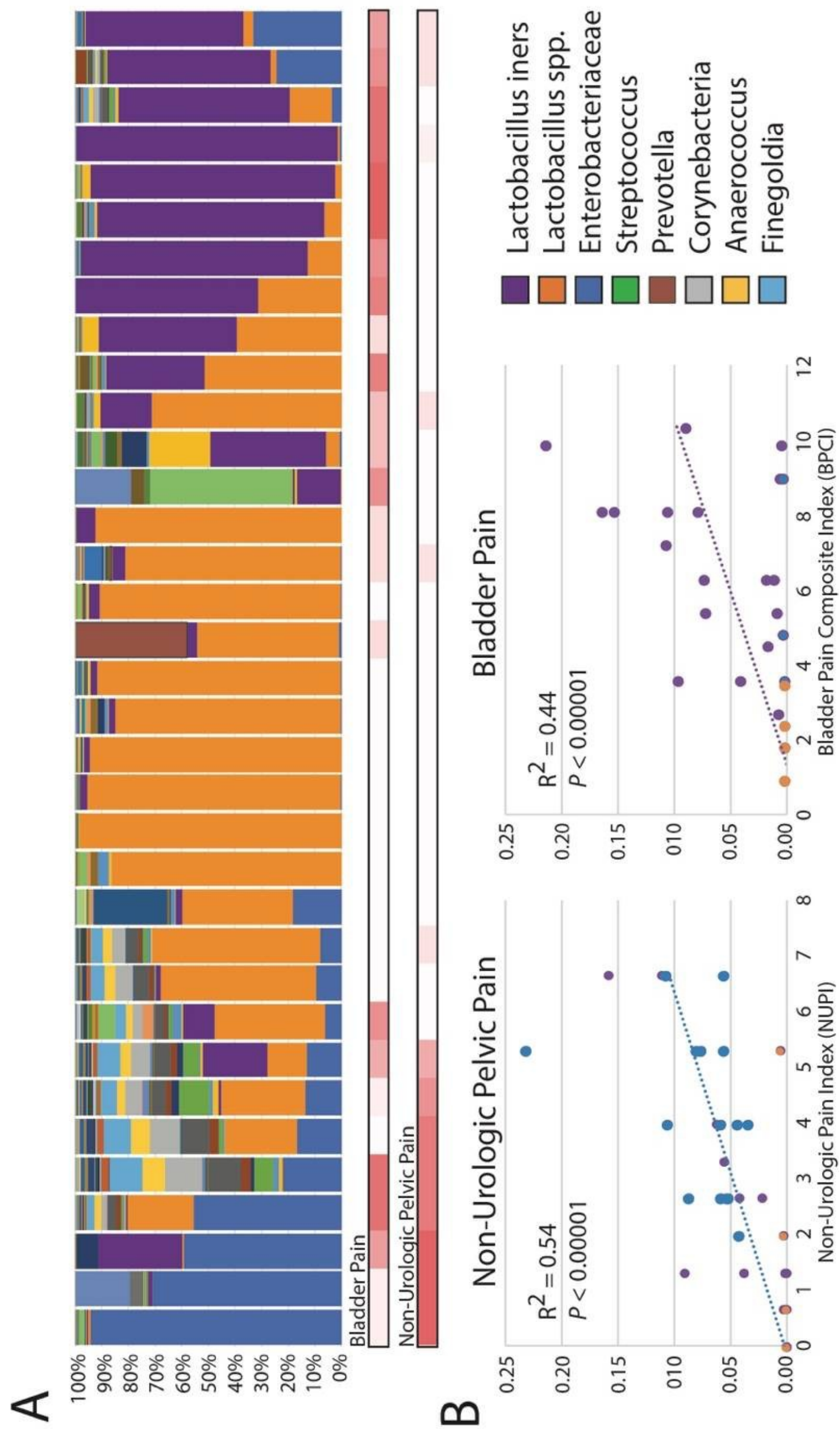

FIG.

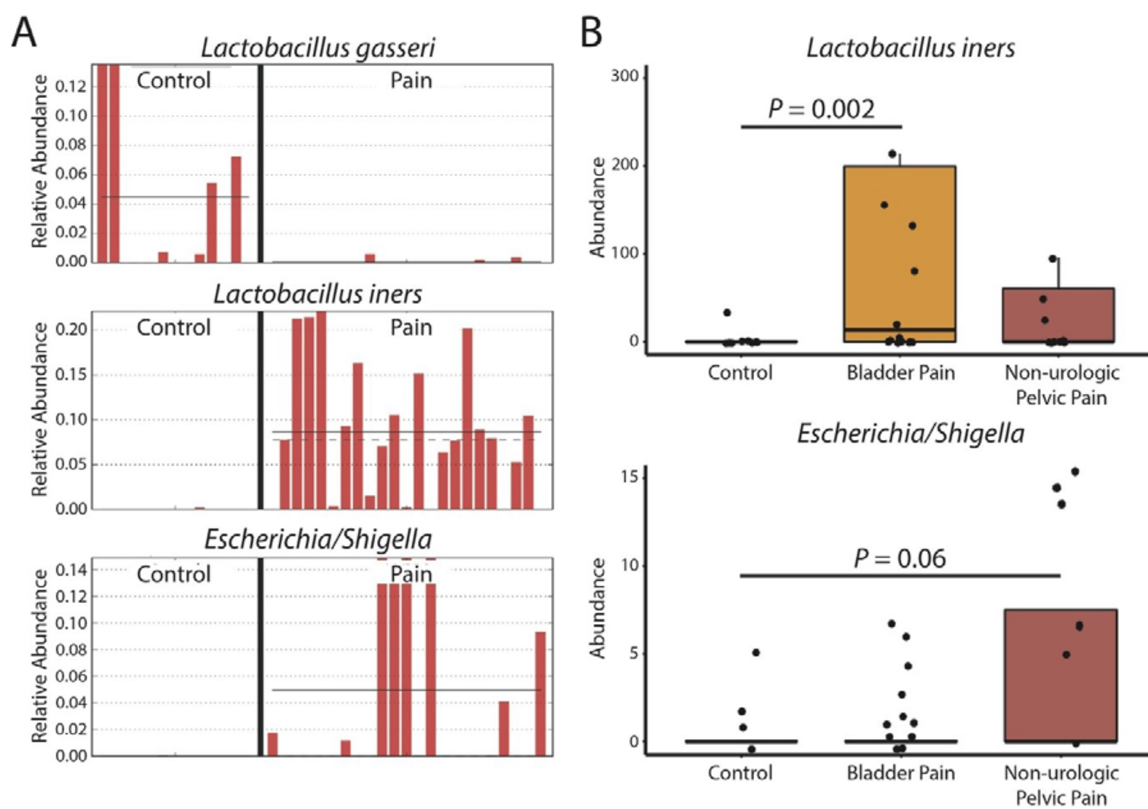

FIG.3

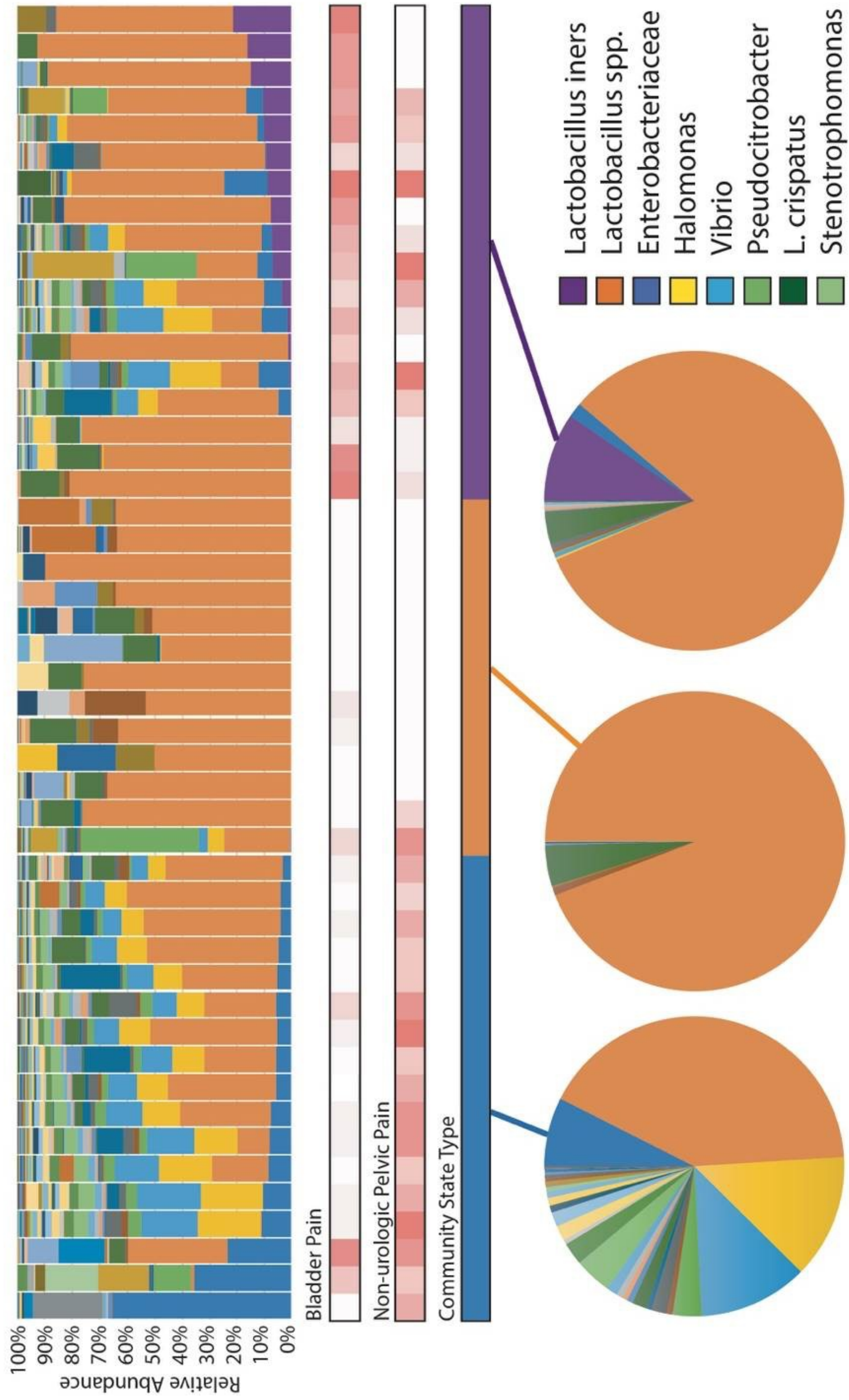

FIG. 4

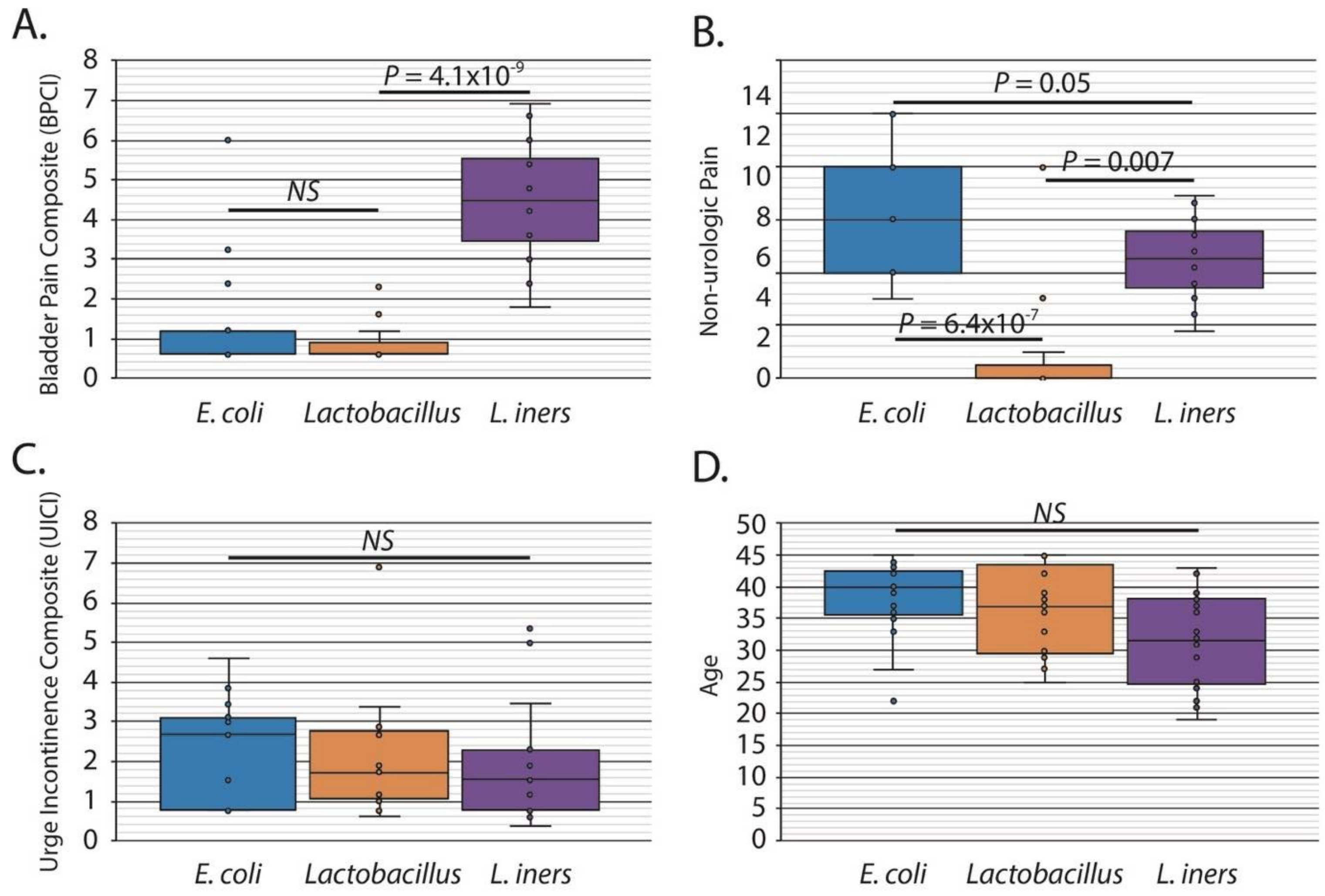

FIG. 6

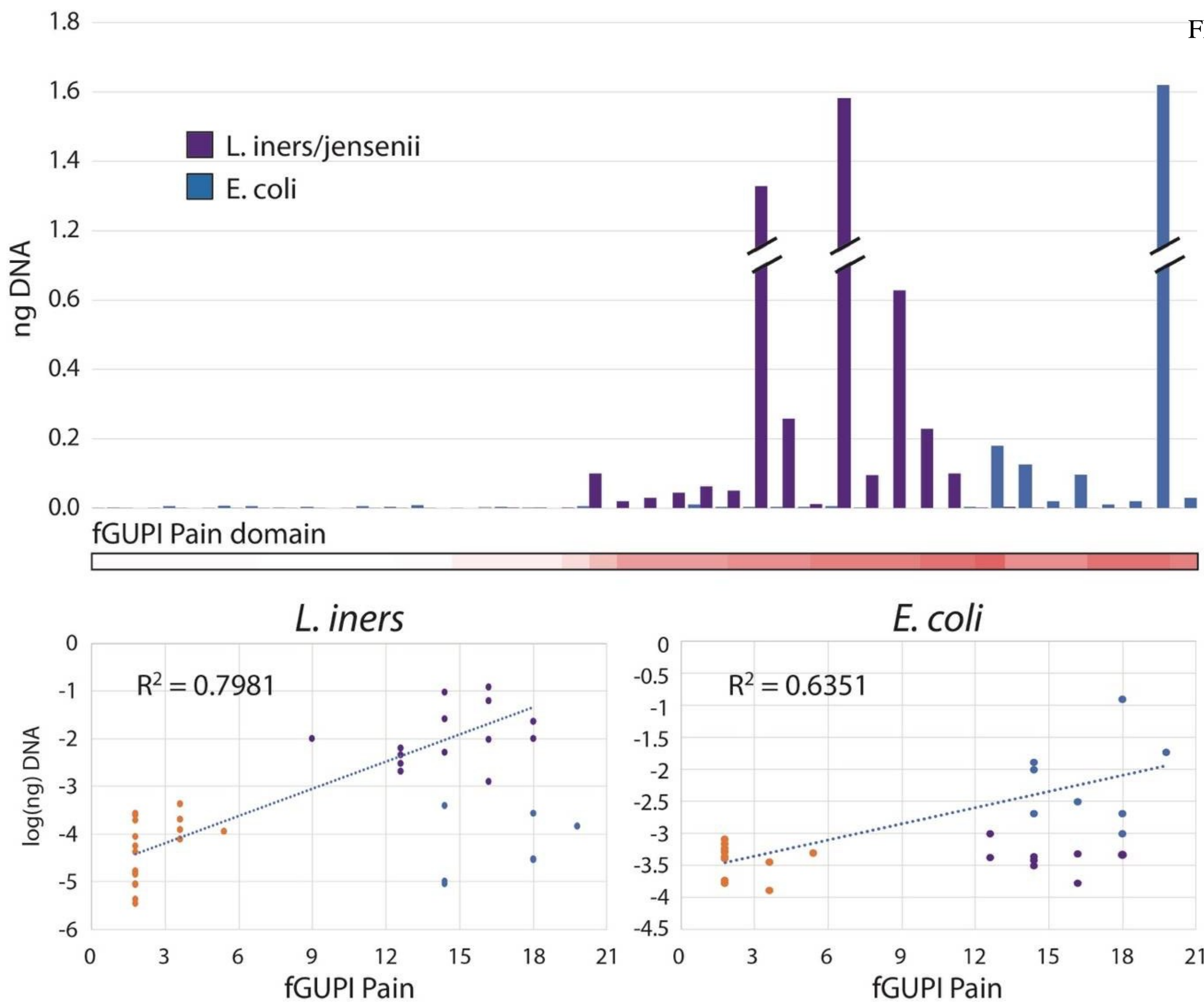

FIG.