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A Genome-Scale DNA Methylation Study in Women with Interstitial Cystitis/Bladder Pain Syndrome

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Abstract

Aims—To assess the feasibility of using voided urine samples to perform a DNA methylation study in females with interstitial cystitis/bladder pain syndrome (IC/BPS) as compared to age- and race-matched controls. A unique methylation profile could lead to a non-invasive, reproducible, and objective biomarker that would aid clinicians in the diagnosis of IC/BPS.

Methods—Nineteen IC/BPS patients and 17 controls were included. IC/BPS patients had an Interstitial Cystitis Symptom Index score of >8; controls had no bladder symptoms. DNA was extracted from pelleted urine sediment. Samples with >500 ng of genomic DNA underwent quantitative DNA methylation assessment using the Illumina Infinium MethylationEPIC BeadChip. Age- and race- matching was applied prior to analysis. Linear regression models were used to compare average methylation between IC/BPS cases and controls at each cytosine guanine dinucleotide site (loci where methylation can occur).

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Results—16 participants (8 IC/BPS age- and race-matched to 8 controls) had adequate DNA for methylation analysis. The median age was 43.5 years (interquartile range 33.8, 65.0), the median BMI was 27.1 (IQR 22.7, 31.4), and 14 were Caucasian (87.5%). A total of 688,417 CpG sites were analyzed. In exploratory pathway analysis utilizing the top 1000 differentially methylated CpG sites, the mitogen-activated protein kinase (MAPK) pathway was overrepresented by member genes.

Conclusions—The results demonstrate the feasibility of using voided urine specimens from women with IC/BPS to perform DNA methylation assessments. Additionally, the data suggest genes within or downstream of the MAPK pathway exhibit altered methylation in IC/BPS.

Keywords

Urine DNA; Urine epigenomics

Introduction

Interstitial cystitis/painful bladder syndrome (IC/BPS) is a debilitating disease that presents with a constellation of symptoms including pain, urinary urgency, frequency, nocturia, and small voided volumes in the absence of other identifiable etiologies.¹ Overall prevalence of IC/BPS is between 2.7% to 6.5% (approximately 3.3 to 7.9 million US women),² and this disorder results in substantial costs³ and impairments in health-related quality of life.⁴ Unfortunately, there is a lack of consensus on the pathophysiology and etiology of this disorder.

The pathogenesis of IC/PBS is likely multifactorial, with current proposed etiologies including urothelial cell dysfunction, immunologic abnormalities, mast cell involvement, neurogenic causes and inhibition of urothelial cell growth by antiproliferative factor (APF).⁵ While several hypotheses exist, the mechanisms of the genesis, progression or spontaneous resolution of this disorder are unknown. Thus, therapies are often empiric, with limited evidence and variable levels of improvement. Unless the pathophysiology of IC/BPS is better understood, treatments for this disorder will remain empiric.⁶

Epigenetic signatures are emerging as potentially powerful diagnostic and investigative tools to elucidate the pathogenesis of disease. DNA methylation constitutes a well-understood epigenetic mechanism that contributes to regulation of gene expression. It involves the covalent addition of a methyl group (-CH₃) to the 5' carbon of a cytosine moiety, generating 5-methylcytosine (5-mC), which occurs predominantly in the context of cytosines that precede guanine (5'-CpG-3') dinucleotides, or CpGs.⁷ DNA methylation has already been well correlated to the severity of inflammation in various chronic disease states such as inflammatory bowel disease.⁸ It is now widely accepted that epigenetic alterations in target tissues can be causal to the development of malignancy, including bladder cancer.⁹ Importantly, in this era of precision medicine, clinicians have looked to epigenetics to further characterize disease states such as colon cancer, and there are already commercially available methylation panels for colon cancer screening.¹⁰

It is possible that epigenetics may also be involved in the chronic symptoms of IC/BPS¹¹ as environmental and bacterial exposures can cause tissue-specific changes in DNA methylation. Prior exposure to a bacterial pathogen could result in altered methylation at a variety of regulatory genes, with long-term changes in gene expression. These epigenetic changes could explain why chronic symptoms, similar to those of bacterial cystitis, persist for years in women with IC/BPS.¹² In support of this assertion, bladder tissue methylation changes have been demonstrated in a chronic cystitis mouse model.¹³ This is a particularly attractive premise because it connects seemingly disparate theories into a single unifying and plausible hypothesis, that altered DNA methylation, detectable in urine sediment, results in changes in gene expression that lead to inflammation and pain in IC/BPS patients as compared to controls. However, the feasibility of epigenetic assessment of urine sediment from women with benign lower urinary tract disorders has not yet been established.

The objective of the study was to assess the feasibility of using voided urine samples to perform a DNA methylation assessment study in female patients with interstitial cystitis/bladder pain syndrome (IC/BPS) as compared to age- and race-matched controls. A unique methylation profile in IC/BPS patients could lead to a non-invasive, reproducible, objective biomarker that would aid clinicians in the diagnosis of this difficult disorder and provide insight into the molecular etiology of this disease.

Materials and Methods

Study population

This study was approved by the Duke University Institutional Review Board (Pro00058689). After informed consent was obtained, patients with IC/BPS and control participants without bladder symptoms completed study information and provided a one-time urine sample. Recruitment occurred between May 2015 and December 2015. All IC/BPS were symptomatic at the time of urine collection and met the AUA clinical guidelines definition of IC/BPS¹⁴ by answering yes to the question “Have you experienced at least moderate burning in your bladder for the last 6 weeks?” In addition, IC/BPS patients were required to score >8 on the Interstitial Cystitis Symptom Index (ICSI),¹⁵ though they were allowed to be on oral therapy for their bladder symptoms. IC/BPS patients also completed a 3-day voiding diary to confirm the presence of urinary symptoms. Control participants were required to score 0 on the ICSI and report “no” on the AUA clinical question as above. All study participants completed the female genitourinary pain index (GUPI).¹⁶ Exclusion criteria included culture proven or treated urinary tract infection in the last 6 weeks, cystoscopy or intravesical therapy in the last 4 weeks, a history of chemotherapeutic treatment of cancer or pelvic radiation, history of augmentation cystoplasty or cystectomy, hematuria that had not been fully evaluated, and pregnancy. Participants were required to have a voided urine volume of at least 50 mL. Urine cultures were sent on all study candidates and participants were excluded if they had a positive result.

Urine collection and DNA extraction

A random voided midstream clean catch urine sample was collected from all study participants. The urine was refrigerated, aliquoted into 50 ml sterile conical tubes with 2.5

mL of AssayAssure® (Sierra Molecular, Incline Village, NV), and transported to the laboratory with icepacks. Times of collection and transportation to the processing laboratory were recorded. The urine was centrifuged at 2000xg for 10 min at 4°C, the supernatant was removed and the pellet was washed with 5 mL PBS. This centrifugation step was repeated and the supernatant was discarded. The pellet was resuspended in 200 µl of PBS. DNA was extracted using the QIAmp DNA Blood Minikit (Qiagen, Germantown, MD). Extracted DNA was stored at -80°C. The DNA yield was estimated using a Nanodrop2000 Spectrophotometer (Thermo Scientific, Waltham, MA).

DNA methylation Analysis

IC/BPS patients and controls were matched for age (± 6 years) and race. DNA concentration was verified with the Quant-iT PicoGreen dsDNA kit (Thermo Scientific, Waltham, MA). A total of 500 ng of DNA was bisulfite converted and hybridized to the Illumina Infinium MethylationEPIC Beadchip (Illumina, San Diego, CA) which simultaneously profiles the methylation status for >850,000 CpG sites at single-nucleotide resolution. Methylation beadchips were processed at the Molecular Genomics Core at the Duke Molecular Physiology Institute (Durham, NC), following standard protocols.

Statistical analysis

Data preprocessing—BeadStudio (Illumina, San Diego, CA) was used to calculate the raw methylation level at each CpG locus. Raw methylation levels were expressed as beta-values [β = intensity of the methylated allele (M)/(100 + intensity of the unmethylated allele (U) + intensity of the methylated allele (M)], which represent approximate percent methylation at each CpG locus. DNA methylation (β) values were logit [i.e., $\log(\frac{\beta}{1-\beta})$] transformed to M values as in previous studies¹⁷, since M values increase true positive rates of statistical comparisons. CpG sites with poorly detected probes in >10% of samples were dropped entirely. Probes were excluded if they contained known SNPs, repeat regions, INDELs, and if they map to multiple genomic regions.¹⁸ This left 688,417 CpG sites for analysis. Subset quantile within-array normalization (SWAN) was used to normalize between the two probe types used by Illumina and adjusted for plate effects by applying the ComBat method.¹⁹

Comparative statistics—A linear regression model, controlling for age, was conducted between the matched cases and controls for each of the remaining 688,417 CpG sites. Demographic and baseline variables of the groups were analyzed with Mann-Whitney U for continuous variables, and Fisher's exact test for categorical variables. All subsequent statistical analyses were conducted within the R statistical computing environment (www.r-project.org, version 3.2.3), utilizing the minfi Bioconductor package and IBM SPSS Statistics version 22.0. Bonferroni correction was applied to account for multiple comparisons, adjusting the level of significance from 0.05 to 7.26×10^{-8} . Additional adjustment for principal components to address potential population structure²⁰ was explored but not retained due to the subjects already being race-matched.

Gene analysis—The DNA methylation measurements of CpG sites were linked to chromosomal position based on NCBI human reference genome hg19 data (<https://www.ncbi.nlm.nih.gov/genome/guide/human/>) and analyzed via the University of Santa Cruz Genome Browser (<http://genome.ucsc.edu/>). The list of genes with differentially methylated CpG sites from pairwise comparisons (between IC/BPS and controls) was imported into DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>) for KEGG pathway mapping. KEGG is an encyclopedia of genes and genomes. The primary objective of the KEGG database project is to assign functional meanings to genes and genomes, both at the molecular and higher levels. Functional annotation clustering is then performed by the software to delineate which biologic pathways have multiple represented sites. (Table S1)

Results

A total of 30 patients with IC/BPS and 24 asymptomatic controls were assessed for eligibility. Details of participant enrollment are presented in Figure 1. Eleven cases and 7 controls were excluded prior to urine DNA processing due to ICSI score <8 for cases (5) and >0 for controls (3), positive urine culture (5 cases; 3 controls) or no urine culture (1 case; 1 control). Of the included 19 IC/BPS cases and 17 controls, 6/19 (31.5%) cases and 7/17 (41.2%) of controls had <500 ng of DNA after extraction. Among the remaining 13 IC/BPS cases and 10 controls, we performed rigorous age-matching within 6 years and the final cohort consisted of 8 cases and 8 age- and race-matched controls.

Of the 16 participants included for analyses, the median age was 43 (IQR 33.8, 65) with a median BMI of 27.1 (IQR 22.7, 31.4). Most patients were white (14/16, 87.5%). Demographic data are presented in Table 1. As expected due to age-matching, there was no difference in median age (in years) between subjects with IC/BPS as compared to controls (43.5 v. 40.0, $p=0.91$). There was also no difference in median BMI (IC/BPS 30.9 v. Controls 25.5; $p=0.13$). Notably, there were more past and current smokers in the IC/BPS group (IC/BPS 87.5% v. controls 25%; $p=0.04$). Of the IC/BPS subjects, 4/8 (50%) had cystoscopy performed as part of their clinical care with histology findings presented in Table 1. Participants were on a variety of medications at time of enrollment (Table 1). Most IC/BPS patients (5/8, 62.5%) had been prescribed antibiotics for presumed UTI in the past, but all antibiotics were taken at least 6 weeks prior to urine collection as per exclusion criteria.

After testing for significant differential methylation at each CpG site, the 688,417 obtained p-values were then corrected for multiple testing. Using the conservative Bonferroni correction, no genome-scale statistically significant differences ($p<7.26\times10^{-8}$) between cases and asymptomatic controls were observed (Figure 2). Likewise, no significant differences were found after adjusting the p-values for false discovery rate (FDR). These results were not unexpected due to the small sample size of the study. We therefore turned to the CpG sites most significantly differentially methylated from regression models, to investigate if any might be linked through specific gene pathways. Such findings could provide biological insight to the methylation differences and furthermore help alleviate concern that significant sites were identified by chance.

After regression modeling, the list of genes with the top 1000 differentially methylated CpG sites from pairwise comparisons was imported into the DAVID Bioinformatics Resources for KEGG pathway mapping. This ultimately encompassed sites with a p-value of <0.002 . (Supplementary Material, Table S1) The pathway most prominently enriched for genes with differentially methylated CpG sites in the dataset was the mitogen-activated protein kinase pathway (MAPK pathway, $p=2.2 \times 10^{-3}$). This pathway contained 22 differentially methylated sites, and 19/22 (86.3%) of the sites were hypomethylated in the IC/BPS patients as compared to controls (Table 2). As the location of the differentially methylated CpG site is important in terms of the effect on transcriptional regulation, Table 2 also denotes the CpG sites' association with either a promoter region, a CpG Island, a CpG shore (<2 kb flanking CpG Islands) and/or a CpG shelf (<2 kb flanking outwards from a CpG shore).

In addition to finding specific pathways significantly enriched for our 'top hit' CpGs, having more than one significant CpG site in the same gene also decreases the likelihood of false discovery.²¹ In this regard there were 28 genes with two or more significant differentially methylated CpG sites. One of these genes is a member of the MAPK pathway: MDS1 and EVI1 complex locus (MECOM). The 3-methylcrotonoyl-CoA carboxylase MCCC1 gene also had 5 differentially methylated CpG sites all of which are promoter-associated.

Discussion

The goal of this study was to demonstrate the feasibility of genome-scale methylation profiling in women with benign lower urinary tract disorders and particularly to assess the utility of this technique in women with IC/BPS. Overall, the study results support feasibility of this approach. Although differences in none of the individual CpG sites met a genome-scale level of significance, the KEGG pathway analysis identified 22 MAPK pathway genes with differentially methylated CpG sites between IC/BPS cases and controls, many of which were associated with a promoter or near a CpG Island.

To our knowledge, this is the first study to evaluate the methylation profiles of DNA from voided urine in patients with IC/BPS. Study participants with IC/BPS were included based on accepted clinical criteria with symptoms determined with validated questionnaires,^{15,22,23} while the controls were confirmed to be asymptomatic using validated criteria. The study also had a rigorous adherence to age- and race-matching and applied a linear regression model to the data to decrease potential confounding from these variables.

The goals of any pilot study are to test logistics and gather information prior to a larger study, to improve quality and efficiency. Although the study is limited by the small sample size, several important lessons were identified that will aid in future larger scale studies (Table 3). Despite utilization of the DNA stabilizing agent AssayAssure® and DNA extraction within one day of initial urine collection, 13/36 (36%) samples had a DNA yield of <500 ng. As patients were only required to void at least 50 mL for utilization of their specimen, we believe this low DNA yield was due to the urine volume and the relative paucity of DNA in the urine (a low biomass substance). In future studies, a higher minimum voided volume of at least 100 mL would be recommended. As the average voided volume of

women with IC/BPS is often low, collection of the first morning void would be considered prudent to obtain this minimum value. Considering the study findings, over-recruitment in future studies of at least 20% to anticipate for insufficient DNA yield should also be considered.

Recent simulation-based studies utilizing the Illumina 450k BeadChip array recommend a sample size of at least 100 case-control matched pairs in order to detect a mean methylation difference of 10% at genome-wide significance ($p=1\times 10^{-6}$) with 80% power.²⁴ Even larger sample sizes are necessary to account for the additional multiple-hypothesis testing that comes with the broader coverage of the MethylationEPIC Beadchip. As this study is the first methylation assessment study in women with IC/BPS, the results of the average methylation differences and standard deviations will help estimate sample sizes for appropriately powered future studies. Given the study's sample size, the genome-scale significance level of 7×10^{-8} required to adjust for multiple testing with the EPIC platform, and average standard deviation for mean β per site of 0.014, we had 80% power to detect an 8% difference in methylation. Given these same parameters, a sample size of 160 per group would be necessary to detect a 1% significantly different level of methylation in a genome-scale study.

Importantly, small changes in methylation can have a strong effect on transcriptional activity with methylation changes of even 1% leading to doubling of transcription.²⁵ Gene expression data to determine the functional impact of changes in DNA methylation on gene expression in the bladder will be very helpful to corroborate any study findings.

In this study, voided urine samples were used as opposed to bladder biopsies. Although this was an important preliminary step in the search for candidate markers, future studies may consider the use of bladder biopsies to better characterize the differences in epigenetic signatures of the intact and sloughed urothelium. Very importantly, the study cohort had more smokers in the IC/BPS group as compared to controls. This was an unanticipated finding. Smoking has been associated with bladder pain in some cross-sectional studies,²⁶ and it is a known environmental factor that can have long-lasting effects on DNA methylation,²⁷ and therefore should be controlled for (along with age and race) in future studies.

Multiple studies have investigated differential expression of genes in the urine sediment and bladder biopsies from patients with IC/BPS.^{5,28} Sensory, inflammatory and apoptotic pathways have all been suggested to play a role in this heterogeneous condition. Exploratory pathway analysis showed multiple sites in the MAPK pathway that exhibited decreased methylation in participants with IC/BPS compared to controls and a few genes in this pathway have more than one differentially methylated CpG site. Although the mechanistic relationship between the MAPK signaling pathway and the pathophysiology of IC/BPS remains to be defined, increased gene expression in this pathway has been reported in previous studies.²⁸ In general, p38 MAP kinase is associated with inhibition of cell growth, inflammation, and with mediating apoptosis in different cell types. Increases in p38 MAP kinase activity have been demonstrated in IC/BPS immortalized urothelial cells,²⁹ and

increased phosphorylation of p38 MAP kinase has been found in the bladder of IC/BPS patients when compared to controls.³⁰

We did not see a difference in any inflammatory pathways between cases and controls as have been demonstrated in other studies amongst patients with urothelial Hunner's ulcers.¹⁵ As we did not use NIDDK inclusion criteria our case patients did not necessarily have urothelial ulcers. It is well known at this point that in a non-ulcerative population there are not the same levels of inflammatory modulators⁷ and thus there may be differences in pathogenesis of an ulcerative and non-ulcerative population.

Although speculative, hypomethylation of CpG sites could lead to increased expression of genes in the MAPK pathway in patients with IC/BPS. This may translate to inhibition of normal urothelial growth and turn-over, therefore explaining long-term symptoms. Future studies would need to not only reproduce the study's findings on a larger scale, but also include methylation-transcription studies using catheterized specimens or biopsied tissue to determine whether the changes in methylation are functional.

Conclusions

We demonstrated the feasibility of DNA methylation profiling from voided urine specimens in women with benign lower urinary tract disorders, such as IC/BPS, and identified several important operational details for future studies. The MAPK pathway had multiple gene members with differentially methylated CpG sites in cases relative to controls, suggesting this pathway may be deregulated by methylation changes in IC/BPS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Study Flow diagram

* Poor DNA yield was defined as <500ng estimated DNA per Nanodrop2000 Spectrophotometer with PicoGreen verification

Figure 2.

Manhattan plot showing genome scale association between IC/BPS cases and controls. The y axis shows the $-\log_{10} P$ values of 688,417 CpG sites, and the x axis shows their chromosomal positions with chromosomes separated by color.

Table 1

Demographics, Historical Therapies and Cystoscopy Findings of Study Cohort

	IC/BPS Cases (n=8)	Controls (n=8)	P-value*
Age (years)	43.5 (35,62)	40.0 (34,61)	0.91
BMI (kg/m ²)	30.9 (26,36)	25.5 (23,27)	0.07
Race (White)	7/8 (87.5)	7/8 (87.5)	0.95
Smoker			0.04
Current	3/8 (37.5)	1/8 (12.5)	
Past	4/8 (40)	1/8 (12.5)	
ICSI Score [†]	14 (12.75,15)	0	
Question 1 (urgency)	4 (1,4,25)	0	
Question 2 (frequency)	4 (4,5)	0	
Question 3 (nocturia)	3 (2,3,25)	0	
Question 4 (bladder pain)	4 (2,75, 4)	0	
GUPI Score	31.5 (30,35.5)	0	
Baseline Bladder Diary		N/A	
Time between voids (hour:min)	1:45 (1:28,2:01)		
Voided volume/void (mL)	150 (100,250)		
Details of Previous Therapy and Evaluation of IC/BPS patients (n=8)			
Previous Therapies			
Elmiron	2/8 (25%)		
Amitriptyline	2/8 (25%)		
Antibiotics (greater than 6 weeks prior to inclusion)	5/8 (62.5%)		
Botox Injection	1/8 (12.5%)		
Cystoscopic Hydrodistension	2/8 (25%)		
Intravesical Instillations	1/8 (12.5%)		
Cystoscopy Findings			
Normal	2/4 (50%)		
Glomerulations	1/4 (25%)		
Ulcerations	1/4 (25%)		
Bladder biopsy			
Benign	3/4 (75%)		
Inflammation with histiocytes	1/4 (25%)		

BMI, Body Mass Index; ICSI, O'Leary Sant Interstitial Cystitis Index; GUPI, Female Genito-urinary Pain Index

Data are presented as median (25%,75%) or n/N (%)

* Analyses performed by Mann-Whitney U for continuous variables and Fischer's exact test for categorical variables

[†] ICSI score range 0–20

GUPI score range 0–45

Table 2
Differentially methylated CpG sites in the Mitogen Activated Protein Kinase (MAPK) Pathway

Gene Name	Chr. #	Average β -Cases	Average β -Controls	Difference β	P-value	Probe ID	CpG Site Position
CPG SITES HYPOMETHYLATED IN CASES							
NR4A1	12	0.0233	0.0331	0.010	3.19E-05	cg07544244	CpG Island
TAOK3	12	0.0672	0.0813	0.014	5.24E-05	cg00545573	
DUSP7	3	0.054	0.0722	0.018	1.06E-04	cg02789309	Promoter
CACNA1C	12	0.5437	0.607	0.063	3.27E-04	cg15740346	
ATF2	2	0.5509	0.7153	0.164	7.94E-04	cg07253311	
MECOM	3	0.0526	0.0789	0.026	8.08E-04	cg13117777	
STK3	8	0.1654	0.7127	0.547	8.16E-04	cg19268168	Promoter
CACNA1H	16	0.8903	0.905	0.015	9.31E-04	cg27000853	CpG Shelf*
FGF9	13	0.05078	0.06723	0.016	1.38E-03	cg10910055	Promoter, CpG Island
MAPK13	6	0.07711	0.08954	0.012	1.53E-03	cg07812763	
RPS6KA2	6	0.92436	0.94628	0.022	1.78E-03	cg05521717	CpG Island
RPS6KA5	14	0.91468	0.93254	0.018	1.97E-03	cg16023770	CpG Shore*
FGF8	10	0.06492	0.09781	0.033	2.22E-03	cg26141857	
NLK	17	0.0298	0.0602	0.030	2.24E-03	cg18046570	Promoter, CpG Shore*
MAPK7	17	0.07563	0.09585	0.020	2.31E-03	cg13602666	CpG Island
GNAI2	7	0.1277	0.1612	0.034	2.35E-03	cg15302705	CpG Island
TAB1	22	0.91902	0.92720	0.008	2.55E-03	cg23265294	
MAP3K3	17	0.93159	0.94708	0.015	2.67E-03	cg16593069	
MAPK8IP3	16	0.85971	0.88377	0.024	2.70E-03	cg06286401	CpG Shore*
CPG SITES HYPERMETHYLATED IN CASES							
PPP3CA	4	0.96440	0.95766	0.007	9.08E-04	cg18872881	
CACNB2	10	0.95859	0.94002	0.019	2.25E-03	cg22892760	Promoter
CACNA2D4	12	0.24740	0.19007	0.057	2.54E-03	cg21746969	

Raw methylation levels were expressed as beta-values [β = intensity of the methylated allele (M)/(100 + intensity of the unmethylated allele (U) + intensity of the methylated allele (M)], which represent approximate percent methylation at each CpG locus. A β -value of 0 corresponds to a completely unmethylated CpG site whereas a β -value of 1 represents complete methylation.

* CpG shore = < 2kb flanking CpG Islands, CpG shelf = <2kb flanking outwards from a CpG shore

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Table 3**Recommendations for Voided Urine Epigenetics Studies: Benign Lower Urinary Tract Disorders**

1. Optimization of urine DNA yield	
a.	Urine sample collection of 100 mL
b.	Consider first morning void
c.	Over-recruitment by ~20%
2. Sample size calculation	
a.	P-value for genome scale significance (to account of multiple testing required with Illumina Infinium EPIC chip) = 7×10^{-8}
b.	Average beta difference between IC/BPS cases and controls = 0.027
c.	Average standard deviation (SD) = 0.014
d.	Example: sample size of 160 per group would be necessary to detect a 1% difference in methylation.
3. Methylation assessment and gene expression analysis	
4. Correlation of DNA methylation from urine sediment and bladder biopsies	
5. Control for age, race and smoking status as significant confounders	