Short- and mid-term temporal variability of the human urinary microbiota: an observational cohort study

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Abstract

Understanding the temporal variability of the microbiome is critical for translating associations of the microbiome with health and disease into clinical practice. The aim of this study is to assess the extent of temporal variability of the human urinary microbiota.

A pair of catheterized or mid-stream urine samples were collected from study participants at 3–40-month interval. DNA was extracted and the bacterial V4 hypervariable region of the 16S rRNA gene was sequenced on the Illumina MiSeq platform. The alpha and beta diversity of paired samples was analyzed using Chao1 and Shannon indices and PERMANOVA.

A total of 63 participants (43 men and 20 women with a mean age of 63.0 and 57.1 years, respectively) were included in the final analysis. An average of 152 ± 128 bacterial operational taxonomic units (OTUs) were identified in each urine sample from the entire cohort. There was an average of 41 ± 32 overlapping OTUs in each sample pair, accounting for 66.3 ± 29.4% of the relative abundance. There was a clear correlation between the number of overlapping OTUs and the relative abundance covered. The difference in Chao1 index between paired samples was statistically significant; the difference in Shannon index was not. Beta diversity did not differ significantly within the paired samples. Neither age nor sex of the participants influenced the variation in community composition. With a longer interval between the collections, the relative abundance covered by the overlapping OTUs changed significantly but not the number of OTUs.

The abundance of bacterial taxa present in both collections fluctuated, but the proportion of these taxa in the community was about two-thirds of the total bacterial community. No significant intraindividual differences in beta diversity were observed between the two urine samples.

Data Summary

The raw DNA sequences together with anonymized sample metadata have been deposited at the NCBI SRA under the accession number PRJNA1093489. Processing scripts are deposited at a public repository https://doi.org/10.5281/zenodo.12556460. Processed sequencing files and tables including full taxonomic assignment are deposited at https://doi.org/10.6084/m9.figshare.26046355.

Importance

This study represents a comprehensive investigation dedicated specifically to the subject of urinary microbiome (UM) temporal stability. Our dataset (n = 63) consists of a relatively large group of patients of one ethnic origin but varying ages and both sexes. Additionally, samples from individual participants are separated by different lengths of time. This approach allows us to assess the effects of three variables on the stability of human UM. Our findings demonstrate that, while the relative abundance of dominant bacteria varies, repeated collections generally share more than 60% of the bacterial community. Furthermore, we observe little variation in the alpha and beta diversity of the microbial community in human urine. These results help to understand the dynamics of human UM and enable interpretation of future studies.

Introduction

The existence of the human urinary microbiota (UM) is now considered a fact. (1,2) Both the lower and upper urinary tract harbor diverse microbial communities, some members of which have been cultured using expanded quantitative urine culture, while others have been detected by next-generation sequencing of the 16S rRNA gene. There is evidence that, in addition to the bacteriome, the UM also includes viruses (the virome) (3)and fungi (the mycobiome)(4), about which very little is known to date.

As far as the niches of the human body are concerned that have been incorporated in the Human Microbiome Project (5), their microbial communities appear to be stable. In the gastrointestinal tract, the composition of the microbiome is generally consistent over time and even after antibiotic exposure or a temporary change in diet, the microbiota returns to its original state. Not only the operational taxonomic units (OTUs) but also the metagenomic profile remain stable within a given individual. (6) Despite the exposure of the skin to various environmental factors, the stability of the community is maintained in the short and long term. A prolonged interval between sampling did not lead to an increase in the variation of the cutaneous microbiota within an individual. (7) In the oral cavity, the subgingival microbiota remains stable to a certain extent, although it varies from person to person and even between different sites of the oral cavity. (8) The stability of the vaginal microbiome over time is more challenging to determine due to the physiologically ever-changing influences of the hormonal cycle, perturbations of the microbiota during pregnancy, etc. (9)

Understanding the temporal variability/stability of the microbiota is crucial for translating associations of the microbiome with health and disease into clinical practice. However, until now it has been unclear how stable the UM is or how reliably a single sample reflects the microbial communities of a bladder over time. The present study aims to fill this knowledge gap and assess the degree of temporal variability of the human UM.

Methods

Population

The population of the present study is based on a cohort of patients with bladder cancer and a control group of subjects without cancer reported previously (10). A total of 107 subjects were asked to provide a second urine sample for the assessment of their urinary microbiota stability. Those willing to re-attend provided a second sample of urine for the assessment of alpha and beta diversity. To expand the study cohort, members of the research staff (healthy volunteers) were asked to provide two samples of urine several months apart. Antibiotic treatment was not administered to any subject six weeks prior to the first urine collection. The study was conducted in accordance with the Declaration of Helsinki after previous approval by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital with Multi-center Competence under the number G-19-01 and informed consent was obtained from all participants prior to enrolment. Participants´ enrollment into the study and sample collection was conducted between 27 May 2019 and 31 January 2023. The STORMS checklist for this study is attached as S1 Table. (11)

Sample collection and handling

The first paired sample was collected either as a mid-stream, clean-catch voided specimen of urine (mid-stream urine, MSU) or obtained via transurethral catheterization under anesthesia before a surgical procedure. The second paired sample was uniformly collected as MSU. An aliquot of each sample was stored at 4°C and processed within 24 hours with standard urine culture; another aliquot was frozen at -20°C on the same day for several months until shipping, DNA extraction and sequencing.

DNA extraction and sequencing

107 DNA extraction and 16S rRNA gene sequencing were performed as previously described (12) at the
108 Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic. Briefly, DNA was
109 extracted from urine samples using Eligene Urine Isolation Kit (Elisabeth Pharmacon, Ref. 90051-50)
110 according to manufacturer's instructions in a random order of patient groups to avoid batch effects.
111 The whole amount of urine was vortexed for 15 s, 10 mL of urine was then centrifuged at 6,000× g for
112 20 min, the supernatant was discarded, and pellet resuspended in 200 µL of molecular grade water,
113 200 µL of MI3 solution, and 20 µL of Proteinase K was added. After 15 s vortexing, the mixture was
114 incubated for 15 min at 65 °C. The lysate was centrifuged at 6,000× g for 5 min. The supernatant was
115 transferred to microtube and 210 µL of MI4 solution added. The lysate was centrifuged for 1 min at
116 13,000× g. Due to a low microbial load of source samples, DNA extraction controls as well as negative
117 controls for PCR reactions were included. Enrichment of microbial DNA was not performed. Yield and
118 purity of extracted DNA were checked using NanoDrop 1000 Spectrophotometer (Thermo Fisher
119 Scientific). Samples with even as low DNA concentration as 5 ng μL^{-1} were included in the
120 amplification step.
121 The primers 515F (5'- GTGCCAGCMGCCGCGGTAA) and 806R (5'- GGACTACHVGGGTWTCTAAT)(13)
122 were used to amplify the hypervariable region V4 of the 16S rRNA gene. Each forward primer was
123 barcoded by a custom sequence of nucleotides designed to multiplexing of different samples. PCR
124 was performed in triplicates, and every reaction contained 5 µL of 5× Q5 Reaction Buffer for Q5 High-
125 Fidelity DNA polymerase (New England Biolabs, Ref. B9027S); 0.25 µL Q5 High-Fidelity DNA
126 polymerase (New England Biolabs, Ref. M0491L); 5 µL of 5× Q5 High GC Enhancer (New England
127 Biolabs, Ref. M0491L); 1.5 µL of BSA (10 mg mL^{-1}, GeneOn, Ref. 209-005W); 0.5 µL of PCR Nucleotide
128 Mix (10 mM, Thermo Fisher Scientific, Ref. R0191); 1 µL of primer 515F (10 µM); 1 µL of primer 806R
129 (10 µM,); 1.0 µL of template DNA and sterile ddH_2O up to 25 µL. Conditions for amplification started
130 at 94 °C for 4 min followed by 25 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 75 s and finished
131 with a final setting of 72 °C for 10 min.

132 The successful PCR was confirmed using agarose gel electrophoresis. Three PCR reactions were
133 pooled together in order to randomize PCR bias and to get higher DNA yield. Pooled samples were
134 purified by MinElute PCR Purification Kit (Qiagen, Ref. 28004) and mixed in an equimolar amount
135 according to the concentration measured on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific).
136 Sequencing libraries were prepared using the TruSeq DNA PCR-Free Kit (Illumina, Ref. 20015962)
137 according to manufacturer's instructions while following official ligation protocol for the kit used.
138 Sequencing was performed on Illumina MiSeq in a 2 × 250 bases sequencing run. Raw fastq files were
139 retrieved and used as input to the bioinformatic pipeline described in the following section.

140 Statistical analyses

141 Demographic and clinical data were analyzed as continuous or categorical variables and reported as
142 mean and standard deviation (SD)/interquartile range (IQR) or counts with percentages as
143 appropriate.
144 The sequencing data were processed using SEED 2.1.2 (14). Pair-end reads were merged using fastq-
145 join (15). Sequences with ambiguous bases were omitted as well as sequences with average quality
146 PHRED score <30. The chimeric sequences were detected and removed using USEARCH 8.1.1861, and
147 clustered into OTUs using the UPARSE algorithm (16) at a 97% similarity level. This step yielded in
148 average 15,107 ± 8,700 sequences per sample (SD, min = 129). To filter out non-bacterial sequences,
149 the most abundant sequence from each OTU was assigned to the closest hit from the GenBank
150 database by NCBI BLAST 2.10.1. Taxonomy was assigned using DECIPHER 2.30.0 (17) and threshold 40
151 with IDTAXA algorithm trained on SILVA SSU database r138 (18). This step yielded in average 11,524 ±
152 8,669 sequences per sample (SD, min = 19).
153 If one of the paired samples showed Goods sequence coverage <85, the pair was excluded from
154 analysis. Global singleton OTUs were removed before analysis of OTUs occurrence and relative
155 abundance of individual OTUs was calculated. Alpha and beta diversity analyses were performed
156 using the packages tidyverse 2.0.0 (19), vegan 2.6-4 (20), QsRutils 0.1.5 (21), GUniFrac 1.8 (22) and
157 tidylog 1.0.2 (23) in R 4.3.1 (24). For alpha diversity analyses, rarefaction was done following (25):

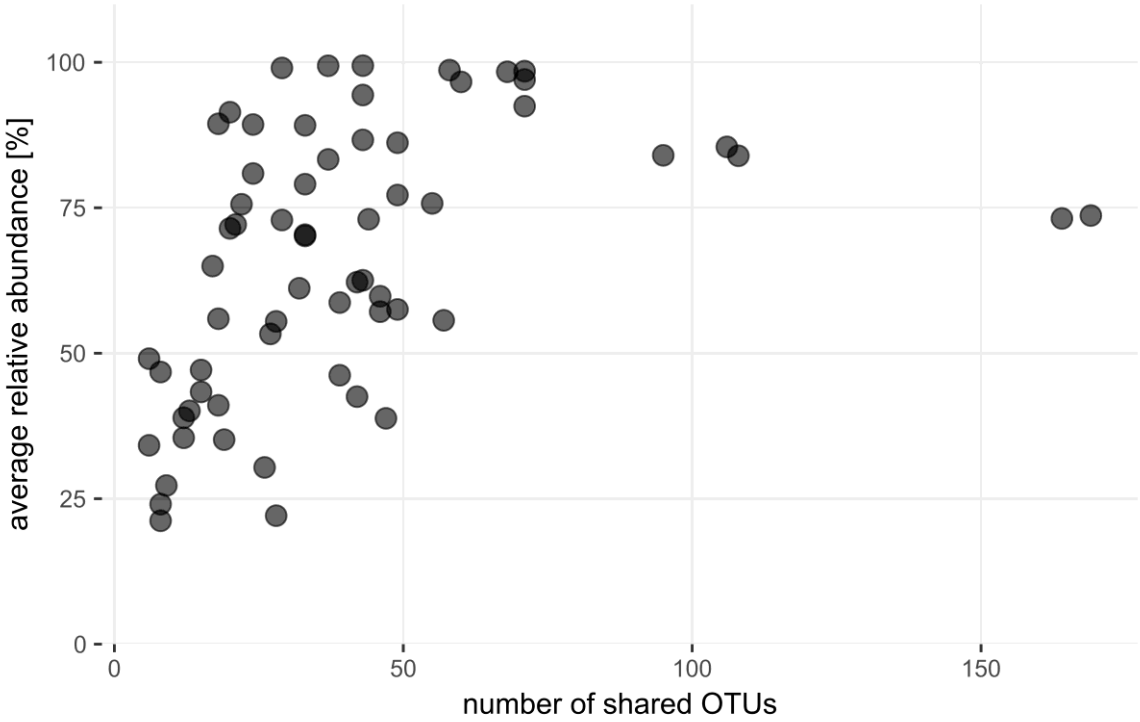
158 each sample was randomly subsampled to 1,000 sequences while excluding patients with at least one
159 sample below this threshold (S2 Table). Subsampling was iterated 1,000 times and per-sample
160 average summary statistics was calculated. The following indices were calculated (26): Chao1
161 (reflecting species richness (22) and Shannon diversity index (reflecting species evenness). Normality
162 was tested by Shapiro–Wilk test, differences among groups were tested by Kruskal-Wallis and
163 Wilcoxon paired test as appropriate and PERMANOVA based on the Bray–Curtis dissimilarity matrix.
164 Temporal trends in the number of shared OTUs and their relative abundance were modelled by
165 linear regression. The results were considered statistically significant at the level $p < 0.05$.
166

167 # Results

168 After exclusion of samples with insufficient Good´s coverage (6 subjects), a total of 63 subjects and
169 their paired samples of urine were included in the analysis (S2 Table). These samples were provided
170 by 43 men (mean age 63.0 ± standard deviation (SD) 15.2 years) and 20 women (mean age 57.1 ±
171 13.6 years). The time gap between the two collections ranged from three to 40 months (median 24.8,
172 IQR 14.9 months).
173 Standard urine culture was negative at 10^5 colony forming units/mL for all samples except one (no.
174 307) at the first sampling. The second sample was culture-negative in 61 of subjects. Among the
175 study participants, 28 were patients with non-muscle invasive bladder cancer (stage Ta-T1)
176 presenting for a transurethral resection of the bladder tumor, 18 were patients admitted for elective
177 surgery for a non-malignant condition (benign prostate hyperplasia or upper urinary stone disease)
178 and 17 were volunteering members of staff who provided two urine samples at least three months
179 apart.
180

181 In each urine sample, a mean of 152 ± 128 OTUs were identified in total bacterial community. In each
182 pair of samples from an individual subject, 41 ± 32 (27 ± 21%) of OTUs were overlapping, i.e.
183 detected in both collections. These OTUs present in both collections covered in average 66.3 ± 29.4%

184 (median 72.7%, IQR 49.7%) of the microbial community in urine samples in terms of relative
185 abundance. There was a clear correlation between the number of overlapping OTUs and the relative
186 abundance they were accounting for (Pearson's $R^2 = 0.43$, $p < 0.001$) (Fig 1).
187
188 **Fig 1: The correlation between the number of overlapping OTUs and the relative abundance they**
189 **are covering.** The more OTUs are shared between two samples of urine taken apart, the higher
190 relative abundance they account for (Pearson's $R^2 = 0.43$, $p<0.001$).



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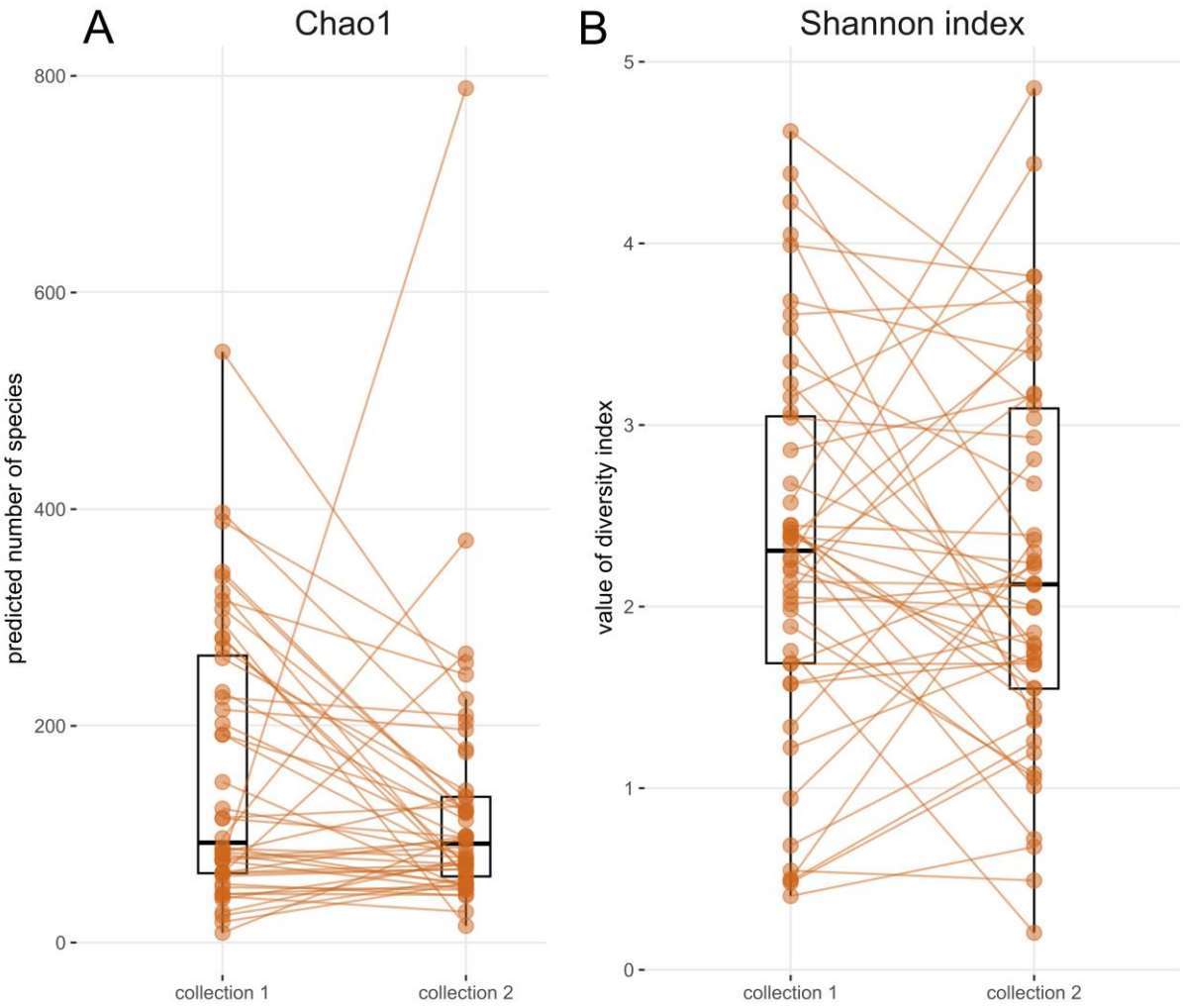
193 Median value of Chao1 index was lower by 15.4 (IQR = 129.6) when comparing the second collection
194 to the first one (Wilcoxon test, $p < 0.05$, Fig 2A). Shannon index showed no significant difference in
195 community evenness between individual collections (Fig 2B). Comparison of groups with and without
196 bladder cancer and groups with and without ureterolithiasis did not show differences in alpha
197 diversity between these health conditions (S3 Table).
198

199 **Fig 2: Microbial community indices of alpha diversity for two collection timepoints**. (A) Chao1 and
200 (B) Shannon index after 1,000× rarefaction. Boxplots show median, upper, and lower quartile, highest
201 and lowest values. The first and second samples from the same subject are connected by orange
202 lines.



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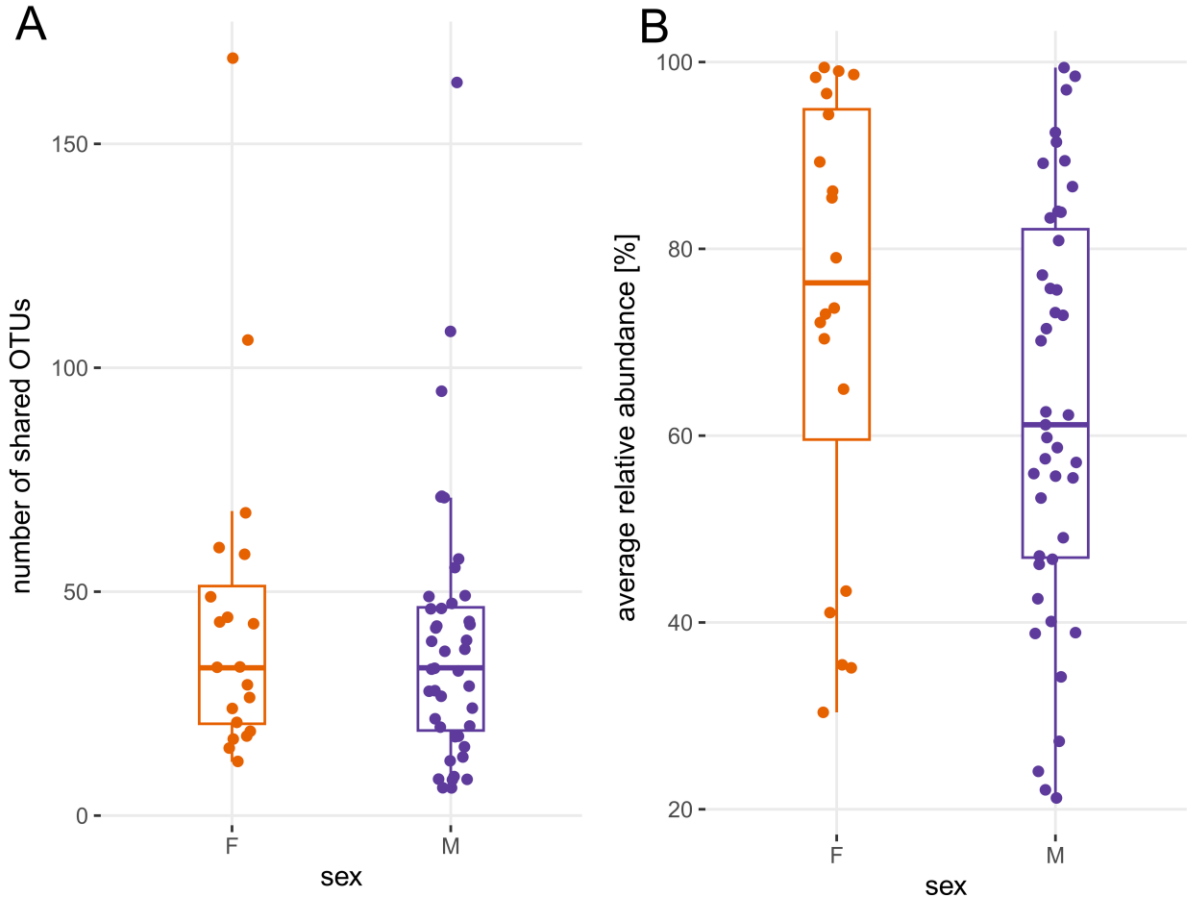
205 In addition to alpha diversity metrics, the sum of bacterial relative abundance represented by
206 overlapping taxa as well as their identity was evaluated in relation to biological sex. There was no
207 difference in the number of overlapping OTUs and the relative abundance of overlapping community
208 between males and females (Figs 3A, 3B) and between patients with bladder cancer and patients
209 without cancer plus healthy volunteers (S1 Fig).

210

211 **Fig 3: Overlap of the bacterial community from the two collection timepoints.** (A) number of
212 overlapping OTUs (e.g. taxa detected in both collections). (B) averaged sum of relative abundances of
213 overlapping taxa from collections at two timepoints ([relative abundance of the overlapping OTUs at
214 first sampling + relative abundance of the overlapping OTUs at second sampling] / 2). Boxplots show
215 median, upper and lower quartile, highest and lowest values.



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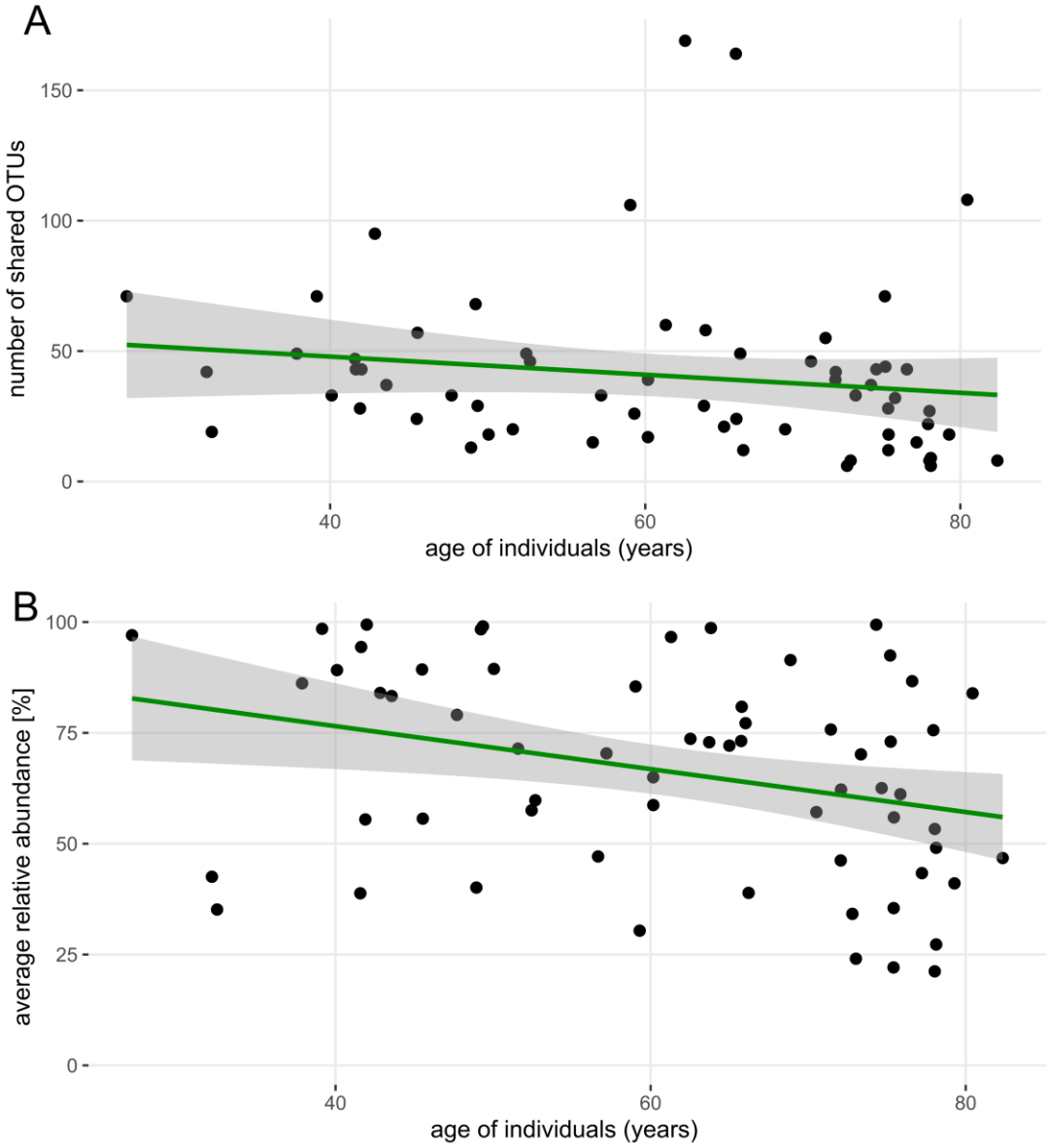
218 There was a non-significant trend towards lower overlap with increasing age of study subjects in both
219 taxa count and relative abundance (Figs 4A and 4B). With a longer interval between the first and
220 second urine collections the number of overlapping OTUs did not change significantly (Fig 5A). There
221 was, however, a significantly decreased mean relative abundance of the overlapping taxa with time
222 ([relative abundance of the overlapping OTUs at first sampling + relative abundance of the
223 overlapping OTUs at second sampling] / 2; $p < 0.05$; Fig 5B).

224

225 **Fig 4: Relationship between taxa representation and age of subjects.** (A) the number of OTUs which
226 were detected in both collections as a function of age. (B) averaged sum of overlapping taxa relative
227 abundance from collections at two timepoints as a function of age. Some urine pairs overlapping taxa
228 represented nearly 100% of the community in both collections. In some cases, the relative abundance
229 of the overlapping taxa was as low as 25%. Shaded area represents linear smoothing with 0.95
230 confidence interval.



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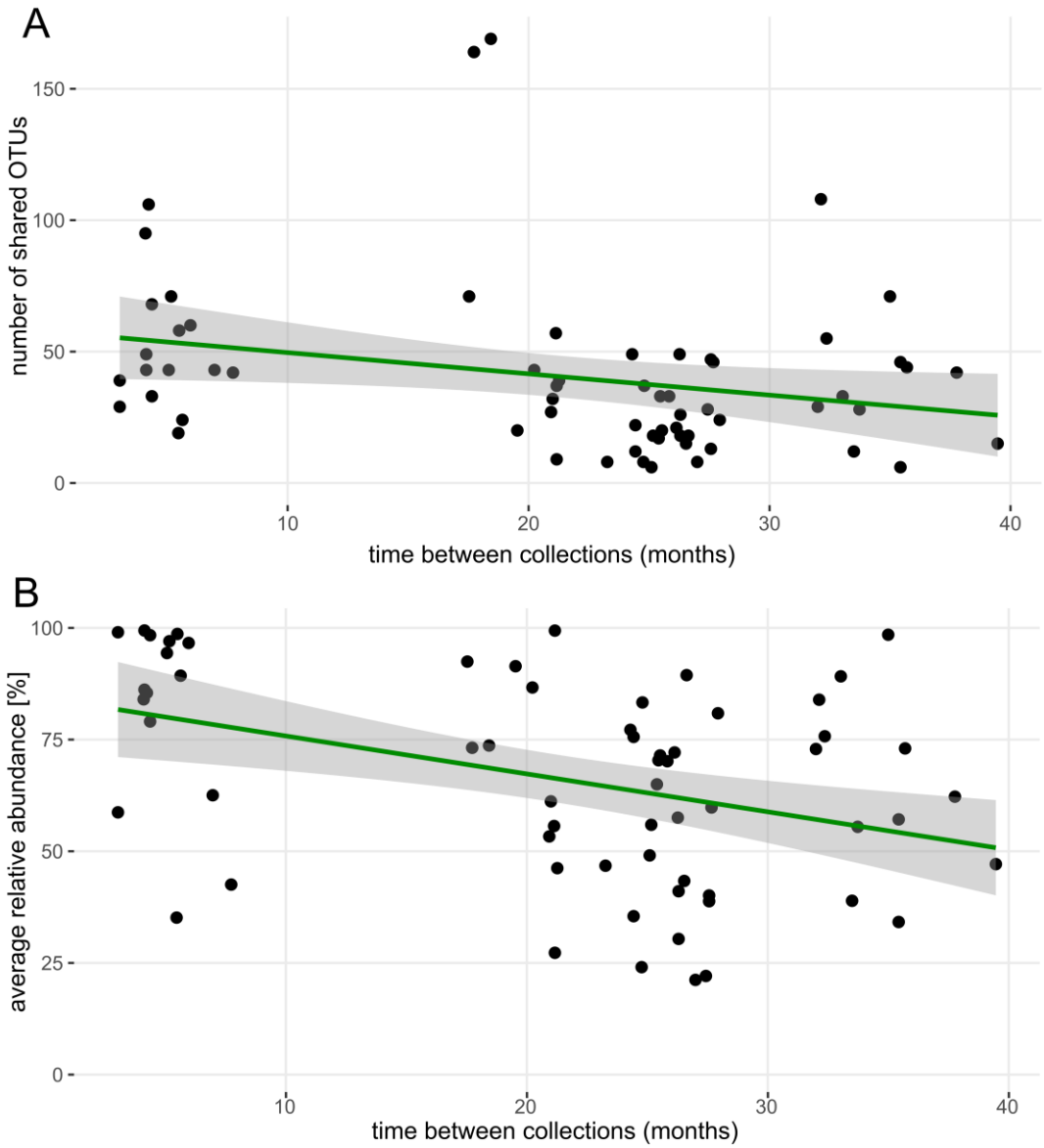
233 **Fig 5: Relationship between overlapping bacterial community and time between collections.** (A)
234 number of OTUs which were detected in both collections as a function of time between collections.

235 (B) averaged sum of overlapping taxa relative abundance as a function of time between collections (p

236 < 0.05). Shaded area represents linear smoothing with 0.95 confidence interval.



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238

239 In terms of beta diversity, the patients' identity was a stronger driver for community composition

240 than the time between collections (R^2 = 0.04 and 0.02, respectively, $p < 0.05$). There were certain

241 OTUs frequently observed in both paired samples and in more than 50% of male and female subjects

242 (Table 1).

243

244 **Table 1. Taxa detected in both the first and second urine sample by frequency.**

Genus (Class)	OTU ID	incidence in males (%)	incidence in females (%)
Staphylococcus (Bacilli)	CL00011	40 (93%)	17 (85%)
unclass. *Enterobacteriaceae (Gammaproteobacteria)*	CL00007	31 (72%)	16 (80%)
Cutibacterium (Actinobacteria)	CL00024	34 (79%)	14 (70%)
Finegoldia (Clostridia)	CL00021	29 (67%)	14 (70%)
Peptoniphilus (Clostridia)	CL00035	27 (63%)	14 (70%)
Corynebacterium (Actinobacteria)	CL00041	31 (72%)	12 (60%)
unclass. *Enterobacteriaceae (Gammaproteobacteria)*	CL00004	27 (63%)	13 (65%)
Streptococcus (Bacilli)	CL00020	28 (65%)	0 (0%)
Fusobacterium (Fusobacteriia)	CL00076	0 (0%)	13 (65%)
Enhydrobacter (Gammaproteobacteria)	CL00050	26 (60%)	0 (0%)
Campylobacter (Campylobacteria)	CL00013	0 (0%)	12 (60%)
Dialister (Negativicutes)	CL00030	0 (0%)	12 (60%)
Gardnerella (Actinobacteria)	CL00003	0 (0%)	11 (55%)
Bacteroidales (Bacteroidia)	CL00006	0 (0%)	11 (55%)
Anaerococcus (Clostridia)	CL00015	23 (53%)	0 (0%)
Corynebacterium (Actinobacteria)	CL00054	22 (51%)	0 (0%)

Taxa detected in both the first and second urine sample with more than 50% prevalence in either sex.

Discussion

251 Since the discovery of the human UM in 2011 (1,2), it has been associated with various pathological
252 conditions. Twelve years later, there is not only a lack of description of normal UM, but also a lack of
253 deeper understanding of UM dynamics. However, this knowledge is crucial to draw conclusions about
254 the role of UM in the onset and development of disease. For example Isali et al. (27) recently pointed
255 out that it is difficult to characterize causality in microbiome research of bladder cancer and even
256 with known composition of microbial taxa, these results cannot be readily translated to therapeutic
257 applications. This study aims to uncover the effects of time on the UM composition using repeated
258 sampling of study participants. Furthermore, we want to characterize the overlap of the microbial
259 community from the same subject over a period of several months.

260 Microbial communities of other human body niches are known to be stable over time. (6–8) For the
261 urinary tract, there has been little evidence on this topic. The first endeavor investigating UM short-
262 term stability on 14 subjects of both sexes and various ages using 16S rRNA gene sequencing found
263 that the voided urinary microbiota composition remained stable on a short-term basis (several days
264 apart). (28) Eight women were observed longitudinally for three months with daily culture of mid-
265 stream urine (MSU) and periurethral swab; UM was undergoing fluctuations associated with
266 menstruation and sexual activity. (29) In a cohort of 10 women who provided mid-stream urine
267 samples 2.5 years apart, high intraindividual variability was observed with only 29% (range 9–42%) of
268 species overlapping from the first and second sample even though the sampling was performed
269 uniformly in the third week of the menstrual cycle. Of note, the authors chose to use an expanded
270 quantitative urine culture (EQUC) protocol instead of 16S rRNA gene sequencing to assess the
271 microbial diversity. (30) In 13 pregnant women whose mid-stream urine samples were collected in
272 the first and second trimester and subjected to 16S rRNA gene sequencing, the UM was stable with
273 *Lactobacillus* being the dominant microorganism in the majority of women. (31)

274

275 In the present study, we show that the number of bacterial taxa overlapping in two urine collections
276 usually does not exceed 50 (Figs 1, 3B, 4A, 5A) and that the relative abundance of these overlapping
277 OTUs averages between 60% and 70% sometimes even reaching 90–100% (Figs 4B and 5B). There
278 was no significant difference between men and women in the number of shared OTUs or in the
279 relative abundance represented by these OTUs (Figs 3A, 3B). The bladder cancer status of patients
280 also had no effect on the number of shared OTUs or their sum of abundance (S1 Fig).

281 Using the Chao1 estimator, we have shown that the richness of the microbial community changes
282 between collections, i.e. there is some variation in the number of resident bacteria in urine samples
283 taken months apart (Fig 2A). The relative abundance of overlapping OTUs may vary considerably (Fig
284 2B) and this phenomenon becomes more significant as the time interval between collections
285 increases (Fig 5B).

286 At the community composition level (beta diversity), greater variability was explained by the subject
287 identity than by the effect of collection time. Therefore, the differences in community composition
288 within a subject were smaller than the differences between individuals.

289 Based on the design of our previous project (10), to which the present study is an extension, 26 of 63
290 (41%) urine pairs contained catheterized urine from the first collection (all second samples were
291 MSU). When MSU was compared to MSU in both samples, there were 47 ± 32 overlapping OTUs;
292 when catheterized urine from the first collection was compared to MSU from the second sampling,
293 the proportion of overlapping OTUs decreased to 32 ± 31 OTUs. This difference was statistically
294 significant ($p = 0.01$) and is consistent with previous research reporting microbial communities in
295 catheterized samples differ from MSU. (12)

296 We have noted that certain OTUs were observed in both paired samples in more than 50% of
297 subjects (Table 1). These might represent candidate OTUs in the quest for a core urinary microbiota.
298 Whether such phenomenon exists is still a matter of debate but of the taxa listed in Table 1, the
299 following have been consistently reported in previous urinary microbiota studies (in alphabetical

order): *Corynebacterium, Finegoldia, Gardnerella, Peptoniphilus, Staphylococcus* and *Streptococcus* (1,32–35). Aerobic subgroup of these taxa (like *Corynebacterium*, *Staphylococcus* and *Streptococcus* are also present in a recent genome collection of bladder-specific isolates and thus their metabolic potential might be assessed. (36) Other taxa like *Peptoniphilus* are more elusive due to their anaerobic growth. The fact that *Lactobacillus* does not feature among the top OTUs in our dataset may be explained by the postmenopausal status of the study female participants (37); indeed, *Lactobacillus* was detected in both paired samples in only 40% of women.

We analyzed the dynamics of the microbial community in individual sample pairs (S2 Fig) to explain very low community overlap in some cases. In some of the pairs with the lowest overlap, a particular OTU was present with a high relative abundance (>50%) in one sample and absent in the other. These OTUs were identified as (in alphabetical order) *Arcanobacterium, Enterobacter/Morganella, Idiomarina, Lactobacillus, Pseudomonas and Ureaplasma*.

Some methodological weaknesses of our study deserve attention: 1) Urine pairs collected at shorter time intervals between collections were from younger subjects, while paired samples from older participants were separated by a longer time gap. This may have led to a systematic bias favoring the statistical significance of the trend seen in Fig 5B; 2) while none of the study subjects had taken antibiotics six weeks prior to the first urine collection, the absence of antibiotic treatment was less rigorously documented at the second urine sampling. Therefore, we cannot rule out the possibility that a shift in the richness or diversity of the UM was due to undocumented antibiotic exposure; 3) patients who harbored urinary stones at first sampling may have been stone-free at second sampling. While these shortcomings weaken the statistical significance of some of our results, they strengthen the claim that UM undergoes only limited change in time.

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Conflicts of interest

The authors declare no competing interests.

Ethical approval

The study was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital in March 2019 (docket no. G-19-01) and extension of the study was approved in May 2021 (docket no. 125699/21).

Author Contributions

JH, VH, and RZ designed the study. JH, VH, PB, PC, and RZ performed sample collection. PB and PC performed lab research. VT and PTD analyzed the data. JH assisted with data interpretation. JH and VT wrote the manuscript.

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344 # References

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346 1. Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, et al. Urine Is Not
347 Sterile: Use of Enhanced Urine Culture Techniques To Detect Resident Bacterial Flora in the
348 Adult Female Bladder. J Clin Microbiol. 2014;52(3):871–6.
349 2. Siddiqui H, Nederbragt AJ, Lagesen K, Jeansson SL, Jakobsen KS. Assessing diversity of the
350 female urine microbiota by high throughput sequencing of 16S rDNA amplicons. BMC
351 Microbiol. 2011;(11):1–12.
352 3. Garretto A, Thomas-White K, Wolfe AJ, Putonti C. Detecting viral genomes in the female
353 urinary microbiome. J Gen Virol. 2018;99:1141–6.
354 4. Ackerman A, Anger JT, Khalique MU, Ackerman JE, Tang J, Kim J, et al. Optimization of DNA
355 extraction from human urinary samples for mycobiome community profiling. PLoS One.
356 2019;14(4):1–26.
357 5. The NIH Human Microbiome Project. Genome Res. 2009;(19):2317–23.
358 6. Mehta RS, Abu-ali GS, Drew DA, Lloyd-price J, Lochhead P, Joshi AD, et al. Stability of the
359 human faecal microbiome in a cohort of adult men. Nat Microbiol. 2018;3(3):347–55.
360 7. Oh J, Byrd AL, Park M, Kong HH, Segre JA. Temporal Stability of the Human Skin Microbiome.
361 Cell. 2016;165(4):854–66.
362 8. Tamashiro R, Strange L, Schnackenberg K, Santos J, Gadalla H, Zhao L, et al. Stability of healthy
363 subgingival microbiome across space and time. Sci Rep [Internet]. 2021;11(1):1–11. Available
364 from: https://doi.org/10.1038/s41598-021-03479-2
365 9. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, et al. The composition and
366 stability of the vaginal microbiota of normal pregnant women is different from that of non-

pregnant women. Microbiome. 2014;2(1):1–19.

10. Hrbáček J, Tláskal V, Čermák P, Hanáček V, Zachoval R. Bladder cancer is associated with decreased urinary microbiota diversity and alterations in microbial community composition. Urol Oncol. 2023 Feb;41(2):107.e15-107.e22.

11. Mirzayi C, Renson A, Genomic Standards Consortium, Massive Analysis and Quality Control Society, Furlanello C, Sansone SA, et al. Reporting guidelines for human microbiome research: the STORMS checklist. Nat Med [Internet]. 2021 [cited 2024 Jun 16];27(11):1885–92. Available from: https://www.nature.com/articles/s41591-021-01552-x

12. Hrbacek J, Morais D, Cermak P, Hanacek V, Zachoval R. Alpha-diversity and microbial community structure of the male urinary microbiota depend on urine sampling method. Sci Rep [Internet]. 2021;11(1). Available from: https://doi.org/10.1038/s41598-021-03292-x

13. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of rjnp diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A. 2011 Mar;108 Suppl(Suppl 1):4516–22.

14. Větrovský T, Baldrian P, Morais D. SEED 2: a user-friendly platfomr for amplicon high-throughput sequencing data analyses. Bioinformatics. 2018;34(13):2292–4.

15. Aronesty E. Comparison of Sequencing Utility Programs. Open Bioinforma J. 2013;7(1):1–8.

16. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013 Oct;10(10):996–8.

17. Wright ES. Using DECIPHER v2.0 to analyze big biological sequence data in R. R J. 2016;8(1):352–9.

18. Murali A, Bhargava A, Wright ES. IDTAXA: a novel approach for accurate taxonomic classification of microbiome sequences. Microbiome. 2018 Aug;6(1):140.

19. Wickham H, Averick M, Bryan J. Welcome to the Tidyverse. J Open Source Softw. 2019;4(43):1686.

20. Oksanen AJ, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. vegan: Community

393 Ecology Package. R package version 2.6-2. Vol. 5, Community Ecology Package. 2022.
394 21. Quensen J. QsRutils: R functions useful for community ecology [Internet]. 2020. Available
395 from: https://github.com/jfq3/QsRutils
396 22. Chen J, Zhang X, Yang L, Zhang L. GUniFrac: Generalized UniFrac distances, distance-based
397 multivariate methods and feature-based univariate methods for microbiome data analysis
398 [Internet]. 2023. Available from: https://cran.r-project.org/package=GUniFrac
399 23. Elbers B. tidylog: Logging for “dplyr” and “tidyr” Functions [Internet]. 2020. Available from:
400 https://cran.r-project.org/package=tidylog
401 24. R Core Team. R: A language and environment for statistical computing. [Internet]. 2022 [cited
402 2021 Sep 22]. Available from: http://www.r-project.org/
403 25. Schloss PD. Waste not, want not: Revisiting the analysis that called into question the practice
404 of rarefaction. mSphere [Internet]. 2024 Jan;30(9). Available from:
405 https://www.biorxiv.org/content/10.1101/2023.06.23.546312v1%0Ahttps://www.biorxiv.org
406 /content/10.1101/2023.06.23.546312v1.abstract
407 26. O’Hara RB. Species richness estimators: How many species can dance on the head of a pin? J
408 Anim Ecol. 2005;74(2):375–86.
409 27. Isali I, Helstrom EK, Uzzo N, Lakshmanan A, Nandwana D, Valentine H, et al. Current Trends
410 and Challenges of Microbiome Research in Bladder Cancer. Curr Oncol Rep [Internet]. 2024
411 [cited 2024 May 30];26(3):292–8. Available from: https://link.springer.com/10.1007/s11912-
412 024-01508-7
413 28. Bundgaard-Nielsen C, Ammitzbøll N, Isse YA, Muqtar A, Jensen AM, Leutscher PDC, et al.
414 Voided Urinary Microbiota Is Stable Over Time but Impacted by Post Void Storage. Front Cell
415 Infect Microbiol. 2020;10(August):435.
416 29. Price TK, Wolff B, Halverson T, Limeira R, Brubaker L, Dong Q, et al. Temporal dynamics of the
417 adult female lower urinary tract microbiota. MBio. 2020;11(2).
418 30. Ksiezarek M, Ugarcina-perovic S, Rocha J, Grosso F, Peixe L. Long-term stability of the

419 urogenital microbiota of asymptomatic European women. BMC Microbiol. 2021;21:64.
420 31. Sung J, Halverson T, Goodman JR, Wolfe AJ, Waters T. 606: Stability of the maternal urinary
421 microbiome across pregnancy. In: American Journal of Obstetrics and Gynecology [Internet].
422 Elsevier Inc.; 2020. p. S388–9. Available from: https://doi.org/10.1016/j.ajog.2019.11.622
423 32. Pearce MM, Hilt EE, Rosenfeld AB, Zilliox MJ, Thomas-White K, Fok C, et al. The female urinary
424 microbiome: A comparison of women with and without urgency urinary incontinence. MBio.
425 2014;5(4).
426 33. Thomas-White KJ, Hilt EE, Fok C. Incontinence Medication Response Relates to the Female
427 Urinary Microbiota. Int Urogynecol J. 2016;27(5):723–33.
428 34. Groah SL, Pérez-Losada M, Caldovic L, Ljungberg IH, Sprague BM, Castro-Nallar E, et al.
429 Redefining Healthy Urine: A Cross-Sectional Exploratory Metagenomic Study of People With
430 and Without Bladder Dysfunction. J Urol. 2016;196(2):579–87.
431 35. Bowie KR, Garzotto M, Orwoll E, Karstens L. BMI and BPH correlate with urinary microbiome
432 diversity and lower urinary tract symptoms in men. bioRxiv. 2023;1–26.
433 36. Du J, Khemmani M, Halverson T, Ene A, Limeira R, Tinawi L, et al. Cataloging the phylogenetic
434 diversity of human bladder bacterial isolates. Genome Biol [Internet]. 2024 [cited 2024 May
435 30];25(1):75. Available from:
436 https://genomebiology.biomedcentral.com/articles/10.1186/s13059-024-03216-8
437 37. Ammitzbøll N, Bau BPJ, Bundgaard-Nielsen C, Villadsen AB, Jensen AM, Leutscher PDC, et al.
438 Pre- and postmenopausal women have different core urinary microbiota. Sci Rep [Internet].
439 2021;11(1):1–10. Available from: https://doi.org/10.1038/s41598-021-81790-8
440

441 **S1 Fig: Overlap of the bacterial community from the two collection timepoints.** (A) number of
442 overlapping OTUs (e.g. taxa detected in both collections). (B) averaged sum of relative abundances of
443 overlapping taxa from collections at two timepoints ([relative abundance of the overlapping OTUs at
444 first sampling + relative abundance of the overlapping OTUs at second sampling] / 2). Boxplots show

445 median, upper and lower quartile, highest and lowest values for groups of patients with and without
446 bladder cancer.
447
448 **S2 Fig: The dynamics of the bacterial community shared among individual sample pairs.** Individual
449 facets represent patients with two collections each, numbers in facet headers represent patient IDs.
450 Shared OTUs are depicted as points and direction of change in their relative abundance between
451 collections is visualized by connecting lines.
452
453 **S1 Table: The STORMS checklist**. Form was downloaded from https://stormsmicrobiome.org.
454
455 **S2 Table: Metadata and individual data points from analysis of 69 subjects which were recruited for**
456 **the study.** CKD-EPI eGFR_ml_min - Glomerular Filtration Rate, RT_MP_in_anam – pelvic radiotherapy
457 in patient history.an
458
459 **S3 Table: Values of alpha diversity for selected groups of subjects.** Average value ± standard
460 deviation is given; upper indices mark pairs with significant differences.
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