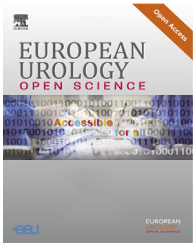
available at www.sciencedirect.com
journal homepage: www.eu-openscience.europeanurology.com



European Association of Urology

Bladder Cancer

Microbiome Profiling in Bladder Cancer Patients Using the First-morning Urine Sample

Carmela Nardelli [a,b], *Achille Aveta* [c], *Savio Domenico Pandolfo* [a,c,d], *Lorella Tripodi* [a,b], *Filippo Russo* [a,b], *Ciro Imbimbo* [c], *Giuseppe Castaldo* [a,b,*], *Lucio Pastore* [a,b]

[a] *CEINGE-Biotecnologie Avanzate Franco Salvatore, Naples, Italy;* [b] *Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Naples, Italy;* [c] *Dipartimento di Neuroscienze, Scienze Riproduttive e Odontostomatologiche, Università degli Studi di Napoli Federico II, Naples, Italy;* [d] *Department of Urology, University of L'Aquila, L'Aquila, Italy*

Article info

Article history:
Accepted November 5, 2023

Associate Editor:
M. Carmen Mir

Keywords:
Urine
Microbiome
Porphyromonas
Bladder cancer
Prostate cancer

Abstract

Background: Several studies support the interplay between the urinary microbiome (ie, urobiome) and bladder cancer (BCa). Specific urinary bacteria may be responsible for chronic inflammation, which in turn promotes carcinogenesis. Different signatures of urobiome in BCa patients were identified depending on tumor type, geographical area, age, and sex.
Objective: We explored the urobiome in BCa patients undergoing transurethral resection of bladder tumor (TURBT), to identify possible predictive biomarkers of cancer.
Design, setting, and participants: The urobiome analysis was conducted in 48 patients (13 females) undergoing TURBT, of whom 30 with BCa (five females) and 18 with benign bladder tumor, analyzing bacterial 16S rRNA by next-generation sequencing in first-morning (FM) urine samples. Forty-three cancer-free individuals and 17 prostate cancer patients were used as controls.
Outcome measurements and statistical analysis: First, we identified the better urine collection procedure to perform the urobiome analysis, comparing bacterial composition between catheterized (CAT) and FM urine samples in TURBT patients. Successively, we observed a specific urobiome in BCa patients rather than controls. A combined pipeline including the DESeq2 and linear discriminant analysis effect size tests was used to identify differential urinary taxa, strictly associated with BCa patients.
Results and limitations: The bacterial composition of CAT and FM urine samples was comparable, so the latter was used for the following analyses. An increased abundance of *Porphyromonas* and *Porphyromonas somerae* was found in BCa patients compared with controls. This signature seems to be more related (p <0.05) to male BCa patients over 50 yr old. Owing to the low biomass of urinary microbiota, several samples were excluded from the study, reducing the number of BCa patients considered.

* Corresponding author. CEINGE-Biotecnologie Avanzate Franco Salvatore, Via Gaetano Salvatore 486, 80145 Naples, Italy. Tel. +39 0813 737 859.
E-mail address: giuseppe.castaldo@unina.it (G. Castaldo).

https://doi.org/10.1016/j.euros.2023.11.003

Conclusions: FM urine samples represent a manageable specimen for a urobiome analysis; *P. somerae* is a specific biomarker of BCa risk.
Patient summary: Our study showed an increased abundance of *Porphyromonas* and *Porphyromonas somerae* in male bladder cancer (BCa) patients, supporting the use of a first-morning urine sample, a less invasive and low-cost collection method, for the urobiome analysis of patients at risk of BCa.

1. Introduction

The development of advanced sequencing technologies, such as 16S rRNA and whole-genome shotgun sequencing, allowed characterization of microbial communities living in several niches of the human body [1]. Microbial changes in specific body districts have been correlated to several pathologies, since microorganisms and their metabolites can alter the functional state of tissues and immune system [2–7]. In addition, the presence of a urinary microbiome overturned the long-standing paradigm that urine is a sterile substance [8]. The whole genome of microbial communities in urine is defined as "urobiome" and plays a key role in the pathogenesis of urological disorders such as interstitial cystitis, urinary incontinence, bladder pain syndrome, overactive bladder syndrome, urinary tract infections, benign prostatic hyperplasia, and urogenital cancer [9–12].

Bladder cancer (BCa) is one of the most common urinary tract–associated malignancies worldwide and non–muscle-invasive BCa is the most frequent BCa, accounting for 70–80% of all diagnosed cases [13]. Besides the factors known to increase BCa risk, such as genetics, tobacco smoking, and occupational exposure to chemicals or toxins, urobiome is emerging as a new actor [8,13]. One of the main mechanisms explaining the interplay between urinary microbiome and BCa is the presence of biofilm-associated bacteria, responsible for chronic inflammation, which in turn promotes carcinogenesis [11]. As previously reported, *Streptococcus* and *Fusobacterium nucleatum* are increased in urine from BCa patients [14]; *F. nucleatum* is a Gram-negative taxon able to trigger a chronic inflammatory response, promoting cancer development [5]. Other studies showed a different microbial composition between genders affected by specific urological disorders, detecting an increased abundance of *Corynebacterium* and *Streptococcus* in men and of *Lactobacillus* in women [15]. In addition to changes in relative abundance, specific taxa were identified in aged individuals regardless of gender [16]. Owing to the absence of standardized urine sample collection, common investigation procedures, and assessment of intra- and interindividual variability [16], it is not surprising to find conflicting data in urobiome studies. In fact, a recent global meta-analysis of urine microbiome reported that the type of urine collection methodology biased the genera abundance because of skin, vaginal, and urethral contamination in the first-morning (FM) catch samples [17].

In the present work, we investigated the urobiome of patients undergoing transurethral resection of bladder tumor (TURBT) from Southern Italy. First of all, we compared bacterial profiling of catheter-collected urine (CAT) samples, with FM urine collected from the same patients undergoing TURBT in order to choose the urine collection procedure. A statistically significant difference was observed between CAT and FM samples in bacterial signature, so we chose FM urine samples for the urobiome analysis. Successively, we received the histological diagnosis of BCa for some TURBT patients. Therefore, we compared the microbiome of BCa patients with control groups using FM urine. We analyzed hypervariable regions (V3-V4-V6) of the 16S ribosomal RNA (rRNA) gene by next-generation sequencing (NGS). We identified a significant increase of *Porphyromonas* in male BCa (mBCa) patients over 50 yr old compared with our control groups over 50 yr old. It could be exploited as a predictive biomarker of BCa and sheds new light on BCa pathogenesis.

2. Patients and methods

2.1. Participant recruitment and sample collection

We studied 108 patients, who were divided into three groups:

1. Forty-eight consecutively recruited patients who underwent TURBT (mean age ± standard deviation [SD] = 69.0 ± 11.6; 13 females). Among these 48 patients, 30 were histologically diagnosed to have BCa (five females) and 18 were diagnosed to have benign urological pathology (bladder benign tumor [BBT]; eight females). The BCa group included patients with different pathological grading: low-grade carcinoma (n = 9; G1) and high-grade carcinoma (n = 21; G3), according to European Association of Urology 2020 guidelines [18].

2. Seventeen subjects affected by histologically proven prostate carcinoma (PK; mean age ± SD = 68.5 ± 9.7) at diagnosis, used as the control group with another pathology.

3. Forty-three volunteers as cancer-free control group (CO; mean age ± SD = 57.5 ± 13.4; 15 females) with no history of BCa or any other type of tumor.

From 34/48 TURBT patients, CAT urine and FM urine (including the first stream) were harvested at the beginning of the TURBT procedure, after careful cleaning of the genital area and surgical draping, and immediately upon endoscope insertion. The remaining TURBT patients did not give the consent for the collection of CAT samples. An FM urine sample was collected from CO and PK patients using a sterile container. Individuals with urinary tract infections, anamnesis of other cancer, or diabetes, or those under treatment with antibiotics or any other drugs in the last month were excluded from the study. Each urine sample was stored at −80°C within 2 h from collection until used.

All TURBT and PK patients came from the Urology Clinic of University Hospital "Federico II" of Naples, while the CO group included only volunteers of research laboratories from CEINGE Biotecnologie Avanzate

Franco Salvatore. All patients were admitted between October 2021 and April 2023. The enrolled patients signed the informed consent to participate in the study, approved by the Ethical Committee of the University Federico II of Naples (n. 191/20) and conducted in accordance with the Helsinki Declaration.

2.2. Bacterial DNA isolation and 16S rRNA sequencing analysis

Bacterial DNA was isolated from all urinary samples using MagPurix bacterial DNA extraction kit (Zinexts Life Science) using the automated system, according to manufacturer instructions. We incubated 1 ml of urine with 220 µl buffer provided by the kit at 56 °C for 30 min. The DNA was eluted in 50 µl of the elution buffer. The Qubit dsDNA HS (high sensitivity) assay kit (Invitrogen Co., Life Sciences) and the TapeStation (Agilent Technologies) were used to evaluate the yield and quality of extracted DNA. The hypervariable V3-V4-V6 regions of the bacterial 16S rRNA gene were analyzed using Microbiota solution B (Arrow Diagnostics) according to manufacturer instructions and as described previously [5,7,9]. The extracted DNA was then used for the amplification of targeted regions; the quality and quantity of the amplicons were evaluated with the TapeStation system and Qubit dsDNA HS assay, respectively. After the preparation of the libraries, we obtained an equimolar pool. Amplicons were sequenced on the MiSeq Illumina sequencing platform (Illumina) using a Nano V2 500-cycle flow cell, loading the pool concentrated to 4.5 pM and 10% Phix. To avoid contaminations, we performed all the analytical steps (from DNA extraction to the library preparation and sequencing), following the previously reported detailed protocol [5] and adding an internal blank negative control. Furthermore, we also used a Gut Microbiome genomic Mix ATCC MSA-1006 (LGC Standards) processed simultaneously with the patients' samples for each run as a positive standard control for the sequencing process.

2.3. Microbiome data processing and statistical analysis

Dedicated bioinformatics software (MicrobAT Suite-SmartSeq) was used to analyze the sequencing raw data (Fastq files). This software automatically assigned the operational taxonomic units (OTUs) according to the Ribosomal Database Project (RDP) database to the sequenced samples, and it returned three comma-separated values (CSV) files: the Metadata file, which contained the name and information of the samples used; the OTU table file, in which the number of reads per single taxon identified was associated to every sample; and the taxonomy table file, which contained the taxonomic information (phylum, class, order, family, genus, and species) of each identified taxon. These three files were used to perform the statistical analysis by the MicrobiomeAnalyst platform (https://www.microbiomeanalyst.ca/). This platform includes different statistical modules for a comprehensive statistical, functional, and integrative analysis of the microbiome data. To analyze marker gene count data, we used the Marker Data Profiling (MDP) module, specifically used for the analysis of the 16S rRNA gene data [19]. After removing unassigned phyla from our OTU table, we filtered taxa having at least 20 counts with 10% of prevalence. To perform community profiling, we normalized data by rarefying and scaling with total sum scaling. The alpha-diversity was measured by using Chao-1, Shannon, and Simpson indices. In order to measure the differences in the community composition between groups, we measured the beta-diversity by using the Bray-Curtis dissimilarity index visualized by principal component analysis.

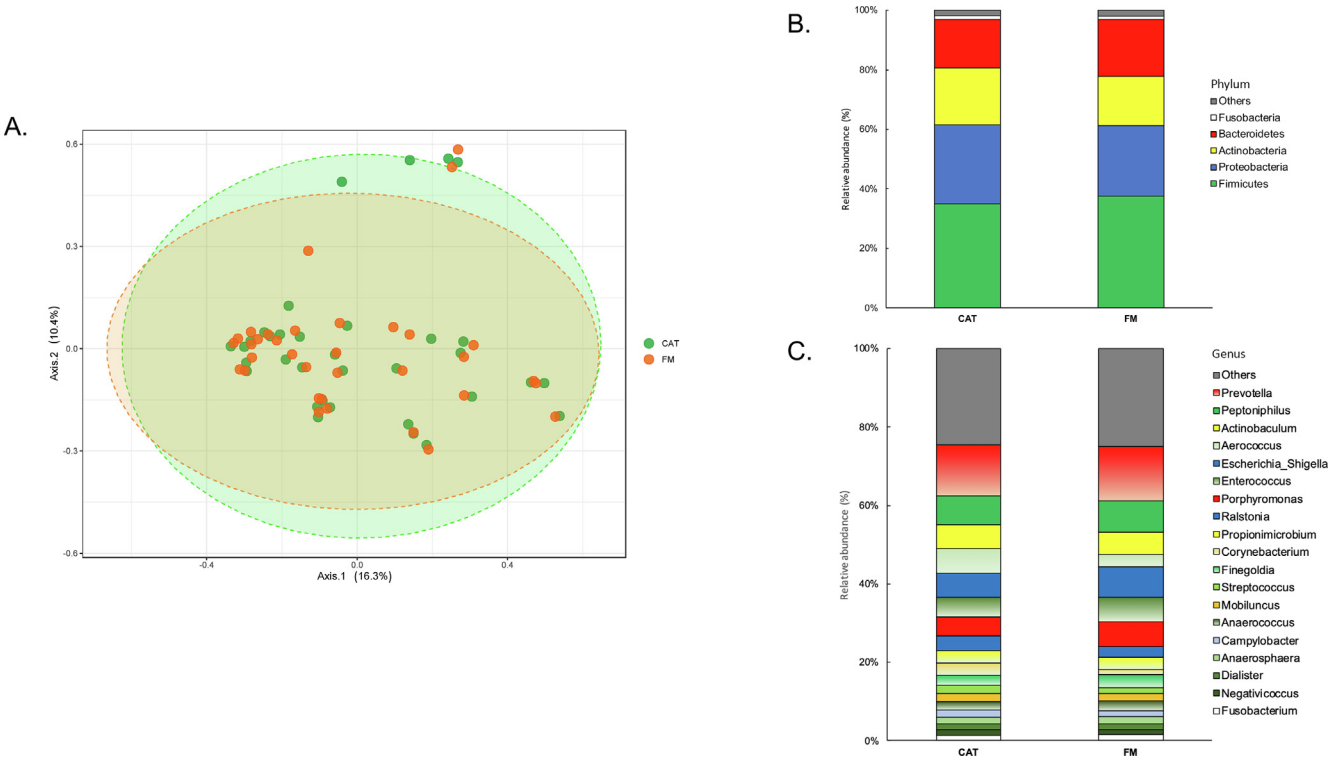
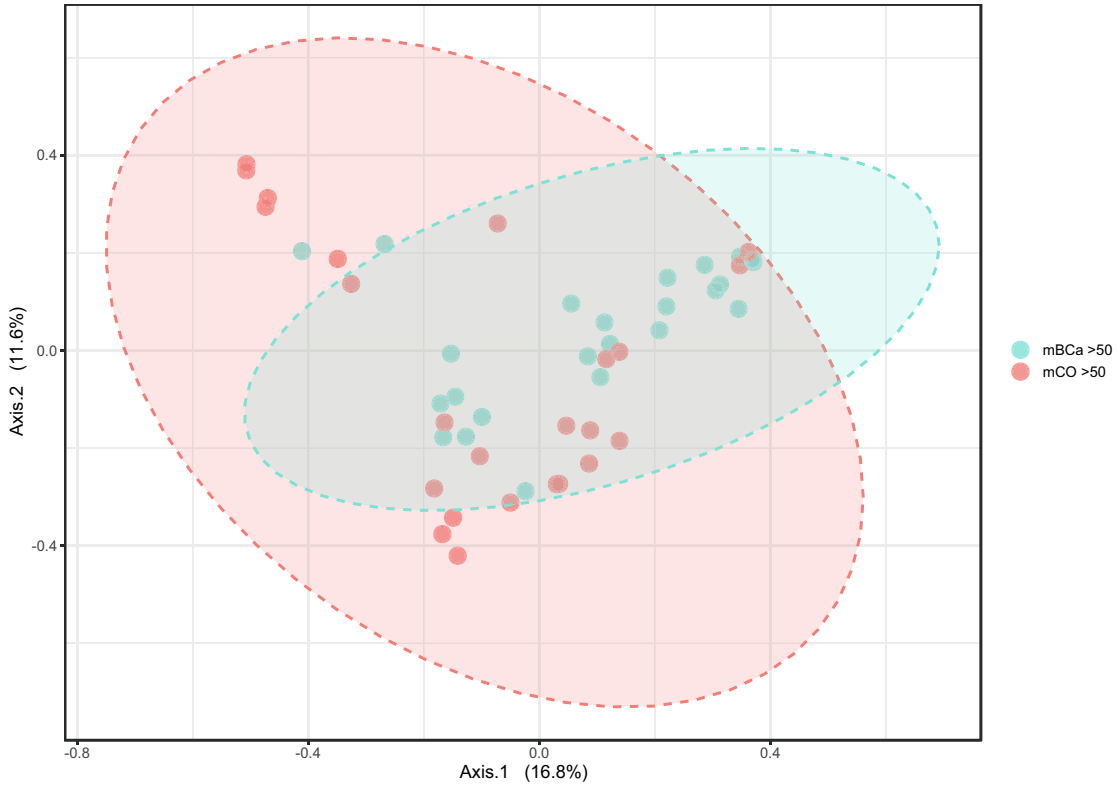
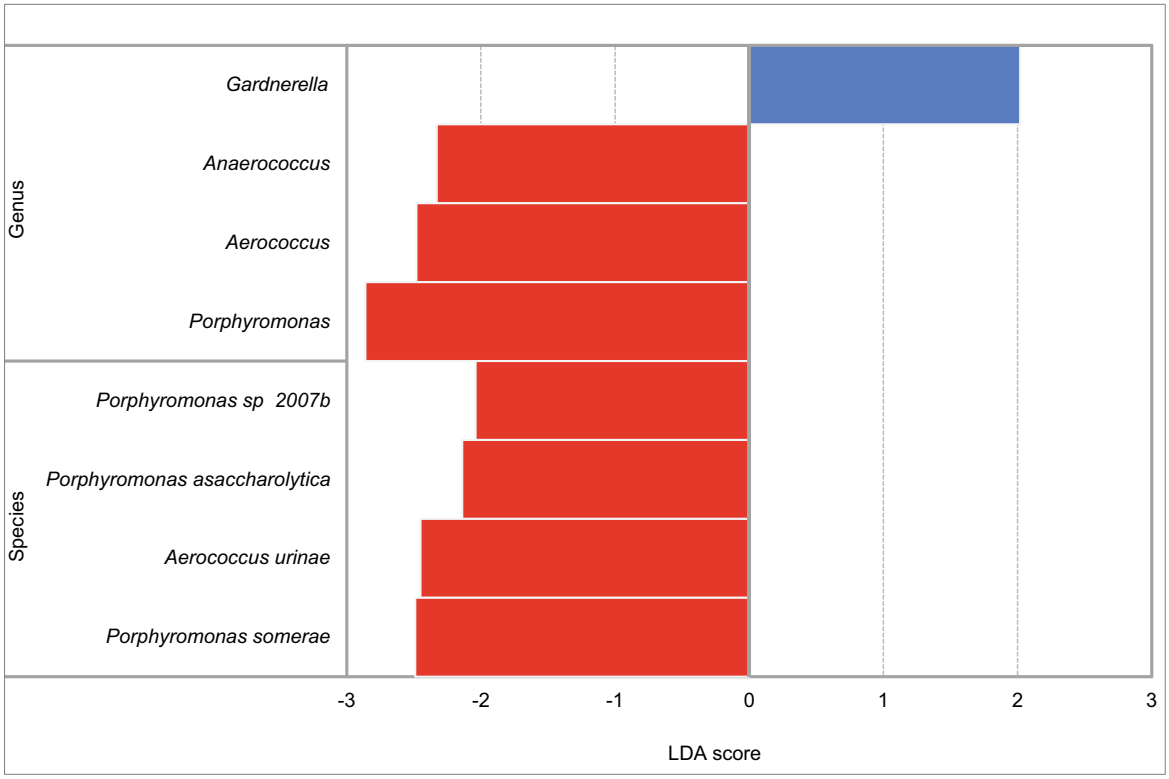


Fig. 1 – A comparison of microbiome profile between CAT and FM urine samples. (A) Principal component analysis evaluated by the Bray-Curtis distance measured the beta-diversity of the bacterial community identified in CAT (green dots) and FM (orange dots) samples. The PERMANOVA test indicated a not significantly different match between the two groups. (B) Phyla with an average relative abundance of >1% in CAT and FM samples are shown: Firmicutes (34.85% vs 37.62%), Proteobacteria (26.78% vs 23.65%), Actinobacteria (19.02% vs 16.60%), Bacteroidetes (16.30% vs 18.99%), Fusobacteria (1.33% vs 1.12%), and other phyla (1.72% vs 2.01%). (C) Genera with an average relative abundance of >1% in CAT and FM samples. CAT = catheterized; FM = first morning; PERMANOVA = permutational multivariate analysis of variance.

A.


0.4
0.0
-0.4
Axis.2 (11.6%)
-0.8
-0.4
0.0
0.4
Axis.1 (16.8%)
mBCa >50
mCO >50

B.


Genus
Gardnerella
Anaerococcus
Aerococcus
Porphyromonas
Species
Porphyromonas sp 2007b
Porphyromonas asaccharolytica
Aerococcus urinae
Porphyromonas somerae
-3
-2
-1
0
1
2
3
LDA score

For a data comparison analysis, we chose a bioinformatic approach that combines the DESeq2 and linear discriminant analysis (LDA) effect size (LEfSe) tests, with no rarefaction and no normalization. A differential abundance analysis was executed using DESeq2 at each taxonomic level, and only taxa with an adjusted p value of <0.05 by false discovery rate were reported. In addition, we performed the LEfSe test to identify possible biomarkers at every taxonomic level. These biomarkers are microbial taxa that differ in abundance between groups, as identified by a Wilcoxon rank-sum test (adjusted $p < 0.05$). To estimate the effect size of each biomarker, the threshold on the logarithmic LDA score for discriminative features was set to 2.0.

The raw NGS data have been deposited in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under project PRJNA981420.

3. Results

3.1. Sequencing data results

We sequenced a total of 142 samples (FM, n = 48; CAT, n = 34; CO, n = 43; and PK, n = 17). The average of read counts per sample was 20 619 (range: 1263–59 419). Taxa assignment was achieved at all taxonomic levels (from phylum to species) with an average percentage of "unassigned species" of 30.58% in all samples. In all the runs, the sequencing of positive standard controls detected similar percentages of taxa, with few differences in the percentages provided by the Gut Microbiome genomic Mix ATCC MSA-1006—ATCC datasheet, confirming good technical and experimental repeatability. In each run, the internal blank negative control confirmed the absence of contaminants except for *Propionibacterium acnes* and *Sphyngomonas sp oral clone AV069* species, identified as contaminants (mean absolute abundance: 298 and 153, respectively) and excluded from the bioinformatic analysis.

3.2. FM urine is a suitable sample for assessing the urobiome

The microbial composition of CAT and FM urine samples from 34 patients undergoing TURBT was compared. Any significant intragroup differences were revealed by alpha-diversity. Beta-diversity showed strong similarity of community composition between the CAT and FM groups (Fig. 1A). The main phyla identified with relative abundance >1% in both samples were, respectively, Firmicutes (34.85% vs 37.62%), Proteobacteria (26.78% vs 23.65%), Actinobacteria (19.02% vs 16.60%), Bacteroidetes (16.30% vs 18.99%), Fusobacteria (1.33% vs 1.12%), and other phyla (1.72% vs 2.01%; Fig. 1B). The main genera identified with relative abundance >1% in both samples are shown in Figure 1C. No difference in bacterial abundance was detected by the DESeq2 analysis at all taxonomic levels (data not shown). Based on this result, we used the FM urine sample to characterize the urobiome in patients undergoing TURBT.

3.3. TURBT patients showed a different urobiome from cancer-free controls

We compared the FM urine microbiome of patients undergoing TURBT (TURBT group, n = 48) with that of cancer-free controls (CO group, n = 43). Despite the alpha-diversity result not being significant, beta-diversity showed a statistically different microbial community between the two groups (permutational multivariate analysis of variance [PERMANOVA], p = 0.001; Supplementary Fig. 1). The differential abundance analysis revealed several increased taxa in the TURBT group with respect to the CO group at family, genus, and species levels (Supplementary Table 1). At genus level, we found that *Actinobaculum* (4.68% vs 1.72%), *Mobilincus* (1.19% vs 0.50%), *Peptoniphilus* (6.38% vs 2.59%), *Porphyromonas* (3.69% vs 0.37%), and *Propionimicrobium* (3.39% vs 0.92%). Conversely, *Lactobacillus* (3.92% vs 10.29%) and *Streptococcus* (2.32% vs 6.17%) were significantly increased in the CO group. At species level, we detected a significant abundance of *Actinobaculum schaalii* (3.06% vs 0.39%), *Porphyromonas somerae* (1.54% vs 0.06%), and *Propionimicrobium lymphophilum* (2.82% vs 0.66%), and a decrease of *Lactobacillus iners* (1.83% vs 7.33%) in TURBT patients (Supplementary Table 1). These results confirmed that TURBT patients have a different urobiome from controls.

3.4. Urobiome of mBCa was specifically characterized by Porphyromonas and P. somerae

Among all patients undergoing TURBT, BCa diagnosis was histologically confirmed in 30 patients, all of whom were over 50 yr old and mainly male patients. Therefore, we focused our urobiome investigation on this subgroup (indicated as mBCa >50 yr; n = 25). Male cancer-free individuals over 50 yr old were used as the control group (mCO >50 yr; n = 24). The measure of beta-diversity showed a significant distance between bacterial communities of the two groups (PERMANOVA, p = 0.001; Fig. 2A). Applying the same combined bioinformatic approach (DESeq2 and LEfSe tests), mBCa >50 yr patients were characterized by an increased relative abundance of *Aerococcus* (3.34% vs 0.28%), *Anaerococcus* (3.21% vs 0.75%), and *Porphyromonas* (5.56% vs 0.20%) genera; on the contrary, a decrease of the *Gardnerella* (0.65% vs 3.23%) genus was observed (Table 1). At species level, the mBCa >50 yr group showed a significant increase of *Aerococcus urinae* (3.08% vs 0.25%), *Porphyromonas asaccharolytica* (1.20% vs 0.09%), *P. somerae* (2.21% vs 0.02%),

Fig. 2 – Community profiling of the urobiome in male BCa (mBCa) patients compared with age- and sex-matched cancer-free controls (mCO). (A) Beta-diversity of bacteria identified in the FM urine microbiome of the mBCa >50 yr group with respect to the mCO >50 yr group. Principal component analysis using the Bray-Curtis distance measure was represented, and the results of the PERMANOVA test indicated a significantly different bacterial composition between two groups: mBCa >50 yr (blue dots) and mCO >50 yr (red dots; p = 0.001). (B) The linear discriminant analysis effect size analysis showing taxa differently abundant (with cutoff LDA score >2 and <–2) at genus and species taxonomic levels, between the mBCa >50 yr and mCO >50 yr groups. Negative and positive LDA scores indicated, respectively, an increased (blue bars) or a decreased (red bars) abundance of bacteria in the mBCa >50 yr and mCO >50 yr groups. BCa = bladder cancer; FM = first morning; LDA = linear discriminant analysis; PERMANOVA = permutational multivariate analysis of variance.

Table 1 – Differential abundance analysis (tested with both the DESeq2 and the LEfSe test) of taxa between male BCa patients older than 50 yr (mBCa >50 yr) and male cancer-free controls older than 50 yr (mCO >50 yr) in FM urine samples

Level	Taxa[a]	Relative abundance (%)		DESeq2	LEfSe	
		mBCa >50 yr (n = 25)	mCO >50 yr (n = 24)	Adjusted p value	Adjusted p value	LDA score
Class	Betaproteobacteria	9.88	0.10	<0.001	0.028	−2.68
	Clostridia	21.93	9.61	0.010	0.048	−3.32
	Gammaproteobacteria	12.79	28.22	0.004	0.028	3.08
Order	Bifidobacteriales	0.68	3.33	0.013	0.011	2.02
	Burkholderiales	9.64	0.05	<0.001	0.017	−2.68
	Clostridiales	21.93	9.61	0.009	0.049	−3.32
Family	Aerococcaceae	3.54	0.29	0.001	0.009	−2.51
	Bifidobacteriaceae	0.68	3.33	0.009	0.009	2.02
	Burkholderiaceae	9.64	0.05	<0.001	0.002	−2.67
	Porphyromonadaceae	6.51	0.32	<0.001	0.005	−2.91
Genus	*Aerococcus*	3.34	0.28	0.007	0.048	−2.48
	Anaerococcus	3.21	0.75	0.016	0.026	−2.33
	Gardnerella	0.65	3.23	0.008	0.012	2.02
	Porphyromonas	5.56	0.20	<0.001	0.003	−2.86
Species	*Aerococcus urinae*	3.08	0.25	<0.001	0.020	−2.45
	Porphyromonas asaccharolytica	1.20	0.09	0.030	0.014	−2.14
	Porphyromonas somerae	2.21	0.02	<0.001	0.003	−2.49
	Porphyromonas sp 2007b	1.04	0.01	<0.001	0.003	−2.04

BCa = bladder cancer; FM = first morning; LDA = linear discriminant analysis; LEfSe = LDA effect size.
[a] Taxa with relative mean abundance of >1% in at least one group (from class to species) were reported when statistically significant at both the DESeq2 and the LEfSe test (p < 0.05). Only taxa with an LDA score of >2.0 threshold were reported.

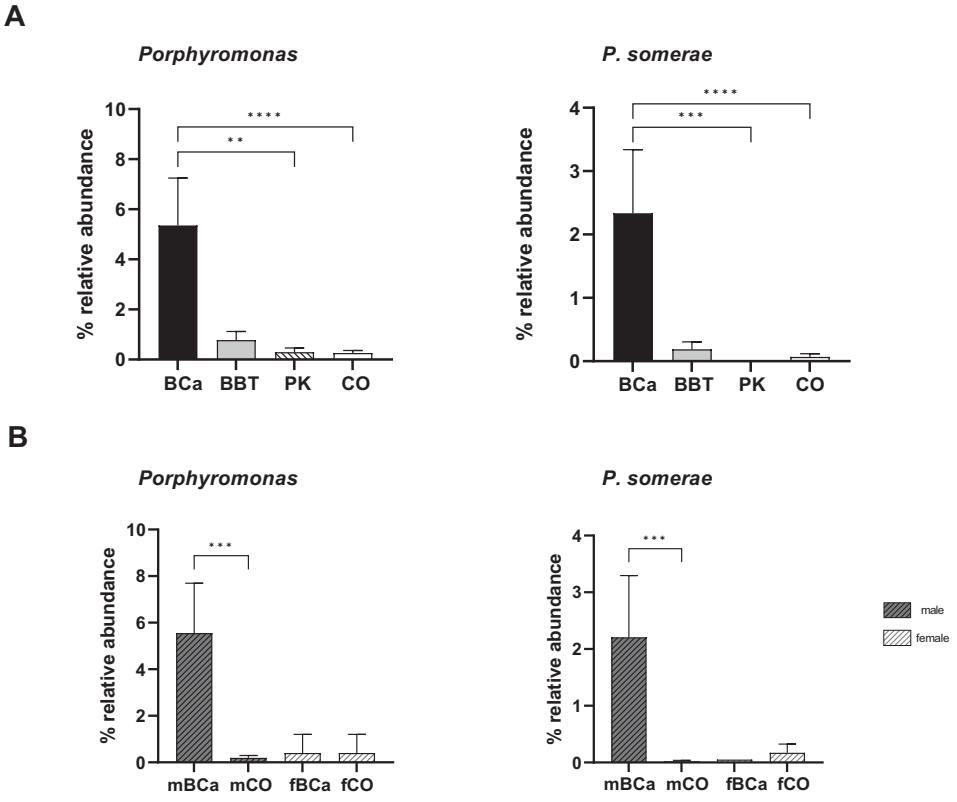


Fig. 3 – Relative abundance of *Porphyromonas* (left) and *Porphyromonas somerae* (right). (A) Comparison of the relative abundance of *Porphyromonas* and *P. somerae* between BCa patients and other three different conditions: benign bladder tumor (BBT), prostate cancer (PK), and cancer-free controls (CO). (B) Comparison of the relative abundance of *Porphyromonas* and *P. somerae* between BCa and CO divided in male (mBCa and mCO, gray striped) and female (fBCa and fCO, white striped) subgroups. All the individuals involved were aged >50 yr. Statistical significance tested with Mann-Whitney *U* test is indicated by the asterisks (**p < 0.0001, ***p < 0.001, and *p < 0.05). BCa = bladder cancer.**

and *Porphyromonas sp 2007b* (1.04% vs 0.01%; Table 1). In Figure 2B, we exclusively reported the bacterial taxa at genus and species levels differentially abundant with an LDA score greater than the threshold set. These data indicated that mBCa >50 yr patients present a particular urobiome, where *Porphyromonas* is definitively more abundant.

In order to confirm whether the increased *Porphyromonas* abundance was actually associated with the BCa

condition, we decided to compare the urobiome of BCa patients (named BCa, n = 30) with three age-matched control groups: prostate cancer group (named PK, n = 17), BBT group (named BBT, n=15), and finally CO group (named CO, n = 35; Fig. 3A). Specifically, BCa showed an increased relative abundance of *Porphyromonas* and *P. somerae* compared with PK ($p < 0.01$ and $p < 0.001$, respectively) and CO ($p < 0.0001$ for both). A similar trend was also observed when comparing BCa with BBT, but the statistical test result was not significant. In particular, in Figure 3B, we observed that the increased abundance of *Porphyromonas* and *P. somerae* was exclusively associated with mBCa patients (n = 25). In fact, the mBCa group showed an increased relative abundance of *Porphyromonas* and *P. somerae* when compared with the mCO subgroup (n = 24, $p < 0.001$ for both). Therefore, we can definitively state that an increase in *Porphyromonas* and *P. somerae* abundance is specific for bladder tumors in male gender. The low number in the female group did not allow for a statistical comparison. Additional analyses will be required to determine their specificity for mBCa and their possible role in the pathogenesis.

4. Discussion

In the present study, we first demonstrated that FM urine is a suitable sample for the analysis of the urinary microbiome, and that no differences were recorded between such samples and CAT urine. This observation supports a general use of FM urine for a urobiome analysis; in fact, standardization of procedures is a relevant issue to compare data obtained from different laboratories that are, at times, discordant. Additional methodological studies on urine collection, and samples processing and storage are required to obtain comparable clinical data. Therefore, in the present study, in order to standardize the analysis reducing the impact of the sample collection variability [16], we used an FM urine sample to compare microbiome data in (1) BCa, (2) PK, and (3) cancer-free individuals. The study revealed that urine samples from mBCa patients were characterized by a peculiar urobiome with an increased abundance of several taxa including *Porphyromonas*.

Despite the association between the urinary microbiome and BCa having become increasingly evident [16], conflicting data have been reported so far, even due to different sample types. In fact, Oresta et al. [20] identified microbial differences between catheterized urine, midstream-voided urine, and bladder washout samples in BCa patients. In particular, an increased abundance of *Veillonella* and *Corynebacterium*, and a decrease of *Ruminococcus* were found in catheterized urine samples from patients with BCa as compared with controls. Compared with catheterized urines, BCa washouts showed specific increases of some taxa, such as *Burkholderiaceae*, whereas midstream urine was enriched with *Streptococcus*. In the same way, Bukavina et al. [17] demonstrated that catheterized urine samples showed lower genera variability combined with a lower contamination than voided urine samples. Thus, to explore the urobiome, many scientists prefer the use of urine samples from a urethral catheter [21]. However, although catheterization avoids the potential contamination by lower urinary tract bacteria, it is an invasive procedure responsible for possible urinary tract infections and is more difficult to be used in a routine setting [21,22]. Since we observed strong similarity of bacterial communities between CAT and FM urine samples, we decided to use the FM urine, a more manageable and accessible specimen.

Therefore, we started a multistep study, first comparing all TURBT patients with all cancer-free controls, revealing a significantly different urobiome. Then, we enucleated BCa from the group of TURBT patients. Considering that gender is a well-known confounding factor that can affect microbiome composition, we focused our investigation on male patients because BCa is more frequent in men [23]. Furthermore, since all male patients were aged >50 yr, we selected cancer-free controls aged >50 yr to exclude age as an impacting factor. Interestingly, in mBCa patients, we found a peculiar microbial composition with an increased abundance of *Porphyromonas*, *Aerococcocus*, and *Anaerococcus* genera. In particular, at species level, we observed the increase of three *Porphyromonas* spp., among which *P. somerae* prevailed. Such urobiome signature seems to be peculiar to BCa, since we did not observe an increase in this bacterial species in FM from other control groups (including prostate cancer patients or cancer-free individuals); however, an increase in *P. somerae* abundance has not been reported in BCa patients analyzed in other studies [11,16].

Porphyromonas is a Gram-negative, obligatorily anaerobic, non–spore-forming, and nonmotile bacterial genus. Several *Porphyromonas* species have already been described to be associated with pathologies in humans: *Porphyromonas gingivalis*, a well-known anaerobe bacterium of the oral cavity, has been involved in the pathogenesis of periodontitis [24], and *P. somerae* has recently been associated with endometrial cancer [25]. *P. somerae* has been described as capable of intracellular invasion of endometrial cancer cells increasing succinate levels, leading to upregulation of HIF-1alpha [25–27]. More specifically, *P. somerae*, invading cells, and increasing succinate can interfere with the normal functioning of host cells, favoring chronic host inflammation in the endometrial tissue. This process may contribute to the onset and progression of cancer by remodeling of extracellular matrix (ECM) and generating oxygen radicals [25]. Furthermore, also *P. gingivalis* is responsible for ECM modification, tight junction disruption, and tissue damage [24]. Considering the genetic similarity between *P. gingivalis* and other *Porphyromonas* species, we may speculate that *P. somerae* may similarly induce host epithelial cell damage, favoring inflammation and triggering bladder carcinogenesis. In the present study, we did not analyze the potential role of *P. somerae* in BCa pathogenesis, but the observed increase in abundance, specific in these patients, encourages us to hypothesize its possible role in BCa onset and/or progression.

The peculiar increase of *Porphyromonas* that we observed in urine from BCa patients may also depend on the higher number of male patients in our study group; in fact, the dif-

ference in the urobiomes of BCa male and female patients is well established [23,28]. Our observations may also be influenced by the geographical area from which our patients have been recruited compared with the data obtained in previous different studies [29], since it is well known that ethnic groups or geographical areas influence microbiome greatly [30]. However, given the strict correlation we found between BCa in male patients and *Porphyromonas*, similar studies in other populations should be performed.

A strength of our study was the choice to simultaneously sequence the three hypervariable regions V3, V4, and V6 of the 16S rRNA gene, with respect to the more frequent use of V3 or V4 regions. Furthermore, the use of a combined pipeline exploiting two different statistical tests (DESeq2 and LEfSe) allowed us to better identify differences in the urobiome of BCa patients. On the contrary, our study is not devoid of limitations. The first limitation consists in the low biomass of urinary microbiota [21], which forced us to exclude several samples due to low DNA extraction yield. A second limit consists in the possible presence of contaminants that could significantly alter the results due to the low biomass of urine samples. To try to overcome this problem, at least in all the analytical steps, we added blank samples from extraction to sequencing, and we eliminated the contaminants from the data analysis.

5. Conclusions

In conclusion, we propose FM urine as an advantageous alternative and less expensive sample as compared with CAT for urobiome profiling. Furthermore, we suggest that *Porphyromonas* could be a possible biomarker to identify patients with an increased BCa risk. Further investigations on larger cohorts of BCa patients are required to confirm such data and clarify the possible pathogenic role of *Porphyromonas* in the development and/or progression of BCa.

Author contributions: Giuseppe Castaldo had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Nardelli, Castaldo, Pastore.
Acquisition of data: Aveta, Pandolfo, Imbimbo.
Analysis and interpretation of data: Nardelli, Tripodi, Russo.
Drafting of the manuscript: Nardelli, Tripodi, Russo, Castaldo, Pastore.
Critical revision of the manuscript for important intellectual content: Nardelli, Castaldo, Pastore.
Statistical analysis: Nardelli, Tripodi, Russo.
Obtaining funding: None.
Administrative, technical, or material support: None.
Supervision: Castaldo, Pastore, Imbimbo.
Other: None.

Financial disclosures: Giuseppe Castaldo certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: None.

Data sharing: The dataset supporting the results of this article was deposited in the SRA under BioProject accession code PRJNA981420.

Ethics statement: All the enrolled patients gave their informed consent to participate in the study, according to the Helsinki Declaration 2013. The study was approved by the Ethics Committee of the "Università degli Studi di Napoli Federico II" (authorization no. 191/20).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euros.2023.11.003.

References

[1] Dekaboruah E, Suryavanshi MV, Chettri D, Verma AK. Human microbiome: an academic update on human body site specific surveillance and its possible role. Arch Microbiol 2020;202:2147–67.
[2] Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet 2012;13:260–70.
[3] Granata I, Nardelli C, D'Argenio V, et al. Duodenal metatranscriptomics to define human and microbial functional alterations associated with severe obesity: a pilot study. Microorganisms 2020;8:1811.
[4] Sacchetti L, Nardelli C. Gut microbiome investigation in celiac disease: from methods to its pathogenetic role. Clin Chem Lab Med 2020;58:340–9.
[5] Nardelli C, Granata I, Nunziato M, et al. 16S rRNA of mucosal colon microbiome and CCL2 circulating levels are potential biomarkers in colorectal cancer. Int J Mol Sci 2021;22:10747.
[6] Nardelli C, Gentile I, Setaro M, et al. Nasopharyngeal microbiome signature in COVID-19 positive patients: can we definitively get a role to *Fusobacterium periodonticum*? Front Cell Infect Microbiol 2021;11:625581.
[7] Nardelli C, Scaglione GL, Testa D, et al. Nasal microbiome in COVID-19: a potential role of *Corynebacterium* in anosmia. Curr Microbiol 2023;80:53.
[8] Shoemaker R, Kim J. Urobiome: an outlook on the metagenome of urological diseases. Investig Clin Urol 2021;62:611–22.
[9] 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–e1314.
[10] 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;129:500–6.
[11] Popović VB, Šitum M, Chow C-E-T, Chan LS, Roje B, Terzić J. The urinary microbiome associated with bladder cancer. Sci Rep 2018;8:12157.
[12] Porter CM, Shrestha E, Peiffer LB, Sfanos KS. The microbiome in prostate inflammation and prostate cancer. Prostate Cancer Prostatic Dis 2018;21:345–54.
[13] Cumberbatch MGK, Jubber I, Black PC, et al. Epidemiology of bladder cancer: a systematic review and contemporary update of risk factors in 2018. Eur Urol 2018;74:784–95.
[14] Andolfi C, Bloodworth JC, Papachristos A, Sweis RF. The urinary microbiome and bladder cancer: susceptibility and immune responsiveness. Bladder Cancer 2020;6:225–35.
[15] Modena BD, Milam R, Harrison F, 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 2017;17:712–23.

[16] Hussein AA, Elsayed AS, Durrani M, et al. Investigating the association between the urinary microbiome and bladder cancer: an exploratory study. Urol Oncol 2021;39:370.e9–e19.
[17] Bukavina L, Isali I, Ginwala R, et al. Global meta-analysis of urine microbiome: colonization of polycyclic aromatic hydrocarbon-degrading bacteria among bladder cancer patients. Eur Urol Oncol 2023;6:190–203.
[18] Babjuk M, Burger M, Capoun O, et al. European Association of Urology guidelines on non–muscle-invasive bladder cancer (Ta, T1, and carcinoma in situ). Eur Urol 2022;81:75–94.
[19] Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 2020;15:799–821.
[20] Oresta B, Braga D, Lazzeri M, et al. The microbiome of catheter collected urine in males with bladder cancer according to disease stage. J Urol 2021;205:86–93.
[21] Perez-Carrasco V, Soriano-Lerma A, Soriano M, Gutiérrez-Fernández J, Garcia-Salcedo JA. Urinary microbiome: yin and yang of the urinary tract. Front Cell Infect Microbiol 2021;11:617002.
[22] Wolfe AJ, Brubaker L. "Sterile urine" and the presence of bacteria. Eur Urol 2015;68:173–4.
[23] Bilski K, Dobruch J, Kozikowski M, Skrzypczyk MA, Oszczudłowski M, Ostrowski J. Urobiome in gender—related diversities of bladder cancer. Int J Mol Sci 2020;21:4488.
[24] Su W, Shi J, Zhao Y, Yan F, Lei L, Li H. *Porphyromonas gingivalis* triggers inflammatory responses in periodontal ligament cells by succinate-succinate dehydrogenase–HIF–1α axis. Biochem Biophys Res Commun 2020;522:184–90.
[25] Crooks TA, Madison JD, Walsh DM, et al. *Porphyromonas somerae* invasion of endometrial cancer cells. Front Microbiol 2021;12:674835.
[26] Walther-António MRS, Chen J, Multinu F, et al. Potential contribution of the uterine microbiome in the development of endometrial cancer. Genome Med 2016;8:122.
[27] Walsh DM, Hokenstad AN, Chen J, et al. Postmenopause as a key factor in the composition of the endometrial cancer microbiome (ECbiome). Sci Rep 2019;9:19213.
[28] Mansour B, Monyók Á, Makra N, et al. Bladder cancer-related microbiota: examining differences in urine and tissue samples. Sci Rep 2020;10:11042.
[29] Zeng J, Zhang G, Chen C, et al. Alterations in urobiome in patients with bladder cancer and implications for clinical outcome: a single-institution study. Front Cell Infect Microbiol 2020;10:555508.
[30] Gupta VK, Paul S, Dutta C. Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. Front Microbiol 2017;8:1162.