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EXAMINATION OF TISSUE AND URINE MICROBIOME COMPOSITION AND HUMAN BETA-DEFENSIN PRODUCTION IN BLADDER CARCINOMA PATIENTS

PhD thesis

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Abbreviations

AJCC	American Joint Committee on Cancer
BC	Bladder cancer
BCG	Bacillus Calmette-Guérin
BPH	Benign prostatic hyperplasia
CFU	Colony forming unit
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CRC	Colorectal Carcinoma
$\Delta\Delta Ct$	Double delta Ct method
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
ELISA	Enzyme linked immunosorbent assay
EQUC	Enhanced Quantitative Urine Culture
ERBT	En Bloc Resection of Bladder Tumor
FDA	US Food and Drug Administration
FGFR3	Fibroblast growth factor receptor 3
G1-G3	Grade1-Grade3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hBD	Human beta-defensin
HG	High grade
HV	Healthy volunteer
IQR	Interquartile Range
ITS	Internal transcribed spacer
LG	Low grade
MFUTH	Markhot Ferenc University Teaching Hospital
MIBC	Muscle-invasive Bladder Cancer
NGS	New Generation Sequencing
NMIBC	Non-muscle-invasive Bladder Cancer
OS	Overall Survival
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death 1
PD-L1	Programmed Cell Death Ligand 1

ProtK	Proteinase K Enzyme
PUNLMP	Papillary Urothelial Neoplasm of Low Malignant Potential
RAS	„Rat sarcoma” common oncogen family
RB1	Retinoblastoma 1
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
RQ	Relative expression
TI1-4	Tissue sample from muscle-invasive bladder cancer 1-4
Tis	Tumor (carcinoma) in situ
TN1-6	Tissue sample from non-muscle-invasive bladder cancer 1-6
TP53	Tumor protein 53
TUR	Transurethral resection
TURBT	Transurethral resection of bladder tumor
UI1-4	Urine sample from muscle-invasive bladder cancer 1-4
UN1-6	Urine sample from non-muscle-invasive bladder cancer 1-6
WHO	World Health Organization

1. Introduction

1.1. Epidemiology

Bladder cancer (BC) ranks as the tenth most frequently diagnosed cancer worldwide (1). Considering both genders, BC accounts for 3% of diagnosed tumors, while it is included in 2.1% of cancer deaths (2). Among men, the incidence of the tumor and the mortality rate due to it are on average four times more common than in women, but the incidence greatly varies in the different regions of the world. **Figure 1** shows that the incidence is the highest in the Southern European region of the world, but when examining the countries individually, the data provided for Greece is outstanding among men, while Hungary has the highest incidence among women (3) in Eastern Europe. The risk of BC evidently increases with age; the age-specific curve rises sharply above the age of fifty (3).

Based on the depth of tumor infiltration, BC is divided into non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). About three-fourths of new BC cases are NMIBC at their initial diagnosis. Generally, patients with NMIBC have a better prognosis than those with MIBC. Previous studies reported that the five-year and ten-year overall survival (OS) rates of MIBC patients were about 50% and 36%, respectively, even with aggressive management. Moreover, the five-year OS rate of metastatic diseases was only 15% (4).

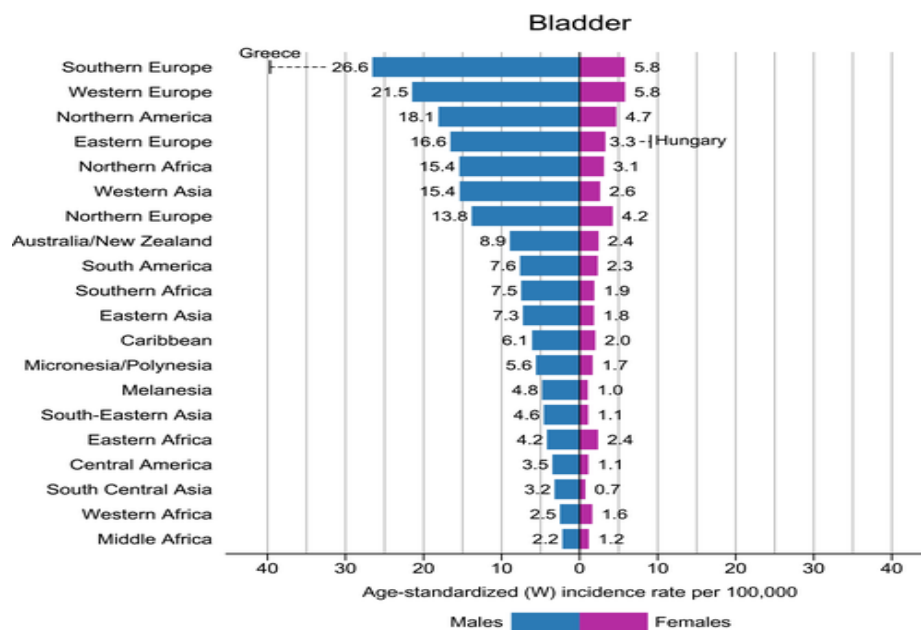


Figure 1: Region-specific and age-standardized incidence rates by gender for BC in 2020 (3).

1.2. Etiopathogenesis and Risk Factors

The pathogenesis of BC is complex and multifactorial either internal, genetic, or external, environmental factors are investigated. Exogenous exposure of the respiratory system, gastrointestinal tract, or skin to carcinogens contributes to the development of BC. The circulating carcinogenic compounds and their metabolites are excreted through the kidneys and spend a shorter or longer time in the urine collected in the bladder. The main source of carcinogenic compounds is tobacco smoke, but the carcinogenic effect of compounds that appear in the natural environment or in workplaces is also important (5, 6). Smoking is a significant factor associated with BC; its harmful effects increase with the intensity and duration of smoking, and this is also true for passive smoking. Tobacco is a rich source of carcinogenic compounds, including polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines, and N-nitroso compounds (7-10). Exposure at work to aromatic amines, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons is the second most important risk factor for BC; exposure to the above compounds can be detected in 10% of diagnosed tumors (11, 12). The role of workplace and environmental pollutants can explain why BC is three times more frequent in industrialized nations than in economically less developed countries (13).

Several nutritional habits, drinking water composition, and alcohol consumption habits have also been investigated for their relationship with the development of BC. Higher vitamin D serum levels have been shown to protect against the development of BC (14). No correlation between regular alcohol consumption and the incidence of BC has been proven (15). However, arsenic in drinking water is a recognized cause of BC, and other drinking water contaminants, such as disinfection byproducts (chlorination) and trihalomethanes, have also been shown to increase the risk of BC (16).

Inherited genetic predisposition plays a role only in 7% of BC cases (17). The best known inherited genetic association with BC has been described as polymorphisms in two detoxification genes, namely, N-acetyltransferase 2 (NAT2) and Glutathione S-transferase μ 1 (GSTM1) (18, 19). NAT2 is a key enzyme in aromatic amine metabolism, and “slow acetylators” undergoing heavy exposure to arylamines and related carcinogens are more likely to develop BC than “rapid acetylators” (20, 21). GSTM1 catalyzes the conjugation of the cellular tripeptide glutathione with a number of electrophilic compounds, including chemical carcinogens or environmental pollutants. Insufficient, low-level GSTM1 function is significantly associated with poor overall survival in BC patients (22).

Increasing evidence suggests that a history of diabetes mellitus (DM) may be associated with an increased risk of bladder cancer (23, 24). Not only the metabolic disease itself but also the drugs used to treat it, such as metformin, rosiglitazone, or pioglitazone, are closely related to the prognosis of BC. In contrast to metformin, which may reduce bladder cancer risk in patients with type 2 diabetes, patients who used a higher dose of pioglitazone or rosiglitazone had a higher incidence of bladder cancer (25-27).

Microbes that cause infection and inflammation in the bladder and those that are present without symptoms also affect the development of bladder cancer. Infection caused by the protozoan *Schistosoma haematobium* is one of the most important players in microbial tumorigenesis due to continuous irritation of the bladder wall and epithelium (28). Cystitis, which occurs much more often in women, can be explained by anatomical conditions favorable to ascending infection. The possibility of pathogenic or commensal microbes ascending to the bladder differs between the two genders. The different composition of the asymptotically present microbiome may result in the difference in BC incidence between the genders (29, 30). The interaction of the chemicals excreted in the urine with the microbes present in the bladder can lead to the neutralization of carcinogenic substances or the formation of harmful metabolites. Both bacteria and their metabolites present in the bladder are one of the many contributors leading to the development and progression of BC (31).

1.3. Staging and Grading of BC

Bladder cancer is divided into non-muscular-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), according to the European Association of Urology (EAU) guidelines. Bladder cancer can also be divided into several histological types – urothelial carcinoma, squamous epithelial carcinoma, and adenocarcinoma –, among which over 90% are urothelial carcinomas (32). Currently, the following differentiations of urothelial carcinoma are used:

1. urothelial carcinoma (more than 90% of all cases);
2. urothelial carcinomas with partial squamous and/or glandular or trophoblastic differentiation;
3. micropapillary urothelial carcinoma;
4. nested variant (including a large nested variant) and microcystic urothelial carcinoma;

5. plasmacytoid, giant cell, signet ring, diffuse, undifferentiated;
6. lymphoepithelioma-like;
7. small-cell carcinomas;
8. sarcomatoid urothelial carcinoma;
9. neuroendocrine variant of urothelial carcinoma;
10. some urothelial carcinomas with other rare differentiations (1).

In clinical practice, knowledge of tumor stage and histological grading is essential for planning treatment and determining prognosis. The American Joint Committee on Cancer (AJCC) Staging Manual, 8th edition, should be the basis for staging urinary bladder tumors (33). Changes and clarifications in the TNM categories are included in **Table 1** based on the review article of Magers et al. (34).

Table 1. TNM classification of bladder cancer (31)

T Primary Tumor	
T _x	primary tumor cannot be assessed
T ₀	no evidence of primary tumor
T _a	non-invasive papillary carcinoma
T _{is}	carcinoma in situ/ flat tumor
T ₁	tumor invades subepithelial connective tissue
T ₂	tumor invades muscle
T _{2a}	tumor invades superficial muscle (inner half)
T _{2b}	tumor invades deep muscle (outer half)
T ₃	tumor invades perivesical tissue
T _{3a}	microscopically
T _{3b}	macroscopically (extravesical mass)
T ₄	tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T _{4a}	tumor invades the prostate stroma, seminal vesicles, the uterus, or the vagina
T _{4b}	tumor invades the pelvic wall or the abdominal wall
N Regional Lymph Nodes	
N _x	regional lymph nodes cannot be assessed
N ₀	no regional lymph nodes metastasis
N ₁	metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac or presacral)
N ₂	metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac or presacral)
N ₃	metastasis in common iliac lymph node(s)
M Distant metastasis	
M ₀	no distant metastasis
M _{1a}	non regional lymph nodes
M _{1b}	other distant metastasis

Over the past forty years, various bladder tumor-grading systems have been introduced, which have generally been well received. The first and most widely accepted system is the 1973 WHO (World Health Organization) system described by Mostofi et al. (35). Based on the atypia and architectural arrangement of the cells, tumors are classified as G1-G2-G3 severity grades. However, the currently recommended system is the WHO's new 2016 classification system, which separates papillary urothelial neoplasms (PUNLMP) of low malignant potential into low grade and high grade tumors (36). However, the 4-grade combination of the two classification systems (LG/G1, LG/G2, HG/G2, and HG/G3) proved to be better than the other classification systems individually. **Figure 2** shows that the combined grading system divided the large group of G2 patients into two subgroups with different prognoses (LG/HG) (37).

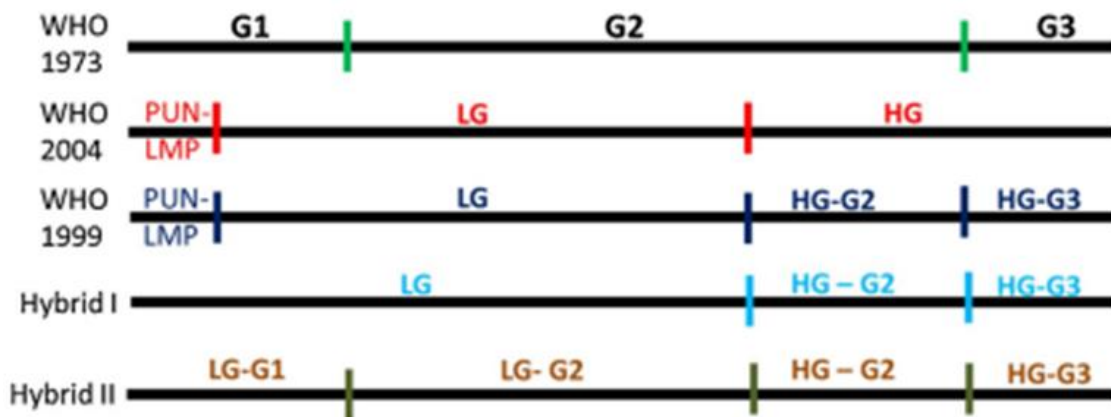


Figure 2: Comparison of five bladder cancer grading systems (36).

PUNLMP = papillary urothelial neoplasm of low malignant potential; LG = low grade; HG = high grade

The graphs show the grade cut-offs of each classification system in parallel. The grade increases from left to right.

1.4. The Molecular Genetic Background of Bladder Tumor Formation

Bladder cancer belongs to a group of molecularly heterogeneous diseases with different clinical courses and different clinical features (38). Several genetic mutation pathways lead to tumor formation and progression. As **Figure 3** shows, in the earliest phase of carcinogenesis, a deletion occurs on chromosome 9 of the urothelial cell.

Fibroblast growth factor receptor 3 (FGFR3) and RAS proto-oncogene mutations lead to hyperplasia and a low grade Ta stage. On the other pathway, tumor protein 53 (TP53) and retinoblastoma 1 (RB1) tumor suppressor mutations lead to dysplasia of the cells, resulting in cancer in situ (CIS). Several additional genetic changes result in the progression of the tumor from the T1 stage to the T2-3-4 stages, which also affect muscle (39).

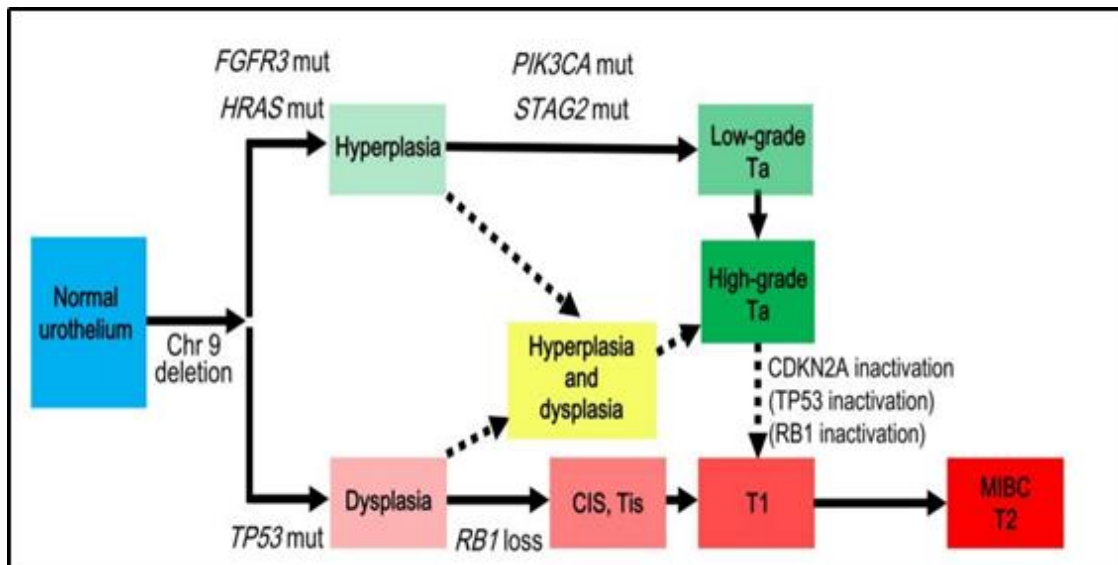


Figure 3: Pathways of the bladder cancer tumorigenesis and tumor progression (38).

1.5. Diagnosis

Painless, bloody urine is the most common symptom of bladder cancer. The nature of haematuria may be clearly visible or detectable only by microscopy, continuous or intermittent, and may contain

blood clots. Urgency, dysuria, increased frequency, and especially irritative voiding symptoms are also characteristic of bladder carcinoma (40). Pelvic pain and symptoms related to urinary tract obstruction occur in more advanced tumors. A palpable pelvic mass can be found with (bi)manual (vaginal and) anal examination in patients with locally advanced cancer (40).

Imaging procedures, including ultrasound, intravenous urography, and computer tomography examinations, play an important role in establishing the diagnosis (41, 42). The latest diagnostic methods, such as multiparametric magnetic resonance imaging (43) or diffusion-weighted imaging (44), as well as the use of artificial intelligence (45) in the

analysis of images, are becoming more and more sensitive and specific procedures in the detection and staging of bladder tumors at an ever earlier stage.

Urine cytology is a non-invasive diagnostic method used in clinical practice during which tumor cells are detected in the urine (46). It has a high sensitivity for high-grade tumors, but the interpretation of cytology can be hindered by, for example, low cell yield, urinary tract infections, and stones (47).

Intensive research is underway in the direction of biomarkers that can be detected in urine. Among the many candidates, the specificity and sensitivity of the methods vary between 50–90% for the detection of bladder cancer and the monitoring of therapy (48–50). Extracellular vesicles (EVs) serve as a promising source of biomarkers for the diagnosis and monitoring of diseases, including cancer. They come directly from tumor cells and contain interesting cargo that is involved in tumor formation. Despite the discovery of EV protein markers and EV genetic markers, which are potential EV biomarkers, further research is needed to prove their clinical utility (51, 52).

Cystoscopy is an effective but invasive tool for detecting bladder cancer. In addition, it has low sensitivity for carcinoma in situ (Tis), and cancers may still be missed as efficiency is operator-dependent (53). Diagnostic technology is constantly being developed, where laser-induced fluorescence, autofluorescence cystoscopy, optical coherence tomography, confocal laser endomicroscopy, and photodynamic diagnostics are in the spotlight (54–57).

1.6. Treatment Options

The traditional classification system for BC is primarily based on pathological parameters. Despite the fact that the recurrence and progression of BC vary widely among different individuals with similar pathological classifications, histological staging and grading provide the basis for assessing the prognosis and choosing the correct therapy. The first step of the available treatment options for BC is transurethral resection of bladder tumors (TURBT). The purpose of TURBT is to remove the cancer tissue and take a histological sample to determine the exact diagnosis, staging, and grading. TURBT is the gold standard in the diagnosis and therapy of non-muscle-invasive bladder cancer (NMIBC). Complete removal of all visible lesions is the crucial procedure in the treatment of NMIBC. Advances in surgical techniques have made it possible to use the En Bloc Resection of Bladder Tumor (ERBT) technique as an alternative to traditional TURBT, which includes finer en bloc sculpting and tumor excision in contrast to the

“piecemeal” resection performed with traditional TURBT (58, 59). When muscle-invasive bladder cancer (MIBC) is suspected, tumors should be removed in separate sections, which include the exophytic part of the tumor, the underlying bladder wall with the detrusor muscle, and the edges of the resection area (60). Depending on the results of the histological examination, additional treatment options include neoadjuvant chemotherapy, radical cystectomy, radiation therapy, chemotherapy, the use of Bacillus Calmette-Guérin (BCG), or checkpoint inhibitors.

1.6.1. Bacillus Calmette-Guérin (BCG)

Albert Calmette and Camille Guérin recultivated colonies originally isolated from pathogenic *Mycobacterium bovis* for thirteen years. In 1921, it was shown that the obtained bacillus was not only non-pathogenic in animal models but also protected against tuberculosis infection in vaccinated animals. BCG is the only commercially available vaccine against tuberculosis to this day (61).

Since 1976, BCG has been used not only as a vaccine against tuberculosis but also as one of the most successful antitumor immunotherapies (62). BCG not only acts against BC by activating the immune system (63), but also directly reacts to cancerous cells, causing oxidative stress, necrosis, or apoptosis (64-66).

Local or systemic side effects occur in approximately 70% of patients, and approximately 5% to 9% of patients discontinue due to side effects and do not complete the intended BCG regimen (67). BCG therapy induces an initial complete response rate of 70–75% in CIS and 55–65% in high-risk papillary tumors. This also means that 25–45% of patients fail treatment in the beginning, and another 40% eventually relapse after initial improvement (68). The cause of the failure of BCG therapy has been investigated by numerous in vitro and in vivo studies (69-73). It has been proven that the increase in the human beta-defensin 2 (hBD2) level in the environment of tumor cells reduces or prevents the uptake of BCG by bladder cancer cells (69, 70, 74).

1.6.2. Immune Checkpoint Inhibitors (ICI)

Immune checkpoints, as normal part of the immune system, provide protection against the destruction of healthy body cells. If T cells recognize a checkpoint protein on the surface of a tumor cell, they unfortunately do not destroy the cancer cell. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, and PD-L1 are these checkpoint proteins that play a role in regulating the immune system. CTLA-4 is involved in

regulating T-cell proliferation early in an immune response, primarily in lymph nodes, while PD-1 suppresses T cells later in an immune response, primarily in peripheral tissues (75). PD-L1 is expressed by tumor cells to contact and inhibit the function and activity of T cells (**Figure 4**). These proteins are important targets for cancer immunotherapy, and blocking them can help activate anti-tumor immune responses (76).

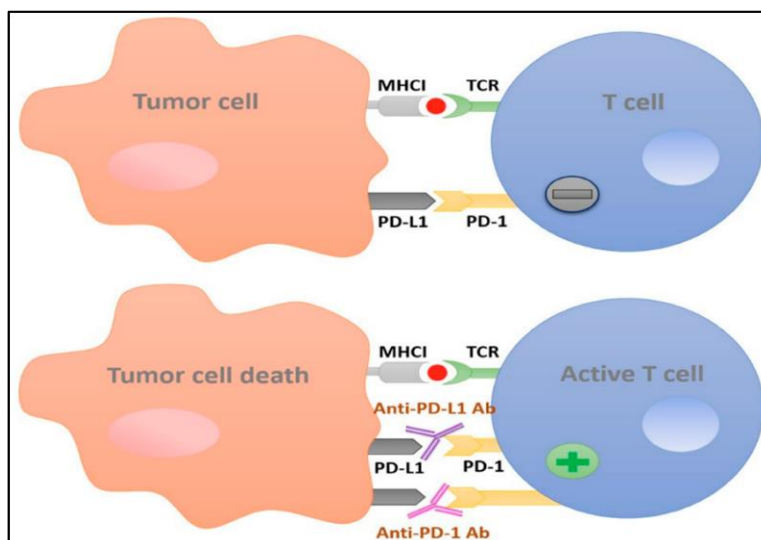


Figure 4: Role of checkpoint proteins in deactivating, and of checkpoint inhibitors in reactivating T cell functions against tumor cells (77).

In the case of cisplatin-ineligible bladder cancer as first-line therapy or as second-line therapy for patients with metastatic urothelial carcinoma, five immune checkpoint inhibitors (ICI) have been approved to date by the US Food and Drug Administration (FDA) (78). PD-1 inhibitory antibodies are the active ingredients of the pembrolizumab and nivolumab ICIs, and PD-L1 inhibition is caused by atezolizumab, avelumab, and durvalumab.

Unfortunately, only 20–40% of patients respond favorably to ICIs. The reasons for failure include among others decreased PDL-1 expression, hypoxia, and interferon- γ (79). Several studies have demonstrated the relationship between gut microbiome composition and ICI efficacy (80, 81).

1.7. Human Beta-Defensins (hBDs) and BC

Human beta-defensins (hBDs) are antimicrobial peptides that play a role in host immunity and mucosal surface protection against fungal, bacterial, and viral pathogens

(82). Human beta-defensin 1 is produced constitutively, while the inducible production of antibacterial hBD2 and hBD3 is affected by bacteria (83). The autonomous defensin production of tumor cells may also amend this state to an unpredictable degree and direction. There is also evidence that hBDs may have a role in cancer, as they can promote or inhibit cancer cell proliferation and migration depending on the origin of the cancer cell (84). As **Figure 5** shows, for example, hBD1 appears to be downregulated in prostate and renal clear cell carcinomas, but upregulated in lung carcinoma. Additionally, hBD1 has been suggested as a candidate tumor suppressor in oral, renal, and prostate cancer (85). In cervix cancer hBD2 and hBD3 have elevated levels, and in this type of cancer, hBD2 and hBD3 increase the migration and proliferation of cancer cells (86). Defensins can also act as chemoattractants for immune cells in the tumor microenvironment.

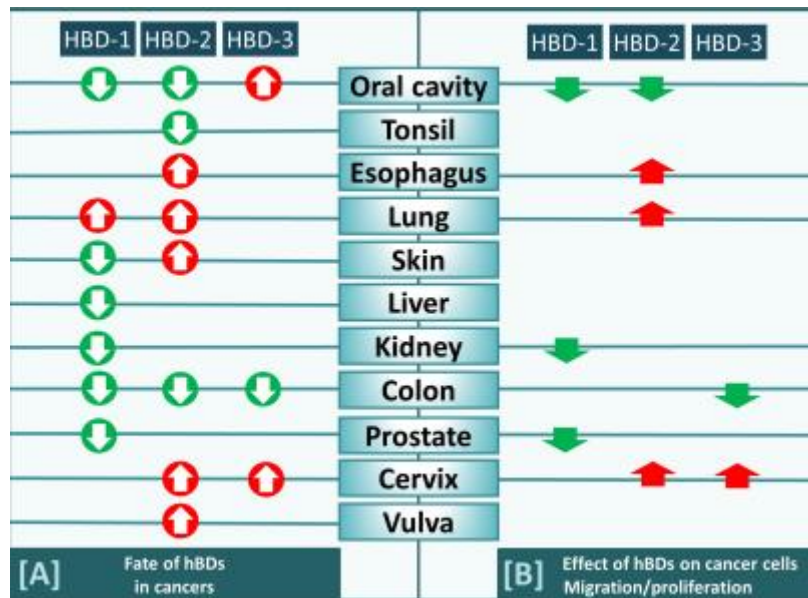


Figure 5: Quantitative change and effect on cancer cell migration and proliferation of human beta-defensins in different cancers (84).

Previous research results on the relationship between hBDs and BC were found only for hBD1 and hBD2. In the case of hBD1, exogenous synthetic hBD1 peptide inhibits bladder cancer cell migration and proliferation (84), and urine-derived hBD1 has been shown to suppress bladder cancer growth (86, 87). hBD1 has also been studied for its potential clinical applications in bladder cancer (88). Previous research results on the relationship between hBD2 and BC described that elevated hBD2 levels inhibit the success of Bacillus Calm  te-Gu  rin (BCG) treatment (69, 70).

1.8. Previous Studies About Urinary Microbiome

Human skin and mucosal surfaces, including those of the gut, lung, and genitourinary tract, are known to be colonized by a diverse collection of eubacteria, archaea, protozoa, yeasts, and viruses. This co-existing and cooperating ecological community made up of taxonomically different microorganisms is called the microbiota. Instead of determining the exact composition of the living microbiota community, it is possible to detect the genetic stock of the microbes present. The gene composition that characterizes the microbiota is called the microbiome (89).

Relying on the results of traditional breeding methods, healthy human urine was considered sterile for a long time. Using the enhanced quantitative urine culture (EQUC) method, already anaerobic, slower-growing bacteria with higher nutrient requirements could be cultured from the urine. The metagenomic analysis using Next Generation Sequencing (NGS) was the next step in the evolution of urine microbiome analysis (**Figure 6**) (90). Using amplicon-based sequence analysis methods (16S rRNA or ITS), the bacteria or fungi present in the sample are identified and quantified at the genus level. The Shotgun sequencing method determines the DNA sequences of any taxon present in the sample, apart from bacteria and fungi, and also provides information about viruses, parasites, or virulence genes, etc.

MICROBIAL IDENTIFICATION METHODS

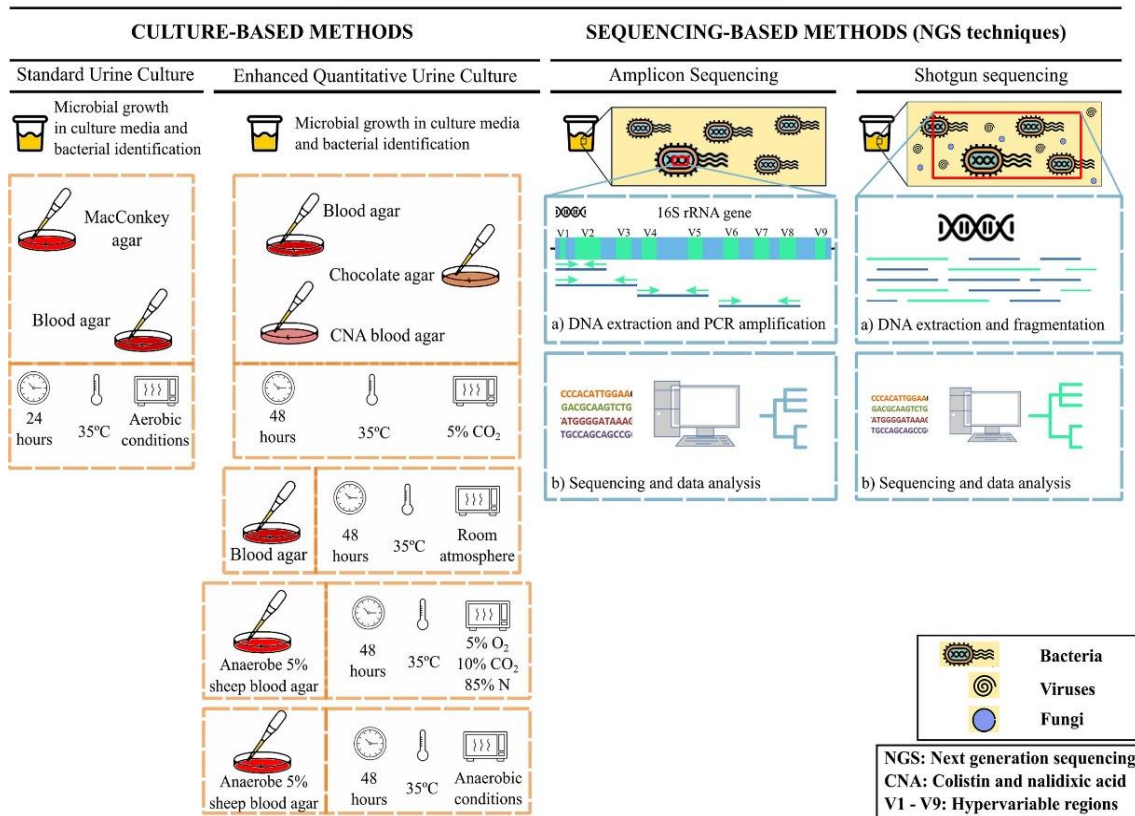


Figure 6: Culture-based and sequencing based microbial identification methods of urine samples (90).

The result of the urine microbial composition test is influenced not only by the method used for detection but also by the possibility of contamination depending on gender, which is related to the sampling procedure and results in large differences. Female voided urine samples contained bacteria of the urinary and genital tract; however, the bacteria assessed by gene sequencing were common in voided, transurethral catheterized, or suprapubic aspirated male urine samples (91). The voided urine of male patients did not adequately characterize the male bladder microbiota, and in contrast to catheterized urine microbiome results, it was not associated with the severity of lower urinary tract symptoms (92). Sampling and detection methods also result in differences, but the composition of the urine microbiome changes with age, gender, diet, antibiotic consumption, and accompanying diseases (93).

Lewis et al. analyzed the composition of the urinary microbiome in thirty-three healthy patients using 16S rRNA sequencing. The research differentiates the core bacteria that are always present in urine and groups them by age and gender (94). Several comparative studies have looked for differences between the composition of the healthy

urinary microbiome and the urinary microbiome associated with urinary tract diseases such as interstitial cystitis (95), benign prostatic hyperplasia (96), chronic prostatitis (97), or prostate cancer (98).

Prior to our investigations, only a few research results were available on bladder carcinoma and the composition of the microbiome. The majority of the results were about the urinary microbiome related to BC (99-101); only one research group at that time characterized the microbiome related to cancerous lesions of the bladder wall (102). To the best of our knowledge, no one before us compared the microbiome composition of catheterized urine and tumor tissue samples.

2. Objectives

1. Our aim was to develop new and reproducible methods for microbiome analysis from urine and removed bladder mucosal tissue samples from patients with prostatic hyperplasia or bladder cancer.

2. Our goal was to determine whether the microbiome composition is different at the distant points of the tumor tissue sample of a given patient or whether it is characteristic of the given patient.

3. By comparing the tissue and urine microbiome results, our aim was to examine whether the urine sample is a suitable test material for the characterization of the bladder tumor microbiome.

4. Our aim was to determine which bacterial genera are more associated with tissue and which with urine.

5. We set out to compare the microbiome composition of cancerous and healthy histological samples in order to determine which taxa are associated with all tissue samples and which taxa are associated only with the cancerous tissue samples.

6. Our aim was to determine whether there are differences between the levels of hBD1, hBD2, and hBD3 in the urine of healthy individuals and patients with prostate hyperplasia or bladder cancer.

7. Our aim was to determine the expression rate of hBD1, hBD2, and hBD3 in bladder carcinoma and healthy mucosa tissue samples.

8. Our aim was to find a correlation between the microbiome composition characteristic of tumor tissue samples and the defensin levels belonging to the given sample.

3. Methods

The methods detailed below were published in our previous articles (103, 104).

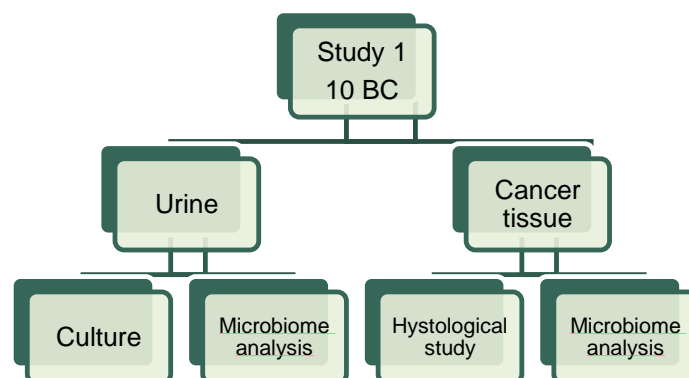
3.1. Ethical Considerations

The whole study was conducted in accordance with the Declaration of Helsinki ethical standards. Protocols for sampling and data management were performed in accordance with the guidelines and regulations of Markhot Ferenc University Teaching Hospital (MFUTH). All research was approved by the Ethics Committee of MFUTH and by the Regional Ethics Committee of Semmelweis University; the permission numbers are SE RKEB 100/2018 and SE-RKEB 100-1/2018/2021. All study participants gave written informed consent to participate in the study and to allow the publication of their anonymized test results.

3.2. Sample Collection

3.2.1. Study Groups

In the first study period, between June 2018 and April 2019, histological and urine samples were collected from 10 patients with bladder carcinoma (BC) for microbiome studies. In the second study period, between April 2021 and September 2021, tissue and urine samples were collected from 55 bladder cancer (BC) patients and from 12 patients with benign prostatic hyperplasia (BPH) for microbiome and defensin measurements. As a negative control for defensin levels, urine samples from 34 healthy volunteers (HV) were also collected (**Figure 7**).



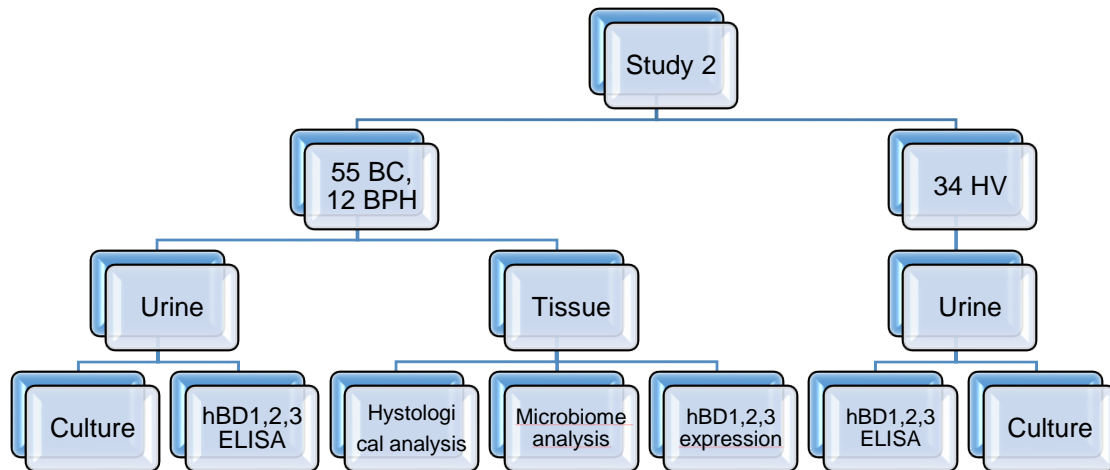


Figure 7: Study design.

BC=Bladder cancer, BPH=benign prostatic hyperplasia, HV=healthy volunteers

3.2.2. Inclusion and Exclusion Criteria

We collected samples from a total of 65 patients with BC and 12 patients with BPH during transurethral resection (TUR) performed at MFUTH. Urine samples were also collected from the same patients during TUR. The members of the BPH group were selected in such a way that they matched the members of the BC group in terms of age and accompanying diseases. Spontaneously excreted urine was collected from 34 HV, employees, or students of Semmelweis University. Exclusion criteria in all study groups were an existing infection or antibiotic or probiotic use in the two months prior to the study.

3.2.3. Sampling Methods

Urine and tissue samples were collected from patients with bladder cancer and prostatic hyperplasia during transurethral resection. We took the greatest possible care to avoid contamination when taking samples. Cathejell Lidocaine, a sterile anesthetizing gel for catheter lubrication, was used with local anesthetic effect for instillation into the urethra before catheterization. After the resectoscope was inserted into the bladder, urine collected through the inner lumen of this device. After this draining of urine, bladder was filled with a sterile 1,5% glycine solution, which provides a safe surgical area without electricity conduction. Urine was drained, and glycine was filled up via the inner sterile lumen of the resectoscope, and only the outer wall of the resectoscope contacted the

urethra. Urine and tissue samples from a given patient were in contact only with the inner lumen of the same device.

From healthy volunteers spontaneously excreted urine was examined.

3.3. Culture Method

Urine samples that were directly collected from the bladder during the TUR were divided for traditional routine culture and microbiome analysis. Fractions for microbiota analysis were centrifuged at 16,000 g for 10 minutes, and supernatants were aliquoted and stored at -80 °C. Fractions for culture were processed in the laboratory of MFUTH. HV urine samples were collected and cultured in the laboratory of the Medical Microbiology Institute of Semmelweis University. Briefly, routine urine culture means inoculation of 10 µl of urine onto blood and eosin-methylenblue agar plates (Biomérieux, France) and 24-h incubation under ambient atmospheric conditions. The level of detection is 100 Colony-forming units per milliliter (CFU/mL).

3.4. DNA Isolation, RNA Isolation

For nucleic acid isolation, tissue samples were prepared by ProtK enzymatic digestion at 56 °C, for 3-5 hours, until the samples became completely liquid. Enzymatic digestion was made with standardized 2-mm-diameter tissue pieces. DNA isolation was performed by the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, USA) from digested tissue and native urine samples. Total RNA was isolated from digested tissue samples by the innuPREP RNA Mini Kit 2.0 (Analytik Jena GmbH, Jena, Germany).

3.5. 16S rRNA Gene Library Preparation and Sequencing

3.5.1. Theoretical Description of the 16S rRNA Library Preparation and Sequencing Method

As a product of DNA isolation from the test materials, in addition to the bacterial DNA, DNA of human or other origin can also be found. By using the sequencing of a targeted gene segment that is present in all bacteria but only contained by bacteria, it is possible to avoid the fact that the metagenome analysis also contains data from other DNA sources. Metagenomic studies are commonly performed by analyzing the gene of prokaryotic 16S ribosomal RNA (16S rRNA), which is approximately 1,500 bp long and

contains nine variable regions interspersed between conserved regions (105). **Figure 8** shows the bacterial gene sequence and two-dimensional structure of 16S rRNA, as well as the targeted V3-V4 region selected for our investigation.

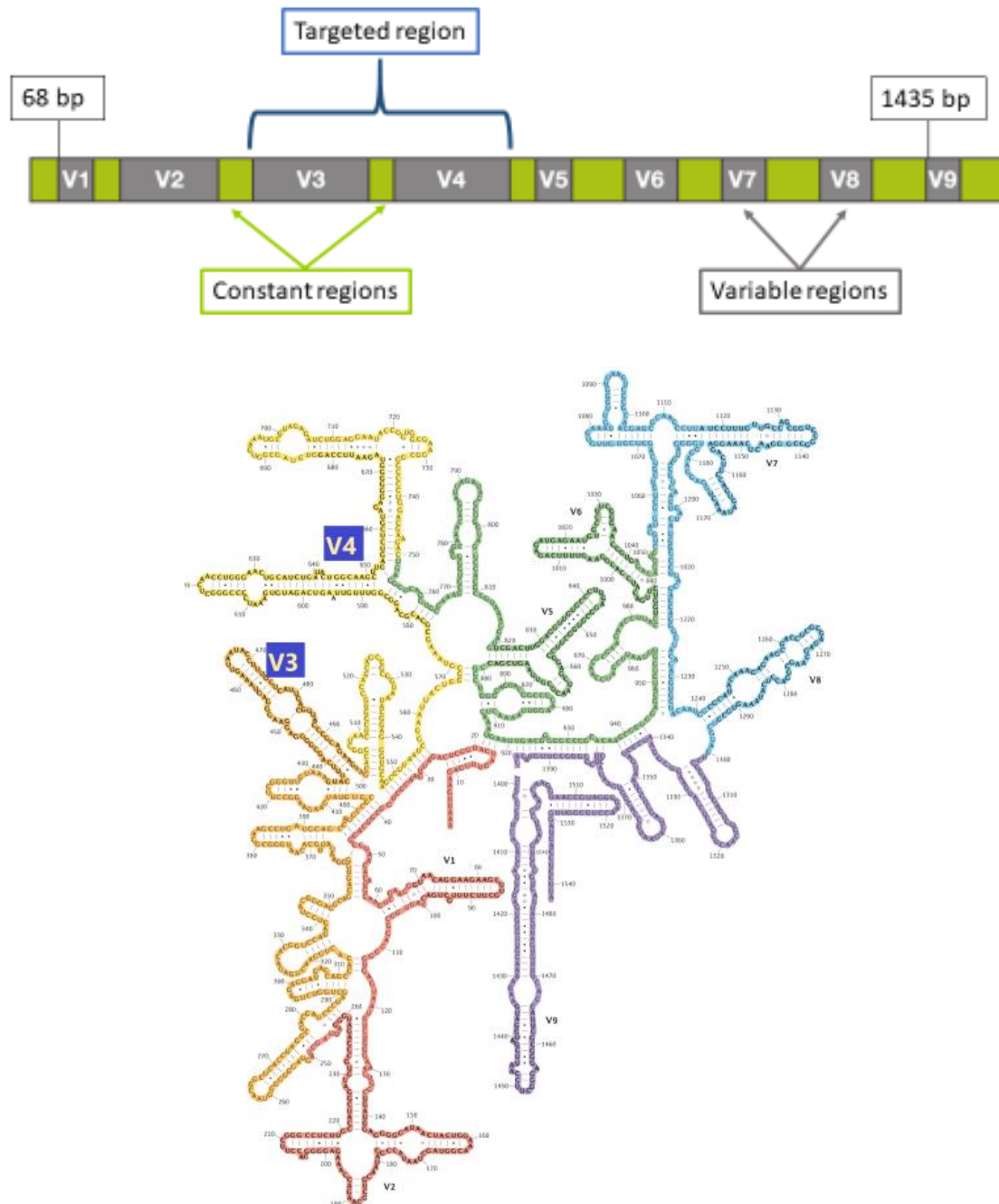


Figure 8: Gene sequence and two-dimensional structure of 16S rRNA (105).

The first step of the metagenome study is the amplicon polymerase chain reaction (PCR), with which the 16S rRNA V3-V4 gene section is amplified. First, PCR clean up purifies the 16S V3 and V4 amplicons away from free primers and primer dimer species.

The second index PCR step — the library preparation — attaches to the amplicon dual indices and adapters for Illumina sequencing. A second PCR clean-up purifies the final library before quantification. Individually indexed samples need to be diluted to the same concentration before pooling; this is supported by library quantification and normalization. Libraries were denatured before Illumina MySeq sample loading.

3.5.2. Materials and Method Used in 16S rRNA Library Preparation and Sequencing

The DNA concentration after isolation was measured using the Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA).

The first amplicon PCR used the following primers with overhang adapters and V3-V4 locus specific sequence (marked with a hyphen).

Forward:

5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-
CCTACGGGNGGCWGCAG–3'

and Reverse:

5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-
GACTACHVGGGTATCTAATCC–3'

The second index PCR used the Nextera XT Index Kit v2 setD (Illumina, San Diego, USA). Both PCRs were performed using the 2x KAPA HiFi HotStart ReadyMix enzyme mix (Roche Diagnostics, Meylan, France). Both PCR products were cleaned up using sparQ PureMag Beads (Quanta BioDesign, Montgomery, USA). PCR and DNA purifications were performed according to Illumina's protocol. PCR product libraries were assessed using the DNA 1000 Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled and sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycles PE) (Illumina, San Diego, CA, USA).

Raw sequencing data were retrieved from Illumina BaseSpace and analyzed using the CosmosId bioinformatics platform (106).

3.6. Defensin Quantitative Measurements

3.6.1. Defensin Expression Assays

Total RNA isolated from tissue samples was used to determine the mRNA levels of defensins and, as a reference, the amount of mRNA transcribed from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. RT-PCR was started with 80–100 ng of RNA, and amplification was performed on qTOWER 3G (Analytic Jena GmbH, Jena, Germany) using the PrimeScript RT reagent kit (Takara Bio, San José, USA). Primers used for defensin and GAPDH expression assays are summarized in **Table 2**.

Table 2: Primers used for defensin and GAPDH expression assays.

Targeted gene	Forward/Reverse	Primer
hBD1	Forward	5'-TTGTCTGAGATGGCCTCAGGTAAC-3'
	Reverse	5'-ATACTTCAAAGCAATTTTCCTTTAT-3'
hBD2	Forward	5'-CCAGCCATCAGCCATGAGGGTCTTG-3'
	Reverse	5'-CATGTCGCAAGTCTCTGATGAGGGAGG-3'
hBD3	Forward	5'-AGCCTAGCAGCTATGAGGATC-3'
	Reverse	5'-CTTCGGCAGCATTTTCGGCCA-3'
GAPDH	Forward	5'-CTACTGGCGCTGGCAAGGCTGT-3'
	Reverse	5'-GCCATGAGGTCCACCACCCTGCTG-3'

The mRNA expression levels of the tested defensins were determined by the double delta Ct ($\Delta\Delta Ct$) method (107). Briefly, the amounts of mRNA transcribed from different target genes were determined by comparing the Ct values obtained during RT-PCR. The higher the level of mRNA transcribed from a tested gene segment, the lower the Ct value (**Figure 9**). The amount of cells in the processed sample is shown by the expression of the housekeeping gene GAPDH, therefore, the expression of the targeted gene is compared to this (ΔCt). The average of the ΔCt values obtained from the tissue samples of BPH patients was used as a control. The relative expression (RQ) of the target (defensin) genes in the BC samples compared to the BPH samples was calculated according to the following formula:

$$\Delta Ct_{BC\text{sample}} = Ct_{\text{def}} - Ct_{GAPDH} \text{ measured from BC sample}$$

$$\Delta Ct_{BPH\text{average}} = \text{average of } Ct_{\text{def}} - Ct_{GAPDH} \text{ measured from BPH samples}$$

$$\Delta\Delta Ct = \Delta Ct_{BC\text{sample}} - \Delta Ct_{BPH\text{average}}$$

$$RQ = 2^{-\Delta\Delta Ct}$$

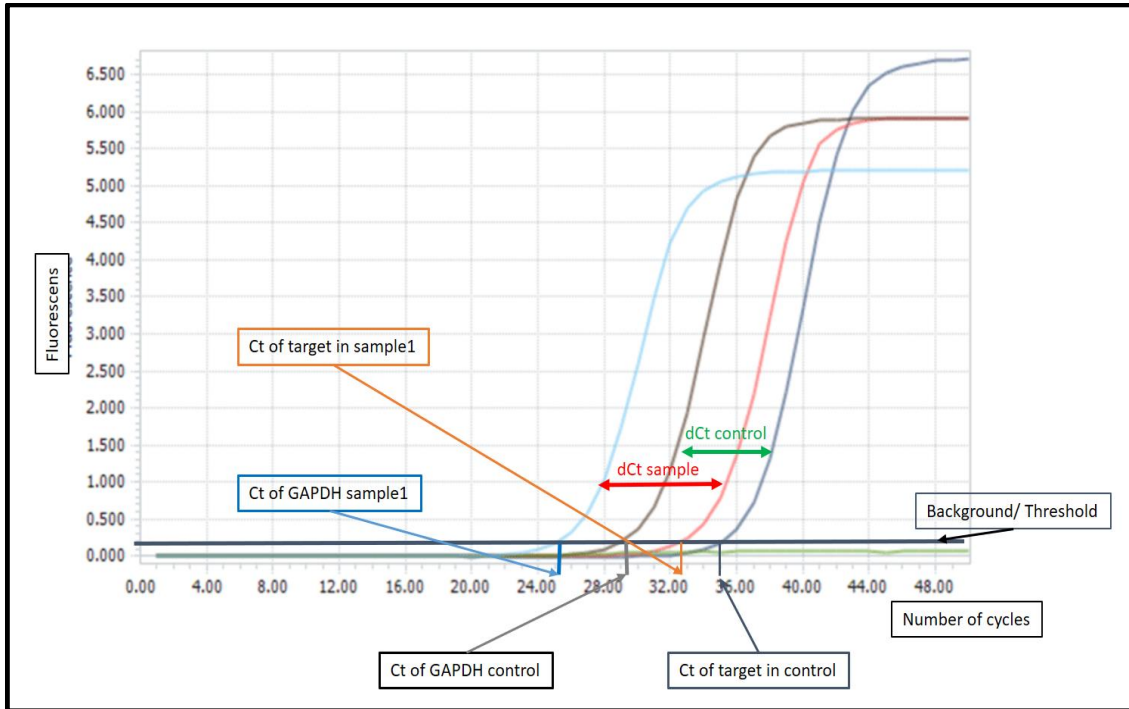


Figure 9: Amplification curves of targeted and GAPDH housekeeping genes.

Data for the calculation of RQ, as dCt sample and dCt control, are represented by arrows between the Ct values within the sample or control.

3.6.2. ELISA (Enzyme-Linked Immunosorbent Assay)

For quantitative measurement of human beta-defensins in urine, the following ELISA kits were used: SEB373Hu for hBD1, SEA072Hu for hBD2, and SEE132Hu for hBD3 (Cloud-Clone Corp., Houston, USA). All diluted standards, samples, and blank wells were measured in duplicate according to manufacturer instructions.

3.7. Statistical Analysis

To find out the difference between urine defensin levels, defensin expression rate, and bacterial taxa abundances measured in the different cohorts' levels of statistical significance ($p < 0.05$) was calculated by the Mann-Whitney U test. Statistical significance

between cohorts was implemented by the Wilcoxon Rank Sum test for Chao1 Alpha diversity and PERMANOVA analysis for Jaccard Beta diversity using the statistical analysis support application of CosmosID (106).

4. Results

4.1. Patient Clinical Data

The characteristics of the patients participating in the first study, in which the urine and tissue microbiome composition were compared, are shown in **Table 3**. Of the five male patients, two had non-muscle-invasive bladder cancer (NMIBC), three had muscle-invasive bladder cancer (MIBC), four of the five female patients had NMIBC, and one had MIBC. The average age of patients was 63.9 years. The table contains the sample identifiers of patients used in the figures, where U stands for urine, T stands for tissue, I stands for muscle-invasive, N stands for non-muscle-invasive. Patients I01, N01, N02, and N03 had such a large tumor that the tissue microbiome analysis was performed on two samples taken from distant locations of the tumor (TI01.1, TI01.2, etc.). **Table 4** shows the data of patients from the second study, in which the relationship between defensins and the microbiome was investigated. The sample identifiers for the 55 bladder cancer patients were BC1-55, for the 12 prostatic hyperplasia patients were BPH1-12, and for the 34 healthy volunteers were HV1-34.

Table 3: Patient data of the first study.

Patient ID	Age	Gender	Stage	Urine sample ID	Tissue sample ID
MIBC1	70	Male	T2	UI1	TI1.1
					TI1.2
MIBC2	80	Male	T3	UI2	TI2
MIBC3	79	Male	T2	UI3	TI3
MIBC4	76	Female	T2	UI4	TI4
NMIBC1	72	Male	T1	UN1	TN1.1
					TN1.2
NMIBC2	54	Male	T1	UN2	TN2.1
					TN2.2
NMIBC3	66	Female	Ta	UN3	TN3.1
					TN3.2
NMIBC4	58	Female	T1	UN4	TN4
NMIBC5	20	Female	T1	UN5	TN5

NMIBC6	64	Female	T1	UN6	TN6
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Table 4: Patient data of the second study.

	Bladder Cancer Group	Benign Prostatic Hyperplasia	Healthy Volunteers
Number of participants	55 (46 in defensin tests)	12	34
Median age of participants (Interquartile Range (IQR))	68.5 (IQR: 14)	71.5 (IQR:19)	51 (IQR:42)
Male/Female	32/14	12	17/17
Diabetes mellitus/ No DM	36/10	8/4	1/33
Hypertension/ Normal blood pressure	29/17	9/3	4/30
Smoker / Non-smoker	28/18	7/5	12/24
Stage	NMIBC:Ta:2, T1:27, MIBC:T2:17,	NA	NA
Grade	G1:23 ,G2:9, G3:14	NA	NA

4.2. Results of Urine Cultures

Using conventional aerobic culture methods, no bacteria were detected in the urine of any patient. As the detection limit was 10^2 CFU/mL, neither bacteria under this limit nor bacteria that cannot be cultured using the traditional aerobic method (e.g., *Ureaplasma sp*) could be detected in this way.

4.3 Comparison of Urine and Cancer Tissue Related Microbiome

4.3.1. Microbiome Alpha Diversity in Urine and Tissue Samples

Using the bioinformatics analysis of urine and tissue samples, we first compared the alpha diversity of different groups of samples. **Figure 10** shows that there was no significant difference in the Shannon alpha diversity values when all urine samples were compared with all tissue samples. In the same way, when comparing all samples from female patients with all male samples, no significant difference in alpha diversity was found at the genus level. The alpha diversity of male and female urine samples also

showed no difference, but the microbiome of female and male histological samples was significantly different in the area of Shannon alpha diversity.

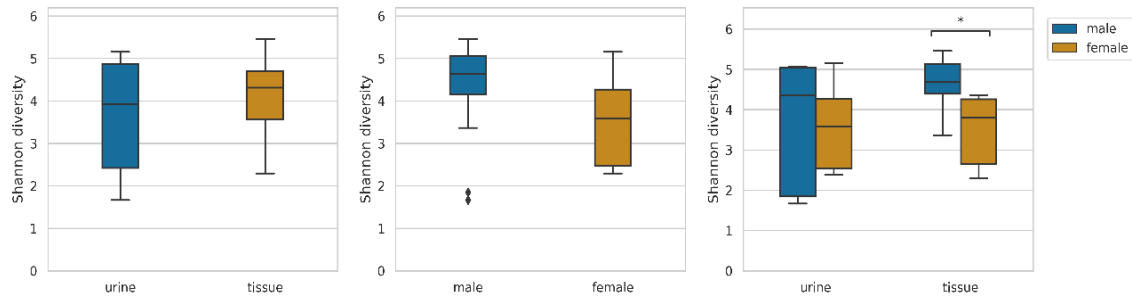


Figure 10: Shannon alfa diversity of urine and tissue microbiome.

No significant difference was observed between urine and tissue samples, between male and female samples, or between the urine samples of males and females. A significant difference was shown only between the Shannon diversity of female and male tissue samples.

4.3.2. Results of Duplicated Tissue Samples' Microbiome Analysis

The tissue samples of four patients were cut, and two pieces located far from each other were separately digested enzymatically. All the isolation of DNA, PCR, library preparation, sequencing, and analysis procedures were done in duplicate. **Figure 11** shows no difference in the abundance of genera detected from sites 1 and 2 of a subject's tissue sample, and clearly demonstrates that the microbiome analysis results were reproducible, and it rigorously characterizes the tissue sample of a given person. **Figure 11** also shows that the abundance of genera in the urine sample of the same patient differs from the results shown in the tissue. However, at the Jaccard β diversity Principal Coordinate Analysis (PCoA) (**Figure 12**), the correlated tissue samples from the same patients show nearly the same β diversity results; they do not cluster with their own urine samples.

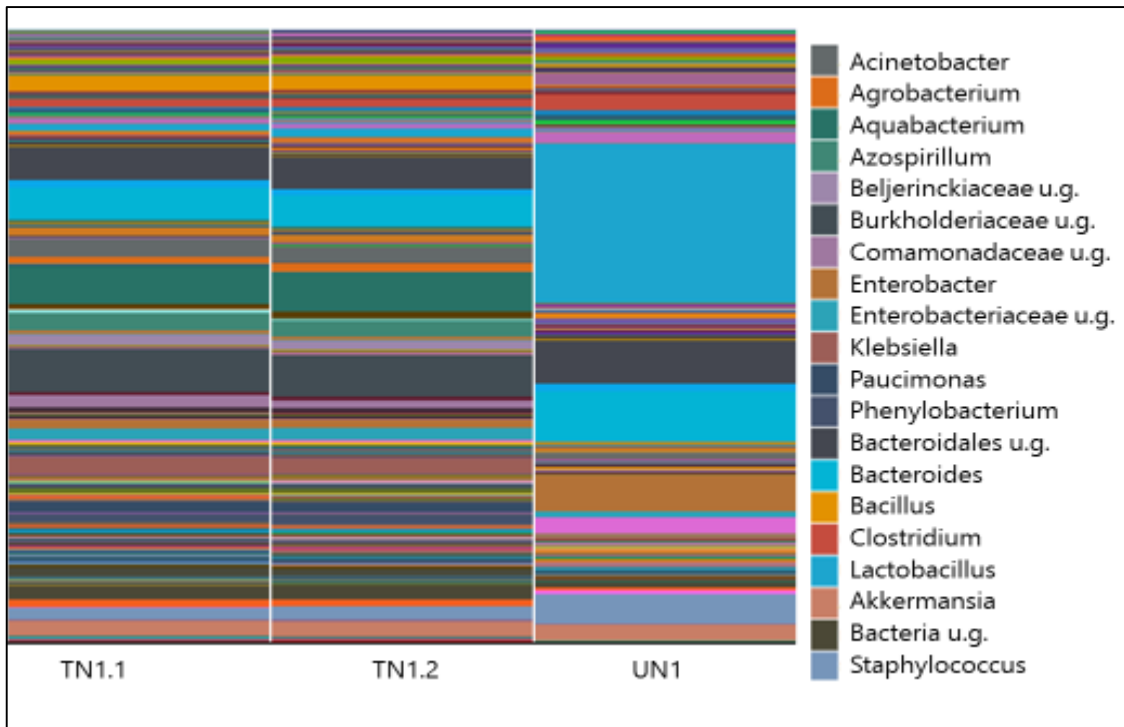


Figure 11: Taxa abundance in tissue (TN1.1 , TN1.2) and in urine sample (UN1) of patient NMIBC1 at genus level.

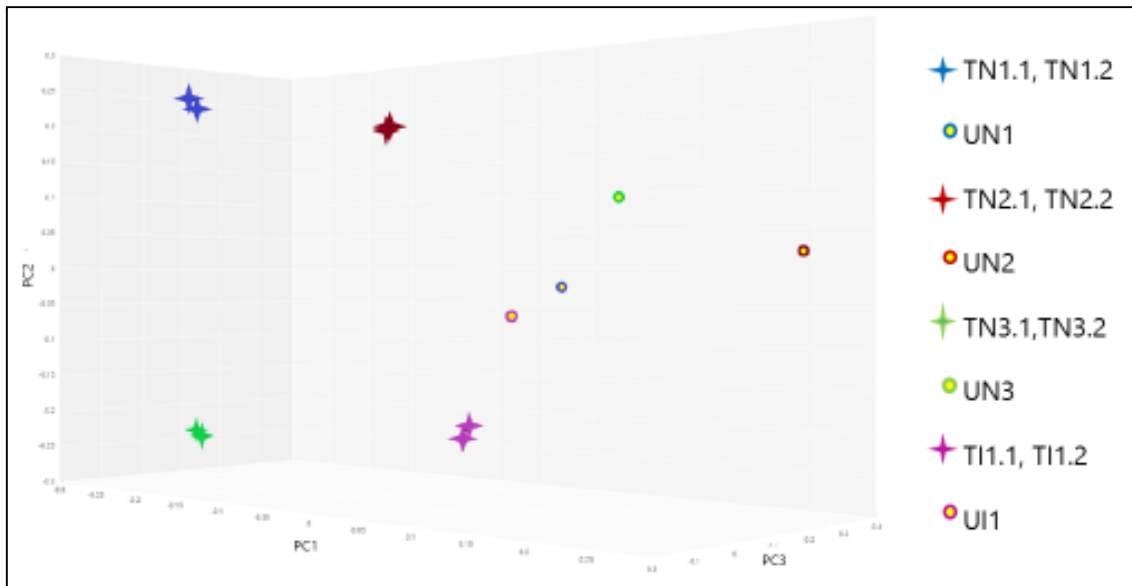


Figure 12: Jaccard β diversity Principal Coordinate Analysis of four duplicated tissue and the related urine sample microbiome.

4.3.3. Taxa Abundances in Urine and Tissue Samples

In urine samples, the most abundant phyla detected were *Firmicutes*, with an abundance of 33%, followed by *Proteobacteria* (29%), *Actinobacteria* (23%), *Cyanobacteria* (7%) and *Bacteroidetes* (4%). In contrast, the order in tissue samples was as follows: *Firmicutes* (34%), *Actinobacteria* (23%), *Proteobacteria* (22%), *Bacteroidetes* (15%), and *Cyanobacteria* (8%). The most abundant genera in urine samples were *Corynebacterium* (12%), *Escherichia-Shigella* (8.7%), *Staphylococcus* (7.8%), *Streptococcus* (6.1%), and *Gardnerella* (5.2%). In tissue samples, the most abundant genera were *Lactobacillus* (9.5%), *Bacteroides* (8.8%), *Staphylococcus* (4.2%), and *Akkermansia* (4.0%). *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella* genera showed remarkably higher median compositional abundance in tissue than in urine samples (**Figure 13**). The heat map (**Figure 14**) shows the co-existence of five genera (namely *Clostridium sensu stricto*, *Akkermansia*, *Bacteroides*, *Enterobacter*, and *Klebsiella*) in tissue samples. The presence of these five genera is characteristic but not general for all samples.

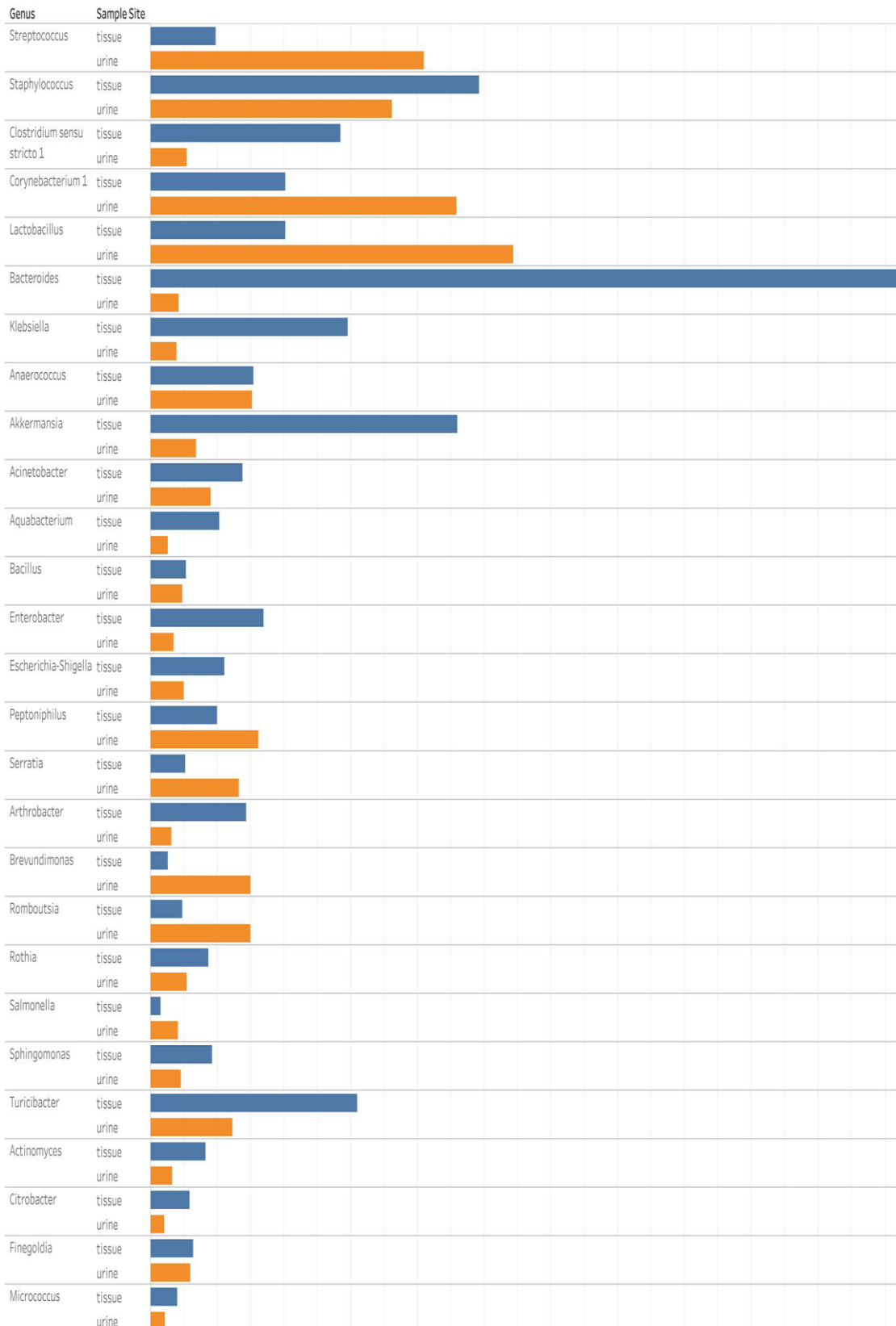


Figure 13: Median abundance of the most abundant genera compared in tissue and urine samples.

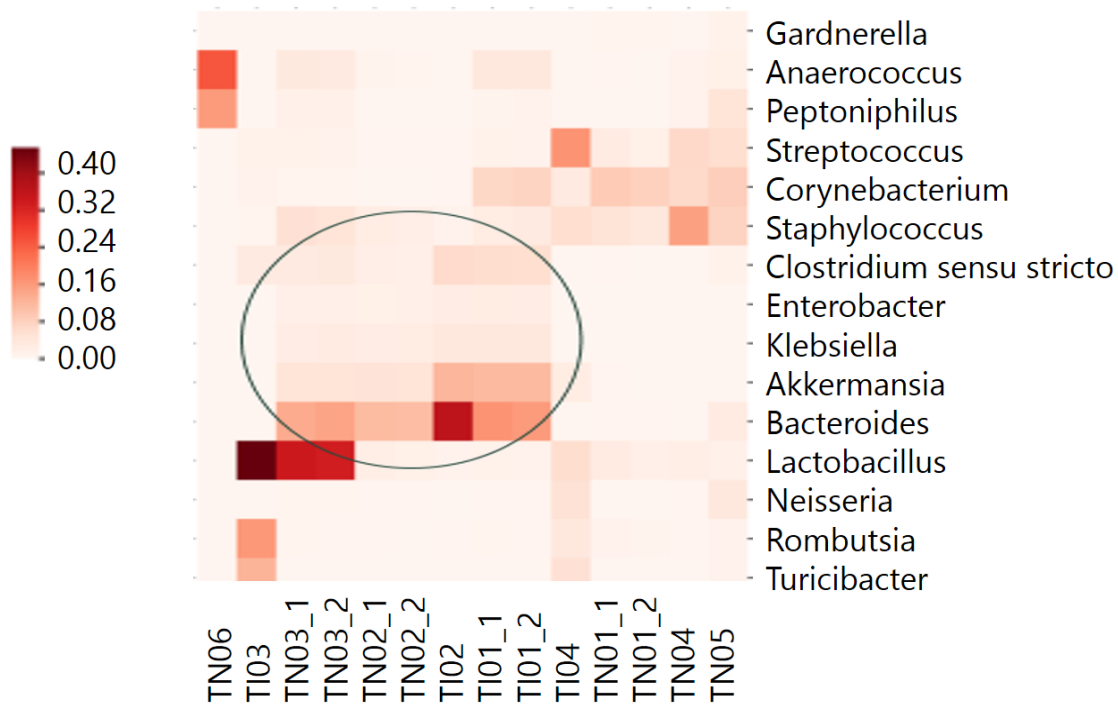


Figure 14: Heat map of the most abundant genera in tissue samples.

4.4. Bladder Tissue Microbiome Composition and Related Human Beta-Defensin Levels

4.4.1. Comparison of Tissue Microbiome Results of BC and BPH Patients

From each of the 67 patients (55 BC and 12 BPH), three smaller pieces of tissue samples were processed separately. After DNA isolation and library preparation of these 201 samples, 16S rRNA sequencing resulted in a total of 40.7 million high-quality reads. However, while the amount of bacterial DNA (median read number 271,525; IQR: 44,506) was significantly higher in BC samples than in BPH bladder urothelial samples (110,083 (IQR: 16,711)) ($p = 0.001$), the alpha diversity of tumor samples' microbiome was still significantly ($p < 0.001$) lower than that of non-tumor samples. The Chao1 alpha diversity index box plot in **Figure 15** shows that the tumor-specific microbiome richness is lower than that of the non-tumor urothelial microbiome at the genus level.

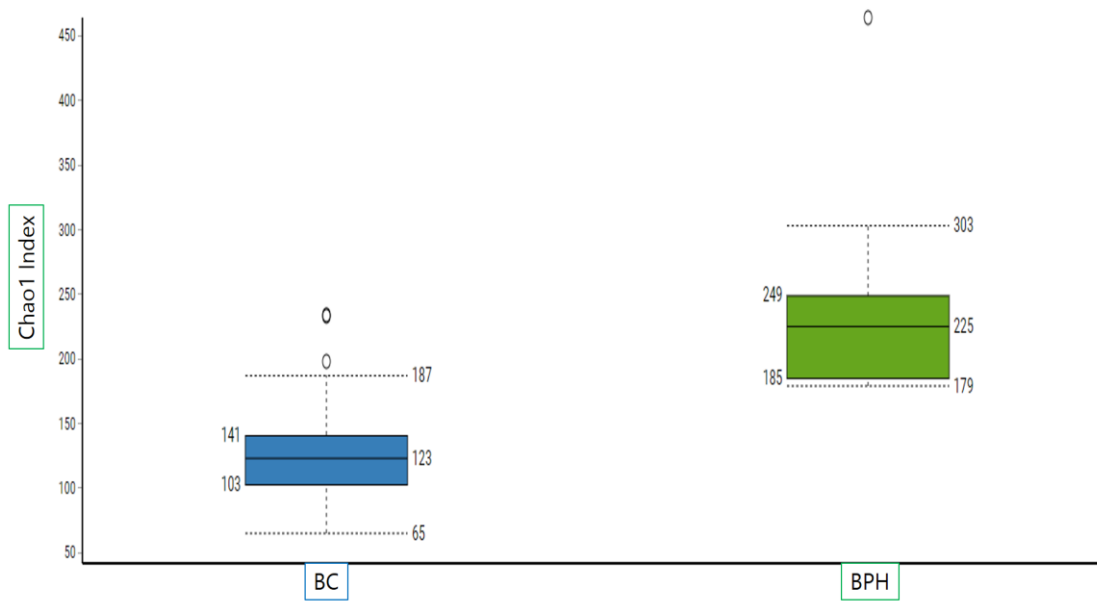


Figure 15: Chao1 alpha diversity box plot.

BC samples genus richness is significantly lower than BPH samples genus richness ($p < 0.001$).

Not only was the richness of genera significantly different between the two study groups, but the underlying compositions of microbial communities also differed significantly, as **Figure 16** shows in the Jaccard beta diversity principal coordinate analysis. BC and BPH samples formed two distinct clusters, and the difference is significant with PERMANOVA statistical analysis ($p = 0.001$).

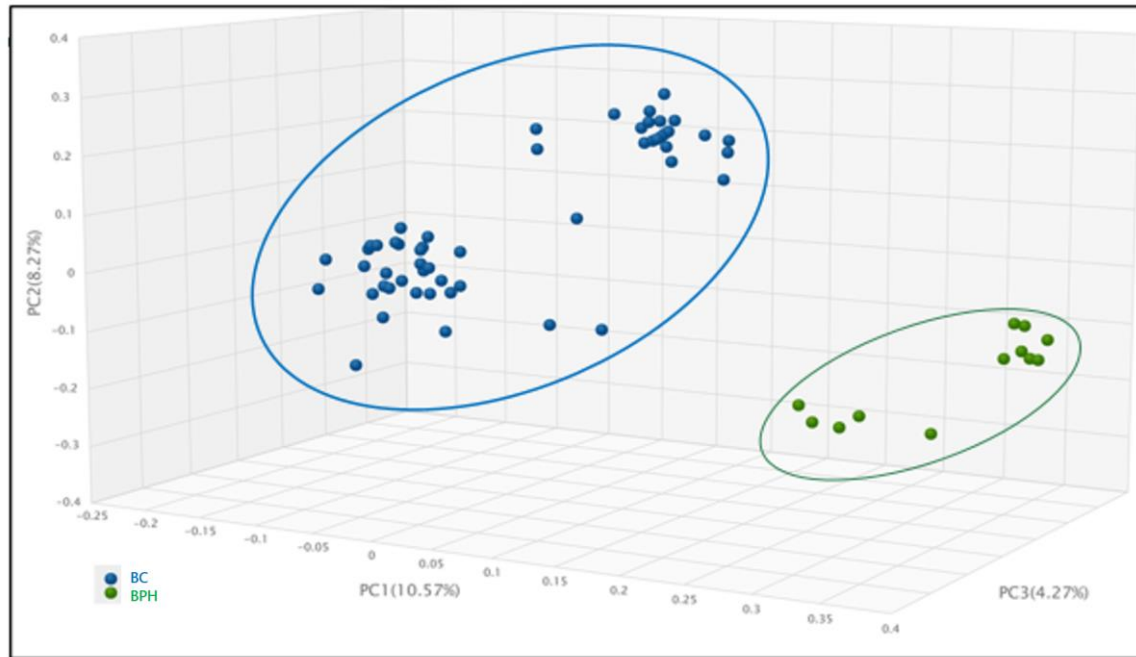


Figure 16: Jaccard Beta diversity principal coordinate analysis.

BC and BPH samples from two distinct clusters.

Figure 17 shows the most abundant phyla in BC and BPH groups. There is no significant difference between the *Firmicutes* phylum abundances (45% vs. 46%) in the two cohorts, but *Proteobacteria* (23% vs. 16%; $p = 0.006$), and *Actinobacteria* (13% vs. 4%; $p < 0.001$) have significantly higher abundances in the BC group than in the BPH one. The only phylum that had a higher abundance in the BPH group than in the BC group was *Bacteroidetes* (30% vs. 11%, $p < 0.001$). Although the *Cyanobacteria* phylum appeared with only a low median abundance in both groups (2% vs. 0.2%), its abundance was significantly higher in the BC group ($p = 0.011$).

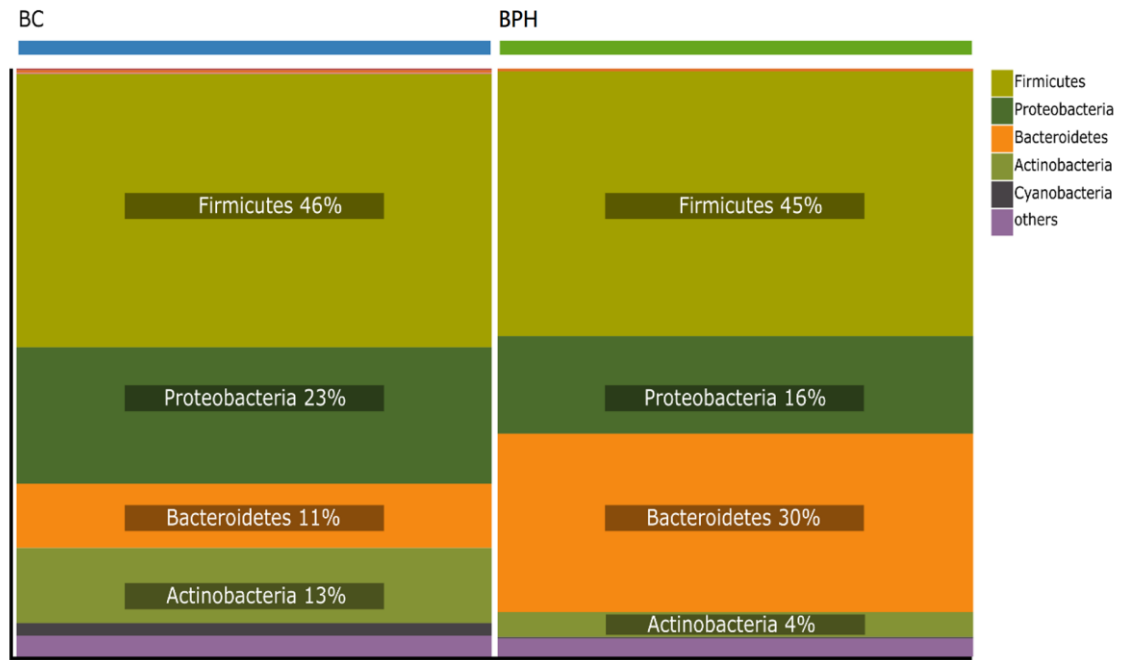


Figure 17: The most abundant taxa in BC and BPH cohorts at phylum level.

At genus level, the most striking differences between BC and BPH groups were in the abundance of *Bacteroides* (3.22% vs. 21.54%; $p < 0.001$), *Faecalibacterium* (1.92% vs. 7.79%; $p < 0.001$) *Staphylococcus* (7.89% vs. 0.59%; $p < 0.001$) and *Corynebacterium* (3.83% vs. 0.63%; $p = 0.001$). Just as there was a significant difference in the *Cyanobacteria* abundance at the phylum level, *Oxyphotobacteria* belonging to the phylum was not a dominant genus in the samples; however, its abundance was significantly higher in the BC group (2.11% vs. 0.07%; $p = 0.024$) (**Figure 18**).

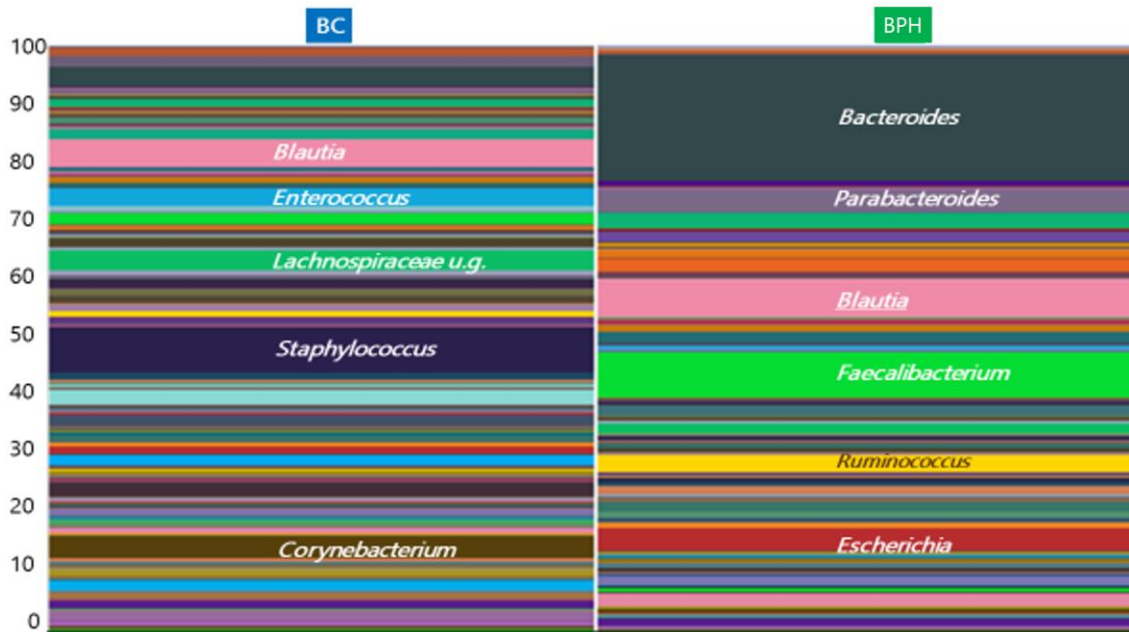


Figure 18: The most abundant taxa in BC and BPH cohorts at genus level.

According to the heatmap shown in **Figure 19**, BC and BPH cohorts and additional nine BC samples are sharply differentiated. Although no bacteria were cultured from any urine sample using the traditional aerobic culture method, for these 9 BC samples, the presence of a current infection could not be ruled out since 1–1 bacterial genera were present in them with exceptionally high abundance. The following genera had high abundance in these samples: BC10: *Streptococcus* (69%), BC17: *Corynebacterium* (93%), BC24, 36: *Gardnerella* (59%, 92%), BC31, 32: *Staphylococcus* (57%, 97%), BC40, 50: *Ureaplasma* (95%, 93%), and BC46: *Lactobacillus* (94%). Samples with an assumed ongoing infection were excluded from further comparative microbiome analyses and defensin expression studies.

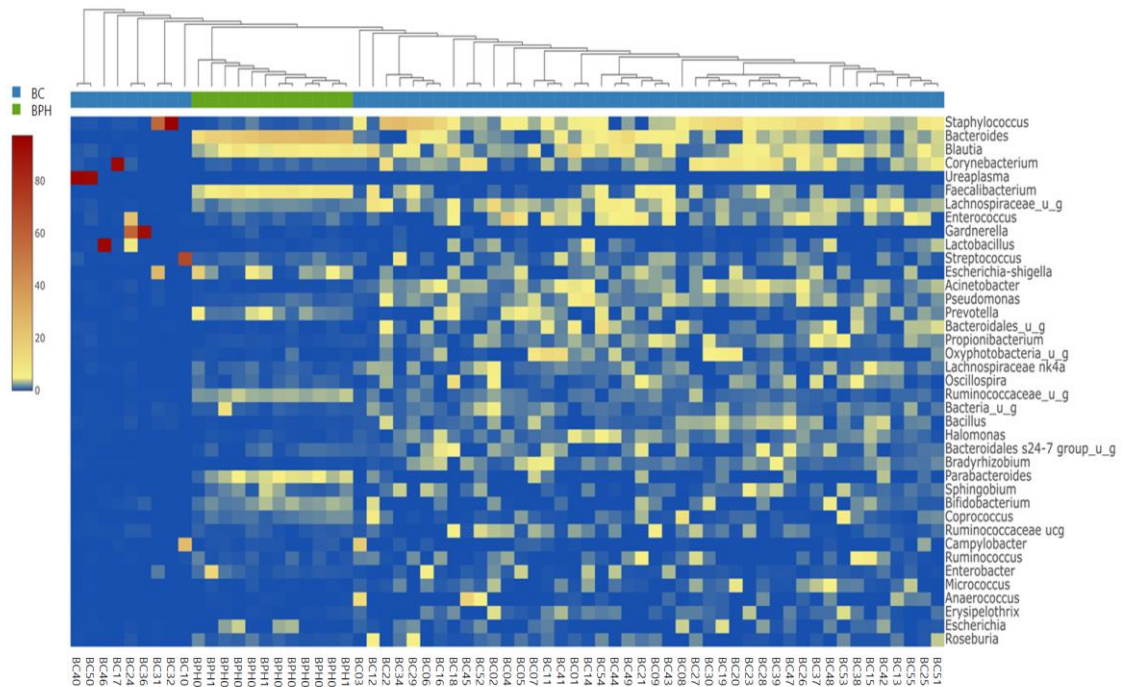


Figure 19: Heatmap visualization of the most abundant taxa at genus level among the BC and BPH patients.

4.4.2. Correlations Between Patient Clinical Data and the Microbiome

We compared the microbiome composition of the cohorts created on the basis of the patients' clinical data. The Jaccard β diversity PCoA analysis on **Figure 20** did not confirm significant differences among the microbiome composition of patient groups according to diagnosed hypertension or diabetes mellitus, smoking habits, or tumor grade and stage classification. The only significant difference was observed among the cohorts of male and female patients.

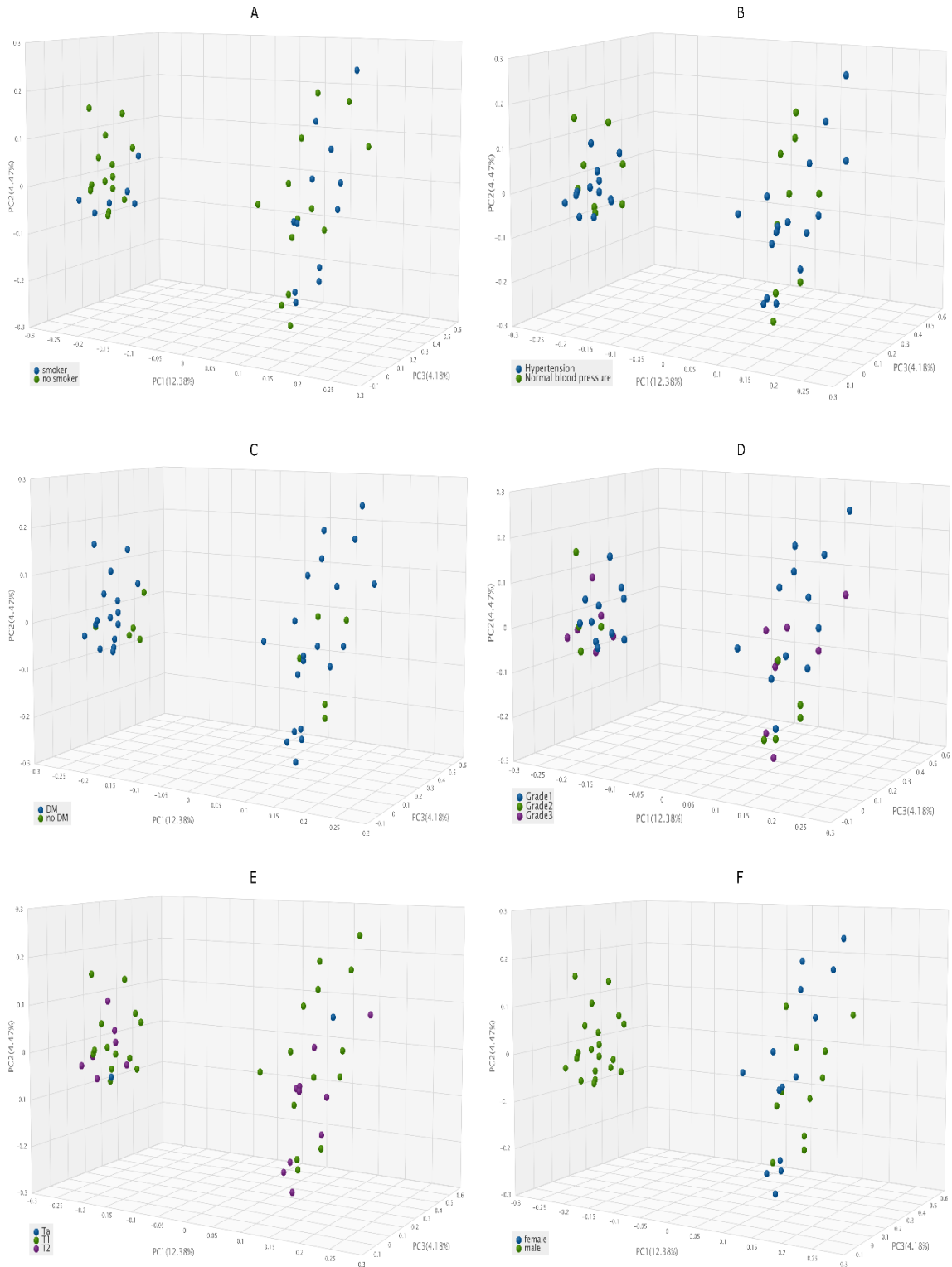


Figure 20: Jaccard beta diversity graph for tumor samples.

In the two distinct groups, the samples of the patients were not arranged according to whether the patients smoked (A), whether they had a diagnosis of hypertension (B) or DM (C), or what the grade (D) and stage (E) of their tumor were.

4.4.3. hBD1-2-3 Level in Urine in Accordance with hBD 1-2-3 Expression in Tissue Samples

The median hBD1 level was 12.59 ng/mL (IQR: 12.04) in the BPH group, 12.33 ng/mL (IQR: 4.09) in the HV group, and 20.28 ng/mL (IQR: 21.44) in the BC group. There was no relevant difference between the urine defensin levels between the BPH and healthy control groups, with a *p* value of 0.65. The median amount of hBD2 was 30.45 pg/mL (IQR: 21.93) in BPH, 31.59 pg/mL (IQR: 28.88) in HV, and 151.69 pg/mL (IQR: 560.89) in the BC group. There was no significant difference between the amounts in urinary hBD2 of the BPH and HV groups. hBD3 levels were 151.96 pg/mL (IQR: 202.36), 186.44 pg/mL (IQR: 198.95), and 653.73 pg/mL (IQR: 1321) in the BPH group, HV group, and BC group, respectively. The amounts of urinary hBD3 in the BPH and HV groups did not show significant differences either. The box plot in **Figure 21** shows that there was no significant difference between the amounts of defensin in the urine of the BPH and HV groups; they differed significantly only from the urinary amounts of defensin in the BC group.

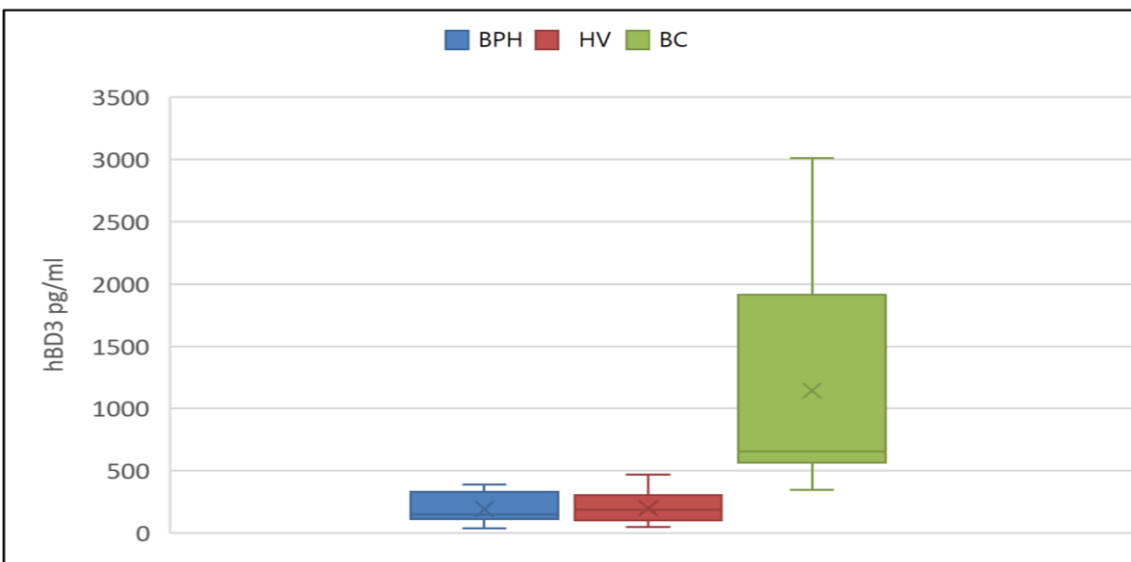
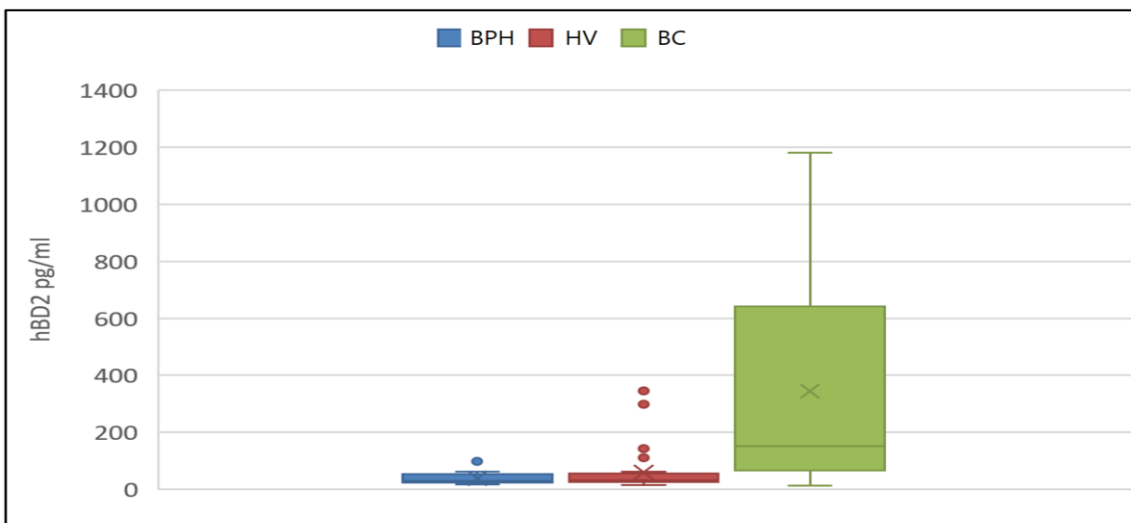
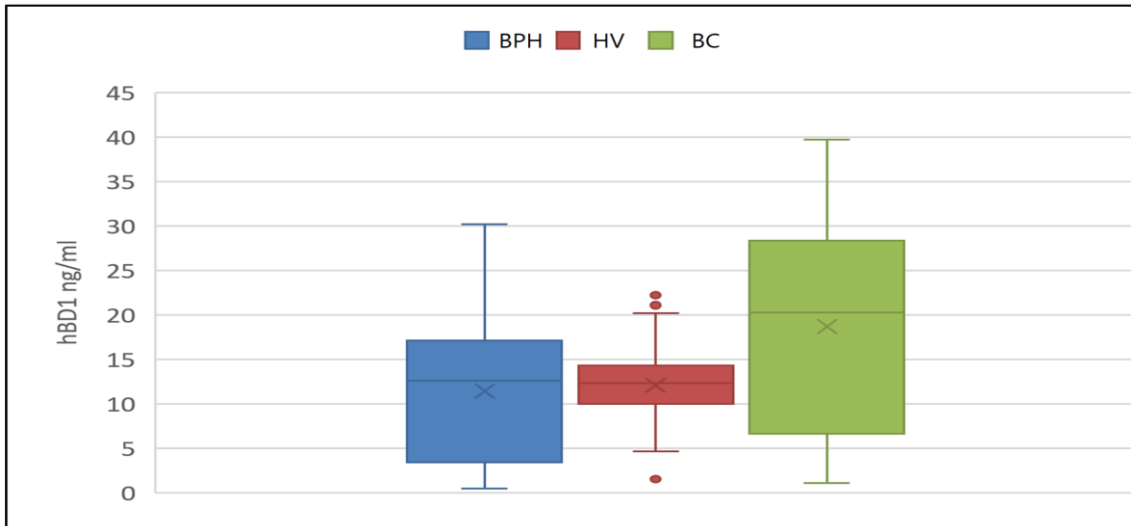


Figure 21: hBD1, hBD2, and hBD3 amounts in the urine of BPH, HV, and BC patients.

Histological samples were only from BC and BPH patients to detect hBD expressions. The BPH patients were exclusively male ones and tended to belong to the older age group, but their urinary defensin levels did not differ from the levels measured in the healthy population. Since there was no significant difference in the amounts of defensin in the urine of the BPH and HV groups, we considered the defensin expression of the tissue samples of the BPH group as a healthy control. In **Figure 22**, the median defensin value of the BPH samples was considered to be one unit. The x-axis of the figure shows multiples of the unit value. Along the y-axis, we plotted the percentage of the BPH and BC samples that represented the given value.

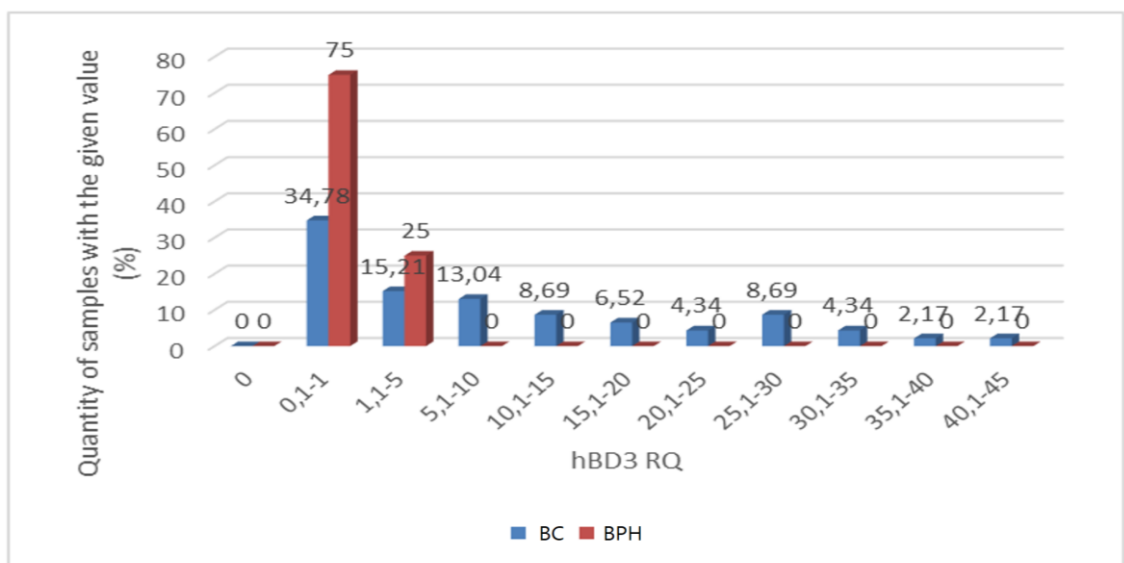
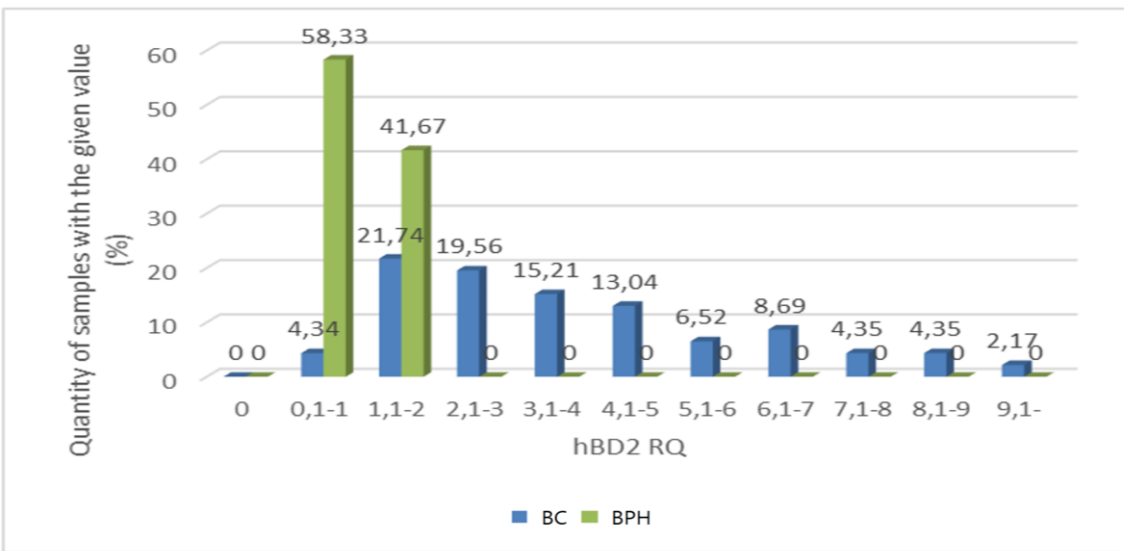
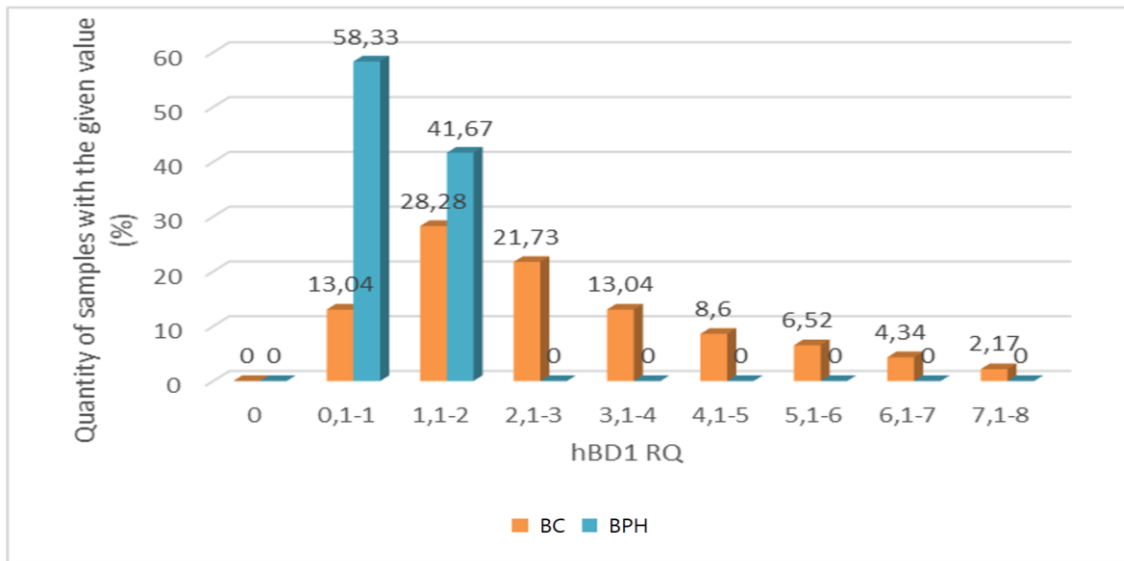


Figure 22: Defensin mRNA expression RQ values from tissue samples.

The amount of hBD1 mRNA expression was elevated in 58% of excised cancer tissue. In 2% of the tumor samples, the expression increased to eight times the mRNA level expressed by healthy tissue. The elevated level of hBD1 mRNA expression was not associated with the increase of urinary hBD1 levels, and even with high mRNA expression in tumor tissue, there were low urinary hBD1 levels. In our opinion, although the tumor tissue produces a large amount of hBD1, the surrounding mucous membrane is genetically determined to produce low levels hBD1, so overall, only a moderate increase in the level of hBD1 can be seen in the urine.

Increased mRNA expression was confirmed in 74% of cancer tissues, and the amount of hBD2 increased in the urine of 78% of BC patients. The hBD2 mRNA expression increased to a maximum of eight times higher than the healthy value, but the amount of hBD2 measurable in the urine could be up to 1000 times higher than the average healthy level. In our opinion, not only the tumor tissue produced hBD2, but the hBD2 production of the other healthy bladder mucosa cells also set the high level of hBD2 measurable in the urine.

The discrepancy between the elevated hBD3 expression of the tumor tissue (up to 40 times compared to healthy) and the hBD3 levels measurable in the urine was even more significant (more than 2500 times the healthy level). Cancer tissue mRNA expression was increased in 50% of the samples, while the amount of hBD3 was multiplied in each urine sample.

4.4.4. Correlations Between hBD Levels and the Composition of the Microbiome

The amount of hBD1 production in healthy cells is genetically determined, microbes appearing in the environment do not affect it. At the same time, it depends on their individual sensitivity at which level of hBD1 the microbes are able to survive. The urinary hBD1 level of the BC patients was partly below and partly above the healthy median value. Only patients with lower hBD1 amounts contained the *Oxyphotobacteria* genus, a member of the *Cyanobacteria* phylum, in their cancer tissue microbiome. The presence of the other bacterial genera is probably influenced by several external factors. A direct correlation was found only between the presence of the *Oxyphotobacteria* genus and the hBD1 level. We did not find any other genus whose abundance depended solely on the abundance of hBD1, hBD2, or hBD3 (**Figures 23 and 24**).

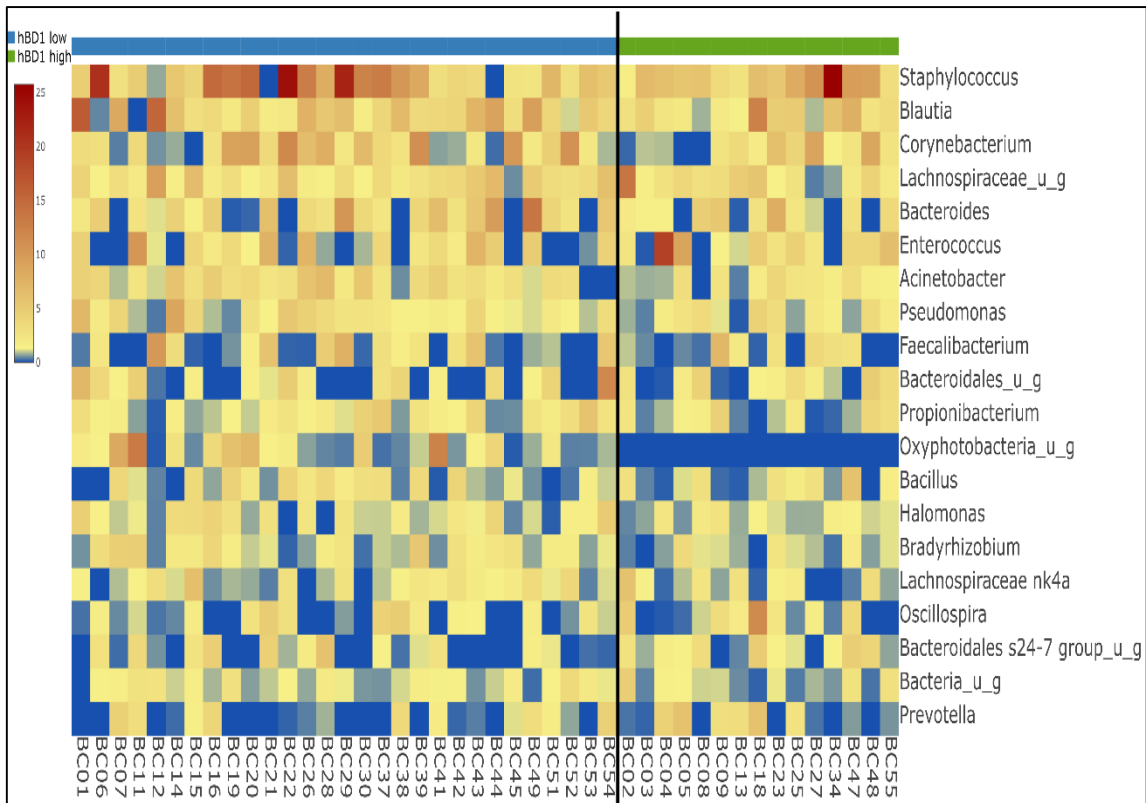


Figure 23: Heatmap visualization about the correlation between the amount of hBD1 in urine and the abundance of different genera in BC tissue microbiome.

Oxyphotobacteria is the only genus whose occurrence differs significantly from the quantitative change in hBD1. We found that *Oxyphotobacteria* was present only at low hBD1 levels.

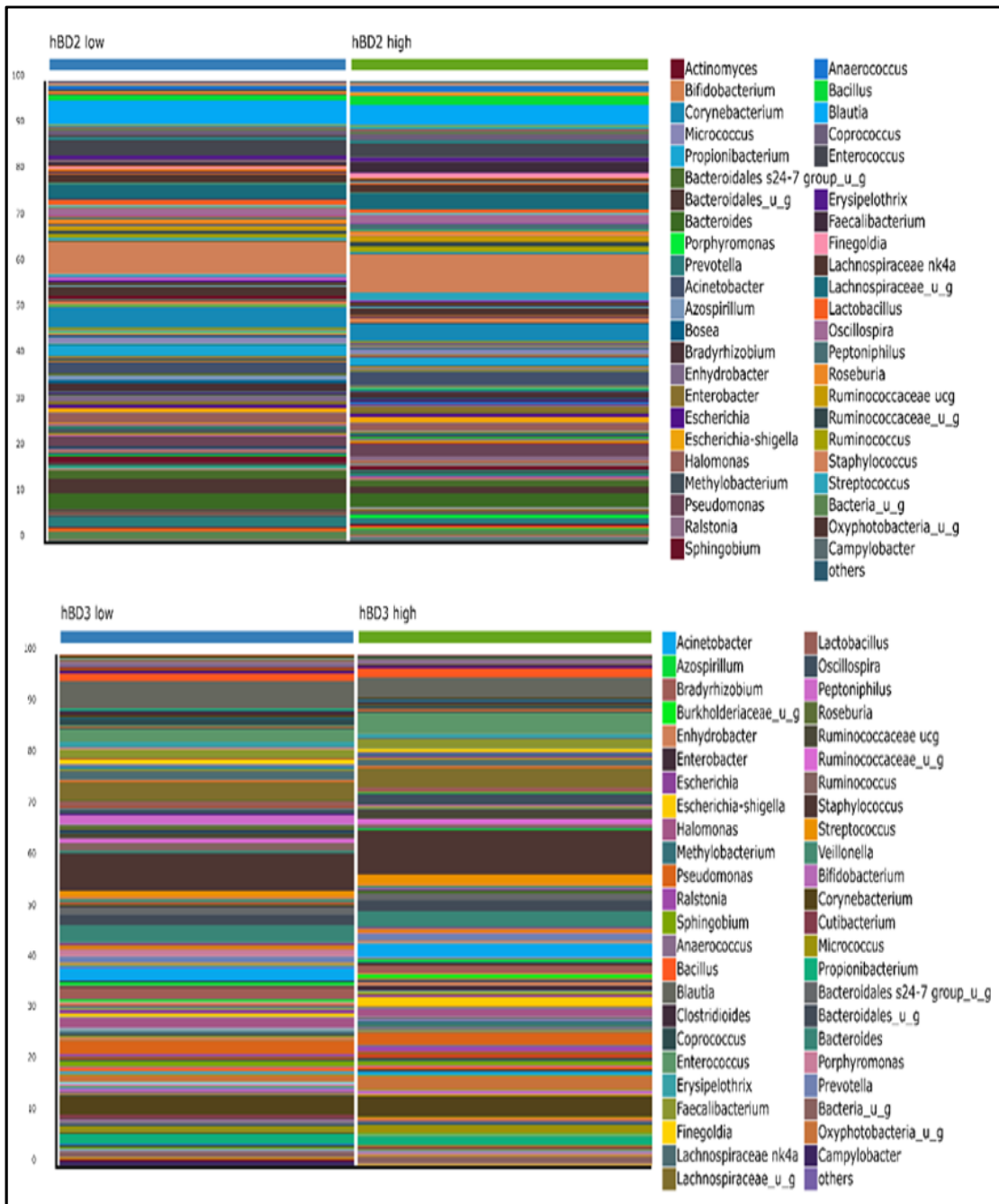


Figure 24: Stacked bar visualization of genera abundance according to different hBD2 or hBD3 amounts.

There is no significant difference due to the urinary level of hBD2 or hBD3 alone in the abundance of the genera in the BC tissue microbiome.

The production of hBD2 and hBD3 is influenced by the environmental microbiome, but together with several other factors, hBD2 and hBD3 levels select among the colonizing microbes. Based on our study, bacterial genera more characteristic of healthy

tissue, such as *Bacteroides*, *Faecalibacterium* and *Parabacteroides*, appeared in the tumor tissue with decreasing abundance as hBD2 and hBD3 levels increased. We did not find any correlation between the abundance of *Blautia*, *Lachnospira*, or *Oxyphotobacteria* and the change in hBD2-hBD3 levels. Associated with common high hBD2 and hBD3 levels, the dominant genera of tumor tissue – *Staphylococcus* and *Corynebacterium* – were present in high abundance in the microbiome of BC patients (Figure 25).

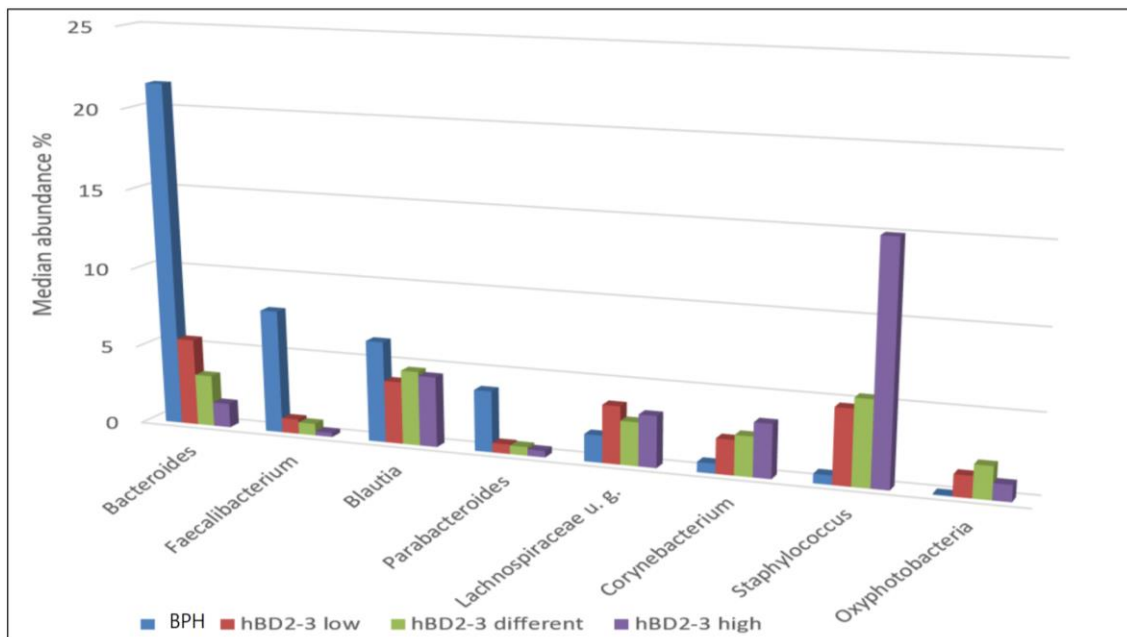


Figure 25: Correlation between the amount of hBD2 and hBD3 in urine and the abundance of the characteristic genera in BPH and BC tissue microbiome.

5. Discussion

Fajkovic et al. (108), who were among the first to study the composition of the urine microbiome using sequencing techniques, established in 2011 that there is a significant difference between male and female urine samples since the abundance of *Actinomycetes* is higher in female urine. These differences could be responsible for the lower incidence of bladder cancer in women (29).

Previous studies looking for a link between BC and the urinary tract microbiome have generally looked at the composition of the urinary microbiome (30, 100, 101, 109, 110). These studies obtained very different results in the comparison of the urine results of healthy and bladder cancer patients. Bucevic Popovič et al. and Moynihan et al. (100, 111) observed no statistically significant differences evaluating the species richness and Simpson index between the urine samples of BC and healthy volunteer patients. In contrast, Wu et al. (101) found a significant increase in bacterial richness and the Chao1 index in the urine of BC patients. Urine samples are usually used in studies of urothelial cancer because of their noninvasive nature, but the differences between the results obtained in these studies draw attention to the fact that the urine sample does not necessarily show an accurate picture of the microbiome associated with the tumor. According to our results, as we also did not find significant differences between diversity values of the transurethral catheterized urine and tissue microbiota (examining male and female samples together), it could be assumed that the urinary microbiome is a fair proxy of the tissue bacterial environment and that it is suitable as a diagnostic and prognostic tool in bladder cancer research. However, our results, which observed significant differences between the richness and Shannon diversity of female and male tissue samples, confirm that transurethral catheterized urine samples are not mirror images of the microbiota at the site of the cancer.

Differences in diversity are based on differences in the abundance of different genera. Xu et al. (99), comparing the urine of six healthy individuals with eight patients with urothelial cancer, observed the enrichment of *Streptococcus* in the urine of cancer patients. In contrast, Bucevic Popovič et al. (100) found *Streptococcus*, *Veillonella* and *Corynebacterium* as the most abundant genera in healthy patients' urine. *Streptococcus*, *Corynebacterium*, and *Fusobacterium* genera were similarly enriched in the urine of our BC patients. Wu et al. (101) found *Acinetobacter*, *Anaerococcus*, *Rubrobacter*, *Sphingobacterium*, *Atoposites*, and *Geobacillus* in the urine of BC patients in high

abundance, while in our BC patients the abundances of *Acinetobacter* and *Anaerococcus* increased depending on the age and gender group, and the other four genera occurred only in minor quantities.

We assume that the composition of the urine microbiome is influenced by gender, age, eating habits, environmental influences, and the current state of health. In the same way, the difference in sampling, such as voided urine, urine taken via a transurethral catheter, or urine taken with a suprapubic puncture, can also cause a big difference in the results (112, 113). In addition to the more or less changing microbiome status of the urine, the composition of the microbiome constantly adhering to the bladder mucosa may show greater constancy. We verified this assumed constancy by matching the microbiome results of multiple tissue samples taken from the given person (104).

At the time of conducting our first study, there was no data in the literature on whether there was a difference in the microbiome composition of bladder cancer tissue and urine. Comparing the urine and tissue samples' microbiomes of ten BC patients, we confirmed the presence of the same genera from the two types of samples, but not only were the abundance values significantly different within one person, but we also did not find any tendency for differences in abundance between urine and tissue when we examined the data of each person. Pederzoli et al. (114) compared urine, cancerous, and healthy tissue samples from 21 male and 8 female patients. According to their results, the urinary microbiome shares >80% of the bacterial families present in the paired bladder tissue, but the comparative results of their studies at the genus level were not reported. Based on our small number of patients (five men and five women), we did not see any difference in bacterial genus abundance between the genders. The most abundant genus in the study of Pederzoli et al. was *Klebsiella* in female urine samples and *Burkholderia* in both male and female cancer tissue samples. Despite the non-negligible individual differences, we found five genera – *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter*, and *Klebsiella* – with a significantly higher abundance in cancerous tissue samples than in the urine samples of BC patients. We suspected that these five genera overrepresented in the tissues, played a role in the development or progression of the tumor. Most of the research results on the relationship between microbiome composition, chronic inflammation, and tumor formation can be found on the topic of colorectal carcinoma (CRC). These results confirmed our assumptions about the bacterial genera associated with the tissue that we detected in high abundance. *Bacteroides* and *Akkermansia* – known as mucin degrader genera – undermine the integrity of the mucosal

barrier and lead to increased inflammation (115, 116). Baxter et al. found a strongly positive correlation in a CRC animal model between an increased tumor burden and the abundance of *Bacteroides* and *Akkermansia* (117). Microbes can play an important role not only in the formation of malignancies but also in their progression or metastasis formation. *Clostridium septicum* appears to have a symbiotic relationship with the growth of already existing tumors but does not appear to initiate carcinogenesis (118). However, the butyrate-producing *Clostridium spp.* inhibits intestinal tumor development (119). Enterobacter can participate in colon tumor initiation and progression through apoptosis inhibition (120). Colibactin, one of the toxins produced by *Klebsiella*, causes DNA double-strand breaks, induces genomic instability, and cell cycle arrest (121). In the presence of *Klebsiella*, the continuous influx of proinflammatory cytokines into the colonic microenvironment also leads to chronic inflammation and epithelial cell proliferation (122). Based on the knowledge that the presence of *Fusobacterium* is high not only in CRC but in oesophagus and pancreatic tumors (123, 124), Bucevic Popovič et al. looked for the presence of *Fusobacterium* DNA in BC urine samples, but it was detectable only in 25% (100). None of our tissue samples contained *Fusobacterium* DNA at the level of detection, but it was represented in all our urine samples. Prior to our research, only Liu et al. (102) performed microbiome detection directly from bladder tumor tissue. They found *Cupriavidus*, *Acinetobacter*, *Anoxybacillus*, *Escherichia-Shigella*, *Geobacillus*, *Pelomonas*, *Ralstonia*, and *Sphingomonas* genera with higher abundance in the tumor relative to healthy tissues (102). In our study, *Cupriavidus* and *Pelomonas* were represented only in two tissue samples, and the urine of these two patients did not contain any detectable amounts of these genera. *Acinetobacter*, *Anoxybacillus*, *Escherichia-Shigella*, *Geobacillus*, *Ralstonia*, and *Sphingomonas* were increasingly represented in our tissue samples compared to the urine samples. The fact that these latter genera, as well as our “five suspects”, are detectable in greater quantities in tumor tissue than in urine strengthened the hypothesis that these bacteria were associated with the tumor-altered tissue. A subsequent study comparing the microbiome of samples taken from cancerous and healthy bladder areas found a significantly higher abundance of *Actinobacteria* in the samples of nonneoplastic bladder mucosa than in tumor tissues (125). In our studies, neither cancerous tissue nor healthy tissue samples contained *Actinobacteria* DNA.

In our second study, we were interested in whether the tissue microbiome of non-cancerous (BPH) patients and BC patients differed, and at what defensin concentrations

these specific bacterial associations were formed. Both the bacteria present and differences in defensin levels have already been reported to have an influence on the success of BC treatment using checkpoint inhibitors, or BCG (69, 70, 126, 127). To date, apart from ours, we have found only 3 publications that examine the microbiome of healthy and tumorous tissue in the case of BC (102, 114, 125).

We have shown distinct differences in both alpha and beta diversity between the BPH and BC tissue microbiome results. Alpha diversity can be used to describe how many different taxa a sample contains, while beta diversity shows what kinds of different taxa make up the different samples. Parra-Grande et al. (125), Liu et al. (102), and Pederzoli et al. (114) all confirmed our results showing that the alpha diversity of the microbiome of non-tumorous tissue is significantly higher than that measured in cancerous tissue. Parra-Grande et al. and Pederzoli et al. did not recognize a significant difference between the beta diversity data, but the results of Liu et al. and our results show two distinct clusters at beta diversity- Mucosa-associated microbial structure in the cancerous tissue group was significantly different from that of the noncancerous tissue group.

Examining only the tumor tissue samples, we found no significant difference when the samples were divided into smoker and non-smoker groups, neither when the division was based on associations with diabetes, hypertension, or tumor grade and stage. Considering both of our studies, comparing 38+5 male and 14+5 female samples, we saw a significant difference in beta diversity according to gender, but Pederzoli et al. did not confirm this with their 21 male and 8 female samples.

As a result of our first study, we hypothesized that the bacterial genera that were present in significantly higher abundance in the tumor tissue than in the urine played a role in the development or progression of the tumor. Our second study, on the other hand, pointed to the fact that *Bacteroides* abundance is also high in non-neoplastic tissue when comparing the mucosal microbiome of BPH and BC samples. Comparing all our results, we now believe that the interpretation is more correct if we say that the *Bacteroides* genus was associated with mucus and not with free urine.

At the genus level, there was a significant difference in the abundance of taxa, whereby *Bacteroides* and *Faecalibacterium* were highly present in healthy tissue, while *Staphylococcus*, *Corynebacterium*, and *Oxyphotobacteria* dominated in tumor samples. The protective effect of *Faecalibacterium*, which has shown low abundance levels in our tumor tissue samples, was described and confirmed by previous studies against colon,

prostate, or even breast cancer, if it was present in high abundance in feces (128-130). *Staphylococcus saprophyticus* is a type of bacteria that can cause urinary tract infections and is known to strongly adhere to human urinary bladder carcinoma cells (131). However, there is no direct evidence linking *Staphylococcus saprophyticus* to bladder cancer. The review article by Wei et al. (132) discusses the association between various tumors and an elevated abundance of *Staphylococcus*. The article mentions a few examples where a high abundance of *Staphylococcus* was confirmed in BC patients (101, 103, 125), but does not establish a causal relationship between the bacteria and cancer. We did not find any data on the abundance of *Corynebacterium* in BC with the examination of tissue samples, but the described result is contradictory: the abundance of *Corynebacterium* in urine samples is lower in the case of BC than in healthy people (100, 113). The *Oxyphotobacteria* genus is a member of the *Cyanobacteria* phylum. Despite the fact that representatives of the *Oxyphotobacteria* genus were not among the most frequently occurring taxa in the samples, according to our studies, the difference in abundance between the BC and BPH groups was significant. *Oxyphotobacteria* are present in all aquatic ecosystems throughout the world, and produce toxic secondary metabolites like microcystins. Negative effects of microcystins – irritation, acute poisoning, tumor promotion, and carcinogenesis – indicate that *Oxyphotobacteria* are relevant medical and economic problems that may affect both fish and fish consumers, including humans (133, 134).

No bacteria were cultured from any of the fifty-five urine samples of the second study, but in nine tissue samples, *Streptococcus*, *Corynebacterium*, *Gardnerella*, *Staphylococcus*, *Ureaplasma*, or *Lactobacillus* were represented with remarkably high abundances. Although we proved in our first study that the urine and tissue microbiomes are different from each other, we assumed that in these cases the explanation was the insufficiency of the detection method. Due to the presence of an existing infection that, eventually, could not be ruled out and may have resulted in a shift in local defensin levels, these samples were excluded from the defensin ELISA and expression tests.

To our best knowledge, our study was the first to look for a correlation between the tissue microbiome of bladder carcinoma patients and human beta-defensin levels that can be measured in urine.

hBD1 is a multifaceted antimicrobial peptide that is coded on chromosome 8p as a tumor-suppressor gene (87). hBD1 is constitutively expressed in urogenital tract epithelia, but dysregulation of hBD1 gene transcription has been demonstrated in several types of

cancer (135). Decreased expression of hBD-1 has been observed in both prostatic and renal carcinomas (136). Although 58% of our tumor tissue samples showed increased hBD1 expression compared to BPH and HV patients, urinary hBD1 levels in BC patients did not increase significantly. Assuming that these patients produce a genetically determined lower amount of the tumor suppressor hBD1 with their healthy mucosal cells, it can be concluded that they have a higher chance of developing a tumor. Another interesting connection is that the tumor-causing *Oxyphotobacteria* were present in the tumor microbiome of BC patients whose urinary hBD1 levels were particularly low.

The hBD2 and hBD3 levels in the urine of BC patients, which were up to several thousand times higher than the healthy levels, could be explained only partially by the increased expression of the tumor tissue. The expression of the latter two antibacterial peptides can be induced, and their quantity can be influenced by bacteria, viruses, chemicals, and many other factors in the environment of the mucosal cells (137). Inducible defensins and microorganisms mutually affect each other's presence in the bladder; and a number of external factors may also affect the resulting balance. The ability to kill gram-negative bacteria is also characteristic of hBD1, 2, and 3. In addition, hBD3 is bactericidal against some gram-positive bacterial strains (138). Our microbiome analysis shows a momentary composition in that it constantly changes, and is influenced by many factors. When examined as individual factors, the increase in hBD2 or hBD3 levels was not correlated with the abundance of bacterial genera, but the combined effect of hBD2 and hBD3 shows an increasingly pronounced difference in the abundance of the genera characteristic of the BPH and BC groups. In this "snapshot" composition, gram-negative bacteria appear with a lower abundance in BC at high levels of hBD2 and 3 compared to the healthy state. With the common hBD2 and 3 levels rising, the abundance of non-tumor-specific genera is reduced (*Bacteroides*, *Parabacteroides*, and *Faecalibacterium*) and the abundance of in-tumor tissue-characteristic genera (*Staphylococcus*, *Corynebacterium*) is elevated.

6. Conclusions

During the research on the therapeutic failures of BC, both the microbiome composition and differences in hBD levels have already been proven to be important causal factors. With our present research, on the one hand, we were looking for a correct research method for examining the microbiome associated with BC, and on the other hand, by examining the relationship between hBD and microbiome changes, we are trying to gain knowledge that can contribute to the planning of more successful individual therapies in the future. The novel observations of our investigations in response to the objectives are as follows:

1. The method developed by our research group is also suitable for examining the microbiome from urine and tissue samples.

2. The microbiome composition is not different at the distant points of the tumor tissue sample, it is characteristic of the given patient.

3. We found that the urine microbiome does not reflect the composition of the microbiome that can be detected in the tissues, and that the constituent taxa differ not only in their proportions but also in their quality between the two sample types. Thus, we found that, due to its non-invasive nature, the more favorable urine sampling is not suitable for examining the relationship between BC and microbiome composition.

4. Based on our first study, the most abundant genera in urine samples were *Lactobacillus*, *Corynebacterium*, *Streptococcus*, and *Staphylococcus*, and in tissue samples, *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter*, and *Klebsiella* genera showed remarkably higher compositional abundance.

5. Our second study, which compared the histological samples of BPH (healthy) and BC patients, found that among the tissue-associated bacteria, the genera *Bacteroides*, *Parabacteroides*, *Blautia*, and *Faecalibacterium* were found in the healthy tissue, while the genera *Staphylococcus*, *Enterococcus*, *Corynebacterium*, and *Lachnospira* were found in the tumor tissue in higher abundance.

6. The increase in hBD1 levels in the urine of BC patients did not follow the increased expression rate of the tumor tissue; therefore, we concluded that the production of hBD1 in the non-tumorous mucosa in these patients is genetically lower than that of the average healthy person.

7. The expression of hBD2 and hBD3 in the tumor tissue also increased compared to the healthy level, but the levels of hBD2 and hBD3 in the urine of BC patients increased

several times more than expected. We conclude that the inducible production of defensin by the non-neoplastic mucosa contributed to this increase.

8. The microbiome composition detectable in the tumor tissue and the defensin levels measurable in the urine are related to each other. The carcinogenic *Oxyphotobacteria* were present at low defensin-1 levels. Along with the increase in hBD2 and hBD3 levels, the abundance of the bacterial genera that are more common in tumors than in healthy tissue also increased.

7. Summary

By sequencing the DNA of samples taken from anatomical areas traditionally considered sterile, the identification of bacterial DNA and the detection of the microbiome became possible. Some previous research has examined the urinary microbiome of patients with bladder cancer.

Human beta-defensins (hBD) are peptides with antibacterial and antitumor effects. The amount of defensins depends on the genetic characteristics of the given person, or the production of some types of defensins is increased by microbes appearing in the environment. An elevated hBD2 level reduces the success of antitumor BCG therapy, and the composition of the microbiome also affects the effectiveness of checkpoint inhibitors.

A 16S rRNA-based microbiome analysis was performed from bladder tissue samples removed for histological examination during bladder carcinoma, or prostatic hypertrophy surgery, as well as from urine samples taken during surgery. We determined the hBD1-2-3 content of the urine using the ELISA method and the hBD1-2-3 expression of the tissue samples using the RT-PCR method.

Urine and histological microbiome results differ from each other: the urine sample is not suitable for examining the relationship between tumor and microbiome. The *Akkermansia*, *Bacteroides*, *Clostridium*, *Enterobacter*, and *Klebsiella* genera showed higher abundances in tissue samples than in urine ones. Among the mucosa-associated bacteria, the *Staphylococcus* and *Corynebacterium* genera were present in the tumor area, while the *Bacteroides* and *Faecalibacterium* genera were present in the healthy mucosa area in higher abundance. There is no significant difference between hBD1-2-3 levels in healthy and prostatic hyperplasia samples, but hBD2 and hBD3 levels are significantly higher in the urine of patients with bladder cancer. hBD1 expression was increased in some of the samples in the tumor tissue, but hBD2 and hBD3 expressions could be produced in larger quantities in the tumor tissue and in the healthy mucosa. Only with low hBD1 urine levels the proven carcinogen *Oxyphotobacteria* genus occurred in tumor samples; with the combined increase of hBD2-3 levels, the abundance of *Bacteroides* and *Faecalibacterium* in the samples decreased, while the abundance of *Corynebacterium* and *Staphylococcus* increased.

For successful individualized therapy of bladder carcinoma, it is necessary to know as precisely as possible the dynamic equilibrium relationship between the microbiome composition and defensin levels.

8. Összefoglalás

A hagyományosan sterilnek tekintett anatómiai területekről vett minták DNS tartalmának szekvenálásával lehetővé vált bakteriális DNS rendszertani beazonosítása és a mikrobiom detektálása. Korábbi kutatások a hólyagrakos betegek vizelet mikrobiomját vizsgálták.

A humán béta defenzinek (hBD) antibakteriális és daganatellenes hatású peptidek. A termelődő defenzinek mennyisége függ az adott személy genetikai adottságaitól, de néhány defenzintípus termelődését fokozzák a környezetben megjelenő mikrobák. A hBD2-szint növekedése csökkenti a daganatellenes BCG terápia sikerét, valamint a mikrobiomösszetétel befolyásolja a checkpoint inhibitorok hatékonyságát.

Hólyagkarcinóma, illetve prosztatata hiperplázia műtét során szövettani vizsgálatra eltávolított hólyag-szövetmintákból, valamint a műtét során vett vizeletmintákból végeztünk 16S rRNS alapú mikrobiomvizsgálatot, meghatároztuk a vizelet hBD1-2-3 tartalmát ELISA módszerrel, valamint a szövetminták hBD1-2-3 expresszióját RT-PCR módszerrel.

A vizelet és szövettani mikrobiom eredmények egymástól eltérőek: a vizeletminta nem alkalmas a daganat és a mikrobiom összefüggéseinek vizsgálatára. Az *Akkermansia*, *Bacteroides*, *Clostridium*, *Enterobacter* és *Klebsiella* genusok magasabb abundanciával rendelkeztek a szöveti mintákban, mint a vizeletben. A nyálkahártya-asszociált baktériumok közül a *Staphylococcus* és *Corynebacterium* genusok a daganatos területen, míg a *Bacteroides* és *Faecalibacterium* genusok az egészséges nyálkahártya területén magasabb abundanciával voltak jelen. A hBD1-2-3 szintek között nincs szignifikáns különbség az egészséges és a prosztatata hiperpláziás mintákban, a hBD2 és hBD3 szintek azonban szignifikánsan magasabbak a hólyag karcinómás betegek vizeletében. A hBD1 expresszió a minták egy részében a tumorszövetben emelkedett, de a hBD2 és hBD3 expressziók a tumorszövetben és az egészséges nyálkahártyán is nagyobb mennyiségben termelődhetnek. Csak az alacsony hBD1 vizelet szintek mellett fordult elő a bizonyítottan daganatkeltő *Oxyphotobacteria* genus a daganatos mintákban. A hBD2-3 szint együttes emelkedésével a minták *Bacteroides*- és *Faecalibacterium*-abundanciája csökkent, *Corynebacterium* és *Staphylococcus* abundanciája növekedett.

A hólyagkarcinóma sikeres, egyénre szabott terápiájához szükséges a mikrobiomösszetétel és a defenzinszintek között fennálló dinamikus egyensúlyi kapcsolat minél pontosabb megismerése.

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10. Bibliography of the Candidates's Publications

Dissertation-related publications

1. **Mansour B**, Monyok A, Makra N, Gajdacs M, Vadnay I, Ligeti B, et al. Bladder cancer-related microbiota: examining differences in urine and tissue samples. *Sci Rep.* 2020;10(1):11042.
2. **Mansour B**, Monyok A, Gajdacs M, Stercz B, Makra N, Penzes K, et al. Bladder Tissue Microbiome Composition in Patients of Bladder Cancer or Benign Prostatic Hyperplasia and Related Human Beta Defensin Levels. *Biomedicines.* 2022;10(7).

Independent publications

3. Monyók Á, Sereg R, Vass I, Vadnay I, Kis Z, Lovasné Avató J, **Mansour B**. Nem izominvazív hólyagtumoros betegek analízise klinikopatológiai szempontól Heves megyében. *Magyar Urológia.* 2022;34(2):58-62.
4. Erdélyi B, **Mansour B**, Kovács I, Kovács P, Monyók Á, Vadnay I, Lovasné Avató J, Tóth E. Nem izominvazív hólyagdaganatok fizikai tulajdonságainak és a WHO 1973 és 20004/2016 grading rendszereinek szerepe a daganatok kiújulásának előrejelzésében. *Magyar Urológia.* 2022;34(2):63-70.

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OPEN

Bladder cancer-related microbiota: examining differences in urine and tissue samples

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The microbiota isolated from the urine of bladder carcinoma patients exhibits significantly increased compositional abundance of some bacterial genera compared to the urine of healthy patients. Our aim was to compare the microbiota composition of cancerous tissues and urine samples collected from the same set of patients in order to improve the accuracy of diagnostic measures. Tissue samples were collected from patients during cancer tissue removal by transurethral resection. In parallel, urine samples were obtained by transurethral resectoscopy from the same patients. The V3–V4 region of the bacterial 16S rRNA gene was sequenced and analyzed using the Kraken pipeline. In the case of four patients, duplicate microbiota analysis from distant parts of the cancerous tissues was highly reproducible, and independent of the site of tissue collection of any given patient. *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella*, as “five suspect genera”, were over-represented in tissue samples compared to the urine. To our knowledge, this is the first study comparing urinary and bladder mucosa-associated microbiota profiles in bladder cancer patients. More accurate characterization of changes in microbiota composition during bladder cancer progression could provide new opportunities in the development of appropriate screening or monitoring methods.

Bladder cancer ranks the ninth most frequently-diagnosed cancer worldwide with 75% cases occurring in men¹. While the main risk factor for bladder cancer is tobacco smoking², exposure to aromatic amines³ or environmental factors such as arsenic in drinking water⁴ have been classified as additional carcinogenic risks. In addition, genetic factors including slow acetylation of *N*-acetyltransferase, a key enzyme in aromatic amines metabolism, are believed to play roles in bladder cancer development⁵.

Urine was traditionally considered sterile; however recent evidence has challenged this dogma by molecular-based detection of microorganisms in urine of healthy individuals⁶. Although the presence of microbes in the urinary tract does not necessarily induce infections, some microbial agents do cause acute infection or chronic inflammation. On the same token, certain commensal strains may control the overgrowth of pathogenic bacterial strains. The association between bladder schistosomiasis infection, inflammation and squamous cell carcinoma is well accepted⁷, but the role of bacteria in the pathophysiology or management of bladder cancer has not been consistently examined. In addition to the genetic characteristics that influence the elimination of chemical carcinogens, the processes can be aggravated or attenuated by the presence of biochemically active microbes. For example, nitrate producing bacteria can mediate the formation of carcinogenic *N*-nitrosamines⁸. Toxins including heavy metals, pesticides, ochratoxins, polycyclic aromatic hydrocarbons or other environmental contaminants are removed from the bloodstream through renal filtration. All these compounds interact with the microbiota during ensuing storage in the bladder. The resulting metabolites can increase or decrease the risk of bladder cancer.

Several factors can influence the difference in incidence and progress of bladder cancer between men and women. The study of de Jong et al. described that male hormones may influence the type of bladder cancer that a patient develops⁹. The urinary microbiota is different in men and women⁶. The anatomical and hormonal

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Patients ID	Age	Gender	Biological characteristic	Urine sample ID	Tissue sample ID	
I01	70	Male	MIBC	UI1	TI01.1	
					TI01.2	
I02	80	Male	MIBC	UI2	TI02	
I03	79	Male	MIBC	UI3	TI03	
I04	76	Female	MIBC	UI4	TI04	
N01	72	Male	NMIBC	UN1	TN01.1	
					TN01.2	
N02	54	Male	NMIBC	UN2	TN02.1	
					TN02.2	
N03	66	Female	NMIBC	UN3	TN03.1	
					TN03.2	
N04	58	Female	NMIBC	UN4	TN04	
N05	20	Female	NMIBC	UN5	TN05	
N06	64	Female	NMIBC	UN6	TN06	
Summary	63.9	Male/female = 5/5	MIBC/NMIBC = 4/6	10	14	24

Table 1. Metadata of patients: MIBC: muscle invasive bladder cancer, NMIBC: non muscle invasive bladder cancer.

differences lead not only to higher incidence of female urinary tract infections, but to different compositions of urinary microbiota between genders. Incidence of female bladder cancer is significantly lower compared to bladder oncogenesis of men. The different microbiota can be one of the hypotheses for the lower incidence of bladder cancers in women^{10,11}.

The urinary microbiota associated with benign urologic conditions from transurethral catheterized urine or from voided urine was reviewed in detail¹⁰. In women voided urine samples contained mixtures of urinary and genital tract bacteria, however the uncultivated bacteria, assessed by 16S rRNA gene sequencing were common in voided, transurethral catheterized or suprapubic aspirated urine samples¹². In men patients with or without lower urinary tract symptoms voided urine does not adequately characterize the male bladder microbiota, and only the detectable bacteria in catheterized urine were associated to the severity of symptoms¹³.

Current knowledge of the microbiota in voided urine or tissue samples of bladder cancer patients is very limited^{14–17}. As far as we know, no studies to date have compared the characteristics of catheterized urine with tissue samples in bladder cancer.

Our aim was to determine whether the microbiota composition in samples from different sites of a given tumor tissue is consistent and to compare the microbiota isolated from the tumor tissue with that from the urine of the same patients.

Results

Using conventional culture methods, no bacteria were detected from the urine of any patient.

From the 39 urine and tissue paired samples collected, 24 samples provided sufficient DNA to meet the sequencing quality criteria. The 24 samples analyzed included 10 urine and 14 tissue samples collected from ten bladder cancer patients. Based on histological examination, four patients had muscle invasive (MIBC) and six patients had non-muscle-invasive (NMIBC) tumors. The male/female ratio was 2:4 in the NMIBC group, and 3:1 in the MIBC group (Table 1).

A total of 3.2 million valid sequences were obtained, resulting in 1.2 million high-quality reads. Within one sample, the median number of reads was 119,268.

Figure 1 shows the observed richness and Shannon diversity at the genus level, comparing the gender and sample size groups. Statistically significant differences were found only between the median of genus richness and Shannon diversity representing female and male tissue samples. Shannon diversity of female patients' transurethral catheterized urine samples were lower than that of male patients (not significant).

From two isolated distant pieces of the tumor, microbiota analysis was performed in four patients. Figure 2, correlation-based reproducibility measurements of bladder cancer tissue microbiota demonstrate that the microbiota analysis results were reproducible, independent of the actual site and highly specific to any given person. Pearson correlation coefficients were 0.98–1, and p values were < 0.05. However, at the Principal Component Analysis (PCA), Fig. 3 the correlated tissue samples do not cluster with their own urine samples. No regularity or tendency was shown regarding dominant microorganisms, there was no rule for how the abundance of genus changes between tissue and urine (Fig. 4).

In urine samples, the most abundant phyla detected were *Firmicutes* with abundance of 33%, followed by *Proteobacteria* (29%), *Actinobacteria* (23%), *Cyanobacteria* (7%) and *Bacteroidetes* (4%). In the tissue samples, the order was as follows: *Firmicutes* (34%), *Actinobacteria* (23%), *Proteobacteria* (22%), *Bacteroidetes* (15%) and *Cyanobacteria* (8%). The most abundant genera in urine samples taken as a whole were *Lactobacillus*, *Corynebacterium*, *Streptococcus* and *Staphylococcus*, with age and gender differences in each samples (Table 2). In tissue samples the most abundant genera were *Bacteroides*, *Akkermansia*, *Klebsiella* and *Clostridium sensu*

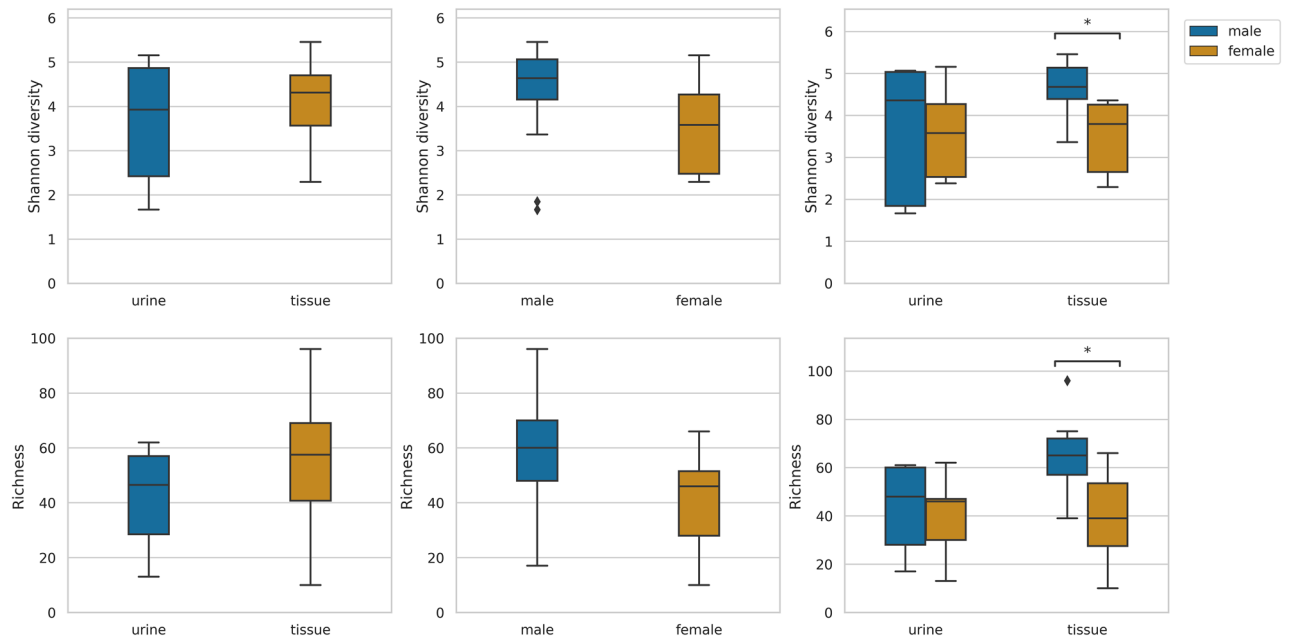


Figure 1. Observed richness and Shannon diversity at genus level from the urine and tissue samples obtained from patients: significant difference was shown only between the median of genus richness and Shannon diversity of female and male tissue samples.

stricto, *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella* genera showed remarkably higher compositional abundance in tissue than in urine samples (Fig. 5).

The Fig. 6 heat map shows the coexistence of five genera in tissue samples. *Clostridium sensu stricto*, *Akkermansia*, *Bacteroides*, *Enterobacter* and *Klebsiella* are characteristic but not general for all samples. No relationship was found between the biological characteristic of cancer [muscle invasive bladder cancer (MIBC) versus non-muscle invasive bladder cancer (NMIBC)] and microbiota composition.

Discussion

Since there were no significant differences between the richness and Shannon diversity values of the transurethral catheterized urine and tissue microbiota, we might even lead to think that a urine microbiota test is suitable as a diagnostic and prognostic tool in bladder tumor research. However, the fact that there are significant differences between the diversity of female and male tissue samples confirms that transurethral catheterized urine samples are not mirror images of the microbiota at the site of the cancer. Clearly, the role of tissue associated bacteria in tumor progression has to be addressed in future studies.

In agreement with the results of Bucevic et al.¹⁵, *Firmicutes* was the most common phylum in both our tissue and urine samples. Two Chinese study groups^{16,17} identified *Proteobacteria* as the most abundant phylum in urine and tissue samples of bladder cancer patients. While none of the earlier reports document *Cyanobacteria* as a significant phylum, in our urine and tissue samples its abundance was 7 and 8%, respectively. Microcystins are toxic products of *Cyanobacteria* that induce hepatocellular cancer and promote migration and invasion of colorectal cancer^{18,19}. Assuming that a change in the gut microbiota also results in a change of the bladder microbiota, different geographical locations and dietary patterns may play a role in the increased presence of *Cyanobacterium* in our samples^{20,21}. Further studies are required to quantify the difference in the amount of microcystin-producing species in the urine of bladder cancer patients and controls. Provided that microcystins also play a role in the development of bladder cancer, we should consider the individual genetic differences in the microcystin detoxification process between cancerous and healthy people²².

Patients with different urinary tract diseases—neurogenic bladder dysfunction, urgency urinary incontinence or interstitial cystitis—reflect altered urinary tract microbiota in contrast to healthy volunteers^{23–25}. Nevertheless, the urinary microbiota of a healthy population is ambiguous, because healthy control participants in the relevant studies belonged to different age groups and different genders. Lewis et al.⁶ propose the existence of a core urinary microbiota—a subset of bacteria present at variable abundances within the clean-catch, mid-stream voided urine—regardless of age and gender. In the age group of 20–49 years our study was limited to one female participant with *Gardnerella*, *Lactobacillus* and *Streptococcus* as the most abundant genera in her urine sample. These bacteria belong to the core urinary microbiota according to the Lewis classification. In the urine samples of our female age group 50–69 years, in addition to the *Peptinophilus*, *Parvimonas*, *Streptococcus*, *Lactobacillus* and *Fastidiosipila* genera belonging to the core, most frequent bacteria observed were *Escherichia-Shigella*, *Actinotignum* and *Williamsia*. In the 76 years old female patient (age group 70+) the most abundant genera, namely *Streptococcus*, *Lactobacillus* and *Corynebacterium*, were all members of the core. The most common

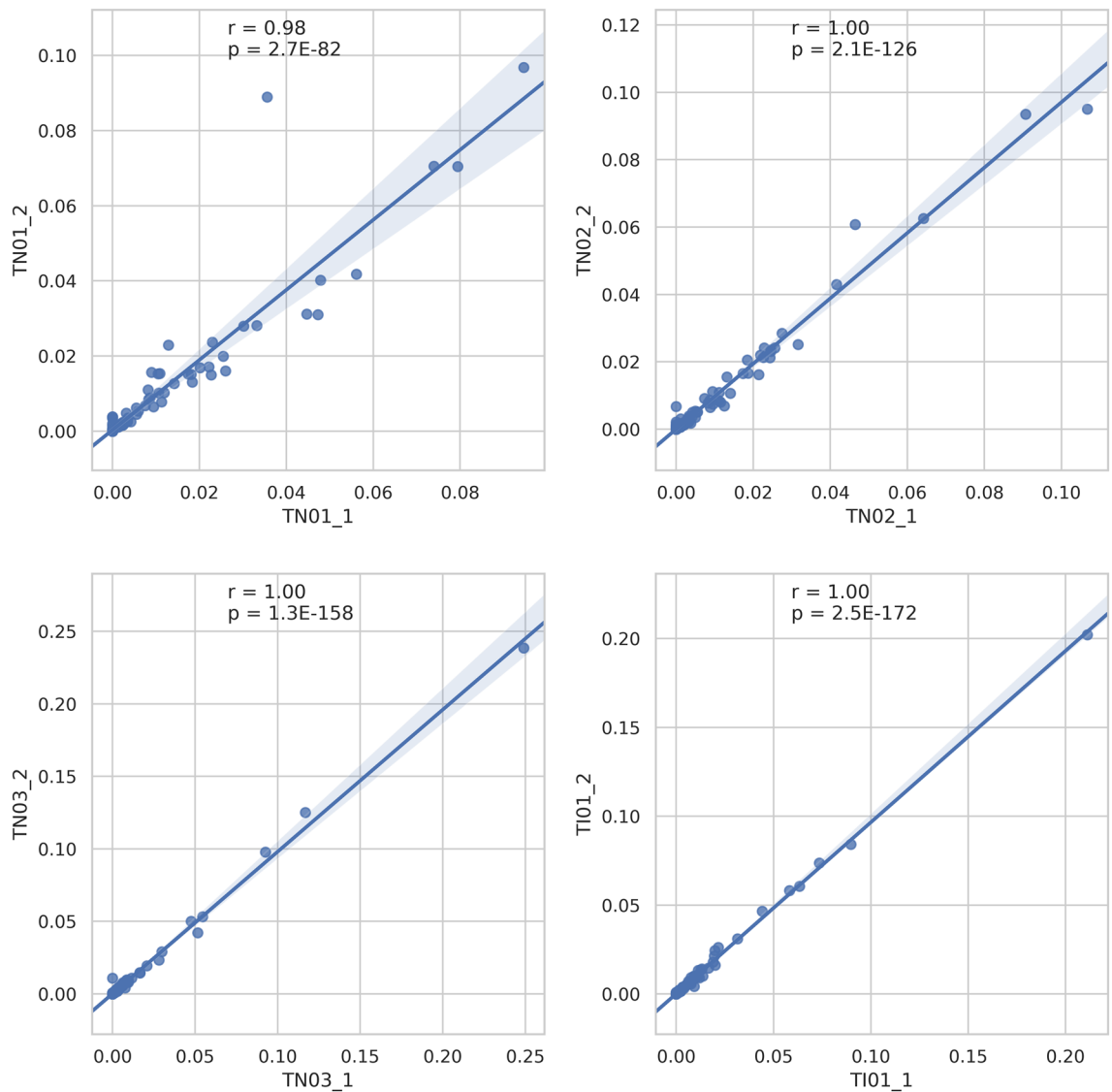


Figure 2. Correlation-based reproducibility of the microbiota from bladder cancer tissue: Scatter plots A, B, C and D show the abundance of genera taken from site 1 and site 2 of a subject (TN01, TN02, TN03, TI01). Pearson correlations were calculated for all the 4 subjects. All correlations show strong statistical significance.

genera in urine of our male patients, regardless of age, was *Anaerococcus*, *Corynebacterium*, *Peptoniphilus*, and *Staphylococcus*, genera belonging to the core and *Streptococcus* outside of the core.

When compared the urine of 6 healthy individuals with 8 patients of urothelial cancer, Xu et al.¹⁴, observed the enrichment of *Streptococcus* in the urine from patients with urothelial carcinoma. In contrast, when Bucevic et al.¹⁵ analyzed 11 healthy and 12 patients with bladder cancer, the most abundant genera in healthy patients' urine were *Streptococcus*, *Veillonella* and *Corynebacterium*, however *Fusobacterium*, *Actinobaculum*, *Facklamia* and *Campylobacter* genera enrichment representing the urine of bladder cancer patients. In the urine of our patients *Streptococcus*, *Corynebacterium* and *Fusobacterium* genera were similarly enriched. Wu et al.¹⁶ found significantly higher relative levels of *Acinetobacter*, *Anaerococcus*, *Rubrobacter*, *Sphingobacterium*, *Atoposites* and *Geobacillus* in urine of cancer patients. While in our patients the abundances of *Acinetobacter* and *Anaerococcus* increased depending on the age and gender group, the other four genera occurred only in minor quantities. Inter-individual variability was observed between urinary microbiotas of the participants in our study. Having said that, the most abundant genera in our samples were those detected by earlier reports. When differences were found, the reason for the alterations may be the low number of samples, ethnicity, gender and/or age. In addition, we collected the urine directly from the bladder by resectoscopy and not by the clean-catch voided urine method.

Inter-individual variability was observed among the microbiota of tissue samples. However, 5 genera, *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella* were over-represented in tissue samples compared to the urine. This difference indicates that these genera are directly associated with the tissue and further studies with larger sample sizes of cancerous bladder mucosa and adjacent normal healthy tissues are needed to clarify the genera associated with the oncogenic transformation process. For now, it is impossible to determine whether the high abundance of these genera is the cause or consequence of tumorigenesis. The link

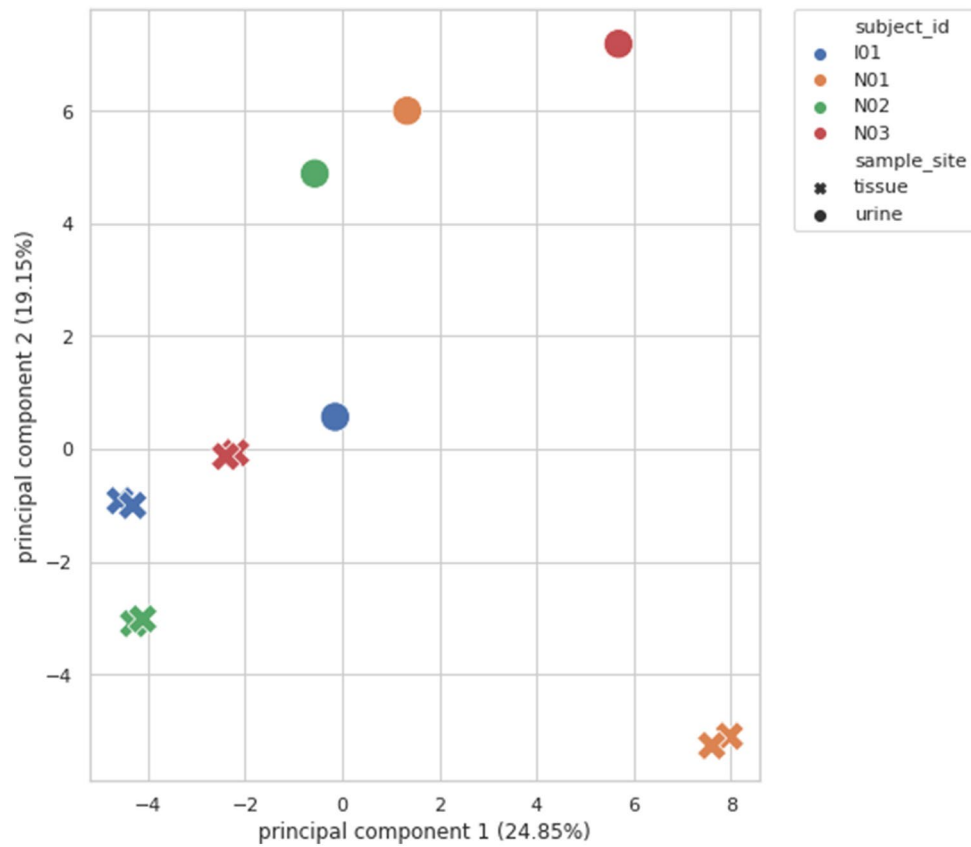


Figure 3. Principal component analysis (PCA) of correlated bladder cancer tissue samples with their respective urine samples: correlated tissue samples did not cluster with their respective urine samples.

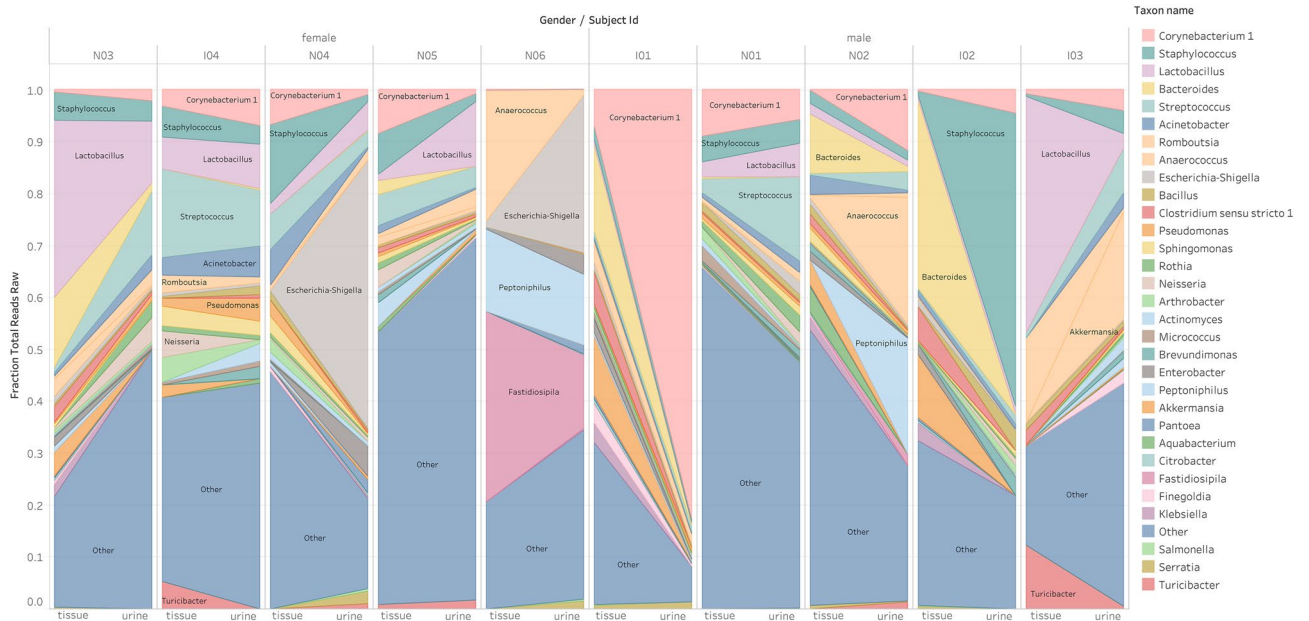


Figure 4. Distribution of genera in the microbiota of the urine and tissue samples obtained from the same patients. Each bar represents the microbiota results from the indicated patient, the most abundant genera are shown on the left and right sides from tissue and urine samples, respectively.

Age group	Female	Male
20–49 years	Akkermansia Atopobium Bifidobacterium Gardnerella Gemella Lactobacillus Romboutsia Staphylococcus Streptococcus Turicibacter	No patient in the study
50–69 years	Actinotignum Enterobacter Escherichia-Shigella Fastidiosipilia Lactobacillus Neisseria Parvimonas Peptoniphilus Streptococcus Williamsia	Anaerococcus Corynebacterium Fastidiosipila Lautropia Peptoniphilus Porphyromonas Prevotella Staphylococcus Streptococcus Varibaculum
70+ years	Acinetobacter Actinomyces Corynebacterium Fusobacterium Lactobacillus Massilia Pseudomonas Sphingomonas Staphylococcus Streptococcus	Anaerococcus Bacillus Brevundimonas Corynebacterium Deinococcus Lactobacillus Peptoniphillus Rubellimicrobium Staphylococcus Streptococcus

Table 2. The most abundant bacterial genera in urine samples by ages and gender of patients. Those highlighted in bold are not members of CORE bacteria in healthy population according to Lewis et al.¹¹.

between chronic inflammation, microbiota and the progression of solid tumors has been established especially for colorectal cancer (CRC). In the CRC animal experiments of Baxter et al.²⁶, the taxa *Bacteroides* and *Akkermansia* were strongly positively correlated with an increased tumor burden, but *Clostridium* was associated with a decreased rate of malignant transformation. *Bacteroides* and *Akkermansia* both are known mucin degraders, they undermine the integrity of the mucosal barrier, leading to increased inflammation^{27,28}. The different species from *Clostridium* genera play different roles in carcinogenesis. The butyrate-producing *Clostridium spp.* inhibits intestinal tumor development²⁹ as much as *Clostridium septicum* does not appear to initiate carcinogenesis but appears to have a symbiotic relationship with the growth of already developing malignancies³⁰. The study of Yurdakul et al. shows that *Enterobacter spp.* may be a clinically important factor for colon cancer initiation and progression due to apoptosis inhibition³¹. The colibactin toxin produced by *Klebsiella pneumonia* is known to cause DNA double-strand breaks inducing genomic instability as well as cell cycle arrest³². The continuous insult of colibactin toxin is supplemented with the influx of proinflammatory cytokines into the colonic microenvironment in the presence of *Klebsiella*, leads to chronic inflammation, and stimulation of epithelial cell proliferation³³. The strong correlation between the appearance of these “five suspect genera” in our samples suggests that this set of bacteria mutually enhances each other’s carcinogenic effects. Fusobacterial DNA has been detected in pancreatic, esophageal or colorectal cancers^{34,35}. Bucevic et al.¹⁵ detected *Fusobacterium* in 25% of urine samples of bladder cancer patients. None of our tissue samples contained *Fusobacterium* DNA at the level of detection, but it was represented in all our urine samples. Liu et al. found a higher abundance of *Cupriavidus*, *Acinetobacter*, *Anoxybacillus*, *Escherichia-Shigella*, *Geobacillus*, *Pelomonas*, *Ralstonia* and *Sphingomonas* genera in the cancerous relative to healthy tissues of the same bladder cancer patients¹⁷. In our study *Cupriavidus* and *Pelomonas* were represented only in tissue samples; the urine of these two patients did not contain any detectable amount of these genera. *Acinetobacter*, *Anoxybacillus*, *Escherichia-Shigella*, *Geobacillus*, *Ralstonia* and *Sphingomonas* were increasingly represented in our tissue samples compared to the urine samples. The fact that these latter genera, and our “five suspects” are detectable in greater quantities in cancerous tissue than in the urine supports the hypothesis that these bacteria were associated with the tumor-altered tissue.

The small number of patients (5 male and 5 female) is a limitation of this study, and restricts the conclusions that may be drawn on the impact of gender differences on microbiota changes in bladder cancer development. Additional, more exhaustive studies are required to obtain increasingly accurate data for the better understanding of cause-effect relationships between microbiota and bladder cancer.

Tumor tissue size limited the ability to perform microbiota analysis in duplicate from each tissue sample.

Using the 16S rRNA sequencing method, analyzed by the Kraken-Bracken pipeline is not validated to infer taxonomic information below the genus level, therefore future studies may harness species-level analysis to identify specific microbes which may have associations with bladder cancer.

Limitation of the study is that without using samples of healthy controls—due to obvious ethical reasons—it is not yet clear whether the „five suspect bacterial genus” are associated exclusively with bladder cancer tissue.

However, this is the first report that evaluates urine samples directly from the bladder, avoiding urethra contamination. Moreover, our study suggests that the bladder-mucosa associated microbiota is different from

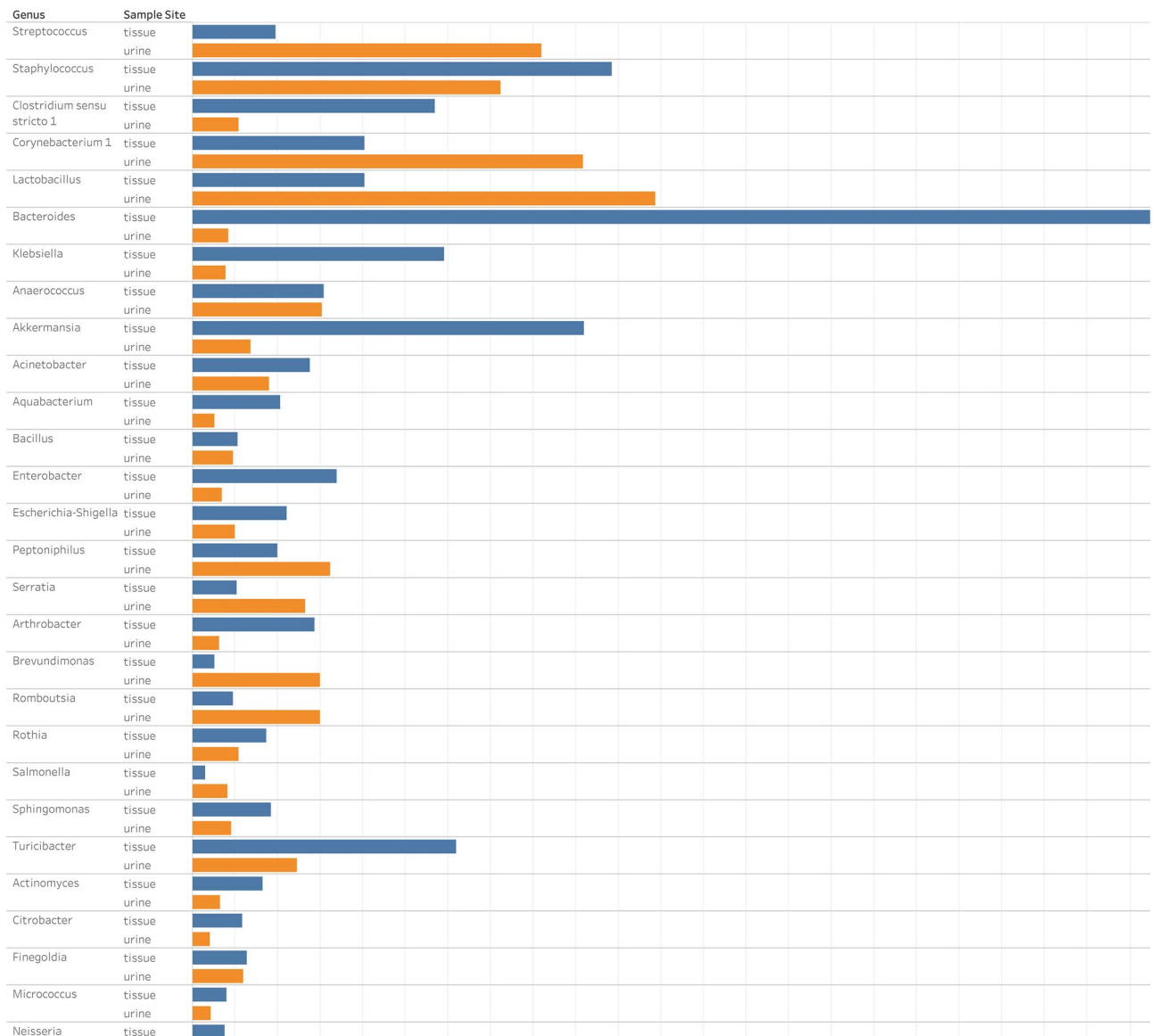


Figure 5. Comparative analysis of median fraction total reads at level of genera detected from the microbiota of the urine and tissue samples obtained from patients.

the urinary microbiota and provides the best test sample to characterize the association between bacteria and carcinogenesis.

Herein, we profiled the bladder mucosa microbiota compared to the urinary microbiota associated with bladder cancer in the most comprehensive study to date. Our study reflected that urine samples provide different results relative to tissue samples during the characterization of the bacteria involved in carcinogenesis. An extension and deepening of these investigations lead to a better understanding of the pathogenesis of bladder cancer, and will provide novel diagnostic and therapeutic options.

Methods

Ethics approval and consent to participate. Sample collection protocols were approved by the Ethics Committee of Markhot Ferenc University Teaching Hospital (MFUTH) and the Ethics Committee of Semmelweis University (SE RKEB: 100/2018). The present study was conducted in accordance with ethical standards that promote and ensure respect and integrity for all human subjects and the Declaration of Helsinki. Patients treated at the MFUTH's Urology Department between May and October 2018 were enrolled in this study, all research was performed in accordance with guideline and regulation of MFUTH, and written informed consent was obtained from all patients.

Sample collection. The study included five male and five female patients with bladder carcinoma. Exclusion criteria were antibiotic or probiotic treatment 2 weeks prior to surgery or recent urinary infection. The

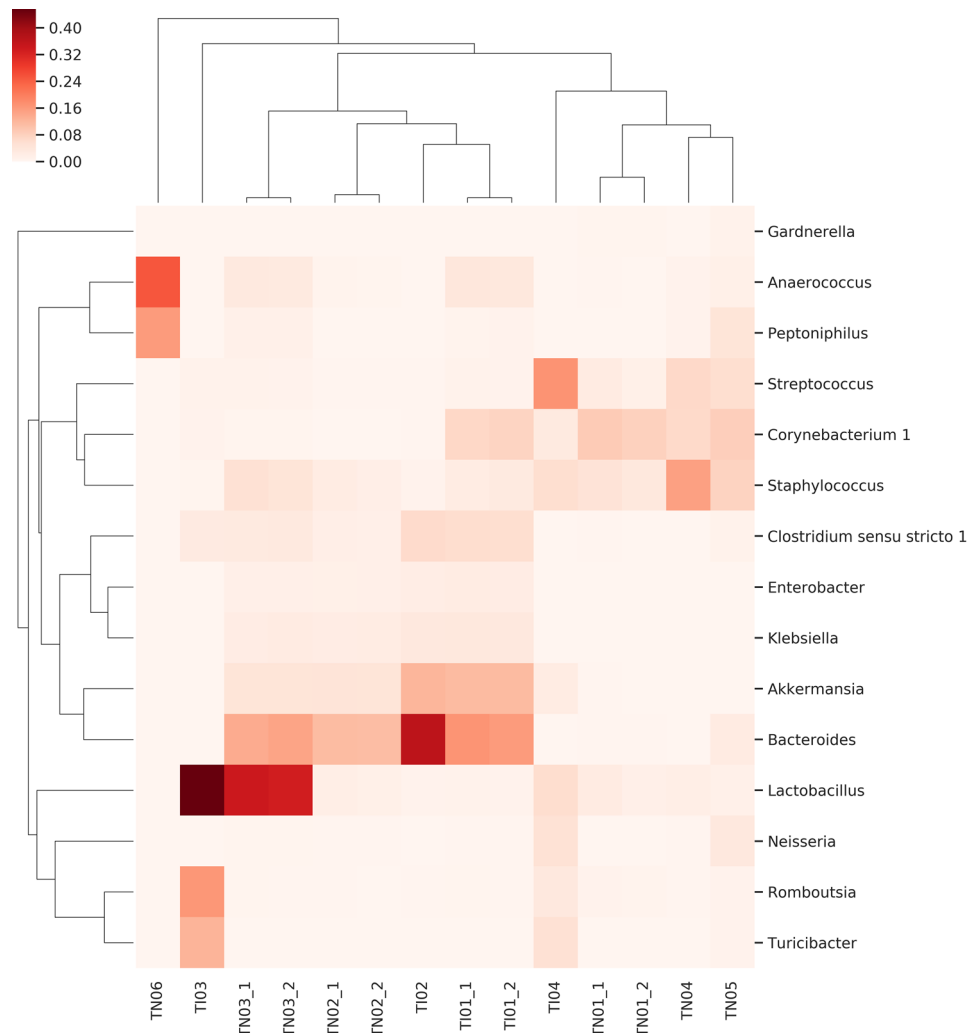


Figure 6. Heat-map of the distribution of co-existing genera in cancer tissue samples obtained from NMIBC (with TN designation) and MIBC (with TI designation) patients. Evidence of a correlation between the biological characteristics of the tumor and the distribution of the bacteria is not confirmed. Regardless of the biological characteristics of the tumor the co-existence of 5 genera (namely *Clostridium sensu stricto*, *Akkermansia*, *Bacteroides*, *Enterobacter* and *Klebsiella*) in tissue samples is characteristic.

patients' clinical parameters, and the metadata of the samples are presented in Table 1. Mucosal tissue samples removed during transurethral resection (TUR) were divided to histological and microbiota analysis. Urine samples were collected directly from the bladder during the TUR surgery and divided for traditional routine culture and microbiota analysis. Routine urine culture involves inoculation of 10 μ l of urine on to blood and eosin-methylene blue agar plates, and 24-h incubation under ambient atmospheric conditions. The level of detection is 100 colony-forming units per milliliter (CFU/ml). Fractions for microbiota analysis were centrifuged at 16,000 g for 10 min, supernatants were aliquoted and stored at -80°C .

DNA isolation, 16S rRNA gene library preparation and MiSeq sequencing. DNA isolation was performed by ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, USA) according to manufacturer's instructions after enzymatic dissolution with ProtK (56°C , 3 h) for the tissue samples. Enzymatic digestion was performed with standardized 2 mm in diameter tissue pieces.

Concentration of genomic DNA was measured using a Qubit2.0 Fluorometer with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial DNA was amplified with tagged primers covering the V3–V4 region of bacterial 16S rRNA gene. PCR and DNA purifications were performed according to Illumina's protocol. PCR product libraries were assessed using DNA 1000 Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (600 cycles PE).

In order to evaluate contribution of extraneous DNA from reagents, extraction negative controls and PCR negative controls were included in every run. To ensure reproducibility, each urine sample was independently extracted and sequenced twice. The full tissue samples of four patients were larger than 10 mm in diameter.

These samples were cut into small pieces, two pieces originally located in a distance were separately digested enzymatically. All ensuing analytical steps, including the isolation of DNA, PCR, library preparation, sequencing and analysis procedures were done in duplicate.

Isolated DNA samples were placed at -80°C until PCR amplification.

Raw sequencing data were retrieved from the Illumina BaseSpace. Quality control (QC) of raw reads was carried out by FastQC and MultiQC³⁶. After QC, low quality reads were trimmed by Trimmomatic³⁷. The first 12 base pairs and consecutive low base calls were removed (phred score < 20) and only reads with minimal length of 36 were kept. The SSU Ref NR 99 database, downloaded on 22/03/2019 (release 132) of SILVA was used³⁸. This contains non-redundant sequences (identity threshold: 99%). The database fasta file was pre-processed and indexed by the Kraken2³⁹ tool (kraken2-build) with k-mer = 31. Kraken2 aligned and classified short-reads and the final estimation of taxon abundances in various taxonomic levels was performed with Bracken⁴⁰.

Richness (number of unique taxa in the sample) and Shannon index (quantifying entropy of the distribution of taxa proportions) were used to quantify alpha diversities in microbiotas. Only those taxa were considered to be positive that had at least support of 50 reads; all others were discarded. Data were processed in python 3.7 with scikit-bio version 0.5.5. Bray–Curtis distance measure was used for assessing the beta-diversities of the microbiota and principal component analysis (PCA) for visualizing microbiotas. Differential abundance testing was done using Wilcoxon rank-sum test (in tissue-urine pairs) and PERMANOVA⁴¹ was used to determine compositional similarities.

Data availability

The datasets generated during the current study are available in the Short Read Archive (SRA) under accession number: PRJNA604455.

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Author contributions

Each author has given final approval of the submitted manuscript. The authors listed below have made substantial contributions to the intellectual content of the manuscript in various sections described below: Conception and design: E.O., B.M. Acquisition of patients' data and samples: B.M., Á.M. Laboratory processing of samples: I.V., N.M. Analysis and interpretation of data: B.L., E.O. Statistical analysis, editing figures: J.J. Drafting of the manuscript: E.O., M.G., D.S. Obtaining funding: E.O.

Competing interests

The authors declare no competing interests.

Additional information

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Article

Bladder Tissue Microbiome Composition in Patients of Bladder Cancer or Benign Prostatic Hyperplasia and Related Human Beta Defensin Levels

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Abstract: Balance between the microbiome associated with bladder mucosa and human beta defensin (HBD) levels in urine is a dynamic, sensitive and host-specific relationship. HBD1—possessing both antitumor and antibacterial activity—is produced constitutively, while the inducible production of antibacterial HBD2 and HBD3 is affected by bacteria. Elevated levels of HBD2 were shown to cause treatment failure in anticancer immunotherapy. Our aim was to assess the relationship between microbiome composition characteristic of tumor tissue, defensin expression and HBD levels measured in urine. Tissue samples for analyses were removed during transurethral resection from 55 bladder carcinoma and 12 prostatic hyperplasia patients. Microbiome analyses were carried out with 16S rRNS sequencing. Levels of HBD mRNA expression were measured with qPCR from the same samples, and urinary amounts of HBD1, 2 and 3 were detected with ELISA in these patients, in addition to 34 healthy volunteers. Mann–Whitney U test, Wilcoxon rank sum test (alpha diversity) and PERMANOVA analysis (beta diversity) were performed. Defensin-levels expressed in the tumor did not clearly determine the amount of defensin measurable in the urine. The antibacterial and antitumor defensin (HBD1) showed decreased levels in cancer patients, while others (HBD2 and 3) were considerably increased. Abundance of *Staphylococcus*, *Corynebacterium* and *Oxyphotobacteria* genera was significantly higher, the abundance of *Faecalibacterium* and *Bacteroides* genera were significantly lower in tumor samples compared to non-tumor samples. *Bacteroides*, *Parabacteroides* and *Faecalibacterium* abundance gradually decreased with the combined increase in HBD2 and HBD3. Higher *Corynebacterium* and *Staphylococcus* abundances were measured together with higher HBD2 and HBD3 urinary levels. Among other factors, defensins and microorganisms also affect the development, progression and treatment options for bladder cancer. To enhance the success of immunotherapies and to develop adjuvant antitumor therapies, it is important to gain insight into the interactions between defensins and the tumor-associated microbiome.

Keywords: bladder cancer; bladder urothelium; prostatic hyperplasia; microbiome; human beta defensins; urine



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1. Introduction

Bladder cancer (BC) is the fourth most common cancer in men and 11th in women with a high prevalence and global incidence. Approximately 3.0% of all new cancer diagnoses and 2.1% of all cancer deaths are due to urinary bladder cancer [1]. Based on stage classification and risk assessment—in addition to surgical removal—therapeutic procedures include Bacillus Calmette–Guérin (BCG) installation, radiation therapy, chemotherapy and the use of checkpoint inhibitors [2].

The most important risk factors for the development of bladder carcinoma (BC) are smoking, exposure to aromatic amines and many other toxic compounds of environmental origin, such as arsenic in drinking water or insecticides [3–5]. These carcinogenic toxins are excreted from the bloodstream through the kidneys and they interact with the microbiota during ensuing storage in the bladder. The resulting metabolites may increase or decrease the risk of BC. Bacteria present in the bladder are one of the many contributors leading to the development and progression of BC [6], with several studies describing microbiome changes associated with BC [7–10].

The physiological balance between the microbiome and mucosal defensin-levels is dynamic, sensitive and host-specific. Members of the microbiome affect the levels of inducible human β -defensin 2 (HBD2) and HBD3, however, the production of HBD1 is constitutive and host-dependent. Mucosal defensin-levels select for bacteria that make up the microbiome, based on strain-specific sensitivity. Defensins, as antibacterial peptides, achieve their bactericidal effect primarily by breaking down the integrity of the outer membrane of Gram-negative bacteria. A healthy individual is characterized by a genetically determined level of HBD1, while the feedback mechanism of the colonizing microbes and the amounts of HBD2–3 that control each other creates the steady state between the microorganisms and antibacterial peptides. The autonomous defensin production of tumor cells may also amend this state to an unpredictable degree and direction.

Several studies have demonstrated the antitumor efficacy of HBD1 against malignant processes in various organs [11–13], as well as its protective effect against BC [14,15]. Chang et al. noted the detrimental effect of increased levels of HBD2 on BCG therapy against BC, and later confirmed the exact mechanism of action through Toll-like receptors [16,17]. It is well-known that not only the change in the gut microbiome affects the effectiveness of the anti-tumor checkpoint inhibitors, such as pembrolizumab or nivolumab, but the bacteria present in the urogenital system can also directly inhibit this therapy [18]. When planning individual therapies, it is essential to have more in-depth knowledge about the components of the bladder tumor microbiome and the HBD levels associated with them.

We previously demonstrated that the microbiome composition of bladder tumor tissue samples is significantly different from the planktonic microbiome content of the urine surrounding the tumor [19]. *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella*, as “five suspect genera”, were over-represented in tumor tissue samples compared to the microbiota composition of the urine. However, taxa with a high abundance in tumor tissue are not necessarily tumor-associated bacteria; they may just occur more often adhering to the mucosal layer. In our present study, we sought to determine whether there is a difference in the microbiome composition of tumor and non-tumor bladder tissue samples, the amounts of HBD1, HBD2 and HBD3 mRNA expressed in them and the levels of defensins found in the urine of cancer and non-cancer patients. We compared the tissue microbiome of BC patients (BC group) with the microbiome of mucosal samples from patients with benign prostatic hyperplasia (PH group), the levels of HBD mRNA expressed in tissue pieces and the levels of defensins measured in the urine of BC and PH patients, compared to healthy volunteers (HV group).

2. Materials and Methods

2.1. Sample Collection

The present microbiome-defensin molecular study included 55 patients with BC and 12 patients with PH. Mucosal tissue samples removed during transurethral resection (TUR)

were divided for histological and microbiome-defensin molecular analysis. Urine samples were collected directly from the bladder during TUR surgery for quantitative defensin detection and traditional routine culture. Spontaneously excreted urine was examined from 34 healthy volunteers. Exclusion criteria were urinary infection, antibiotic or probiotic treatment two months prior to surgery. The characteristics of the study participants are presented in Supplementary Table S1. Members of the PH group were chosen in such a way that they matched the members of the BC group in most characteristics (with the exception of gender).

2.2. DNA Isolation, 16S rRNA Gene Library Preparation and MiSeq Sequencing

From tissue samples DNA isolation was performed by ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA), after enzymatic dissolution with ProtK (56 °C, 5 h). Bacterial DNA was amplified with tagged primers covering the V3–V4 region of bacterial 16S rRNA gene. PCR and DNA purifications were performed according to Illumina's protocol. PCR product libraries were assessed using DNA 1000 Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (600 cycles PE).

To evaluate the contribution of extraneous DNA from reagents, extraction negative controls and PCR negative controls were included in every run. To ensure reproducibility, all analysis procedures were done in triplicate from 3 separately isolated DNA samples from each patient. Raw sequencing data were retrieved from Illumina BaseSpace and data were analyzed using the CosmosID [20] bioinformatics platform.

2.3. Defensin Expression Assays

Total RNA from tissue samples was isolated by innuPREP RNA Mini Kit 2.0 (Analytik Jena GmbH, Jena, Germany) according to the manufacturer's instructions after enzymatic dissolution with ProtK (56 °C, 5 h). Furthermore, 80–100 ng of RNA was used for RT-PCR assay performed using the PrimeScript RT reagent kit (Takara Bio, San Jose, CA, USA) and the resulting cDNA was amplified on a qTOWER 3G (Analytik Jena GmbH, Jena, Germany) instrument in the presence of selected primers. The following primers were used for defensin expression assays: HBD1: 5'-TTG TCT GAG ATG GCC TCA GGT AAC-3' forward, 5'-ATA CTT CAA AAG CAA TTT TCC TTT AT-3' reverse, HBD2: 5'-CCAGCCATCAGCCATGAGGGTCTTG-3' forward, 5'-CAT GTC GCA AGT CTC TGA TGA GGG AGG-3' reverse, HBD3: 5'-AGC CTA GCA GCT ATG AGG ATC-3' forward and 5'-CTT CGG CAG CAT TTT CGG CCA-3' reverse. The primers for the GAPDH housekeeping gene were 5'-CTA CTG GCG CTG GCA AGG CTG T-3' forward and 5'-GCC ATG AGG TCC ACC ACC CTG CTG-3' reverse.

Relative changes in mRNA expression were calculated by using the double delta Ct ($\Delta\Delta Ct$) method [21]. ΔCt of each tumor and PH sample was normalized, calculating the difference between their gene of interest Ct value (HBD1, HBD2 or HBD3) and their Ct value for the GAPDH housekeeping gene. $\Delta\Delta Ct$ values were calculated using the median ΔCt value of PH samples considered as controls. The relative expression (RQ) fold change was calculated as $2^{-\Delta\Delta Ct}$.

2.4. ELISA (Enzyme Linked Immunosorbent Assay)

For quantitative measurement of human beta defensins in urine, the following ELISA kits were used, according to manufacturer instructions: SEB373Hu for HBD1, SEA072Hu for HBD2 and SEE132Hu for HBD3 (Cloud-Clone Corp., Houston, TX, USA). All diluted standards, samples and blank wells were measured in duplicate.

2.5. Statistical Analysis

Levels of statistical significance ($p < 0.05$) for the difference between urine defensin levels, defensin expression rate and bacterial taxa abundances measured in the different cohorts was calculated by Mann–Whitney U test. Statistical significance between cohorts

were implemented by Wilcoxon rank sum test for Chao1 alpha diversity and PERMANOVA analysis for Jaccard principal coordinate analysis (PCoA) beta diversity using the statistical analysis support application, CosmosID [20].

2.6. Ethical Considerations

Sample collection protocols were approved by the Ethics Committee (EC) of Markhot Ferenc University Teaching Hospital (MFUTH) and EC of Semmelweis University (SE RKEB: 100/2018/100-1/2018/2021). The study was conducted in accordance with the Declaration of Helsinki ethical standards that promote and ensure respect and integrity for all human subjects. Patients treated at the MFUTH's Urology Department between January and July of 2021 were enrolled in this study. The healthy volunteers (HV) were employees and students at Semmelweis University who volunteered for the urine test. All research was performed in accordance with guidelines and regulations of MFUTH. Written informed consent was obtained from all patients.

All research was performed in accordance with guidelines and regulations of MFUTH. Written informed consent was obtained from all patients to participate in the study. All study participants gave written informed consent that data from their personal test results could be published. All data and test results in the manuscript cannot be linked to the individual participants, all tests were anonymized.

3. Results

From 55 BC and 12 PH tissue samples, three smaller pieces were processed separately, for a total of $n = 201$ tested samples from 67 patients. Following 16S rRNA sequencing, a total of 52.8 million valid bacterial sequences were obtained, resulting in 40.7 million high-quality reads. The median number of reads in BC tissue samples was significantly higher (271,525 (IQR: 44,506)) ($p = 0.001$) than in PH bladder urothelial samples (110,083 (IQR: 16,711)).

Although BC tissues contained higher amounts of bacterial DNA than PH urothelial samples, alpha diversity of the tumor-specific microbiome was still significantly ($p < 0.001$) lower than that of non-tumor samples (Figure 1A). Beta diversity PCoA also showed significant ($p = 0.001$) difference between the two cohorts (Figure 1B). At phylum level, the most abundant taxa in the BC and PH groups were Firmicutes (46% vs. 45%; $p > 0.05$), Proteobacteria (23% vs. 16%; $p = 0.006$), Actinobacteria (13% vs. 4%; $p < 0.001$) and Bacteroidetes (11% vs. 30%; $p < 0.001$) (Figure 1C). Median abundance of bacteria belonging to the Cyanobacteria phylum was significantly higher in the BC group ($p = 0.011$). The most striking differences between BC and PH groups were in the abundance of Staphylococcus (7.89% vs. 0.59%; $p < 0.001$), Corynebacterium (3.83% vs. 0.63%; $p = 0.001$), Faecalibacterium (1.92% vs. 7.79%; $p < 0.001$) and Bacteroides (3.22% vs. 21.54%; $p < 0.001$) (Figure 1D). Although Oxyphotobacteria (from the Cyanobacteria phylum) did not belong to the predominant bacterial genus in the microbiome, there was significant difference in their abundance between the BC and PH groups (2.11% vs. 0.07%; $p = 0.024$).

Each urine sample collected directly from the bladder during TUR surgery was negative by traditional routine aerobic culture. Samples of the PH and BC groups are sharply differentiated on the heatmap (Figure 2); additionally, in 9 BC samples, specific genera showed a remarkably high presence. These samples contained the following genera: BC 10: Streptococcus (69%), BC 17: Corynebacterium (93%), BC 24, 36: Gardnerella (59%, 92%), BC 31, 32: Staphylococcus (57%, 97%), BC 40, 50: Ureaplasma (95%, 93%) and BC 46: Lactobacillus (94%). Samples with these extreme outlier values—assuming an ongoing infection—were excluded from further comparative microbiome analyses and defensin expression studies.

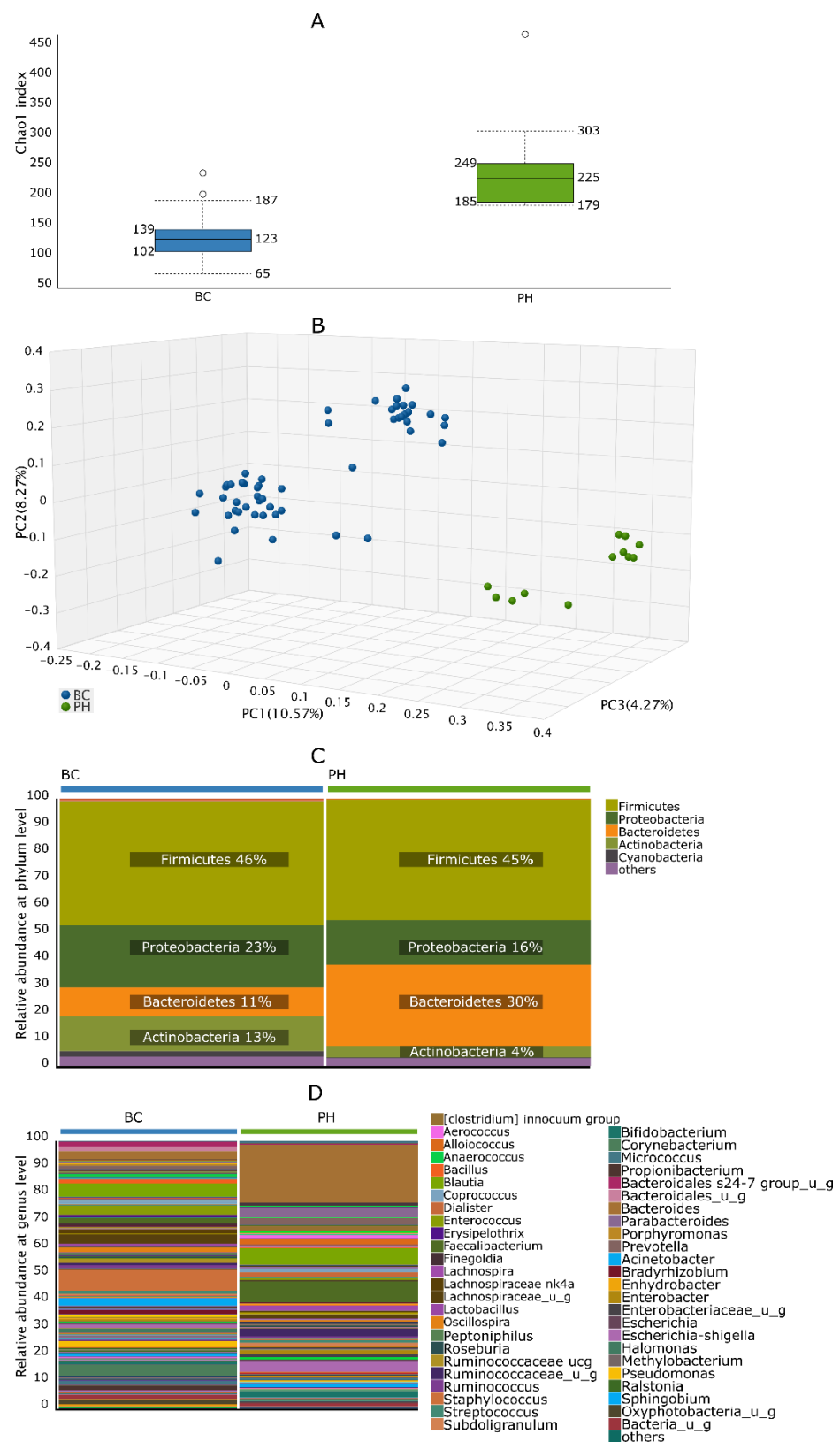


Figure 1. Comparison of tissue microbiome composition of bladder cancer (BC) and prostatic hyperplasia (PH) patients. (A) Chao1 alpha diversity at genus level, (B) Jaccard beta diversity at genus level, (C) taxa abundance in cohorts at phylum level, (D) taxa abundance in cohorts at genus level.

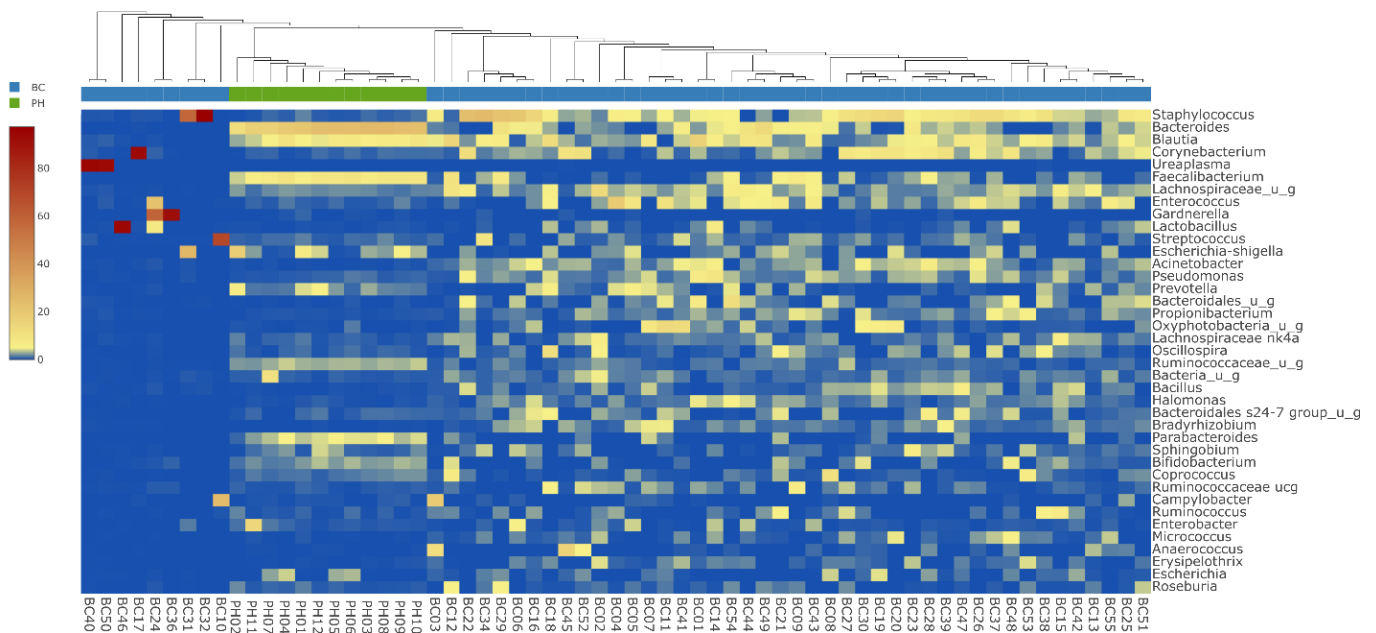


Figure 2. Heatmap visualization of the most abundant taxa at genus level among the BC and PH patients. The samples of the PH and BC groups are sharply separated; additionally, 9 samples of the BC group were detached in which some specific genera showed a remarkably high presence.

After grouping the samples of the BC group by underlying patient characteristics, Figure 3A–E shows the microbiome beta diversity results with Jaccard PCoA: no significant differences were shown according to smoking habits, diagnosed hypertension or diabetes mellitus or tumor grade and stage classification. Significant differences were shown only in the case of beta diversity between samples from males and females ($p = 0.001$) (Figure 3F).

Compared to the median ΔCt value of PH samples—considered as a control group—the RQs of HBD1, HBD2 and HBD3 mRNA of PH patients and BC patients showed curves that were not normally distributed. Furthermore, 59% of samples from BC patients expressed higher amounts of HBD1. Additionally, higher HBD2 or HBD3 expression was detected in 74% or 50% of BC samples, respectively. Compared to the median values of controls, the largest differences in gene expression values in the BC group were 7-fold for HBD1, 9-fold for HBD2 and 45-fold for HBD3, respectively.

Defensin mRNA expression RQ values and the amounts of HBDs measured in urine samples are shown in Figure 4A–E. The median amount of HBD1 was 12.59 ng/mL (IQR: 12.04), 12.33 ng/mL (IQR: 4.09) and 20.28 ng/mL (IQR: 21.44) in the PH group, HV group and BC group, respectively. Median HBD2 levels were 30.45 pg/mL (IQR: 21.93), 31.59 pg/mL (IQR: 28.88) and 151.69 pg/mL (IQR: 560.89) in the PH group, HV group and BC group, respectively. Finally, HBD3 levels were 151.96 pg/mL (IQR: 202.36), 186.44 pg/mL (IQR: 198.95) and 653.73 pg/mL (IQR: 1321) in the PH group, HV group and BC group, respectively. There were no relevant differences between the urine defensin levels between the PH and healthy control groups, with p values 0.65 for HBD1, 0.77 for HBD2 and 0.36 for HBD3, respectively. The HBD1 value in the BC group did not differ significantly from the PH ($p = 0.97$) or HV ($p = 0.99$) groups. In contrast, there were significant differences between the HBD2 values of the BC group, and the PH ($p < 0.001$) or HV ($p < 0.001$) groups. Additionally, HBD3 levels of BC group patients differed significantly from the PH ($p < 0.001$) or HV ($p < 0.001$) group. The increase in urinary HBD1 levels (in 32% of the samples) was not associated with the elevated amount of HBD1 mRNA expressed (58%) in excised tumor tissue; even with high mRNA expression in tumor tissue, there were low urinary HBD1 levels. Elevated tissue HBD2 mRNA expression was present in 74% of samples, and 78% of urine samples contained higher amounts of HBD2. This discrepancy

was even greater for HBD3, where tumor tissue mRNA expression was increased in 50% of the samples, while the amount of HBD3 was multiplied in each urine sample.

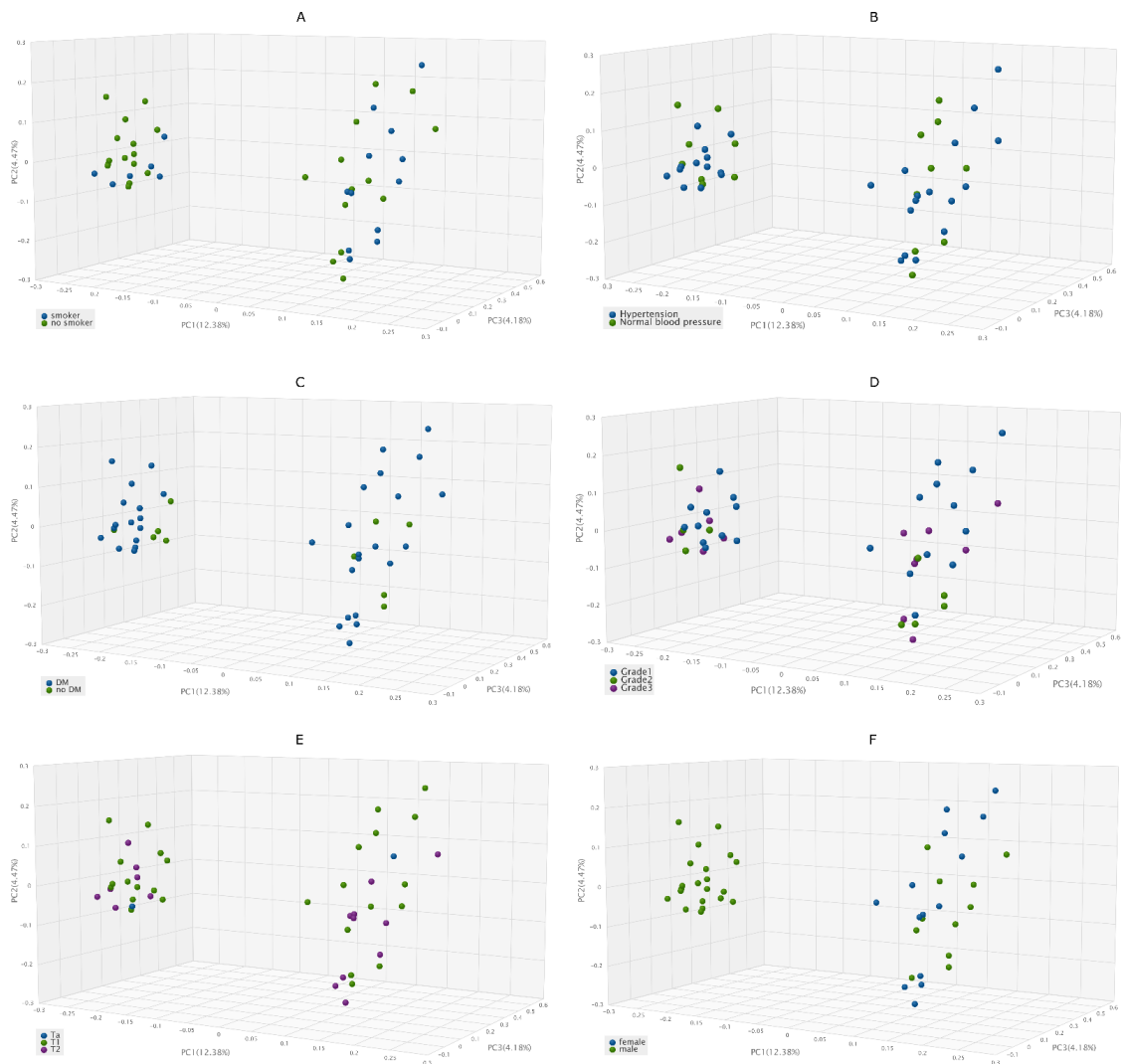


Figure 3. Relationship between characteristics of BC patients and microbiome beta diversity The Jaccard beta diversity graph for tumor samples shows two well-separated clusters, but the following properties alone do not show any correlation with cluster classification, (A) smoking habit, (B) hypertension, (C) diabetes mellitus, (D) grade of the cancer, (E) stage of the cancer. (F) There is a significant difference in the classification of female and male tissue samples into microbiome clusters.

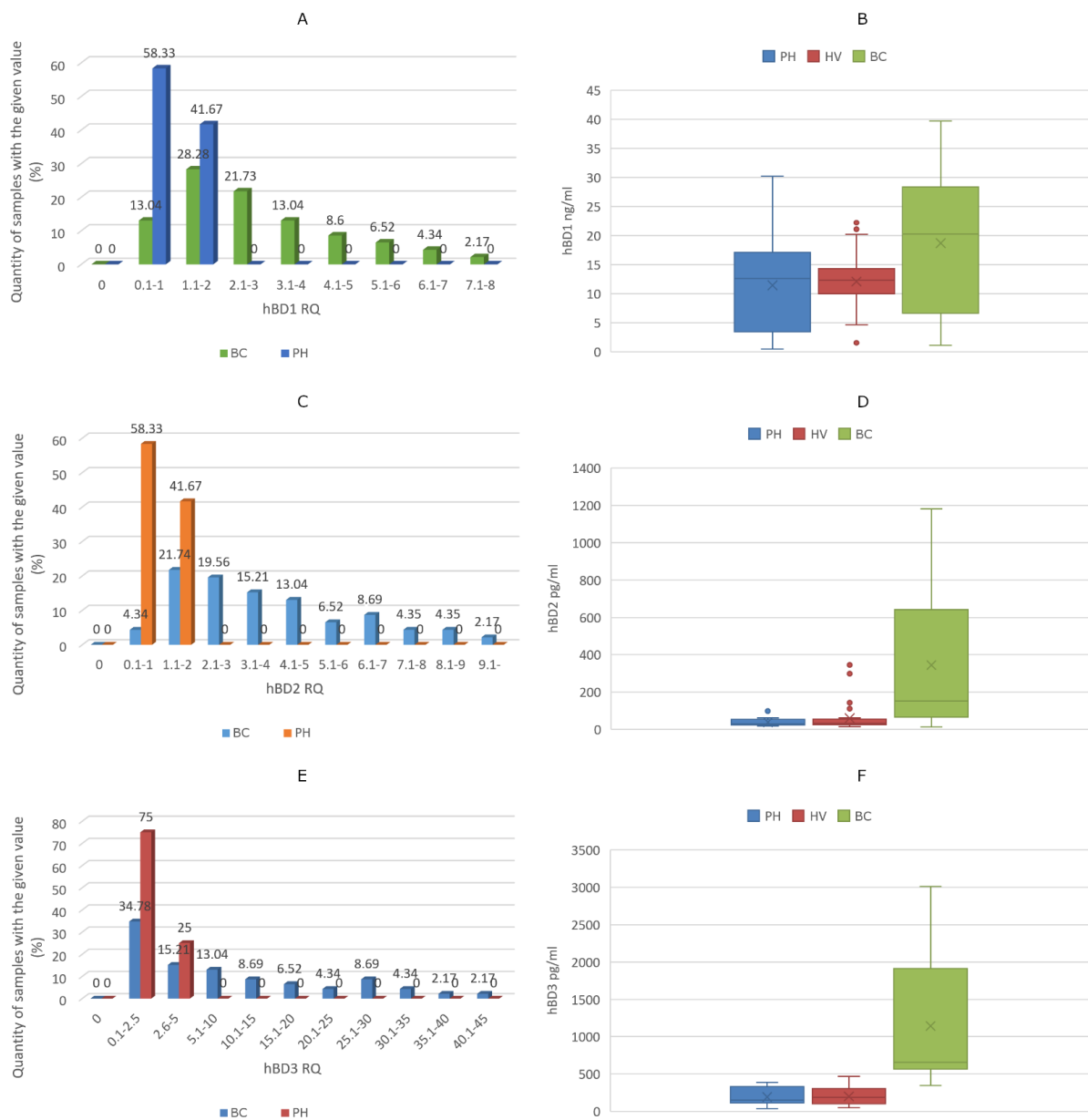


Figure 4. Defensin mRNA expression RQ values from tissue samples and the amounts of HBDs measured in the urine samples. (A) HBD1 RQ in tissue samples, (B) HBD1 levels in urine samples, (C) HBD2 RQ in tissue samples, (D) HBD2 levels in urine samples, (E) HBD3 RQ in tissue samples, (F) HBD3 levels in urine samples. BC: bladder cancer patients, PH: prostatic hyperplasia patients, HV: healthy volunteers.

The Oxyphotobacteria genus, a member of the Cyanobacteria phylum, presents only in the tissue microbiome of patients with low urinary HBD1 values. With the exception of Oxyphotobacteria, no other genus abundance showed an association with either HBD1, HBD2 or HBD3 levels separately (Figure 5A–C). In addition to the combined quantitative change in HBD2 and HBD3, there was a clear trend in the change in abundance of Bacteroides, Parabacteroides, Faecalibacterium, Corynebacterium and Staphylococcus genera (Figure 5D). Compared to the comparator PH group, the tumor-specific low levels of Bacteroides, Parabacteroides and Faecalibacterium gradually decreased with the combined increase in HBD2 and HBD3. Higher Corynebacterium and Staphylococcus abundance in BC samples increased in parallel with an increase in HBD2 and HBD3 levels. No such association was found regarding the abundance of Blautia, Lachnospiraceae and Oxyphotobacteria genera.

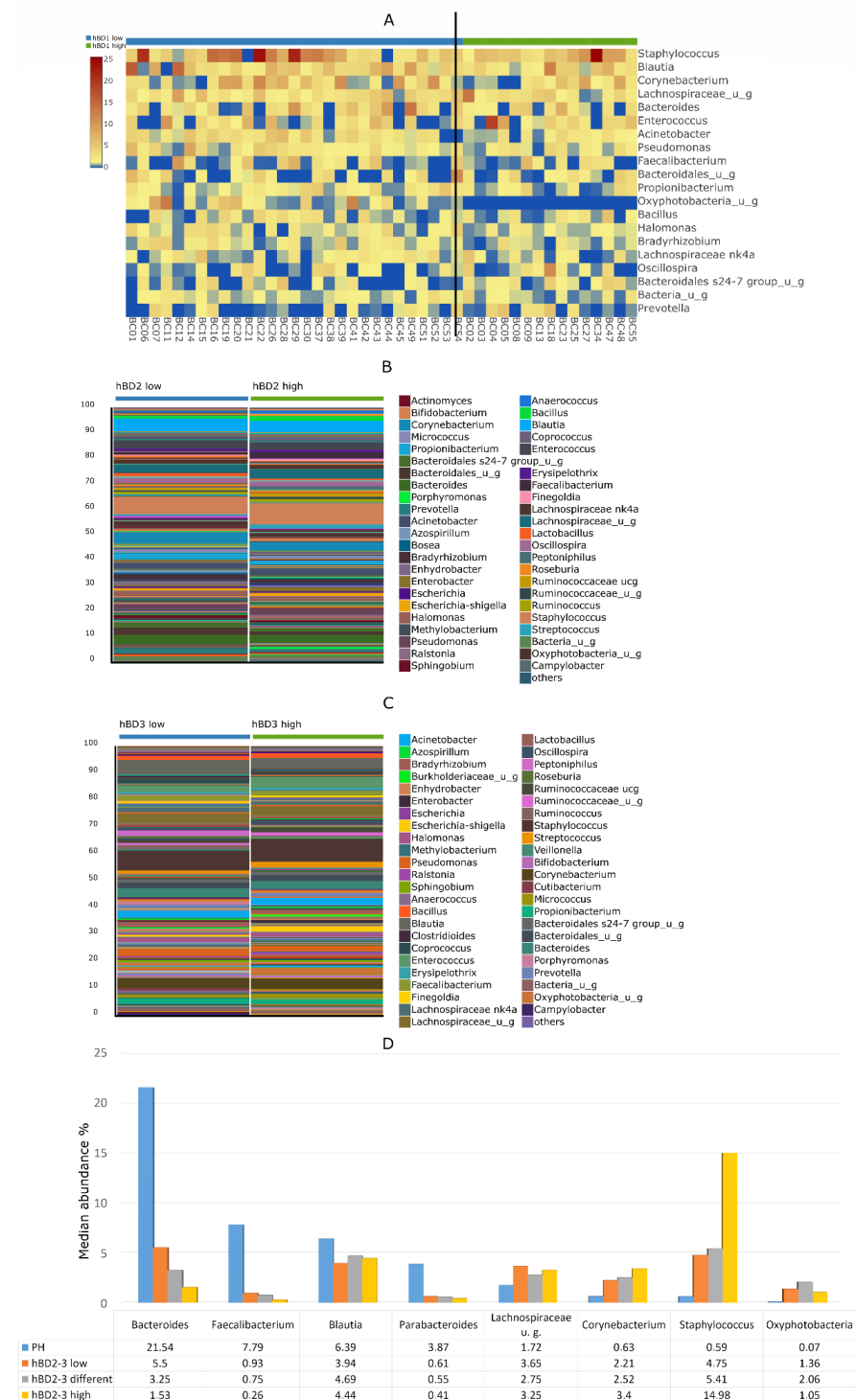


Figure 5. Correlation between the amount of defensins in urine and the abundance of the characteristic genera in BC tissue microbiome. (A) Oxyphotobacteria is the only genus whose occurrence differs significantly from the quantitative change in HBD1, Oxyphotobacteria is present only at low HBD1 levels. There is no significant difference due to the amount of (B) HBD2 or (C) HBD3 alone in the abundance of the genera in the BC tissue microbiome. (D) The combined increase in HBD2 and HBD3 levels reduces the abundance of non-tumor specific genera (Bacteroides, Parabacteroides, Faecalibacterium) and increases the abundance of more common in-tumor tissue genera (Staphylococcus, Corynebacterium).

4. Discussion

BCG (*Mycobacterium bovis*, Bacillus Calmette–Guérin) has been used as one of the most successful anti-tumor immunotherapies since 1976 [22]. This specific strain of the *Mycobacterium* genus not only works against BC by activating the immune system, but also directly reacts to the tumor cells, causing apoptosis [23], necrosis [24] or oxidative stress [25]. One possible reason for the lower incidence of BC in women may be the significantly higher incidence of *Actinomyces* (including the *Mycobacterium* genus) in the female urinary microbiome [26]. Only a fraction of the microbes present in the bladder were previously detectable by conventional urine culture methods, however, advances in molecular techniques have made it possible to quantify and qualitatively assess a large number of bacterial DNAs from urine or bladder tissue [7,8,10,27–30]. An individual's pre-existing bladder microbiome may have a role not only in the development of BC [30], or in protection against cancer formation [26], but also in their response to immunotherapy [7]. The surrounding microbiome also plays a role in treatment failure with BCG intravesical instillation or with anti-PD-1/PD-L1 immune checkpoint inhibitors. Thus, the higher amount of HBD2 induced by the bacteria present in the microbiome impairs the efficacy of BCG therapy [16,17,31]. Chen et al. demonstrated a significant relationship between the amount of *Leptotrichia*, *Roseomonas*, *Propionibacterium*, *Prevotella* and *Massilia* genera in the microbiome of BC tissue and the PD-L1 expression of tumor cells [18]. Based on the experience of retrospective studies, it was found that the effectiveness of various checkpoint inhibitors was significantly reduced by different antibiotics when used in parallel; this effect may also be explained by distinct changes in microbial composition that are detrimental to therapeutic effectiveness of these anticancer medications [32,33].

Although no bacteria were cultured from any of the urine samples, nine tissue samples contained *Streptococcus*, *Corynebacterium*, *Gardnerella*, *Staphylococcus*, *Ureaplasma* or *Lactobacillus* in remarkably high rates. There may be several reasons for the discrepancy, such as the difference between the tissue and the urine microbiome, or more likely, the unsuitability of conventional culture conditions. As an existing urinary tract infection—that could result in a shift in defensin values—could not be ruled out in these samples, defensin expression and ELISA assays were not performed on these samples.

As a result of our previous research, we have demonstrated that the microbiome composition of tumor tissue and the surrounding urine shows characteristic differences [19]. In our previous study, we hypothesized a role in tumor formation or progression, based on the much higher incidence of *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella* in tumor tissue compared to urine. We now interpret our results that the *Bacteroides* genus is associated with the mucosa rather than free urine, as in our current study we examined whether the composition of mucosa-associated bacteria differs in tumor (BC) and non-tumor (PH) tissue samples. We have shown pronounced differences in both alpha and beta diversity between the two sample types. In BC patients, the abundance of *Staphylococcus*, *Corynebacterium* and *Oxyphotobacteria* genera was noted, while in PH patients, *Faecalibacterium* and *Bacteroides* abundances were significantly higher. Reports show that *Faecalibacterium*—which has shown low abundance levels in tumor tissue—has a protective effect against colon, prostate or even breast cancer, if present in high abundance in feces [34–36].

In contrast to Ma et al., who found a significant difference in the urinary microbiome beta diversity of BC patients between smokers and non-smokers [37], there was no difference examined in our tissue samples. We also found no difference in tissue microbiome beta diversity based on associations with diabetes, hypertension or tumor grade and stage. Pedersoli et al. investigated gender-related differences in the urine of BC and healthy patients, and in tumor and healthy tissue samples [38]. Confirming our previous study [19], only around 80% of the bacterial taxa were found in the urine, compared to what was detected in the tissue. In our previous study [19] with a small number of samples, a significant difference was observed in the tissue samples of five males and five females in both α and β diversity of the microbiome. In our present study, the microbiome β diversity of the 32

male and 14 female tumor samples also showed significant differences; on the other hand, Pederzoli et al. examined 21 male and 8 female tumor samples and found no significant differences in their microbial compositions.

To the best of our knowledge, this is the first study that looks for a correlation between defensin-levels and the composition of the tissue microbiome in patients with BC. Nienhouse et al. found that patients with a lower amount of HBD1 have a higher risk of postoperative urinary infection, especially for infections caused by Gram-negative bacteria, while no similar correlation was found in the case of HBD2 [39]. In the HV group, only urinary HBD levels were determined, but as there were no significant differences between the PH and HV groups, defensin expression values of the PH group were used as a negative control to characterize the expression of tumor tissues. HBD1 is constitutively expressed in urogenital tract epithelia; however, its altered expression has been described in multiple human cancers [15]. Although 58% of our tumor tissue samples showed increased HBD1 expression, HBD1 content of patients' urine was not significantly elevated. It is hypothesized that these patients genetically produce less HBD1 in the healthy area of the urinary tract. As HBD1 is a natural tumor inhibitor [14,15], these patients may be genetically more prone to developing tumors. Moreover, the amount of *Oxyphotobacteria*—which is a confirmed tumor-causing *Cyanobacterium* genus [40]—was significantly higher in the BC group, and its presence was associated with low HBD1 levels.

BC cells also showed higher HBD2 and HBD3 expression-levels, but the urinary levels of defensins were also substantially higher, thus, the expression of inducible defensins is also likely to be elevated in other urothelial cells. Microorganisms and inducible defensins mutually affect each other's presence in the bladder; the resulting balance may also be affected by a number of external factors. Examination of HBD2 or HBD3 levels individually showed no association with microbial composition, but the combined effect of HBD2 and HBD3 [41] shows an increasingly pronounced difference in the abundance of the genera characteristic for the PH and BC groups; i.e., the higher the HBD2 and 3 levels, the greater the differences in the microbiome composition between the two groups. HBD3 is also bactericidal against some Gram-positive species, in addition to its ability to kill Gram-negative bacteria, which is also characteristic of HBD1–2 [42]. Our present study also highlights the phenomenon that, with high HBD2–3 levels, Gram-negative bacteria appear with a lower abundance compared to the healthy state. This situation may be considered as a “snapshot”, possibly showing a new state of equilibrium characteristic of the tumor, but possibly an episode in a vicious cycle of ever-deteriorating conditions in microbiome composition.

5. Conclusions

Among other factors, defensins and microorganisms also affect the development, progression and treatment options for bladder cancer. The amount of defensins measured in the urine depends not only on the expression levels in the tumor, but also in other areas of the urinary tract. Based on our findings, there is a significant difference between tumor and non-tumor microbiome composition. In addition, the urine cancer patients showed decreased levels of antitumor HBD1, and increased levels of HBD2 and HBD3, which also corresponded to the characteristic microbial composition of the tumors. To enhance the success of immunotherapies and to develop adjuvant antitumor therapies, it is important to gain insight into the interactions between defensins and the tumor-associated microbiome.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomedicines10071758/s1>, Table S1: The characteristics of the study participants.

Author Contributions: Conceptualization, B.M., Á.M. and E.O.; data curation, B.M., Á.M., M.G., I.V. and E.O.; funding acquisition, M.G.; methodology, B.S., N.M., K.P., I.V. and E.O.; project administration, B.M.; supervision, D.S.; writing—original draft, M.G., D.S. and E.O.; writing—review and editing, B.M., Á.M., M.G., B.S., N.M., K.P., I.V., D.S. and E.O. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Sample collection protocols were approved by the Ethics Committee (EC) of Markhot Ferenc University Teaching Hospital (MFUTH) and EC of Semmelweis University (SE RKEB: 100/2018/100-1/2018/2021). The study was conducted in accordance with the Declaration of Helsinki ethical standards that promote and ensure respect and integrity for all human subjects.

Informed Consent Statement: All research was performed in accordance with guidelines and regulations of MFUTH, written informed consent was obtained from all patients to participate in the study. All study participants gave written informed consent that data from their personal test results could be published. All data and test results in the manuscript cannot be linked to the individual participants, all tests were anonymized.

Data Availability Statement: The datasets generated and analysed during the current study are available in the SRA repository: SRA/ PRJNA 809202/www.ncbi.nlm.nih.gov (accessed on 22 February 2022).

Conflicts of Interest: The authors declare no conflict of interest, monetary or otherwise. The authors alone are responsible for the content and writing of this article.

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