Review

The Urinary Microbiome and Bladder Cancer: Susceptibility and Immune Responsiveness

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Abstract. Bladder cancer is a highly prevalent disease worldwide and is associated with a high mortality rate. Across all stages of bladder cancer, immunotherapy has now become the cornerstone of treatment. The commensal microbiome has become a major focus of research given its impact on numerous states of human health and disease. Many links between commensal microbes and immune function have been reported. Recently a commensal urinary microbiome has been identified and characterized in healthy individuals by several research groups. The urinary microbiome is now emerging as an important factor influencing bladder cancer development and therapeutic responsiveness. In this report, we identify findings from important clinical and mechanistic studies on the urinary microbiome and future opportunities to impact prevention and treatment of bladder cancer.

Keywords: Microbiome, bladder cancer, NMIBC, BCG, immunotherapy

INTRODUCTION

Bladder cancer is a common malignancy with approximately 400,000 new cases and 150,000 deaths occurring annually [1]. Histological types of bladder cancer include urothelial carcinoma, adenocarcinoma, small cell, plasmacytoid, and squamous cell carcinoma. In industrialized countries, urothelial carcinoma accounts for more than 90% of all histological types and is associated with 5-year survival ranging between 30% and 70%, based on clinical stage [4]. A rise in prevalence and mortality is expected due to environmental exposures, smoking, and increased life expectancy [5]. Bladder cancer can be grouped into two main categories with different outcomes and molecular profiles: non-muscle invasive (NMIBC) and muscle invasive bladder cancer (MIBC). NMIBC, particularly Ta and T1, accounts for 70–80% of all diagnosed cases. Standard treatment is transurethral resection of bladder tumor (TURBT). In intermediate- or high-risk disease, TURBT is followed by intravesical immunotherapy with Bacillus Calmette-Guerin.
(BCG) or intravesical chemotherapy (i.e. mitomycin C) [6]. Recently, pembrolizumab, the anti-PD-1 antibody was approved for NMIBC that is BCG-unresponsive. NMIBC has high recurrence rates (up to 52% in five years) and can progress to MIBC [7]. Thus, early stage disease still requires lifelong monitoring with periodic cystoscopy and urinary cytology, making NMIBC one of the most expensive cancers to manage [8]. MIBC treatment consists of radical cystectomy with perioperative chemotherapy or bladder-sparing approaches involving transurethral resection of bladder tumor followed by chemoradiotherapy. For metastatic bladder cancer standard treatments include platinum-based chemotherapy, FGFR-targeted therapy, enfortumab-vedotin, and immune checkpoint inhibitors targeting the PD-1 pathway. Despite advances in management of urothelial cancer, there remains a critical need to develop new therapies and identify factors that have a role in cancer onset, progression, and recurrence.

Emerging data have discredited the historical view that the urine and bladder are sterile in healthy individuals [9–12]. Modern culture and sequencing techniques have now enabled the detection of microbes throughout the urinary system [13–16]. The concept that healthy urine is sterile dates back to the mid-nineteenth century when early microbiologists such as Louis Pasteur found that urine contained in sealed vials did not become clouded, which suggested an absence of bacteria. Over subsequent decades, culture techniques in clinical laboratories were optimized for detection of specific pathogenic bacteria such as E. Coli. Thus, lack of growth in bacterial cultures had been erroneously linked to sterility. More recently, however, enhanced culture techniques, 16S ribosomal RNA (rRNA) sequencing, and whole-genome shotgun sequencing have provided robust evidence for the existence of a commensal urinary microbiome [14–16]. An emerging focus of bladder cancer research is now aimed at understanding how the commensal urinary microbiome can influence susceptibility to bladder cancer development and its impact on treatment efficacy through modulation of the anti-cancer immune response.

**URINARY MICROBIOME IN HEALTHY INDIVIDUALS**

The bladder was notably not included within the Human Microbiome Project, but several studies from healthy individuals have now shown that urine contains bacteria not routinely cultivated by clinical microbiology laboratories (Fig. 1). These bacteria can be identified by expanded culture techniques and nucleic acid sequencing [11–13]. Although the numbers of studies are limited, some found significant differences between the urinary microbiota of men and women [11, 17]. This finding is not unexpected given the differences in anatomical structure, hormones, and local defenses. Curtiss et al. studied the microbiome of 79 healthy women to identify changes related to age and menopausal status [10]. The authors found a greater incidence of *Lactobacillus* in the bladder microbiome of premenopausal women than post-menopausal women, with a trend towards decreased numbers of different genera in-post menopausal specimens. It is known that declining levels of estrogen during menopause induces vulvovaginal atrophy, which impairs the defense against invading pathogens and is also thought to contribute to the increased risk for urinary tract infections (UTI). Incomplete emptying of the urinary bladder after voiding is another factor thought to increase the risk of recurrent UTI. Residual urine and reduction in urine flow in the absence of estrogen impairs the mechanical clearance of bacteria and eases pathogens to colonize the bladder [18]. These findings may also explain differences in the commensal urine microbiome. Lewis et al. [11] suggested the presence of a core microbiome, defined as a subset of bac-teria that is regularly present in the bladder, with samples are grouped by age. Notably, the genera *Jonquetella, Parvimonas, Proteiniphilum,* and *Saccharofermentans* appeared exclusively in the >70 age group. The reason these genera would colonize the urinary tract of individuals older than 70 years of age is not fully understood. In addition to age, non-modifiable host factors such as sex and genetics may influence the innate immune response and, therefore, have a role in the type of bacterial colonization [19]. This process might encompass human urinary tract adaptation to accommodate certain bacterial species, for example, through expression of specific receptors, as well as mutations in bacteria enabling adherence to the uroepithelium and survival. The implication of inherited phenotypes of innate immunity affecting bacterial colonization of the urinary tract has been supported by investigations looking for a genetic correlation between family members with recurrent UTI [20]. These studies have identified polymorphisms and expression patterns in genes such as CXCR1, which are linked with susceptibility to urinary infection. Further studies are needed to determine the role
The healthy bladder is home to a wide assortment of bacterial species. A summary of specific species identified from published data is represented in this figure. As in with other commensal microbial niches in the body, the composition of bacterial communities in the bladder has a high level of inter-patient variability. Phenotypes have now been correlated with particular bacterial species and richness and diversity of bacterial species present in the bladder.

Studies investigating the continuum of health and disease states have further indicated that microbial populations are capable of influencing urological conditions. The precise nature and role of the most relevant microbes remain under investigation, but their potential involvement has now become more apparent. The impact of microbes on bladder cancer carcinogenesis is perhaps most clear from the long-standing observation that squamous cell carcinoma of the bladder is linked with urogenital schistosomiasis [2]. S. haematobium has been consistently reported to be associated with this type of bladder cancer. Its pathogenic role may occur through several mechanisms, such as epithelium damage, chronic inflammation, and oxidative stress [3]. As of today, only few studies have reported a detailed analysis of urinary microenvironment of urothelial bladder cancer. Results of the available studies are summarized in Table 1. Xu et al. [21] compared urine microbiota of healthy individuals and patients with bladder cancer. Their preliminary results showed an enrichment of Streptococcus in urine from patients with urothelial carcinoma. Streptococcus abundance was near zero in almost all healthy patients. In cancer samples where Streptococcus abundance was low, Pseudomonas or Anaerococcus were the most abundant genera. Unfortunately, the study was limited by the very small sample size and limited discussion of methodology. A similar study compared bacterial communities between urine samples of healthy individuals and cancer patients [22]. The authors found that the most abundant phylum in both groups was Firmicutes, followed by Actinobacteria, Bacteroidetes and Proteobacteria. They identified operational taxonomic units (OTUs) belonging to genus Fusobacterium to be more abundant in the bladder cancer group. An
### Table 1
Available studies on urine microbiome

| Reference | Diagnostics | Clinical trials |
|-----------|-------------|-----------------|
| Xu W et al. | To compare microbiome in urine specimens between healthy individuals and urothelial carcinoma patients | To investigate the safety and preventive effect of fermented milk products containing Lactobacillus casei against bladder cancer |
| Bučević Popović V et al. | To characterize urinary microbiome of bladder cancer patients and compare it with that of healthy controls | To assess the role of oral administration of a preparation of the probiotic agent Lactobacillus casei in prevention of NMIBC recurrence comparing standard intravesical epirubicin with epirubicin plus 1-year oral intake of Lactobacillus casei strain. |
| Wu Pe et al. | To characterize the role of microbiome of urinary microbiota associated with bladder cancer in male patients | To evaluate the role of oral administration of a preparation of Lactobacillus preparation (BLP) in patients with superficial BC. Follow up study of the 1992 trial. |
| Aso Y et al. | To investigate the safety and preventive effect after TUR-BT of an orally administered Lactobacillus preparation (BLP) in patients with superficial BC. Follow up study of the 1992 trial. |
| Ohashi Y et al. | To characterize urinary microbiome of bladder cancer patients and compare it with that of healthy controls | To investigate the safety and preventive effect after TUR-BT of an orally administered Lactobacillus preparation (BLP) in patients with superficial BC. Follow up study of the 1992 trial. |
| Naito S et al. | To characterize urinary microbiome of bladder cancer patients and compare it with that of healthy controls | To investigate the safety and preventive effect after TUR-BT of an orally administered Lactobacillus preparation (BLP) in patients with superficial BC. Follow up study of the 1992 trial. |

**Sample size**

| Aim | Healthy (n = 6) | Healthy controls (n = 18) | Treatment group (n = 23) | Cases (n = 180) | Treatment group (n = 100) | BLP group vs Placebo |
|-----|----------------|--------------------------|--------------------------|----------------|--------------------------|---------------------|
| Urothelial carcinoma (n = 8) | Healthy (n = 11) | Healthy controls (n = 18) | Control group (n = 25) | Controls (n = 445) | Control group (n = 102) | Tot of 138 patients |

**Methods**

| Aim | Healthy (n = 6) | Healthy controls (n = 18) | Treatment group (n = 23) | Cases (n = 180) | Treatment group (n = 100) | BLP group vs Placebo |
|-----|----------------|--------------------------|--------------------------|----------------|--------------------------|---------------------|
| Urothelial carcinoma (n = 8) | Healthy (n = 11) | Healthy controls (n = 18) | Control group (n = 25) | Controls (n = 445) | Control group (n = 102) | Tot of 138 patients |

**Species**

| Aim | Healthy (n = 6) | Healthy controls (n = 18) | Treatment group (n = 23) | Cases (n = 180) | Treatment group (n = 100) | BLP group vs Placebo |
|-----|----------------|--------------------------|--------------------------|----------------|--------------------------|---------------------|
| Urothelial carcinoma (n = 8) | Healthy (n = 11) | Healthy controls (n = 18) | Control group (n = 25) | Controls (n = 445) | Control group (n = 102) | Tot of 138 patients |

**Table 1 (Continued)**
Table 1
(Continued)

| Conclusion | Reference | Diagnostics | Clinical trials |
|------------|-----------|-------------|-----------------|
| Urothelial carcinoma may be associated with altered microbiota, as urines from cancer patients were enriched with Streptococcus, while abundance was near zero in healthy volunteers | Xu W et al. [27] (1992) | Firmicutes, Actinobacteria, Bac- teroi- des and Proteobacteria were common in both groups. However, OTUs belonging to genus Fusobacterium were more abundant in the bladder cancer patients. An additional PCR analysis of 42 bladder cancer tissues, detected Fusobacterium nucleatum sequences in 26% of the samples. On the other hand, OTUs from genera Veillonella, Streptococcus and Corynebacterium were more abundant in healthy controls. | Oral administration of Lactobacillus preparation is useful for the prevention of the recurrence of superficial bladder cancer. Recurrence-free interval post TUR-BT was 1.8-fold prolonged in the treatment group compared to the control group. No adverse side effect was observed. |
| | | | Co-administration of intravesical epirubicin and oral Lactobacillus casei is a promising method for prevention of NMIBC recurrence, as a 15% absolute reduction in long-term tumor recurrence was reported. |
| | | | BLP administration seemed to offer beneficial effects in preventing recurrence of superficial bladder cancer |

Comments: Small sample size, Small sample size, Small sample size, Small sample size, Potential confounding factors, Higher dropout rate (approximately 3.5-fold) in the treatment group.

An independent group of 42 bladder cancer tissues was analyzed and confirmed *Fusobacterium nucleatum* sequences could be detected by protein chain reaction in 11 samples. The genera *Veillonella*, *Streptococcus* and *Corynebacterium* were more abundant in healthy urine [22]. More recently, patients with blad-
TREATMENT FOR NON-MUSCLE INVASIVE BLADDER CANCER

Intravesical immunotherapy with Bacillus Calmette-Guerin (BCG), a live attenuated strain of Mycobacterium bovis, is the standard-of-care for adjuvant treatment for NMIBC with a high-risk of progression. It has also been recommended for treatment of intermediate-risk NMIBC [6]. The mechanisms by which BCG immunotherapy mediates tumor immunity have been widely studied, though remain incompletely understood. Findings from preclinical and clinical studies demonstrate that a robust local inflammatory response to BCG involving the following:

a. BCG attachment to the urothelium. Animal studies suggest that BCG binds through the

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interaction between molecules expressed in the bacterial wall and fibronectin in the urothelium [34].

b. BCG internalization. Whether BCG is internalized by bladder cancer cells has long been disputed. BCG can be found in the urine of both mice and humans in the hours following intravesical instillation [35, 36]. However, BCG disappeared rapidly within days. Durek et al. [36] measured the level of mycobacterial DNA in the urine, showing a significant decrease during the 6 days following instillation. The method of internalization of BCG by urothelial cells has been controversial. However, recent studies have identified macropinocytosis as the endocytic process by which BCG is internalized by urothelial cancer cells. This is a process dependent on activation of the Ras and PI3K–PTEN pathways upstream of the kinase PAK1 [37]. It has been hypothesized that efficacy of BCG therapy depends on its uptake by bladder cancer cells due to the presence of oncogenic aberrations in the Ras and PI3K–PTEN pathways, which leads to activation of macropinocytosis. However, activating mutations in these two pathways are present only in a subset of bladder cancers [37, 38].

c. Induction of innate immune response. BCG immunotherapy induces both local and systemic immune responses, prompting the activation of urothelial and antigen-presenting cells (APCs). The production of cytokines and chemokines attracts granulocytes and mononuclear cells [39]. In vitro studies using human urothelial carcinoma cell lines demonstrated that BCG induces upregulation of cytokine production, including IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF) [40–43]. In human studies, cytokines and chemokines found in the urine after BCG treatment include IL-1, IL-8, IL-15, IL-18, and GM-CSF [41, 42, 44]. One consequence of these reactions is the formation of typical epithelioid granulomas in the bladder wall [9, 39, 45, 46].

d. Induction of adaptive immunity. BCG antigens are presented on the cell surface of APCs via class II MHC [39, 47, 48]. These molecules interact with CD4+ T cell receptors, leading to activation and differentiation to a primarily T helper 1 (Th1) [49]. Cytotoxic CD8+ T lymphocytes recognize tumor cells through antigen presentation via MHC class I. Their activation is facilitated by Th1 cells and mediated by IFN-γ [50]. The balance between Th1 and Th2 cells determines the success of BCG treatment, with a Th1 cell response being associated with successful BCG immunotherapy [43, 50–53]. The necessity of T cells in response to BCG immunotherapy is well established, as athymic nude mice bearing bladder tumors showed no response to BCG instillation [54]. Retrospective analysis of clinical trial data showed that 5-year recurrence-free survival was significantly improved in patients with high-risk NMIBC who had a positive purified protein derivative (PPD) skin test before intravesical BCG therapy, compared to those with a negative PPD test (80% vs 45%) [35]. Even though these data suggest that BCG vaccination might improve the therapeutic response to BCG immunotherapy, further studies are needed to assess the relevance of these findings in the clinical practice.

Despite the many research efforts, the mechanism of action of BCG to control proliferation of cancer cells is still unclear, as well as the mechanisms behind bladder cancer recurrence. Unravelling this puzzle is crucial to determining how new therapies should aim to induce specific tumor microenvironments and antigen responses. The commensal urinary microbiome may be an important link involved in the efficacy of BCG immunotherapy.

POTENTIAL INTERACTIONS BETWEEN URINARY MICROBIOME AND BCG IMMUNOTHERAPY FOR NMIBC

BCG is thought to work by stimulating the immune response through attachment of fibronectin, gaining access into the bladder cells. As many different bacteria are able to adhere to fibronectin, it is possible that specific commensal bacteria may saturate the binding sites used by BCG. This would decrease BCG efficacy and potentially downregulate the strong cytotoxic response needed to remove tumor cells. Conversely, as described earlier in this review, some bacteria, such as Lactobacillus, can induce antiproliferative and cytotoxic effects, contributing to the antineoplastic effect [29]. Probiotics may provide some benefit in the treatment of bladder cancer, as shown by studies where participants consumed fermented milk products and probiotics, achieving reduction in bladder cancer incidence and recurrence
It is compelling that *Lactobacillus* spp. may provide some beneficial role in treatment and prevention of bladder cancer. Our group has recently reported data from a study in which we characterized the role of the urine microbiome in 31 patients with high-risk NMIBC undergoing BCG treatment [56]. DNA was extracted and 16S sequencing data were generated using Illumina paired-end sequencing. In this cohort, 22 (71%) were male and 9 (29%) female, with a median age of 69 years and a range of 46–87 years. There was no difference in recurrence rates between males and females. Proteobacteria was the most abundant phylum, with an incidence of 58% (18 patients). An analysis of the OTUs, based on distance matrix computation, showed a significant difference between patients with and without recurrence (Bonferroni-corrected, \( P = 0.017 \)). The Enterobacteriales order was significantly more abundant in patients with recurrence, while Lactobacillales were more abundant in patients without recurrence. The preliminary results of our study demonstrated the feasibility of analyzing the urinary microbiome in patients with bladder cancer undergoing TURBT and BCG therapy, with the prospect of a possible response prediction to treatment. Data indicate that patients who develop recurrence have significant differences in the abundance of specific bacterial orders at baseline compared with patients without recurrence. Importantly, there are many clinical and biological factors that influence the commensal microbiome and identifying causality can be very challenging (Fig. 2).

**CONCLUSIONS AND FUTURE DIRECTIONS**

A major expansion in knowledge has occurred regarding the impact of the microbiome and its functional role in health and disease. Yet, many more questions have now emerged, resulting in further growth of research efforts, including studies on the microbiome in human cancers. As the concept of urine sterility has now been refuted in several studies, the urine microbiome has become an attractive focus of investigation in bladder cancer due to its proximity to the disease. Several studies have already reported associations with bladder cancer looking at single time points of data. Going forward it will also be important to also track microbial evolution in a longitudinal manner. The microbiome likely has differential impacts on bladder cancer each stage of its development and likely modulates the endogenous anti-tumor immune response. Thus, it has potential utility as a biomarker and therapeutic in bladder cancer, especially given that immune checkpoint therapy now is approved in both early and late stage disease. Direct instillation of probiotics could be a potential strategy to impact the bladder microenvironment, but modification might also be possible through indirect mechanisms. For instance, manipulation of the gut flora with oral agents or fecal microbial transplant may result in systemic effects on immune response or circulating metabolites. Another important direction of future inquiry will be the characterization of the relationship between the urine microbiome and other commensal bacteria in the gut, skin, and other niches. It also remains unknown whether differences exist between the microbial populations of urine derived from the upper tract versus the bladder, or between the microbes potentially present within a bladder tumor versus within normal bladder tissue. A bowel-derived urinary diversion represents another unique environmental niche that is unique to bladder cancer patients and warrants further study. Finally, the complexity of data being generated will likely necessitate the
application of advanced computational techniques incorporating clinical or other biological variables into sophisticated statistical models or machine learning algorithms. Using these approaches, research on the commensal microbiome has the potential to unlock more effective methods for diagnosis and treatment of patients with bladder cancer.

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AUTHOR CONTRIBUTIONS

CA: performance of work, writing the article
JCB: performance of work, interpretation and analysis of data
AP: performance of work, interpretation and analysis of data
RFS: conception, performance of work, interpretation and analysis of data, writing the article

ETHICAL CONSIDERATIONS

This study, as a literature review is exempt from any requirement for Institutional Review Board approval.

CONFLICT OF INTEREST

RFS reports consulting/honoraria from Aduro, AstraZeneca, BMS, Exelixis, Eisai, Janssen, Mirati, and Puma.

The other authors have no conflicts of interest to declare.

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