EUROPEAN UROLOGY ONCOLOGY xxx (xxxx) xxx–xxx

available at www.sciencedirect.com
journal homepage: euoncology.europeanurology.com

European Association of Urology

Microbiomes of Urine and the Prostate Are Linked to Human Prostate Cancer Risk Groups

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Article info

Article history:
Received 13 September 2021
Received in Revised form
8 March 2022
Accepted March 29, 2022

Associate Editor:
Alberto Briganti

Stat Editor:
Andrew Vickers

Keywords:
Anaerobic bacteria
Cancer progression
Metagenomic sequencing
Metastases
Microbiota
Microbiome
Prognosis
Prostate cancer
Urine

Abstract

Background: Bacteria play a suspected role in the development of several cancer types, and associations between the presence of particular bacteria and prostate cancer have been reported.
Objective: To provide improved characterisation of the prostate and urine microbiome and to investigate the prognostic potential of the bacteria present.
Design, setting, and participants: Microbiome profiles were interrogated in sample collections of patient urine (sediment microscopy: n = 318, 16S ribosomal amplicon sequencing: n = 46; and extracellular vesicle RNA-seq: n = 40) and cancer tissue (n = 204).
Outcome measurements and statistical analysis: Microbiomes were assessed using anaerobic culture, population-level 16S analysis, RNA-seq, and whole genome DNA sequencing.
Results and limitations: We demonstrate an association between the presence of bacteria in urine sediments and higher D'Amico risk prostate cancer (discovery, n = 215 patients, $p < 0.001$; validation, n = 103, $p < 0.001$, χ^2 test for trend). Characterisation of the bacterial community led to the (1) identification of four novel bacteria (*Porphyromonas* sp. nov., *Varibaculum* sp. nov., *Peptoniphilus* sp. nov., and *Fenollaria* sp. nov.) that were frequently found in patient urine, and (2) definition of a patient subgroup associated with metastasis development (p = 0.015, log-rank test). The presence of five specific anaerobic genera, which includes three of the novel isolates, was associated with cancer risk group, in urine sediment (p = 0.045, log-rank test), urine extracellular vesicles (p = 0.039), and cancer tissue (p = 0.035), with a meta-analysis

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https://doi.org/10.1016/j.euo.2022.03.006
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Please cite this article as: R. Hurst, E. Meader, A. Gihawi et al., Microbiomes of Urine and the Prostate Are Linked to Human Prostate Cancer Risk Groups, Eur Urol Oncol (2022), https://doi.org/10.1016/j.euo.2022.03.006

16S ribosomal amplicon sequencing

hazard ratio for disease progression of 2.60 (95% confidence interval: 1.39–4.85; p = 0.003; Cox regression). A limitation is that functional links to cancer development are not yet established.

Conclusions: This study characterises prostate and urine microbiomes, and indicates that specific anaerobic bacteria genera have prognostic potential.

Patient summary: In this study, we investigated the presence of bacteria in patient urine and the prostate. We identified four novel bacteria and suggest a potential prognostic utility for the microbiome in prostate cancer.

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1. Introduction

Prostate cancer is the most common nonskin malignancy in men in developed countries, with over 250,000 deaths annually worldwide [1]. The clinical course of prostate cancer is highly heterogeneous, and critical decisions are made about the likelihood of aggressive disease based on information obtained at presentation, including histopathological Gleason score determined following biopsy [2]. Determining urinary biomarkers to identify aggressive prostate cancer is an area of growing interest. Material secreted by the prostate gland appears in the urine, and reflux of urine into the prostate is well established, supporting the existence of a prostate-urine loop [3–5]. Urine biomarkers identified include assessment of gene methylation [6] or gene expression profiles including PCA3, and various gene combinations [7–9]. However, none of these tests are in widespread clinical use, and the challenge remains to find a combination of biomarkers and clinical data that, at initial patient assessment, can reliably predict prostate cancer risk groups and disease progression.

Genetic inheritance and ethnicity have established roles [10,11] in prostate cancer development, while chronic inflammation has also been proposed as an aetiological factor [12–14]. *Helicobacter pylori* has an established role in the development of gastric cancer [15], stimulating the search for microbial involvement in the development of other cancers. Bacteria are known to be present in the urogenital tract and in prostate tissue [12,16], and bacteria isolated from the prostate can cause inflammation in animal models [12–14]. Encouragingly, microbes present in prostate tissue differ between patients with different Gleason grades [17], and there are links between the presence of prostate cancer and distinct microbial profiles of the urine [13,18] and the gastrointestinal tract [18–20].

In this study, we used fluorescent microscopy, anaerobic culture, 16S ribosomal amplicon sequencing, mRNA sequencing, and whole genome DNA sequencing to detect bacteria in urine after digital rectal examination (DRE) and prostate cancer tissue. We (1) use the nonbiased "tree of life" [21] methodology to isolate and classify novel bacteria, and (2) search for associations between the presence of bacteria and prostate cancer risk groups.

2. Patients and methods

Detailed methods can be found in the Supplementary material.

2.1. Patient recruitment and specimen collection

Ethical approval was obtained from the local research ethics committee (12/EE/0058). Patients were categorised into clinical groups (Supplementary material), and prostate cancers were stratified according to D'Amico risk group [22]. Urine samples were collected (from April 2012 to January 2015) [7] after DRE (prostate massage three strokes per lobe from the base to the apex), prior to biopsy, from patients undergoing assessment for prostate cancer or haematuria at the Norfolk and Norwich University Hospital, Norwich, UK, and processed immediately using sterile techniques. Urine sediments and extracellular vesicle fractions were prepared as described previously [7], with an additional step for the detection of bacteria by microscopy (Supplementary material). Prostate secretions (100–400 μl) were collected (from May 2017 to February 2020) via manual compression of the excised prostate <20 min after prostatectomy. Samples were processed immediately, snap frozen on dry ice, and stored at −80°C.

2.2. Metagenomics, 16S ribosomal amplicon DNA sequencing, and RNA-seq metatranscriptomics

DNA extraction from urine sediment was similar to the method of Yu and Morrison [23], with repeated bead-beating extraction to maximise bacterial DNA yield. Bacterial 16S DNA was amplified and sequenced (V1-V3/V3-V5 hypervariable regions). Controls included no template controls, elution buffer controls, and blank bead-beating extraction samples. Quantitative polymerase chain reaction (qPCR) assays detected several bacterial genera and species. Urine extracellular vesicle total RNA was extracted as described previously [7] from 40 urine samples and were sequenced and processed with the SEPATH [24] pipeline.

2.3. Detection of bacteria in International Cancer Genome Consortium prostate tissue whole genome sequences

Unmapped reads from human-aligned whole genome sequencing data (International Cancer Genome Consortium [ICGC] prostate cancer tissue n = 204, collected from March 2004 to June 2014) were classified using a curated BWA database containing GRCh38, 75 study isolates, and strains frequently identified by Kraken (Supplementary material). Reads were filtered to have a minimum mapping quality of 20 and 50 bp minimum alignment, and were subject to complexity filtering. Assemblies with ≥200 bp of their genome covered were considered present in the sample.

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2.4. Isolate anaerobic culture, whole genome sequencing and assembly, and phylogenetic and metabolic pathway analyses

Urine or prostate secretion samples were inoculated into pre-reduced PY broth or *Brucella* blood agar plates with 5% sheep blood and vitamin K1/hemin supplementation (Beckton Dickinson GmbH, Heidelberg, Germany), and grown in an anaerobic cabinet supplied with 5% hydrogen, 10% CO2, and 85% nitrogen at 37°C. Pure colonies were picked and prepared for DNA extraction, and sequenced with Nextera XT library preparation on Illumina MiSeq (San Diego, CA, USA) using V3 reagents (2 × 300 bp). MinION nanopore sequencing (Oxford Nanopore Technologies, Oxford, UK) was used on three novel species for hybrid assembly (Unicycler). A phylogenetic analysis was carried out as described previously [21], using multiple sequence alignments of 16 ribosomal proteins from isolates and known strains. Metabolic pathways were predicted using InterProScan REST api v5.29-68.053 (Supplementary material).

2.5. Statistical analysis

For the urine sediment microscopy dataset (n = 318), patients were categorised into clinical groups including low-, intermediate-, and high-risk prostate cancer (for further details, see the Supplementary material) [22]. Further data for each cohort including clinical characteristics are provided in Supplementary Tables 1–4. Follow-up for the clinical cohorts was over 3–4 yr (median 2.7 yr) and up to 6 yr after sample collection (median 5.2 yr) for the 16S and RNA-seq datasets, respectively. For the cancer tissue dataset (n = 204), follow-up data were up to 9.8 yr (median 3.5 yr; Supplementary material). Progression events were the detection of prostate cancer metastasis or prostate-specific antigen (PSA) biochemical failure following initial treatment (two PSA tests ≥0.2 ng/ml). Survival analyses include Kaplan-Meier curves, Cox proportional hazard models, and the log-rank test. A random-effect meta-analysis based on log hazard ratios was carried out with metagen function (meta R package; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Bacteria in urine are associated with increased risk of prostate cancer

Examination of post-DRE urine sediments revealed background DNA staining of bacteria in ~50% samples, supported by scanning electron microscopy and 16S ribosomal RNA gene detection (Supplementary Figs. 1A, 1B, and 2, and Supplementary Table 5). To further investigate this observation, we analysed urine samples from men undergoing assessment for prostate cancer (n = 300) or from a haematuria clinic (n = 18). Background DNA staining of bacteria was more common from men with intermediate and high D'Amico risk and advanced prostate cancer (Table 1), with a statistically significant association between the presence of bacteria and increased risk of prostate cancer (χ^2 test for trend in proportions: discovery set, p < 0.001, n = 215; validation, p < 0.001, n = 103). A similar significant association with the presence of bacteria was observed in each of the component parts of risk groups, including PSA, Gleason score, and clinical stage (Supplementary Fig. 3). For the combined data set, large aggregates of bacteria in urine were also significantly associated with increased prostate cancer risk (p = 0.006; Supplementary Fig. 2). The bacteria also appeared, in some cases, to be intracellular within human cells (Supplementary Fig. 4).

Table 1 – Presence of bacteria in urine cell sediment and association with groups with increased risk of prostate cancer[a]

Category	Percentage positive for microorganisms	Negative count	Positive count
Discovery set			
Normal PSA range	31	18	8
Low-risk PCa	46	6	5
Intermediate-risk PCa	64	20	36
High-risk PCa	88	4	29
Advanced PCa	83	2	10
Atypia/HG-PIN	26	14	5
Raised PSA negative biopsy	40	35	23
Validation set			
Normal PSA range	23	10	3
Low-risk PCa	17	5	1
Intermediate-risk PCa	77	6	20
High-risk PCa	75	4	12
Advanced PCa	100	0	6
Atypia/HG-PIN	30	7	3
Raised PSA negative biopsy	27	19	7

DRE = digital rectal examination; HG-PIN = high-grade prostatic intraepithelial neoplasia; PCa = prostate cancer; PSA = prostate-specific antigen.

[a] A significant correlation of clinical D'Amico risk group and advanced disease (PSA >100 ng/ml) with the presence of background DAPI-stained bacteria fluorescence in post-DRE urine was observed. Data are presented as percentage positive for bacteria/micro-organisms. Samples examined by microscopy were divided into a discovery (two-thirds of samples; n = 215) and a validation (one-third of samples; n = 103) dataset by random assignment, stratified by clinical group: the discovery set (χ^2 test for trend in proportions, p < 0.001) and the validation set (χ^2 test for trend in proportions, positive trend, p < 0.001). Statistical analyses were performed on the first five groups. Data on atypia/HG-PIN and raised PSA negative biopsy were included for comparison.

3.2. Culture confirms new species of bacteria from the urine of prostate cancer patients

To identify the bacteria involved, we applied 16S sequencing on urine sediments from 46 men (24 with a diagnosis of prostate cancer) using accepted protocols and controls to avoid contamination [25,26]. The bacterial community structure identified revealed 1614 bacterial operational taxonomic units (OTUs). No significant association was found between the number of OTUs detected and prostate cancer risk groups: an average of 168 OTUs (range: 64–265) in samples from noncancer patients, 130 OTUs (range: 67–237) in samples from low/intermediate-risk prostate cancer patients, and 171 OTUs (range: 81–290) in samples from high-risk and advanced prostate cancer patients. Many OTUs lacked assignment at levels lower than genus or family level. We found no exact matches of these unassigned OTU sequences in the NCBI dataset, suggesting that post-DRE urine contains novel bacterial species. Therefore, we attempted to culture them.

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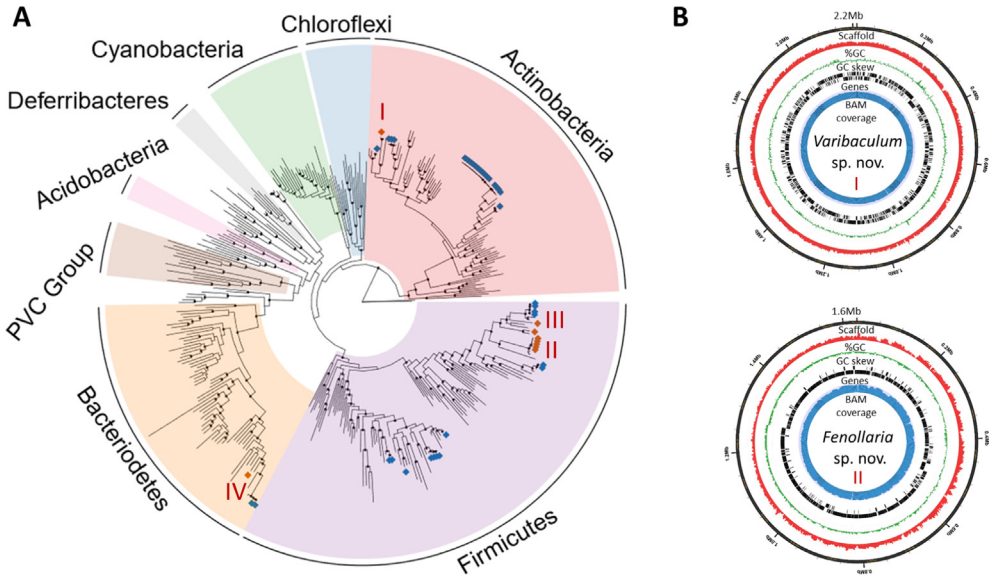

Fig. 1 – Phylogenetic tree and novel bacteria. (A) Cultured fastidious anaerobes were isolated from urine and the prostate, and their genomes were decoded using Illumina and Nanopore DNA sequencing. Each bacterial strain was positioned on the phylogenetic tree as described in the Patients and methods section. Bacteria with a known identification are highlighted with blue diamonds, while novel species are highlighted with orange diamonds (I: *V. prostatecancerukia* sp. nov., II: *F. sporofastidiosus* sp. nov., III: *P. rachelemmaiella* sp. nov., and IV: *P. bobii* sp. nov.). (B) Genome representation for two of the novel species. Data are given for: I, *Varibaculum* sp. nov. isolate 39, 2.2 Mb, GC content, 53%; II, *Fenollaria* sp. nov. isolate 24, 1.6 Mb, GC content, 36%; III, *Peptoniphilus* sp. nov. isolate 23, 1.9 Mb, GC content 49%; and IV, *Porphyromonas* sp. nov. isolate 6C, 2.2 Mb, GC content 56% (other isolates are in Supplementary Tables 6 and 7, and Supplementary Figures 6A–D). PVC = *Planctomycetes*, *Verrucomicrobiae*, *Chlamydiae* group.

We applied a fastidious anaerobic culture protocol for culturing bacteria previously considered to be "unculturable" [27] to post-DRE urine sediments and to prostate fluid secretions obtained by squeezing the prostate after prostatectomy. Previous studies identified anaerobic bacteria in prostate tissue [28,29] but have not fully characterised the species present. In this study, strict anaerobic culture protocols yielded 39 bacterial isolates from post-DRE urine (Supplementary Table 6) and eight isolates from prostate cancer secretions. Assembly of whole genome sequencing data (Illumina sequencing for all isolates and Oxford Nanopore sequencing for candidate novel species) resulted in one to 515 contigs per isolate. Most anaerobic bacterial isolates from post-DRE urine sediments were from the phyla *Firmicutes*, class *Clostridia*, including genera *Peptoniphilus*, *Fenollaria*, and *Anaerococcus* (Supplementary Table 6). Sixteen isolates of *Propionimicrobium lymphophilium* from three different urine samples demonstrated considerable genetic variation (Supplementary Fig. 5). Prostate secretions yielded bacteria from the genera *Porphyromonas*, *Staphylococcus*, *Streptococcus*, and *Cutibacterium* (Supplementary Table 6).

A higher-resolution phylogenetic analysis was performed by aligning selected full-length ribosomal gene protein sequences from unclassified isolates to the same genes from known bacterial species (Fig. 1A) [21]. This allowed us to identify four novel species (Table 2). The novel species, defined as sequence similarity <97% to the closest published assemblies [30], were from the phyla *Firmicutes* (*Fenollaria sporofastidiosus* sp. nov. and *Peptoniphilus rachelemmaiella* sp. nov.), *Actinobacteria* (*Varibaculum prostatecancerukia* sp. nov.), and *Bacteroidetes* (*Porphyromonas bobii* sp. nov.). Further details on novel species and isolates are provided in Supplementary Figures 6A–D and Supplementary Table 7.

We confirmed the presence of all four novel species in urine cell sediment samples with in-house qPCR assays (6–65% of samples; Supplementary Table 8). Two novel species (*Peptoniphilus* sp. nov. and *Varibaculum* sp. nov.) were detected by qPCR in prostate tissue (2.8–8.6%), and all four novel species were detected in prostate secretions

Table 2 – Novel bacteria species isolated[a]

Novel species isolated, bacteria ID	Novel species belonging to phyla	Novel species belonging to class	Novel species belonging to genus	Reference novel strain ID
Fenollaria sp. nov.	*Firmicutes*	*Clostridia*	*Fenollaria*	*F. sporofastidiosus* sp. nov. (EMRHCC_24)
Peptoniphilus sp. nov.	*Firmicutes*	*Clostridia*	*Peptoniphilus*	*P. rachelemmaiella* sp. nov. (EMRHCC_23)
Varibaculum sp. nov.	*Actinobacteria*	*Actinobacteria*	*Varibaculum*	*V. prostatecancerukia* sp. nov. (EMRHCC_39)
Porphyromonas sp. nov.	*Bacteroidetes*	*Bacteroidia*	*Porphyromonas*	*P. bobii* sp. nov. (EMRHCC_6C)

[a] Novel species isolated from clinical samples, including taxonomy, and proposed new species name, novel strain ID, are given. *Fenollaria* sp. nov., *Peptoniphilus* sp. nov., and *Varibaculum* sp. nov. were isolated from urine, and *Porphyromonas* sp. nov. was isolated from prostate secretion fluid (further details regarding the novel species are provided in Supplementary Table 7 and Supplementary Fig. 6).

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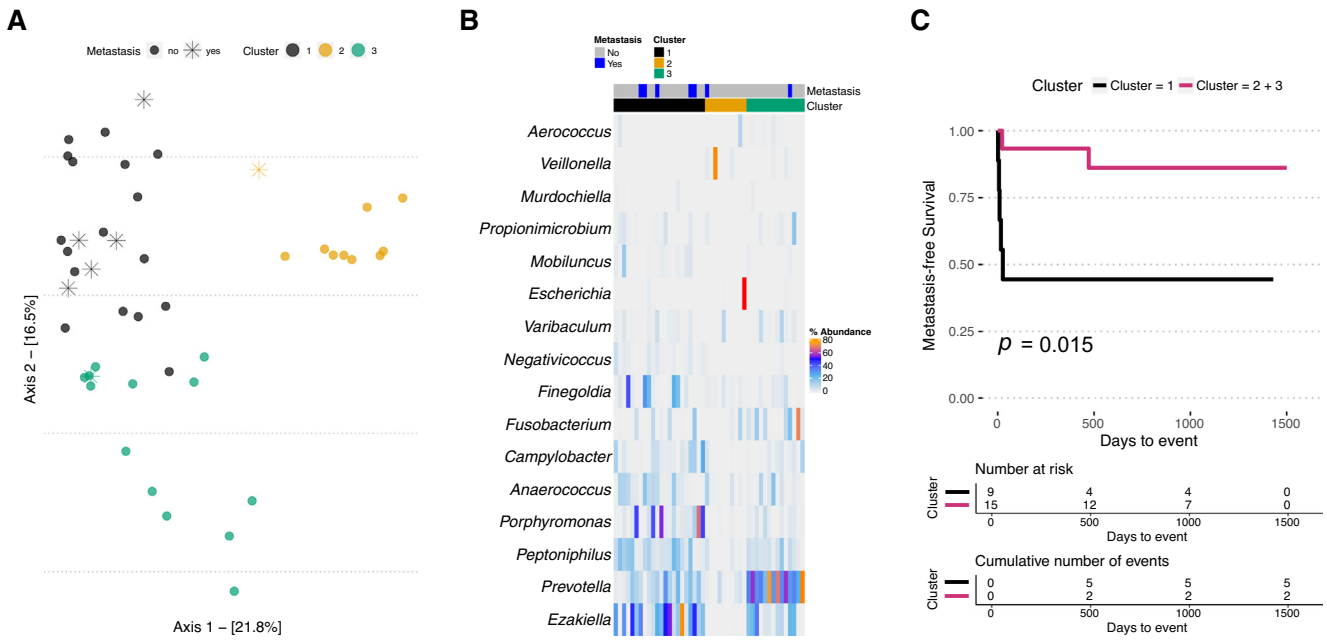

Fig. 2 – Presence and composition of urine microbiota identify participants with a poorer prognosis. The figure presents an analysis of 16S OTU sequence from urine sediments. (A) Principal coordinate analysis (Manhattan distance) of family-level OTU data from urine sediments from 46 patients undergoing assessment for prostate cancer. Clustering with *k*-means suggested three clusters: cluster 1 (black), cluster 2 (yellow), and cluster 3 (green). Samples from patients who developed skeletal metastases are indicated with diamonds. (B) Heatmap demonstrating a variety of bacterial genera selected to demonstrate differences across the three family-level clusters. (C) Kaplan-Meier analysis investigating metastasis-free survival: cluster 1 (black); clusters 2 plus 3 (pink). OTU = operational taxonomic unit.

(2.8–17%). This is consistent with evidence for a prostate-urine reflux loop where there is an exchange of bacteria between urine and the prostate [3–5].

3.3. *Sequencing with 16S ribosomal amplicon identifies bacterial genera potentially associated with prostate cancer risk groups*

Having identified putative novel species, we investigated 16S OTUs. Clustering on the relative abundance of the 16S OTUs at the family level from 46 men, using *k*-means on principal coordinate analysis, revealed three clusters (Fig. 2A and 2B, and Supplementary Fig. 7). The first three principal coordinates explained 49% of variance (Fig. 2A and Supplementary Fig. 7A–C). Patients demonstrating metastases at investigation or during follow-up were over-represented in cluster 1 (Fig. 2A, diamonds, and Supplementary Fig. 7) compared with the other two clusters (p = 0.015, log-rank test; Fig. 2C).

We identified eight genera with significantly higher abundance in cluster 1 (metastatic group) relative to the rest (Supplementary Fig. 7D, Supplementary Table 9, and Supplementary material). Four genera were selected for further study based on significance and value of the median relative abundance of cluster 1 (Supplementary material). These were the strict anaerobes *Fenollaria/Ezakiella, Peptoniphilus*, *Porphyromonas*, and *Anaerococcus*. *Fusobacterium,* another anaerobe detected in the 16S amplicon data, was also included due to growing evidence of association with the development of a range of cancers [31,32]. Co-occurrence plots of the genera in the urine sediment 16S data demonstrated that several of these strict anaerobes are commonly found together in high-risk and advanced/metastatic disease (Supplementary Fig. 8). The five selected bacteria genera (Table 3 and Supplementary Table 10) are referred to as the anaerobic bacteria biomarker set (ABBS) and includes three of the novel isolates.

Table 3 – Anaerobic bacteria biomarker set (ABBS): bacteria associated with poor prognosis[a]

Anaerobic bacteria biomarker set: ABBS bacteria genera	ABBS belonging to phylum	ABBS belonging to class	ABBS belonging to order	Novel species and known species isolated by anaerobic culture belonging to ABBS genera
Fenollaria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Fenollaria* sp. nov.◆
Peptoniphilus	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptoniphilus* sp. nov.◆; *Peptoniphilus harei*
Anaerococcus	*Firmicutes*	*Clostridia*	*Clostridiales*	*Anaerococcus prevotii*
Porphyromonas	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonas* sp. nov.◆; *Porphyromonas asaccharolytica*
Fusobacterium	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacterium nucleatum*

[a] ABBS bacteria taxonomy, novel species isolated in this study (◆).

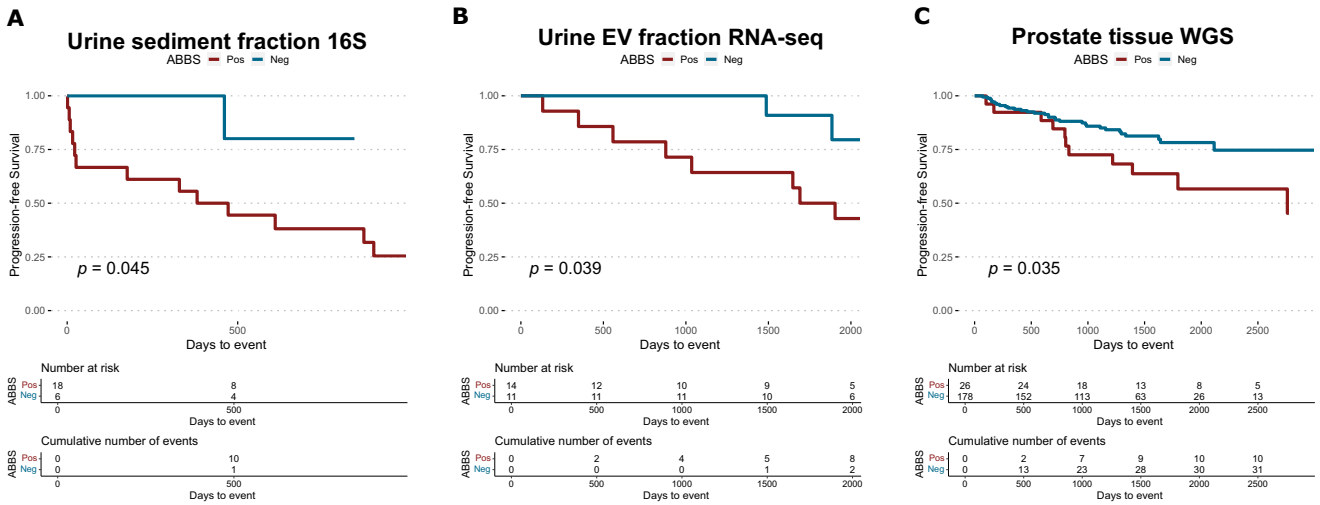

Fig. 3 – Anaerobic bacteria biomarker set (ABBS) from the prostate-urine reflux loop is significantly associated with more rapid progression. Kaplan-Meier analysis investigating progression-free survival is shown. The presence of the following genera was used to partition sample sets: *Fenollaria* (including hits to *Ezakiella* due to closely related 16S sequences to *Fenollaria*), *Peptoniphilus*, *Porphyromonas*, *Anaerococcus*, and *Fusobacterium*. These genera collectively constitute the ABBS in this study. Data were collected from the following: (A) urine cell sediment fraction 16S sequencing (14 total events: 13 from ABBS$^+$ samples and one from ABBS$^-$ samples); (B) RNA sequencing of urine extracellular vesicle (EV) fraction (13 total events: 11 from ABBS$^+$ samples and two from ABBS$^-$ samples); and (C) whole human genome ICGC DNA sequence data from prostate cancer tissue (WGS; 42 total events: 12 from ABBS$^+$ samples and 31 from ABBS$^-$ samples). All *p* values are calculated using the log-rank test. Curves were truncated if there were fewer than four samples in a subgroup or ten samples overall. Univariate cox proportional hazard models are summarised in Supplementary Figure 9. The hazard ratios (95% confidence intervals [CIs]; *p* values) are, respectively, as follows: 6.18 (95% CI: 0.81–47.33; *p* = 0.023), 4.41 (95% CI: 0.95–20.53; *p* = 0.059), and 2.07 (95% CI: 1.04–4.15; *p* = 0.040). A meta-analysis of these three models gave an HR of 2.60 (95% CI: 1.39–4.85; *p* = 0.003). A forest plot of this meta-analysis is available in Supplementary Figure 9. HR = hazard ratio; ICGC = International Cancer Genome Consortium; Neg = negative; Pos = positive; WGS = whole genome sequencing.

3.4. Use of the ABBS from the prostate-urine reflux loop as a prognostic biomarker

Two fractions were produced from processing urine: the sedimentary faction that was used to detect bacterial DNA fluorescence staining and generate 16S OTU data, and a supernatant fraction that contains prostate-derived extracellular vesicles. Both 16S OTU data (n = 24) from urine sediment and RNA–seq data of the urine extracellular vesicle supernatant fraction (n = 25) demonstrated more clinically aggressive cancer when at least one ABBS genus was detected (p = 0.045 and p = 0.039, respectively; log-rank test; Fig. 3A and 3B).

The 16S and RNA–seq investigations are limited by small sample sizes, and so for validation, we examined a much larger and entirely independent prostate cancer tissue whole genome sequencing dataset for the association of bacterial genera (Supplementary Tables 4 and 11) with clinical outcome after prostatectomy (n = 204). Although these are human cancer genome data, bacterial DNA is concomitantly sequenced if present. There is a significantly high rate of biochemical recurrence in donors with at least one of the ABBS genera (log-rank p = 0.035; Fig. 3C).

Combining the three data sets in a meta-analysis gives a hazard ratio for disease progression of 2.60 (95% confidence interval [CI]: 1.39–4.85; p = 0.003; Cox proportional hazard regression; Supplementary Fig. 9). The ICGC dataset was additionally subject to a multivariable analysis including covariates: PSA at radical prostatectomy, age at diagnosis, tumour size at diagnosis, and Gleason score (Supplementary material). The predicted hazard ratio for the multivariable analysis was 2.02 (95% CI: 0.97–4.2, p = 0.061). Overall, these results indicate that detection of anaerobic bacteria that comprise the ABBS in the urinary tract may constitute a prognostic test for prostate cancer biochemical failure.

To explore common biological features of ABBS bacteria, we used assemblies to predict genes and their function. We found the following genes enriched in ABBS compared with non-ABBS isolates that are potentially relevant to cancer development (Supplementary Fig. 10): (1) components of metabolic pathways that can convert cholesterol to androstenedione, an immediate precursor for testosterone that is required for prostate cancer growth [33]; (2) flavin-dependent bacterial-specific thymidylate synthase; (3) a predicted citrate lyase complex (reduced citrate is a known predictor of cancer aggression in prostate cancer [34]); and (4) the glycine cleavage complex and components of the pathway for biotin synthesis that can impact host metabolic pathways [12,34–36]. We currently have no evidence of causality.

4. Discussion

A review published in 2019 [12] describes the association of the microbiome with prostate pathologies but concluded that major difficulties remain: sampling contamination, obtaining effective control tissue, and classifying the often-novel bacteria involved. Addressing these concerns, we implemented several improvements. Firstly, we used protocols to minimise contamination during OTU data generation [25,37]. Secondly, we used strictly anaerobic culture conditions (Supplementary Fig. 11) leading to the isolation of novel bacteria and then their qPCR detection in urine

and the prostate. We also used short and long read DNA sequencing to obtain complete genomes for three of the novel bacteria. Overall, our results provide more complete characterisation of the urine and prostate microbiomes, and provided a solid foundation for examining the relationships between the presence of specific bacteria and clinical outcome.

Several separate lines of evidence support the role of bacteria as a prognostic marker of disease progression. We demonstrated a significant correlation between groups with increased risk of prostate cancer and the presence of bacteria as determined by the fluorescence microscopic detection of bacterial cell DNA, both in discovery ($n = 215$ patients, $p < 0.001$) and validation ($n = 103$, $p < 0.001$) datasets. Secondly, the principal coordinate analysis of 16S OTU data identified a cluster of patients with a higher incidence of metastatic disease. This observation led to the development of the ABBS consisting of five genera of strictly anaerobic bacteria (Table 3). Thirdly, an analysis of RNA-seq libraries prepared from the extracellular vesicle fraction of urine and of OTU data from the urine sedimentary fraction provided indicative results supporting the importance of the ABBS. Remarkably, ABBS importance was validated by a distinct detection technology in tissue, namely, by interrogating a large ($n = 204$) dataset whereby whole genome DNA sequencing captured information from bacteria present in prostatectomy tumour samples. Taken together, these studies provide a strong case for a role of specific anaerobic bacteria (ABBS) present in the prostate-urine reflux loop in predicting aggressive prostate cancer.

Recent studies undertaken by others [38,39] and by our own laboratory [40] have provided comprehensive analyses of microbiomes and viromes associated with human cancers. A consistent observation is that microbiomes present in cancer tissue or blood can act as diagnostic markers across multiple cancer types [38,39]. Analyses of published datasets [38,39] indicated that ABBS bacteria were also present in other cancer types (results not shown); hence, their relevance in determining aggression may extend beyond prostate cancer. We also provide predicted functions of the ABBS, adding to previous studies on prostate cancer that have investigated the association between the presence of micro-organisms and inflammation [14,29], and identifying a variety of molecular mechanisms that are of potential interest for tumour progression and therapeutic exploitation. Further research is needed to determine whether ABBS-specific bacteria may cause cancer, the pathogenetic processes involved, if they do, and subsequently to identify potential treatment options that would eradicate the anaerobic pathogens.

5. Conclusions

In conclusion, our results establish the importance of bacteria present in urine and the prostate as potential prognostic markers and, when considered together with data from other studies [12–14,16,18], provide a starting point for future investigations into the roles of bacteria in prostate cancer pathogenesis and evolution.

***Author contributions*:** Colin S. Cooper had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Hurst, Meader, Clark, Kay, Webb, Manley, Curley, Walker, Kumar, Schmidt, Yazbek-Hanna, Rochester, Mills, Ball.
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Obtaining funding: Hurst, Clark, O'Grady, Brewer, Wain, Cooper.
Administrative, technical, or material support: Curley, Webb, Walker.
Supervision: Hurst, Clark, O'Grady, Brewer, Wain, Cooper.
Other: None.

Financial disclosures: Colin S. Cooper certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: Colin S. Cooper, Daniel S. Brewer, Rachel Hurst, Ghanasyam Rallapalli, Abraham Gihawi, John Wain, Justin O'Grady, and Emma Meader are coinventors on a patent application (UK Patent Application No. 2200682.9) from the University of East Anglia/UEA Enterprises Limited regarding the application of ABBS genera in prostate cancer.

***Funding/Support and role of the sponsor*:** This work was funded by Prostate Cancer UK (research grant ref RIA15-ST2-029, MA-ETNA19-003), the Bob Champion Cancer Trust, and Big C Cancer Charity (ref 16-09R). We are grateful for and acknowledge support from the Masonic Charitable Foundation Successor to the Grand Charity, Movember, the Prostate Cancer Research Centre, the King Family, and the Stephen Hargrave Trust. We also acknowledge funding from Cancer Research UK and Dallaglio Foundation that supported the ICGC project (research grant ref. C5047/A22530). John Wain and Justin O'Grady were funded by the BBSRC Institute Strategic Programme Microbes in the Food Chain BB/R012504/1 and its constituent project BBS/E/F/000PR10349. Maria Traka was funded by the BBSRC Core Capability Grant BB/CCG1860/1 and its constituent project BBS/E/F/00044600 Food Databanks National Capability. The authors confirm that the funding organisations did not inform or influence the design and conduct of the study; collection, management, analysis, and interpretation of the data; or the preparation, review, or approval of the manuscript.

***Acknowledgements*:** The authors acknowledge the staff in the Urology Department at the Norfolk and Norwich University Hospital, in particular, Elizabeth Rooney, Andrea Clayton, and Robert Collins, for facilitating the research. The authors also acknowledge Kim Findlay at the John Innes Centre Bioimaging facility for expertise with the scanning electron microscopy and Dr. Paul Thomas at the Henry Wellcome Laboratory for cell imaging for advice with microscopy at UEA. We are grateful for the advice

regarding the culture for NCBI entry: *Sporobacterium WAL_1855D* taxonomy ID 507843 from Dr. Sydney Finegold, Nisha Patel, and Dr. Paul Lawson. Much of the computational reported in this manuscript was carried out on the high performance computing cluster supported by the Research and Specialist Computing Support service at the University of East Anglia. The authors would especially like to thank the participants who have donated their time and samples for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euo.2022.03.006.

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