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Bacteriophages of the lower urinary tract

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13 **Abstract** | The discovery of bacteria in the female urinary bladder has fundamentally changed current
14 dogma regarding the urinary tract and related urinary disorders. Previous research characterized many
15 of the bacterial components of the female urinary tract, but the viral fraction of this community is
16 largely unknown. Viruses within the human microbiota far outnumber bacterial cells, with the most
17 abundant viruses being those that infect bacteria (bacteriophages). Similar to observations within the
18 microbiota of the gut and oral cavity, preliminary surveys of the urinary tract and bladder microbiota
19 indicate a rich diversity of uncharacterized bacteriophage (phage) species. Phages are vital members of
20 the microbiota, having critical roles in shaping bacterial metabolism and community structure. Despite
21 the fact that phages have been discovered in the urinary tract, such as phages that infect *Escherichia*
22 *coli*, sampling them is challenging owing to low biomass, possible contamination when using noninvasive
23 methods, and the invasiveness of methods that reduce the potential for contamination. Phages could
24 influence bladder health, but an understanding of the association between phage communities,
25 bacterial populations, and bladder health is in its infancy. However, evidence suggests that phages can
26 defend the host against pathogenic bacteria and, therefore, modulation of the microbiome using phages
27 has therapeutic potential for lower urinary tract symptoms. Furthermore, as natural predators of
28 bacteria, phages have garnered renewed interest for their use as antimicrobial agents, for instance in
29 the treatment of urinary tract infections.

30

- 31 • Phages are abundant members of the microbiota of the lower urinary tract.
- 32 • Active or lytic phages have been isolated from urine samples, but the majority of phages within
33 the urinary microbiota persist through dormant infections, the lysogenic life cycle.
- 34 • Evidence suggests that phages have a role in modulating the composition of the urinary
35 microbiota, similar to that observed in microbiota of other organs of the human body.
- 36 • Phage therapy, or the use of phages to treat pathogenic bacterial infections, is an active area of
37 research within urology, given their potential use to treat urinary tract infections.

38

39

40 Introduction

41 Bacteriophages (phages) are ubiquitous viruses that infect bacteria; they are the most abundant
42 biological entities, far exceeding even bacteria¹. Surveys of the marine environment have resulted in an
43 estimated prevalence of 10³⁰ phages in the oceans alone, meaning that for each bacterial cell in the
44 ocean there are ~10 phages¹. In addition to their abundance within the marine environment², phages
45 are prevalent within the soil^{3,4} and in freshwater⁵. Phages have even been isolated from some of the
46 most inhospitable conditions, including desert sands⁶, sea ice⁷, and the depths of the ocean⁸. In
47 addition to our surroundings, phages are abundant in and on plants⁹ and within the bodies of insects
48 and mammals^{10,11}. Indeed, the human gut alone is home to an estimated 2 trillion phages¹², greatly
49 exceeding the number of eukaryotic viruses in our bodies¹³. Although phages are unable to infect
50 eukaryotic cells, evidence has been reported of direct interactions, such as direct transcytosis of phages
51 across cell layers of the gut, lung, liver, kidney and brain¹⁵, between phages and human cells^{14,15}.
52 However, the full extent of direct and indirect effects of phages is unknown, as researchers have only
53 just begun to speculate about the roles of phages in the human body, including the human immune and
54 central nervous systems¹².

55 As phage genomes can be dsDNA, ssDNA, double-stranded RNA (dsRNA), or ssRNA, linear or circular,
56 and even segmented, sequencing phage populations often is limited by the genomic nucleic acid
57 extraction protocol and might need amplification before sequencing¹⁶. Amplification can introduce
58 biases such as quantitative biases, the preferential amplification of ssDNA viruses, stochastic biases,
59 systemic biases¹⁷⁻¹⁹. Nevertheless, whole-genome sequencing technologies have enabled researchers to
60 identify new phage species. In contrast to cellular organisms, no universal marker exists for phages
61 because no gene is conserved within all phages. To identify phages, researchers often target genes that
62 encode structural proteins such as phylogenetic markers²⁰. However, these signature sequences are far
63 from comprehensive²¹. Only a small fraction of phage sequence diversity is represented in extant
64 sequence databases, and it is heavily biased for sequences of phages with DNA genomes that infect
65 bacterial species that are routinely studied in the laboratory, such as *E. coli*, *Pseudomonas* spp., and
66 *Bacillus* spp.²²⁻²⁴. Metagenomics, the high-throughput sequencing of mixed, complex communities of
67 microbes, has enabled exploration of the diversity of phages on Earth²⁻⁵ and the human body²⁵⁻³¹,
68 including in the lower urinary tract³²⁻³⁶.

69 The Human Microbiome Project (HMP), which characterized human microbiota using 16S ribosomal RNA
70 sequencing and metagenomic whole-genome shotgun sequencing, revolutionized our understanding of
71 bacteria that inhabit the human body³⁷. However, the bladder was not included in the HMP
72 publications, which focused on the oral cavity, nasal cavity, skin, gastrointestinal tract, and vagina^{37,38}. In
73 the absence of a urinary tract infection (UTI), the bladder was thought to be sterile and so it was not
74 included in the HMP³⁹. This dogma resulted, in part, from the widespread use of standard clinical
75 microbiology urine culture protocols, which are designed to detect common fast-growing pathogens
76 with basic nutrient needs and no aversion to oxygen (especially *E. coli*). Thus, the standard protocol does
77 not detect anaerobes, slow-growing bacteria, or bacteria with complex needs. However, diverse
78 bacterial and fungal species have been detected in urine obtained directly from the bladder via
79 transurethral catheterization (herein catheterization) or suprapubic aspiration that is negative using
80 standard culture using an enhanced urine culture method called expanded quantitative urine culture
81 (EQUC) and/or DNA sequencing methods, such as *Lactobacillus*, *Corynebacterium*, *Streptococcus*,
82 *Actinomyces*, *Staphylococcus*, *Aerococcus*, *Gardnerella*, *Saccharomyces* and *Candida* spp.⁴⁰⁻⁴⁹. These and

83 other studies of the bladder microbiome and microbiota have revealed associations of bladder bacteria
84 with postoperative UTIs^{50,51}, urgency urinary incontinence (UUI)^{44,45,47}, and response to overactive
85 bladder treatment⁵². For instance, the microbiome of women with UUI had increased levels of
86 *Gardnerella* spp. and decreased *Lactobacillus* spp. relative to the microbiome of women without UUI⁴⁴.
87 Some bacteria are even associated with the lack of symptoms and an abundance of *L. iners* seems to
88 provide protection against post-instrumentation UTI^{46,50,51,53,54}. These results suggest that the bladder
89 might possess its own protective microbiota and that dysbiosis results in disorders, such as UTI and UUI
90^{55,56}. An effort to generate a genomic catalogue of bacteria isolated from the bladder that was published
91 in 2018 revealed that the genomes of bladder bacteria are quite distinct from bacteria isolated from the
92 gut, but somewhat similar to those of the vagina⁵⁷. This suggests an interlinked female urogenital
93 microbiota, i.e. strains resident of the vaginal community could be transferred to the urinary tract and
94 vice versa.

95 Although the HMP focused on characterizing the bacterial fraction of the human microbiota, sequencing
96 of some viral genomes was unavoidable because viral DNA was present in the samples and because
97 prophage DNA (a stage in the lysogenic cycle of temperate phages when the phage genome is either
98 integrated into the host genome or remains in the cytoplasm as a self-replicating plasmid) was present
99 within the bacteria⁵⁸. Subsequent to the original initiative, bacterial and viral communities within the
100 five niches studied in the HMP were extensively investigated⁵⁹, most notably the communities
101 inhabiting the gastrointestinal tract as it is a high biomass niche and can be studied using stool samples
102 as a proxy. These viral communities include both eukaryotic viruses and phages. The gut virome (the
103 viral component of the microbiome) has been the focus of numerous studies²⁵⁻³¹, each leading to the
104 same conclusion: phages are key members of the gut microbiota. A core phage community exists within
105 the gut of healthy individuals³⁰ and disruption of this core phage community (dysbiosis) has been
106 associated with certain gastrointestinal symptoms and disease, such as Crohn's disease and ulcerative
107 colitis^{27,29-31,60,61}. Within the gut, seven phage taxa were found to be associated with type 2 diabetes,
108 establishing a type 2 diabetes-specific gut phage community⁶². Other studies have characterized the
109 viromes of the body sites included in the HMP⁶³⁻⁶⁸; the data from these studies are publicly available
110 (**Table 1**). Like the gut, associations between phage communities and patient symptoms and/or disease
111 have been identified in these other body sites. For example, phages within the oral cavity have been
112 linked to periodontitis⁶⁵. In contrast to the sites of the HMP, investigation of the phage communities
113 within other niches of the human body has only recently begun. These associations within the gut and
114 oral cavity are active areas of investigation as the extent to which phages modulate the human
115 microbiota remains an open question. Furthermore, phage communities and their putative role in
116 disease and/or symptoms have yet to be determined in the other niches studied within the HMP.
117 Although the investigation of the urinary microbiota was launched independent and subsequent to the
118 HMP, considerable progress has been made in characterizing this niche. Investigations into the phages
119 of the lower urinary tract present challenges unique to this niche, but have greatly benefited from the
120 work of their predecessors exploring the viromes of the five HMP niches.

121 Studies of the bacterial communities of both the lower urinary tract in women and men have revealed a
122 diverse community of species. Furthermore, studies of the urinary microbiome have documented
123 clinical relevance of bladder bacteria; including associations with urinary symptom levels, treatment
124 response, and UTI risk^{42,44,45,51-54,69-72}. Phages are the most abundant biological entities in the human
125 body, and given observations made in other organs phages are probably vital members of the lower

126 urinary tract microbiota with the potential contribution to urinary symptoms and/or disease. Their role
127 in the lower urinary tract is largely unknown. In this Review, we describe the current knowledge of
128 phages within the urinary tract and their possible contribution to urinary tract health. We provide a brief
129 introduction to phages followed by a discussion of both culture-based and culture-independent studies
130 of the viruses of the urinary tract. We present some of the challenges in studying the urinary microbiota.
131 Finally, we consider the clinical relevance and applications of phages. Both historical and current
132 applications of phage therapy for lower urinary tract infections and other disorders are discussed.

133

134 [H1] The phage life cycle

135 Phages have three distinct, generally well-characterized life cycles for propagation and reproduction:
136 lytic, lysogenic and chronic ⁷². All phages infect their bacterial host by binding to surface receptors, a
137 process called adsorption (**Fig. 1**); these receptor binding proteins are often quite specific, leading to a
138 narrow range of hosts (strains or species) that a particular phage can infect ⁷³. Following adsorption into
139 the host cell, the phage injects its DNA or RNA genome into the host's cytoplasm. In the lytic cycle, the
140 phage genome replicates and phage proteins are synthesized ⁷⁴. For double-stranded DNA (dsDNA)
141 phages, DNA is inserted into the protein procapsid, whereas for single-stranded DNA (ssDNA) and single-
142 stranded RNA (ssRNA) phages, the capsid is formed around the nucleic acid (**Fig. 1**). The bacterium's cell
143 wall breaks ('bursts'); phage proteins called holins can form holes in the cytoplasmic membrane or
144 spanins can degrade the outer membrane ⁷⁴, and the phage progeny disperse into the surrounding
145 environment. Some phages are obligately lytic, but others, called temperate phages, can alternate
146 between the lysogenic and lytic cycles. In the lysogenic cycle, the phage genome is either integrated into
147 the host genome or remains in the cytoplasm as a self-replicating plasmid ⁷⁵ (**Fig. 1**). The phage genome
148 (now called a prophage) generally replicates in synchrony with the host chromosome, some phage
149 genes are also known expressed by the bacterium. ⁷⁵ Temperate phages, such as the model phage λ , are
150 capable of going through the lytic and lysogenic cycles. Their prophages can remain dormant for
151 generations until, often, an environmental cue, such as host starvation, change in nutrients,
152 temperature ^{75,76}, triggers entry into the lytic cycle — a process known as induction. This switch between
153 the lysogenic and lytic cycle can also be determined by the phage-produced peptide communication
154 system (the 'arbitrium' system), in which progeny phage lysogenize when this peptide is abundant
155 within the environment ⁷⁷. In addition to the lytic and lysogenic cycles, phages can reproduce by chronic
156 infection; in this process phages, for example the filamentous phage M13, are shed from the bacterial
157 cell without killing the host cell ⁷⁸. The majority of known phages can be associated with one of these
158 three life cycles, but additional modes of infection and reproduction, such as pseudolysogeny, have
159 been described ^{72,76,78}.

160 Given these multiple mechanisms of infection and persistence, unsurprisingly phages can have profound
161 effects on microbial communities (**Fig. 1**). Phages can transform a microbial community through
162 predation (lysis) ⁷⁹⁻⁸¹. Furthermore, phages can affect bacterial diversity within a community ⁸²⁻⁸⁵,
163 including adaptation in susceptible host species such as loci associated with phage resistance ^{86,87}.
164 Coevolving lytic phages can increase diversity within bacterial populations by selecting for multiple
165 modes of resistance ⁸⁴. Phages have also been shown to alter apparent competition among bacterial
166 strains ⁸⁴. Exposure to temperate phages can increase bacterial virulence (a process referred to as
167 lysogenic conversion) ^{88,89} by, for example, encoding toxins ^{90,91}. Case reports detail shiga toxin-

168 producing *Escherichia coli* strains, most commonly associated with enteric infections, found within the
169 urine of individuals with UTIs⁹²⁻⁹⁴. In this example, the shiga toxin is carried by a phage, integrated
170 within the *E. coli* genome. Thus, lysogeny can be beneficial for the bacterial host⁹⁵. Some temperate
171 phages can transfer genetic material from one cell to another (the process of transduction (Fig 2))
172 because they integrate their genome into their host's genome; this process can benefit the recipient
173 host cell. Indeed, temperate phages are well known to mediate horizontal gene transfer (HGT) and have
174 helped spread virulence and/or resistance factors through bacterial communities⁹⁶. Similarly, lytic
175 phages can also transfer bacterial DNA via transduction⁹⁷. Data exist that support both frequent and
176 infrequent phage-mediated spread of antibiotic resistance genes⁹⁸⁻¹⁰¹. Phages also can contribute to
177 HGT indirectly; for example a 2017 study identified two 'superspreader' phages, which are phages that
178 promote extensive plasmid transformation¹⁰². In this scenario, phage lysis spreads intact host plasmids,
179 enabling HGT via transformation. The two superspreader phages discovered were 50-times more
180 efficient in dispersing antibiotic resistance genes¹⁰². Given the large genetic diversity present within
181 phage communities¹⁰³, investigation of the complexities of phage-host dynamics is in very early stages
182 ^{22,31,104,105}.

183

184 [H1] Viruses of the urinary tract

185 Viruses are abundant members of the human microbiota, found throughout the body including the
186 urinary tract. These viruses include those that infect human cells (eukaryotic viruses, Box 1) as well as
187 both lytic and lysogenic phages.

188 [H2] Eukaryotic viruses

189 The urinary tract harbours a diverse eukaryotic viral community, including adenoviruses, anelloviruses,
190 papillomaviruses, and polyomaviruses^{32-36,106-116}. Adenoviruses can be detected in urine¹⁰⁶, and can
191 range from limited, localized infections in otherwise healthy individuals to severe and potentially fatal
192 infections in immunocompromised individuals¹⁰⁷. Torque teno virus (TTV), also referred to as small
193 anellovirus, has largely been studied in relation to immunodeficiency in renal transplant recipients¹⁰⁸.
194 Rani et al.³² collected midstream clean-catch urine from 22 kidney transplant recipients; whole-genome
195 sequencing was conducted for the urinary viromes of these samples and 108 different subtypes of TTV
196 were detected. The most prevalent eukaryotic viruses in urine samples are human polyomavirus 1 (BK
197 virus) and 2 (JC virus)¹⁰⁹. Both of these polyomaviruses seem to have little effect on healthy individuals,
198 but each can lead to nephropathy and hemorrhagic cystitis in immunocompromised populations^{110,111}.
199 Metagenomic sequencing of the bladder microbiome (both the bacterial and viral fractions) of 30
200 individuals enabled reconstruction of the full JC virus genome in five of the samples³³. JC virus and other
201 polyomaviruses have also been detected in other viromes from urine samples (obtained using an
202 undescribed voided urine collection method)³⁴. Human papillomaviruses (HPVs) also have been
203 detected in voided urine¹¹² and bladder tissue^{113,114}. Certain HPV genotypes have been attributed to
204 condylomata acuminatum of the bladder^{115,116}, but these high-risk genotypes associated with cervical
205 cancer are rare. In one investigation of the urinary virome, 95% of the 20 participants sampled had HPV
206 sequences detected in their urine³⁵; for eight patients, these samples were collected via intermittent
207 catheterization and the for the others, an undescribed voided urine collection method was used. In the
208 case of the latter, whether contamination from either the skin microbiota or the vaginal microbiota,

209 both of which are known to contain HPV ¹¹⁷, occurred is unknown. However, eukaryotic viruses are
210 estimated to represent just a small fraction of the urinary virome ³³⁻³⁶.

211

212 [H2] Lytic phages

213 Lytic phages have been isolated directly from urine on numerous occasions. The first phage from urine
214 was isolated by the co-discoverer of bacteriophages Félix d'Hérelle in 1917 when he observed that this
215 invisible microbe lysed the Shiga bacillus, despite not knowing exactly what a phage was at the time ¹¹⁸.
216 A century later, two studies isolated phages capable of infecting *Pseudomonas aeruginosa* from urine
217 samples ^{119,120}. Transmission electron microscopy (TEM) of the isolated phages provided information
218 about the phages' morphology, which includes a siphophage, a tailed phage with a long, thin, often
219 flexible tail structure, ¹¹⁹ and two tailless phages (Fig 3) ¹²⁰. A fourth *Pseudomonas*-infecting phage,
220 harvested from a bacterial isolate from a urine sample collected via catheterization has been discovered
221 (Johnson et al., in preparation). This phage is capable of lysing *P. aeruginosa* PAO1. Coliphages, or
222 phages that infect *E. coli*, have also been isolated from voided and catheterized urine samples. Dallas
223 and Kingsbery ¹²¹ found 100,000 colony-forming units (CFU) /ml of bacterial growth in routinely plated
224 urine samples (collected using an unknown method) and, upon closer inspection, phage plaques.
225 Furthermore, four coliphages were isolated from clinical urine samples and their morphologies were
226 determined to be siphophages using TEM ¹¹⁹. A further seven coliphages were isolated from the bladder
227 of four women with UUI (in urine collected via catheterization) ¹²². From the complete genomes of these
228 seven coliphages, six (phages Greed, Sloth, Envy, Pride, Gluttony, and Lust) resemble coliphages that
229 were isolated from cattle slurry ¹²³. This observation similarity suggests that the human urinary virome
230 might include strains found within other hosts, having a regulatory role in the urinary microbiota. The
231 seventh coliphage identified, phage Wrath, most closely resembles a lysogenic *Bacillus* phage sequence.
232 TEM images suggested that these phages had siphophage morphology (Fig 4). Testing of the host range
233 of the phage Greed showed that in addition to its ability to lyse the laboratory strains *E. coli* C and K-12,
234 it is also capable of infecting and lysing some *E. coli* strains isolated from urine samples, including the
235 uropathogen *E. coli* CFT073 ¹²⁴. Thus, within the urinary microbiota, Greed might be effective in
236 thwarting the proliferation of uropathogenic *E. coli* strains. However, the lytic phage population is only
237 one part of the phage community within the lower urinary tract.

238

239 [H2] Lysogenic phages

240 Lysogenic phage communities have been routinely under-reported in the human body, a direct result of
241 the methods used to collect and sequence viral isolates ¹²⁵. In fact, evidence suggests that lysogenic
242 phages are the most abundant phage type within the gut microbial community ¹²⁶. Similar observations
243 have been made within the bladder. Several prophages have been identified within *E. coli* isolates
244 collected from catheterized urine from the bladder ¹²⁷. Numerous prophage sequences have been
245 identified within the genomes of four *Gardnerella* strains isolated from urine specimens obtained via
246 catheterization from the bladders of adult women with UUI, although a lytic *Gardnerella*-infecting phage
247 has yet to be isolated, possibly owing to the challenges of growing this phage in the laboratory, and is a
248 currently unexplored area of phage biology ¹²⁸. Analysis of these four genomes and other publicly
249 available *Gardnerella* genomes revealed that phage infections were pervasive within the urinary

250 microbiota¹²⁸. This examination of lysogenic phages was then expanded to include 181 bacterial
251 isolates, which was representative of the phylogenetic diversity within the bladder¹²⁹. These samples
252 were collected from women with or without lower urinary tract symptoms. Over 400 phage sequences
253 were identified; the majority (86%) of these bacterial isolates harboured one or more lysogenic phages
254¹²⁹. Furthermore, many (57%) of the phages identified in this study¹²⁹ exhibited no sequence similarity
255 to any known phages, indicative of a vast unexplored phage population residing in the bladder.

256 To date, three published studies have employed a metagenomic approach to sequence the viral fraction
257 of the urinary microbiota. The first study, conducted by Santiago-Rodriguez et al.³⁵, sought to determine
258 whether the urinary virome was affected by urinary tract health status. The viral fraction (eukaryotic
259 viruses and extracellular phages) of urine collected from 10 individuals with and 10 individuals without a
260 diagnosed UTI were sequenced. For each cohort, samples were collected either via catheterization or
261 voided urine from five men and five women. As previous research has shown, clean-catch studies of
262 voided urine from women routinely contain bacterial taxa from vaginal contamination⁴¹. Furthermore,
263 the bacterial taxa of the male and female urinary microbiota are not identical^{130,131}. Only 27% of the
264 viral sequences produced in this study were homologous to known viruses, the majority (>99%) of which
265 represented phage genes³⁵, again suggesting a large unexplored phage community within the urinary
266 tract. In a second study, urine samples were collected via the voided mid-stream clean-catch method
267 from 14 men and eight women who received a kidney transplant³² and the viral fraction was isolated
268 and sequenced. Phages are present in these viromes, but this study did not mention phages and
269 sequence data is not publicly available; instead, the authors focused solely on eukaryotic viruses. The
270 subsequent study of Thannesberger et al.³⁴ also found a large phage community, but the authors
271 concluded that the phage community primarily consisted of relatives of known species, the majority
272 resembling Chlamydia microviruses, which infect *Chlamydia* spp. This study included two healthy
273 individuals and four individuals with human cytomegalovirus (CMV) infections. However, information
274 about how the urine was collected or demographics of the patients was not provided. This omission,
275 compounded by the small sample size, limits our ability to frame the study's results with respect to
276 other virome studies.

277 Viral diversity within the urinary tract also has been studied by sequencing the entire urinary microbiota.
278 In 2018, Moustafa et al.³⁶ published a study in which metagenomic sequencing was performed for urine
279 samples from 49 individuals with suspected UTIs (collected via the clean-catch method). As this study
280 did not select for the viral fraction, most of the sequenced data corresponded to bacterial genetic
281 material. Nevertheless, viral — primarily phage — sequences were detected³⁶. Similar to the study
282 conducted by Santiago-Rodriguez and colleagues³⁵, this study examined samples from individuals with
283 UTIs and detected sequences homologous to those from phages that infect bacteria commonly found
284 within the urinary tract and associated with UTIs, including those of the genera *Escherichia*,
285 *Enterococcus*, *Lactobacillus* and *Pseudomonas*³⁶. Abundant bacterial species harbouring prophages
286 would result in an abundance of phages; thus, one would expect to identify phages infectious of UTI-
287 associated bacterial taxa. In a similar approach, sequencing was undertaken of urine collected using
288 catheterization from 10 asymptomatic women and 20 women with overactive bladder³³. Partial and
289 complete viral genomes were reconstructed in 12 of the 30 samples sequenced, including the complete
290 genomes of novel phage strains³³. Partial and complete phage genomes also exhibited sequence
291 homology to previously characterized lytic or lysogenic phages that infect *Gardnerella*, *Lactobacillus* and
292 *Streptococcus* species. These bacterial species are dominant members of the urinary microbiota of

293 healthy women as well as women with overactive bladder symptoms⁴⁴; thus, one would expect to
294 readily identify phage infection of these taxa. In sequencing both the bacterial and viral members of the
295 microbiota, associations between phages and their hosts can be inferred. As both of these studies have
296 highlighted, phages that infect dominant bacterial taxa within the urinary microbiota can be identified
297^{33,36}. One can, therefore, postulate that novel phage sequences (phages that do not share sequence
298 homology with any known, sequenced phage or prophage sequence) are infectious of a bacterial taxa
299 within that same individual's urinary microbiota, whereas more prolific phage species, which are
300 representative of more deeply sequenced viral sequences, are probably infectious of dominant bacterial
301 taxa.

302

303 Culture-based and culture-independent studies have revealed a large, active phage population within
304 the lower urinary tract. The diversity present has yet to be comprehensively catalogued, but the
305 consistent finding that the majority of phage sequences detected do not resemble known, sequenced
306 phages suggests a novel community within the urinary tract. In parallel to continued efforts to catalogue
307 this community, future studies should conduct comparisons of the urinary virome to the viromes of
308 other areas of the human body. In particular, comparisons to the gut virome are warranted given
309 emerging evidence that viruses of the gut have been found elsewhere in the body¹².

310

311 **[H1] Challenges of studying bladder phages**

312 The bladder has orders of magnitude less microbial biomass than the gastrointestinal tract, oral cavity or
313 vagina^{43,132,133}. DNA concentrations are often low, a challenge faced by both those studying the bacterial
314 and those investigating the viral constituents of the bladder¹³⁴. Thus, two of the metagenomic studies of
315 the urinary virome employed amplification before sequencing^{34,35}. This technique is efficient for
316 increasing viral genomic material, but these amplification methods have well documented biases¹⁷⁻¹⁹.
317 For instance, multiple displacement amplification (MDA) can increase the DNA template concentration
318 for sequencing, but small circular viral genomes are over-amplified. Both MDA and single-primer
319 amplification (SISPA) methods have also been found to under-amplify viral genomes with GC contents at
320 the extremes¹³⁵. Perhaps of greater concern are the methods by which urine is collected and the
321 anatomical microbiota that the collected urine represents. The method of urine collection is a frequently
322 debated and investigated topic in the field^{55,130,134}, owing to the need to balance the invasiveness of
323 procedures during collection and the purity of the sample obtained. This debate is not unique to the
324 bladder, urinary tract, or urine; biopsies and stool samples give quantitatively and qualitatively different
325 results for the gut¹³⁶ and methods of sampling the gut microbiota are still being refined^{137,138}. Studies of
326 voided urine have routinely observed vaginal contamination of clean-catch samples^{36,48}. Virome studies
327 by Santiago-Rodriguez and colleagues³⁵, Rani and colleagues³², and Moustafa and colleagues³⁶
328 investigated voided urine samples. Thus, whether the viruses detected resided in the bladder and/or in
329 the urethra, vagina, or skin remains unknown. Another study, in which the bacterial communities in the
330 bladder were obtained via paired samples by catheterization and suprapubic aspiration from women
331 were compared showed that both methods did not isolate microbial communities that resembled the
332 skin or vaginal microbiomes and successfully avoided vulvovaginal contaminants⁴¹. Moreover, the
333 communities identified by the two methods were similar⁴¹. Although a similar study has not been
334 conducted comparing the virome of urines collected via catheterization and suprapubic aspiration, one

335 would assume that both represent the same community of the bladder microbiome. Thus, catheterized
336 urine samples have a lower probability of contaminants than voided urine samples¹²⁷. Study of less-
337 invasive methods for collection is an ongoing pursuit¹³⁹. In a 2019 study, the use of the non-invasive
338 Peezy midstream device (Forte Medical) was tested¹⁴⁰. The results showed that voided urine collected
339 by the Peezy was less prone to contamination, having a bacterial abundance distinct from the
340 periurethra¹⁴⁰. This device is a promising step towards a sampling method that is less-invasive than
341 catheterization, which is the current best method for sampling the bladder's microbiota.

342

343 [H1] Phages and urinary tract health

344 The associations between phage communities, bacterial populations, and the human host are not yet
345 fully understood. Some evidence suggests that phages might contribute to human health¹⁴¹, in
346 particular the gut in which they have been suggest to have roles including maintaining a stable bacterial
347 community within the gut and providing an innate defense to pathogenic species^{27,29-31,60}. These studies
348 of the gut will probably inform future studies of the urinary tract and other niches of the human body,
349 providing a model for conducting such studies and expanding our knowledge of phage genetic diversity
350 within the human body. Paralleling those discoveries of associations between phage communities of the
351 gut and GI symptoms, associations have also been made within the bladder: variation was observed in
352 the abundance of lysogenic phages in bacteria isolated from asymptomatic individuals and those with
353 overactive bladder, in which the microbiota of women with OAB included more *Lactobacillus* phages
354 than the microbiota of women without OAB¹²⁹. However, notably, the *Lactobacillus* species between
355 these two cohorts varied which might be contributing to the observed difference and, therefore,
356 warrants further investigation¹²⁹. Variation, determined via the β diversity statistic and principle
357 component analysis, was not found in the extracellular phage populations of individuals with or without
358 UTI symptoms³⁵. Although the bacterial communities differ between individuals with and without UTIs,
359 the virome does not seem to change in response, suggesting that UTI symptoms are not associated with
360 changes in the virome³⁵. Further investigation of this observation is needed as the sample size was
361 limited. However, importantly, understanding of the diversity of phages within the urinary tract has only
362 just begun, in contrast to the gut phage communities. Cataloguing the phage community in both
363 asymptomatic and symptomatic individuals is a critical first step in understanding if and how phages
364 contribute to urinary tract health. All of the aforementioned studies discovered a large collection of
365 novel viral sequences indicative of a unique genetic diversity present within the urinary tract. Further
366 investigation of phage–bacteria dynamics in the bladder and urinary tract could reveal indicators for
367 early detection of symptoms.

368 Phages could also offer a defense to the human host against pathogenic bacteria. Studies of the gut
369 communities have revealed unexpected ways in which phages interact with human cells, organs, and
370 immune system^{12,29}. The prevalence of phages on the mucosal surfaces of the gut might confer a direct
371 benefit to the human host by protecting the epithelium from bacteria¹⁴². This study's findings suggest
372 that phages and mucosal surfaces have coevolved such that phages bind to mucosal glycoproteins; this
373 phage mucosal layer reduces adherence of bacterial pathogens¹⁴². Changes in the mucosal phage
374 population have been associated with ulcerative colitis⁶¹. Evidence also suggests that phages have
375 increased virulence to bacteria when human cells are present¹⁴³. In this study, phages were found to
376 reduce *Clostridium difficile* numbers more efficiently in the presence of human cells¹⁴³. Furthermore,

377 phages can interact directly with human cells. Studies have found that the wild type T4 phage and its
378 substrain HAP1 can bind to cancer cell membranes and inhibit or attenuate melanoma tumour growth
379 ¹⁴⁴. Although phages cannot infect eukaryotic cells, there are several means in which they can enter
380 eukaryotic cells. A phage could be a passenger, as a cell of an invasive bacterial species that harbours a
381 phage could enter a eukaryotic cell ^{145,146}. Alternatively, eukaryotic cells can take up free phages by
382 endocytosis ^{145,147}. A *Staphylococcus*-infecting phage was capable of infecting bovine mammary epithelial
383 cells and clearing intracellular *S. aureus* ¹⁴⁷. One study showed that phages are capable of penetrating
384 epithelial cell layers via endocytosis with an estimated 31 billion phage particles passing through these
385 layers of the gut into the body daily ¹⁵. Given this observation, in a study of the urinary virome,
386 comparison with the gut virome should be considered in order to identify if urinary phages originated
387 from the gut. Within the human body, phages can modulate immune responses ¹⁴⁸. For instance, T4
388 phages mediated inhibition of T-cell proliferation via the CD3 complex ¹⁴⁹ in vitro and stimulated of
389 humoral responses in mice in vitro and in vivo ¹⁵⁰. Phage-mediated immunoregulation holds promise,
390 such as for attenuating the expression of proinflammatory cytokines during UTIs ¹⁵¹. The mechanisms by
391 which phages interact with the immune system remains an active area of investigation ¹⁴⁸.

392 Appreciation is growing of the therapeutic potential of modulating the human microbiome. Induction
393 and release of temperate phages can lyse sensitive competitor strains or lysogenize other cells ^{152,153}. For
394 instance, the gut bacterium *Enterococcus faecalis*, which has also been associated with UTIs, uses its
395 prophages to colonize when competing strains are present ¹⁵². Alternatively, an individual's bacterial
396 infection can be treated with obligately lytic phages, known as phage therapy. In the face of the
397 increasing threat of antibiotic-resistant bacterial strains, phage therapy has regained interest ¹⁵⁴. Phage
398 therapy was a promising area of UTI treatment in the early 20th century. For instance, in a 1928 report,
399 phages isolated from sewage were 90% efficient in lysing *E. coli* and *P. aeruginosa* strains isolated from
400 catheterized urine samples ¹⁵⁵. The USA and Western Europe abandoned phage therapy when
401 antibiotics became commercially available (amongst other reasons) ¹⁵⁶; this area of research and
402 treatment continued in Eastern European countries. Phage therapy is a publicly available treatment for
403 individuals with UTIs in Russia, Poland, and the Democratic Republic of Georgia. In one study ¹⁵⁷, 41 *E.*
404 *coli* and 9 *Klebsiella pneumoniae* strains isolated from individuals with UTI were challenged with phages
405 from collections from the Democratic Republic of Georgia. Only one *E. coli* isolate was resistant to the
406 individual phages and phage cocktails tested, and one phage was capable of lysing all *K. pneumoniae*
407 strains. Similar efficiencies have been observed for other bacterial species that cause UTI symptoms. A
408 single patient, for whom gentamicin, ceftazidime, ciprofloxacin and meropenem were unable to clear
409 the root cause of the UTI (*P. aeruginosa*) for > 2 years, was successfully treated with a combination of 6
410 phages from the Eliava Institute in Tbilisi collection ¹⁵⁸. Phage treatment was administered via
411 catheterization every 12 h for 10 days, and meropenem was administered starting on day 6 through 30
412 and urine samples were negative 1 year later ¹⁵⁸. A 2-year long clinical trial of bacteriophages for treating
413 UTI in patients undergoing transurethral resection of the prostate (NCT03140085) at the Tzulukidze
414 National Center of Urology (Tbilisi, Georgia) concluded in 2017. Participants were treated with either an
415 antibiotic, a phage (bacteriophage Pyo and adapted substrains of Pyo), or a placebo, the latter two were
416 administered via catheterization for 7 days ^{159,160}. The study was unable to draw any statistically reliable
417 conclusions, but it did conclude that phage treatment of UTIs might be effective and safe ¹⁶¹. Phages
418 have also been explored for their potential use in pretreating long-term catheters with phages to
419 minimize bacterial biofilm development and catheter blockage, which can cause catheter-associated
420 UTIs (CAUTIs) ¹⁶². Catheters have been pretreated with phages that infect *P. aeruginosa* ¹⁶³, *Proteus*

421 *mirabilis*¹⁶⁴, and *E. coli*¹⁶⁵ with varied success. The pretreatment of catheters with two phages were
422 found to considerably reduce *P. mirabilis* biofilms for up to 168 hours post treatment¹⁶⁴.

423 Increased understanding of phage, microbiota, and human host interactions is imperative for the
424 feasibility of phage therapy of urinary tract symptoms and infections. Phage therapy has the potential to
425 combat antibiotic-resistant bacterial infections, and anecdotal evidence of its success certainly warrants
426 further investigation¹⁶⁶. Phage therapy has already proven effective in the treatment of bacterial
427 infections in other areas of the human body. In the highly publicized case of a life-threatening
428 *Acinetobacter baumannii* infection, all modern antibiotics were found to be ineffective and over a
429 hundred phages were tested before the few phages capable of saving the patient's life were found¹⁶⁷.
430 Phage–drug cocktails are promising as well; for instance, such a cocktail was used to clear a vascular
431 graft *P. aeruginosa* infection¹⁶⁸. The *Pseudomonas* phage OMKO1, used in combination with
432 ceftazidime, was able to completely clear the infection as bacteria resistant to the phage were more
433 sensitive to ceftazidime and vice versa¹⁶⁸. All phages infect their bacterial host by binding to surface
434 receptors, a process called adsorption (**Fig. 1**); these receptor binding proteins are often quite specific,
435 leading to a narrow range of hosts (strains or species) that a particular phage can infect⁷³. This
436 specificity is in direct contrast to broad-spectrum antibiotics and has the benefit of targeting the
437 pathogen with no effect to commensal bacteria. However, this specificity means that phage
438 therapeutics will probably have to be developed on a patient-by-patient basis. In the aforementioned *A.*
439 *baumannii* case, nearly 100 *A. baumannii* phages (selected from a larger collection of phages known to
440 infect multi-drug resistant *A. baumannii* strains) were screened against clinical isolates from the patient;
441 the vast majority of the phages tested had no effect against the clinical isolates¹⁶⁷. A phage therapy
442 effective for a larger patient population ($n>1$) will, therefore, probably be a cocktail of phages, including
443 phages capable of infecting different strains. Phage cocktails also provide the benefit of outpacing
444 pathogen evolution, a strategy similar to that employed for the vascular graft infection¹⁶⁸.

445

446 Given the rise of antibiotic-resistance, phage therapy is a promising replacement or augmentation to
447 antibiotic treatment for infections throughout the body, including infections of the urinary tract. Several
448 clinical trials are or have been conducted, including the recent trial for UTIs^{160,161}, the results of which
449 provide critical data for moving forward

450

451 **[H1] Conclusions**

452 Whole-genome sequencing and new enhanced culture methods have been of great benefit to the study
453 of the microorganisms within the bladder and the rest of the lower urinary tract, but considerable work
454 remains to be done. An ongoing debate is occurring surrounding the potential presence of vulvovaginal
455 and/or skin bacterial contaminants in urine samples of the urinary microbiota, and the same
456 conversation is relevant to the new study of the lower urinary tract virome. Most of the studies
457 discussed herein, with a few exceptions^{35,122,128,129}, have used voided urine for isolation of lytic phages
458 or sequencing of the urinary virome. To the best of our knowledge, the phage populations of adjacent
459 anatomical locations have yet to be investigated so the rate of incidence of viral contamination is
460 unknown. As we have just begun to explore the phage communities within the urinary tract, such
461 considerations must be kept in mind. More samples of the urinary virome must be sequenced to

462 determine if, like in the gut³⁰, a core phage community exists within the bladder, the urethra, the
463 periurethral niche and adjacent urogenital niches. Only through such efforts can we fully ascertain what
464 a healthy and an unhealthy phage community consists of. Whether a shift from the lysogenic life cycle to
465 the lytic cycle is a cause or consequence of bacterial community dysbiosis or urinary symptoms in
466 currently unknown. Studies such as those by Moustafa et al.³⁶ and Garretto et al.³³ will be particularly
467 powerful in capturing the dynamics between phages and their hosts, increasing understanding of their
468 interactions. These studies should become increasingly attainable as the costs of sequencing continue to
469 decline

470 Knowledge of the phage communities within the lower urinary tract and their role in urinary tract health
471 is a vital first step in the development of new strategies to treat urinary symptoms and infections.
472 However, critical to effective and reliable phage therapy strategies is the understanding of the extant
473 beneficial microbiota. Phage therapies should ideally cause minimal to no disturbance of this
474 community. In contrast to broad-spectrum antibiotics, phages can be directed very narrowly toward a
475 specific pathogen within the community. Given the observed novelty of many of the phages sequenced
476 from urine and from the bladder^{35,129}, perhaps the genomes of the modifiers of urinary tract health
477 have already been sequenced. Our understanding of the phage population of the urinary tract is in its
478 infancy and future studies will highlight new areas of investigation.

479

480

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486

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849 **Competing interests**

850 The authors declare no competing interests.

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856 **Author contributions**

857 A.G. and T.M.-E. researched data for the article, all authors made substantial contribution to discussion
858 of content, wrote the article and reviewed and edited the manuscript before submission.

859

860 **Tables**

861

Factor	Airway	Gastrointestinal tract	Oral cavity	Skin	Urogenital tract and/or vagina
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Total number of metagenomic viral contigs	268	29,107	48,904	2,461	422
Unique viral clusters*	70	3,645	6,963	210	68
Total number of genomes	107	510	886	491	101

862 **Table 1.** Current number of viral sequences from virome studies of HMP anatomical sites.

863 *Clusters correspond to genetically distinct groups. (Data retrieved from the Integrated Microbial
864 Genomes/ Virus (IMG/VR) system⁹⁰.)

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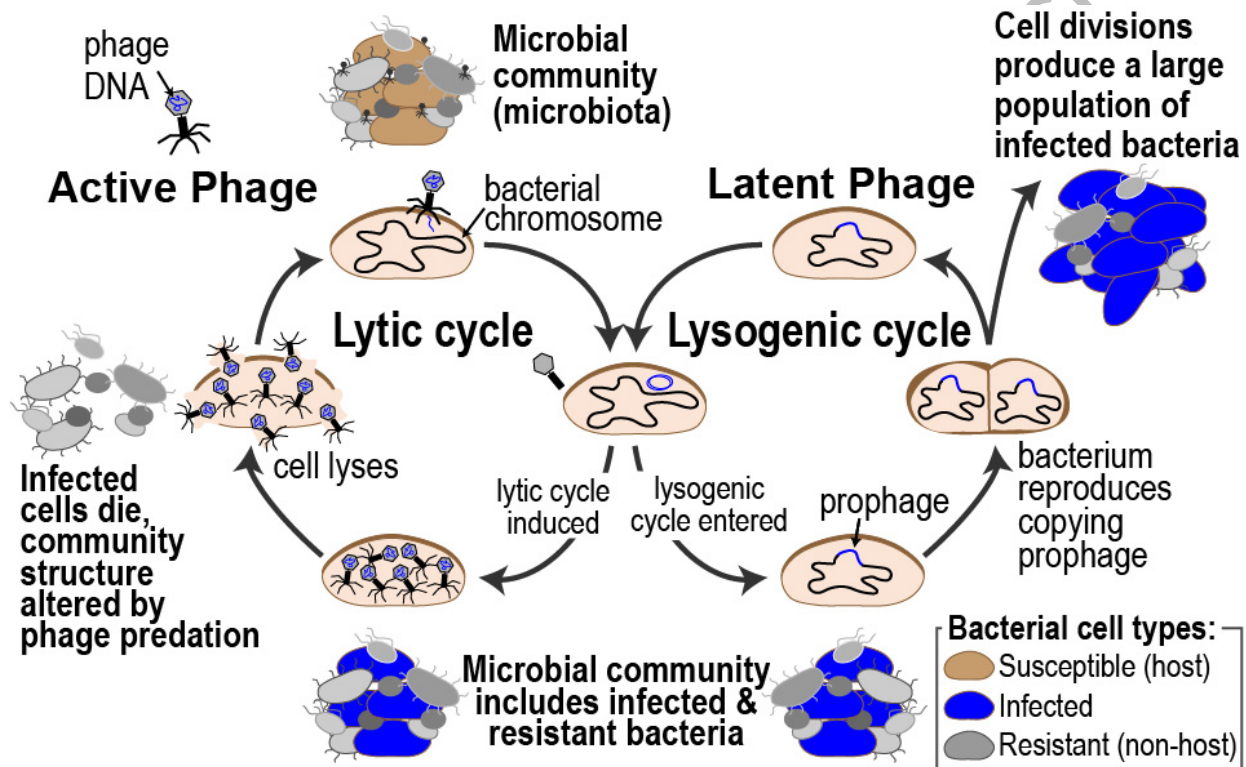
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869 **Figures**

870 **Fig. 1.** Lytic and lysogenic cycles of phages and their impact on microbiota. Active phages infect a
 871 susceptible bacterial host, binding to surface receptors, and inject their DNA entering into the lytic or
 872 lysogenic life cycle. In the lytic cycle (left), the phage genome replicates, producing mature virions. The
 873 phage virions then burst the host cell and diffuse through the surrounding environment. Thus, the
 874 susceptible bacteria within the microbiota are killed, leaving resistant (or non-host) bacteria. Within the
 875 lysogenic cycle (right), the phage genome either integrates into the bacterial genome (prophage) or
 876 persists as an extrachromosomal plasmid. As the infected bacterial cell reproduces, the phage genome is
 877 also replicated. Cell divisions produce a population of bacterial cells harbouring the phage genomic
 878 material. Environmental factors can induce a lysogenic (or latent) phage to enter the lytic cycle.



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 880 [Reproduced by *Nature Urology Reviews*]

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883 **Fig. 2** The process of transduction. In transduction, bacterial DNA is transferred from one cell to another
 884 by phages. Adapted with permission from Sirha et al. *Nature Reviews Urology* 15, 750–776 (2018) 169.

885 [Not shown here]

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 887

888 **Fig. 3** Phage tail morphologies. Siphoviridae families have a baseplate at the distal end of the tail to
889 which receptor-binding proteins (RBPs), such as tail fibres and tail spikes, are attached, tailless phages
890 are just a capsid. Adapted with permission from Nobrega et al. Nature Reviews Microbiology 16, 760–
891 773 (2018)¹⁷⁰.

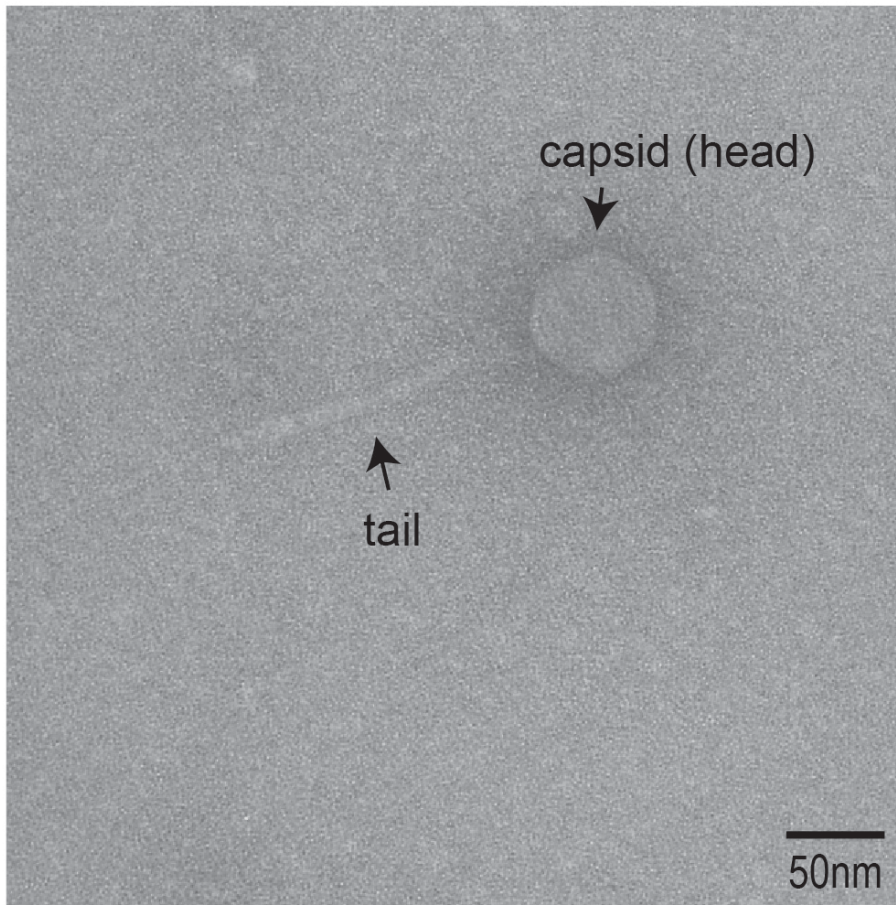
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896 **Fig. 4.** Bacteriophage Greed, isolated from catheterized urine microbiome sample. The phage's capsid
897 (head) containing the phage genomic material can be seen, as well as the phage's tail structure. Tail
898 fibers are not visible. The scale bar represents 50 nm. Samples were positively stained with 2% (wt/vol)
899 uranyl acetate and observed at 80 kV using a Hitachi H-600 transmission electron microscope (TEM).



900