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1 **Current challenges and future opportunities of phage therapy**

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3

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8

9 **One sentence summary:**

10 The remarkable potential of phage therapy for the control of antibiotic resistant infections
11 within the One Health approach, the challenges currently faced and the potential solutions
12 in development.

13

14

15 **ABSTRACT**

16 Antibiotic resistance is a major public health challenge worldwide, whose implications
17 for global health might be devastating if novel antibacterial strategies are not quickly
18 developed. As natural predators of bacteria, (bacterio)phages may play an essential role
19 in escaping such a dreadful future. The rising problem of antibiotic resistance has
20 revived the interest in phage therapy and important developments have been achieved
21 over the last years. But where do we stand today and what can we expect from phage
22 therapy in the future? This is the question we set to answer in this review. Here, we
23 scour the outcomes of human phage therapy clinical trials and case reports and address
24 the major barriers that stand in the way of using phages in clinical settings. We
25 particularly address the potential of phage resistance to hinder phage therapy and
26 discuss future avenues to explore the full capacity of phage therapy.

27

28

29 **Keywords:** clinical trials, phage resistance, phage engineering, phage cocktails,
30 regulatory framework, One-Health

31 INTRODUCTION

32 The discovery of antibiotics in 1928 and their introduction in clinical practice has
33 revolutionized the field of medicine. Since then and for decades, antibiotics were used to
34 treat a wide range of severe infections, saving millions of lives (Davies and Davies 2010).
35 However, nobody predicted what was about to come a few decades later. As a
36 consequence of antibiotic overuse and misuse, bacteria managed to develop multiple
37 antibiotic resistance mechanisms, and the golden age of antibiotics has come to an end
38 (Davies and Davies 2010; Malik and Bhattacharyya 2019). We are currently facing a
39 post-antibiotic era, in which common infections or minor injuries can become fatal (WHO
40 2014). Recent reports state that more than 2.8 million antibiotic-resistant infections occur
41 each year in the United States and that more than 35,000 people die as a result (Centers
42 for Disease Control). In Europe, approximately 33,000 people die every year from
43 antibiotic-resistant infections (Cassini *et al.* 2019). If no action is taken, the World Health
44 Organization estimates that drug-resistant infections could kill about 10 million people
45 per year by 2050. The search and development of new and effective antibacterial
46 compounds is urgently required to avoid such a threatening future, and (bacterio)phages
47 might play a major role in tackling this global crisis.

48 Phages are bacterial viruses and the most abundant entities on Earth (Clokic *et al.* 2011;
49 Fernández *et al.* 2019). While the use of phages in human therapy begun soon after their
50 discovery by Frederick Twort and Félix d'Hérelle over a century ago, their application in
51 clinical practice in Western countries was quickly overshadowed by the introduction of
52 antibiotics (Chanishvili 2012; Gordillo Altamirano and Barr 2019). In places such as
53 Georgia and Poland, phage therapy remained active until today, mostly via two major
54 phage therapy centres: the Eliava Institute of Bacteriophages, Microbiology and Virology
55 (Tbilisi, Georgia) and the Ludwik Hirsfeld Institute of Immunology and Experimental

56 Therapy (Wroclaw, Poland) (Rohde, Wittmann and Kutter 2018). Many patients with
57 antibiotic-resistant infections are traveling from multiple places in the world to these
58 centres to receive individualized phage treatments as a last hope (Rohde, Wittmann and
59 Kutter 2018). Despite all the success cases of patients treated with phages documented to
60 date, the introduction of phage therapy in Western countries still faces major obstacles,
61 especially regulatory issues (Fauconnier 2019). Now, efforts to make phage therapy
62 widely available are ongoing and a number of clinical trials are being conducted in Europe
63 and in the United States (Sybesma *et al.* 2018; Fauconnier 2019). In this review, we will
64 first discuss the current state of phage therapy in the Western world and then address the
65 major challenges faced by phage therapy and the future opportunities in this field.

66

67 **THE CURRENT STATUS OF PHAGE THERAPY**

68 The clinical use of phages to treat a wide range of infections begun in the early 1920s.
69 However, inconsistent results reported about phage trials during the 1930s and a lack of
70 controls and inappropriate characterization, production and purification of phage
71 preparations raised important concerns about the safety and efficacy of this therapy
72 (Gordillo Altamirano and Barr 2019). As such, phage therapy remained active only in a
73 few countries of Eastern Europe, where studies have provided substantial evidence of the
74 efficacy of phages to treat certain infections with no adverse effects reported
75 (Sulakvelidze, Alavidze and Morris 2001; McCallin and Brüssow 2017). Still, the lack of
76 confirmation in line with evidence-based medicine, i.e. clinical trials, fuels the reluctance
77 of regulatory agencies and clinicians from Western countries on the use of phage therapy
78 (Sybesma *et al.* 2018). To establish phage therapy as a feasible alternative to antibiotics,
79 clear efficacy data from randomized controlled clinical trials is required (McCallin *et al.*

80 2019). To tackle this situation, an increasing number of clinical trials have been carried
81 out over the last years but only a few are currently completed (Furfaro, Payne and Chang
82 2018; Rohde, Wittmann and Kutter 2018; Sybesma *et al.* 2018).

83 In 2009, Wright *et al.* reported a randomized, double-blind, placebo-controlled phase I/II
84 clinical trial approved by both UK Medicines and Healthcare products Regulatory
85 Agency (MHRA) and the Central Office for Research Ethics Committees (COREC)
86 (Wright *et al.* 2009). This trial was carried out on 24 patients with chronic otitis to assess
87 the efficacy and safety of a phage preparation composed of six phages for the treatment
88 of otitis caused by antibiotic-resistant *Pseudomonas aeruginosa*. By the end of the trial
89 (day 42), all the clinical indicators (e.g. inflammation, ulceration, discharge type and
90 quantity, and odour) improved in patients treated with phages, but only three of the 12
91 patients receiving phage treatment were apparently cured. Importantly, no serious adverse
92 effects were reported (Wright *et al.* 2009). Also in 2009, Rhoads and colleagues reported
93 another randomized, double-blind controlled study that addressed the safety (and not
94 efficacy) of a phage cocktail targeting *P. aeruginosa*, *Staphylococcus aureus* and
95 *Escherichia coli* for the treatment of venous leg ulcers (VLU) (Rhoads *et al.* 2009). This
96 first phage therapy trial in the United States involved 42 patients with VLU. Patients were
97 topically treated with either phage cocktail or saline solution (control) for 12 weeks with
98 a follow-up period of up to 24 weeks. No adverse effects were associated with phage
99 treatment, but no significant differences were found on the rate and frequency of healing
100 between phage-treated and control groups. This is not surprising as the phages were not
101 tested for infectivity on the bacteria causing the VLU. According to the authors, the
102 efficacy of the phage preparation should be evaluated in a phase II efficacy trial with a
103 larger sample and with wounds infected with bacteria susceptible to the phage cocktail
104 (Rhoads *et al.* 2009).

105 The largest clinical trial on phage therapy conducted in Europe and performed under both
106 Good Manufacturing Practices (GMP) and Good Clinical Practices (GCP) was the
107 PhagoBurn trial, launched in 2013. In this multicentre randomized controlled phase I/II
108 clinical trial , 27 patients suffering from burn wound infections were recruited from
109 hospitals located in France and Belgium to be randomly treated with phage therapy (a
110 cocktail of 12 lytic phages) or standard care (1% sulfadiazine silver emulsion cream) to
111 compare the efficacy and tolerability of both treatments in patients with wounds infected
112 by *P. aeruginosa* (Jault *et al.* 2019). Both treatments were topically administered for
113 seven days with a 14 days follow-up period. Overall, the phage cocktail was able to
114 decrease bacterial burden in burn wounds but the progress was slower than in the control
115 group (standard treatment). On the positive side, no adverse effects were found in the
116 phage-treated group. The limited efficacy of the phage cocktail was reported to be caused
117 by a significantly drop of the phage titre after GMP manufacturing, leading the
118 participants to receive a much lower concentration of phages than initially estimated.
119 More importantly, the susceptibility of wound bacteria to the phage cocktail was not
120 assessed prior to treatment. In those patients in which phage treatment failed, bacteria
121 were later found to be resistant to low phage doses (Jault *et al.* 2019).

122 Nestlé (Switzerland) also performed a phase I/II trial in collaboration with the Dhaka
123 Hospital of the International Centre for Diarrheal Disease Research, Bangladesh (Sarker
124 *et al.* 2016). This randomized double-blind, placebo-controlled trial was conducted
125 between 2009 and 2011 to assess the safety and efficacy of oral administration of a T4-
126 like phage cocktail or a placebo, in children hospitalized with acute bacterial diarrhoea.
127 Although the oral coliphages could reach the intestine, no phage replication was observed,
128 and the treatment had no beneficial effects. At the time, the authors attributed the failure
129 to improve diarrheal outcome to the low host range coverage of the phage cocktail (i.e.

130 some strains were not infected) and also the need of higher oral phage doses (Sarker *et al.*
131 2016). Indeed, oral application of phages without any protection (e.g. encapsulation of
132 the phages or neutralization of the stomach acid) prior to administration reduces the phage
133 numbers reaching the intestine to levels that might be insufficient for a visible therapeutic
134 effect. Later, it was also found that *E. coli* was not the main cause of acute bacterial
135 diarrhoea, and therefore even an efficient phage treatment of *E. coli* would not result in
136 improved diarrheal outcome (Satter *et al.* 2017; Nelson *et al.* 2018). This Nestlé trial and
137 the clinical trial developed by Rhoads *et al.* highlight the importance of identifying the
138 etiologic agent(s) causing infection and of checking for phage susceptibility prior to
139 treatment. Therefore, phage therapy clinical trials must be carefully designed to avoid
140 potential problems that might impair the outcome of the treatment. Recently, Ooi *et al.*
141 reported a clinical trial aiming to assess the safety, tolerability and preliminary efficacy
142 of a phage cocktail composed of three lytic phages, applied intranasally in patients with
143 recalcitrant chronic rhinosinusitis (CRS) caused by *S. aureus* (Ooi *et al.* 2019). In this
144 open label, phase I clinical trial, only patients carrying a clinical isolate sensitive to the
145 phage cocktail were considered. Overall, the twice-daily intranasal irrigation of phages
146 was safe and well tolerated by the nine patients through the 14 days treatment, with no
147 serious adverse events reported. While the preliminary efficacy observations seem
148 promising (two of the nine patients had eradication of infection), the authors highlighted
149 the need for a randomized clinical trial to determine the optimal dose regimen and
150 demonstrate the efficacy of the phage cocktail (Ooi *et al.* 2019). The high safety of phage
151 therapy has already been reported in multiple patients from the phage therapy unit in
152 Poland (Międzybrodzki *et al.* 2012; Rogóż *et al.* 2019).

153 While most clinical trials have failed to provide unequivocal evidence of the efficacy of
154 phage therapy, the number of case studies in which phage therapy was successfully used

155 to treat life-threatening infections is increasing (Table 1) (Sybesma *et al.* 2018; McCallin
156 *et al.* 2019). Some of these successful cases have reached the media (Dedrick *et al.* 2019;
157 Strathdee, Patterson and Barker 2019), fostering the interest of the global community in
158 this therapy. One of these newsworthy cases concerned a 68-year-old man who suffered
159 from necrotizing pancreatitis complicated by an *Acinetobacter baumannii* multidrug-
160 resistant infection (Schooley *et al.* 2017). Despite multiple rounds of antibiotic treatments,
161 the patient condition rapidly deteriorated over time. Therefore, the *A. baumannii* strain
162 isolated from the patient was used to screen for phages in two different laboratories,
163 which made possible to compose phage cocktails tailored for the patient. Phage
164 administration (via catheters into the abdominal cavity and also intravenously) rapidly
165 reverted the clinical condition of the patient by clearing the infection (Schooley *et al.*
166 2017). Phage therapy documentaries have also been broadcasted on television in many
167 countries (Djebara *et al.* 2019). As a consequence, the Queen Astrid military hospital in
168 Brussels, Belgium, has experienced a huge increase in external phage therapy requests
169 since 2017 (Djebara *et al.* 2019). The majority of these requests were initiated by the
170 patients themselves and came mostly from the Netherlands followed by Belgium and
171 France. Among the 260 phage therapy requests received by the hospital between 2013
172 and 2018, only 15 patients, who were infected with bacterial pathogens susceptible to the
173 available phages, received treatment but these data were not yet reported (Djebara *et al.*
174 2019).

175 The rising interest in phage therapy by patients and physicians and the consequent
176 increase of requests for phages from all over the world highlights a growing need for the
177 establishment of phage banks with well characterized phages that could facilitate access
178 by the international community. Some phage banks have already been established, such
179 as the Félix d'Hérelle Reference Center for Bacterial Viruses at the University of Laval

180 (Québec, Canada), the Leibniz Institute DSMZ German Collection of Microorganisms
181 and Cell Cultures (Braunschweig, Germany), the Bacteriophage Bank of Korea (Yongin,
182 South Korea), the American Type Culture Collection (ATCC) Bacteriophage Collection
183 (Virginia, USA), the National Collection of Types Cultures (NCTC) Bacteriophage
184 Collection (Salisbury, UK) (McCallin *et al.* 2019; Sacher 2019), and the Fagenbank
185 (Delft, Netherlands). It is important that phage researchers feed these global phage banks
186 to have a larger coverage of (pathogenic) bacterial species.

187

188 **CURRENT CHALLENGES IN PHAGE THERAPY**

189 **Quality and safety requirements**

190 The success of phage therapy is highly dependent on the safety of phage preparations,
191 which raises manufacturing and formulation challenges (Fig. 1A). For broad medical
192 applications, phages would need to be produced in large scale under Good Manufacturing
193 Practices (GMP) approved by regulatory agencies (Regulski, Champion-Arnaud and
194 Gabard 2018). Although the production of phages for therapy must comply with the strict
195 regulations that are usually applied for pharmaceutical products to ensure the high quality
196 standards appropriate for their intended use, no clear guidelines were yet developed
197 specifically for phage manufacturing (Mutti and Corsini 2019). To address this issue, a
198 group of phage researchers have set some quality and safety requirements for sustainable
199 phage therapy products (Pirnay *et al.* 2015). One of the requirements is to avoid phages
200 encoding for lysogeny, virulence factors or antibiotic resistance. However, this might
201 limit the use of phage therapy in some fastidious bacteria for which no strictly virulent
202 phages have been found so far, such as *Clostridium difficile* (Hargreaves and Clokie

203 2014). The presence of impurities such as endotoxins in phage preparations should also
204 be avoided or be below a threshold (Pirnay *et al.* 2015). Several purification methods
205 have been developed and optimized to remove these toxic elements from phage
206 preparations (Hietala *et al.* 2019), but none has reached optimal results so far.

207 It is important to note that as phages are biological entities, the development of robust
208 manufacturing processes in compliance with GMP is also essential to avoid variability
209 among phage preparations (García *et al.* 2019; Mutti and Corsini 2019). Another
210 important aspect is the quality control of phage stock preparations. This should be
211 regularly assessed by checking for their stability (shelf life), sterility and cytotoxicity, as
212 well as by performing periodic pH measurements (Merabishvili *et al.* 2009; Pirnay *et al.*
213 2015). Although recent progress in phage manufacturing has revitalized phage therapy in
214 Western countries, there is still a long way to go before a general approval is reached for
215 the use of phage therapy (Regulski, Champion-Arnaud and Gabard 2018).

216

217 **Stability of phage preparations**

218 The stability of phage preparations is a key requirement for successful treatment and also
219 for the regulation of phages as pharmaceuticals. A potential phage candidate for therapy
220 should have a good shelf life, i.e., it should be stored in a formulation that ensures activity
221 without significant drop in phage titre during processing and long-term storage (Fig. 1B),
222 as such decrease might compromise the outcome of the treatment (Malik *et al.* 2017;
223 Merabishvili, Pirnay and De Vos 2018; Jault *et al.* 2019). Several strategies have been
224 developed and optimized to improve phage stability and the most common include spray-
225 drying, freeze drying, extrusion dripping methods, emulsion and polymerisation

226 techniques (Malik *et al.* 2017). However, phage stability in different formulations (e.g.
227 liquids, gels, powders) is highly variable, especially among different phage types (Leung
228 *et al.* 2017; Gonzalez-Menendez *et al.* 2018; Merabishvili, Pirnay and De Vos 2018). An
229 alternative strategy to improve the storage shelf life of phages is their encapsulation on
230 different matrices such as liposomes, alginate, cellulose or other polymers (Malik *et al.*
231 2017; Cortés *et al.* 2018). Phage encapsulation strategies are important not only to achieve
232 longer shelf life but also for therapeutic purposes. Because treatment efficacy highly
233 depends on phage concentration at the site of infection, protecting phages from the harsh
234 conditions found in the human body is vital to avoid phage inactivation during treatment
235 due to e.g. low pH or clearance mechanisms associated with the immune system (Malik
236 *et al.* 2017; Dąbrowska 2019). In fact, the immune system plays a crucial role in phage
237 clearance or inactivation from animal and human bodies. Most studies on the immune
238 response to phages have focused on the development of phage-specific antibodies
239 (adaptive immunity). These have been shown in many cases to decrease the circulation
240 of phages, but other studies have reported no antibody formation or no effect of the
241 formed antibodies on the ability of phages to clear the infection (Dąbrowska 2019). *In*
242 *vitro* and *in vivo* studies have demonstrated the ability of encapsulated phages to persist
243 for longer periods at low pH, enhancing the efficacy of oral administration in animal
244 models (Yongsheng *et al.* 2008; Ma *et al.* 2012; Colom *et al.* 2017; Otero *et al.* 2019;
245 Vinner *et al.* 2019). More studies are required to understand protection given by
246 encapsulated strategies against immune clearance of phages. The protection of phages is
247 also important for certain combined therapies that can inactivate phages when applied
248 together and impair the outcome of the treatment. As an example, burn wound care
249 products and their active ingredients usually exhibit high acidity that can negatively affect
250 the activity of phages in wounds (Merabishvili *et al.* 2017).

251 Another issue of phage stability is the occurrence of spontaneous mutations in phage
252 stocks stored for long periods or accumulated during phage production and manufacturing,
253 which can impair viral fitness (Drake 1966; Botka *et al.* 2019). Although difficult, it
254 would be helpful to predict phage evolution during production to set up a manufacturing
255 process that would minimize the mutation rates in phage genomes (García *et al.* 2019).

256

257 **Fast phage screening methods**

258 Due to the high specificity of phage activity, finding a phage that targets a particular strain
259 often requires the screening of large phage collections (Fig. 1C). The most traditional
260 method to detect phage activity against a strain is the double layer agar (DLA) method,
261 in which different phages are spotted on top of a lawn of the bacteria of interest (Cornax
262 *et al.* 1990; Kropinski *et al.* 2009). Depending on the growth rate of the particular strain
263 to target, results may take up to 48h to show and therefore the DLA method is not
264 convenient in a therapeutic context where fast diagnosis is crucial. High-throughput and
265 fast-screening methods are desirable to rapidly identify phage(s) able to efficiently infect
266 the target strain(s).

267 Multiple methods have been developed for the detection and quantification of phages, via
268 direct or indirect measurements, but few seem to have application in a clinical setting.
269 For example, real-time PCR (qPCR) methodologies (Del Rio *et al.* 2008; Ly-Chatain *et*
270 *al.* 2011) have been developed for fast and sensitive detection of phages and for the
271 identification of infection via detection of increasing phage concentrations. But qPCR
272 methods require a set of primers and optimized conditions for (almost) every phage,

273 which is neither high-throughput nor feasible when testing large (and fast expanding)
274 phage collections against a target strain.

275 Flow cytometry has also been used to reveal phage infection via detection of cells with
276 low-density cell walls (Michelsen *et al.* 2007). Low-density cell walls have been observed
277 as a consequence of phage infection in *Lactococcus lactis*. The method allows for fast
278 and early detection of phage infection, but is low-throughput and most likely not universal
279 for all bacterial species and/or phages. Some other works have detected phage
280 propagation indirectly via measuring enzyme release from bacterial cells due to phage-
281 induced cell lysis. Intracellular enzymes such as adenylate kinase and adenosine 5'-
282 triphosphate (ATP) or β -galactosidase have been tested as measurements of infection by
283 *E. coli* phages (Stanek and Falkinham 2001; Guzmán Luna *et al.* 2009). Enzyme release
284 is detected by the generation of a bioluminescence or colour signal after cleavage of a
285 specific substrate. These assays are highly sensitive, generating a detectable signal in a
286 short time (≈ 3 h) even when starting with a low phage amount. Such methods are
287 compatible with high-throughput and, in theory, work with any phage but may need to be
288 optimized (e.g. enzyme/substrate selected) for each bacterial species.

289 The aptitude of surface plasmon resonance (SPR) techniques to measure and quantify
290 molecules bound to surfaces was explored to study the interaction between phages and
291 bacterial host (García-Aljaro *et al.* 2008). For this method, bacteria are immobilized on
292 gold sensor chips using avidin-biotin, and binding of phages to the bacteria and
293 consequent bacterial lysis can be detected and measured with high sensitivity in just 2 h.
294 As it is, however, the method is not compatible with high-throughput screening as only a
295 strain-phage pair can be tested simultaneously. A microfluidics adaptation of the method,
296 in which multiple channels are created to test multiple phages simultaneously, could
297 provide an interesting solution.

298 Cell respiration can also be used as a reporter for cellular growth and, consequently, for
299 phage infection. Using this principle, Henry and colleagues developed the OmniLog™
300 system, in which cell respiration is measured using redox chemistry via reduction of a
301 tetrazolium dye that produces a colour change measured in microtiter plates (Henry *et al.*
302 2012). Successful phage infection is detected by a reduction in colour due to reduced
303 bacterial growth and respiration. Such method is simple and high-throughput, but might
304 be limited to aerobic bacteria.

305 A simple approach was also recently suggested based on the analysis of optical density
306 kinetics in bacterial cultures for the detection and quantification of phages (Rajnovic,
307 Muñoz-Berbel and Mas 2019). This method detects phages at low amounts with a
308 response time of 3.5 h, and is susceptible of miniaturization and automation for high-
309 throughput applications that can be implemented in routine analysis. A possible drawback
310 is that it relies solely on a change in optical density of the bacterial culture, which is not
311 always observable for lytic phages.

312 In the future, a simple and fast high-throughput method for phage screening should be
313 established and implemented in clinical settings and in phage banks, if phage therapy is
314 to be widely used as a treatment option.

315

316 **Efficacy of phages against biofilms**

317 In nature and in the human body, bacteria are most often found in the form of a biofilm.
318 A biofilm can be defined as a population of bacteria attached to a surface and embedded
319 within a self-produced matrix (Hobley *et al.* 2015). In biofilms, bacterial cells closely
320 collaborate as a strategy for survival and persistence in harsh environments (Costerton *et*

321 *al.* 1995), e.g. providing increased tolerance to antibiotics (Costerton, Stewart and
322 Greenberg 1999; Stewart and Costerton 2001). Phage-bacteria interactions have been
323 mostly studied in planktonic cultures, but these interactions have been shown quite
324 distinct for bacteria in a biofilm form. Studies have revealed the therapeutic potential of
325 phages to control both mono-species (Curtin and Donlan 2006; Fu *et al.* 2010; Alves *et*
326 *al.* 2015; Melo *et al.* 2016) and dual-species biofilms (Sillankorva, Neubauer and Azeredo
327 2010; Gutiérrez *et al.* 2015b; Lehman and Donlan 2015), but multiple works have also
328 unveiled the impressive complexity and diversity of phage-biofilm interactions.

329 Within biofilms, bacteria are protected by a matrix composed mainly of polysaccharides,
330 lipids, extracellular DNA, and proteins (Hobley *et al.* 2015; Seviour *et al.* 2019). The
331 matrix is a major factor influencing the ability of a phage to successfully disturb a biofilm
332 (Darch *et al.* 2017), via several suggested mechanisms. The matrix can adsorb phages
333 (Bull *et al.* 2018) or simply form a physical barrier for phage diffusion (González *et al.*
334 2018; Dunsing *et al.* 2019), preventing phages from reaching and infecting the living cells
335 within the biofilm (Fig. 1D). Phages have, however, developed strategies to counteract
336 the limiting effects of the matrix on their activity (Pires *et al.* 2017a). Many phages
337 encode polysaccharide-degrading enzymes known as depolymerases, which are used to
338 degrade capsular polysaccharides of bacteria and thereby give the phage access to its
339 receptor on the bacterial cell surface. Some depolymerases can also degrade
340 exopolysaccharides of the biofilm matrix and improve access of the phages to the
341 bacterial cells (Harper *et al.* 2014; Gutiérrez *et al.* 2015a). The activity of depolymerases
342 tends to be very specific for a certain polysaccharide type, and the use of a phage cocktail
343 encoding for different depolymerases may represent a good treatment solution, and even
344 enhance the activity of other non-depolymerase producing phages (Schmerer, Molineux
345 and Bull 2014).

346 The spatial organization of the biofilm is also a determinant factor for phage infection.
347 To form a biofilm, cells organize so that localized niches are created with distinct nutrient
348 availability and consequently with bacteria of distinct motility, metabolic state, and gene
349 expression, all of which affect the capacity of phages to infect biofilm cells. The diffusion
350 of the phage through the biofilm is limited by the close proximity of the cells, which may
351 cause multiple phages to infect the same host cell and decrease the number of progeny
352 phages the cell generates (Taylor, Penington and Weitz 2017). Still, it is also possible that
353 local infection of a biofilm leads to a significant disruption of the biofilm structure,
354 ultimately leading to its dispersal and easier removal.

355 The establishment of nutrient gradients often leads to the generation of dormant persister
356 cells in the deeper layers of the biofilm, where nutrient resources are scarce. Phages
357 infecting these metabolically inactive cells are expected to be unable to propagate as they,
358 in principle, cannot use the (inactive) replication machinery of the cell (Łoś *et al.* 2007;
359 Pearl *et al.* 2008). However, a *Staphylococcus* infecting phage was recently shown
360 capable of propagating in dormant staphylococcal cells, a feature expected to be present
361 in other phages yet to discover (Melo *et al.* 2018; Tkhilaishvili *et al.* 2018). Additionally,
362 phages can remain within the persister cells until they exit the state of dormancy, being
363 then able to propagate as normal (Pearl *et al.* 2008).

364 Gene expression in biofilms is frequently controlled by quorum sensing, which involves
365 the use of extracellular signal molecules that sense population density to coordinate gene
366 expression (Ng and Bassler 2009). Quorum sensing can be used by bacteria to respond to
367 phage infections, for example by regulating expression of CRISPR-Cas systems
368 (Patterson *et al.* 2016; Høyland-Kroghsbo *et al.* 2017) and of phage receptors (Høyland-
369 Kroghsbo, Maerkedahl and Svenningsen 2013; Tan, Svenningsen and Middelboe 2015),
370 and also by regulating the production of biofilm matrix (Parsek and Greenberg 2005).

371 Some phages have developed strategies to exploit the bacterial quorum sensing system to
372 guide their lysis-lysogeny decision either by encoding receptors for the bacterial quorum
373 sensing molecules (Silpe and Bassler 2019) or by expressing their own extracellular
374 signalling molecules once inside the bacteria (Erez *et al.* 2017) . By sensing the bacterial
375 population, phages can sense a favourable or unfavourable environment for lytic
376 development.

377 Biofilms are also known to release outer membrane vesicles (OMVs) in high number.
378 These OMVs may contain outer membrane proteins used as receptors by some phages,
379 and therefore work as a decoy for phage infection, protecting biofilm cells from phages
380 (Manning and Kuehn 2011; Reyes-Robles *et al.* 2018). Nevertheless, phages that use
381 receptors other than outer membrane proteins (e.g. lipopolysaccharides) are not affected
382 by such strategy.

383 Dispersion of bacteria from a biofilm for colonization of a new niche is an important step
384 of the biofilm life cycle. Phages may be interesting solutions to control the spreading step
385 of a biofilm infection, as some phages unable to eradicate a biofilm can still inhibit
386 dispersal of migrating bacteria and the establishment of new colonies (Darch *et al.* 2017).

387 Most *in vitro* work in biofilms has been performed using single strains. Natural biofilms,
388 however, are often multi-strain or multi-species, which significantly affects the biofilm
389 spatial organization and the interaction with phages. The specific outcome of phage
390 infection in a multi-species biofilm seems to strongly depend on the bacterial species
391 composing the biofilm (e.g. whether they establish synergist or antagonist interactions).
392 Some studies have reported the ability of phages to target the susceptible host in the
393 biofilm independently of the presence of a non-susceptible strain (Harcombe and Bull
394 2005; Kay *et al.* 2011; Gutiérrez *et al.* 2015a). A few works, however, suggest the

395 presence of insensitive strains to provide spatial structure-associated protection to the
396 sensitive bacteria against phage infection, thereby reducing the efficacy of phage
397 treatment (Tait, Skillman and Sutherland 2002; Testa *et al.* 2019). Broad host range
398 phages (Kim *et al.* 2012) as well as phages carrying depolymerases (Pei and Lamas-
399 Samanamud 2014) may be particularly efficient against multispecies biofilms. In the
400 latter case, the diversity and heterogeneous distribution of exopolysaccharides on a multi-
401 species biofilm may limit depolymerase activity.

402 The complexity of phage-biofilm interactions is increased by evidence of promoted
403 biofilm formation induced by exposure to certain phages (Lacqua *et al.* 2006; Tan, Dahl
404 and Middelboe 2015; Henriksen *et al.* 2019). Two scenarios have been proposed for this
405 phenomenon. In the first scenario, changes in biofilm are thought to occur as a
406 consequence of the specific bacterial receptor used by the phage. Mutations in these
407 receptors occur as a response to infection and may lead to changes in the biofilm cells
408 that result in increased biofilm formation (Scanlan and Buckling 2012; Fernández *et al.*
409 2017; Henriksen *et al.* 2019). The second scenario suggests that some phages may benefit
410 from increased biofilm formation, with entrapment of phages in the biofilm matrix
411 providing protection against harsh environmental factors (Agún *et al.* 2018; Gabiatti *et*
412 *al.* 2018). In this scenario, an increase of the biofilm is beneficial for both bacteria and
413 phage.

414 Overall, the potential of phages to control biofilm infections is clear. However, the
415 complexity and diversity of phage-biofilm interactions limit broad conclusions and call
416 for more research before phage therapy becomes a real solution for biofilm-related
417 infections.

418

419 **Evolution of bacterial resistance to phages**

420 One of the major concerns in phage therapy is the possible emergence of bacteriophage-
421 insensitive mutants (BIMs) that could hamper the success of this therapy (Fig. 1E). Over
422 the last years, several studies have addressed the problem of bacterial resistance to phages,
423 demonstrating that the emergence of phage-resistant mutants is frequent and almost
424 unavoidable (Oechslin 2018; McCallin and Oechslin 2019). The resistance mechanisms
425 used by bacteria to counter-attack phage evasion include, among others: (i) prevention of
426 phage adsorption by loss or modification of bacterial receptors; (ii) prevention of phage
427 DNA entry by superinfection exclusion systems; (iii) degradation of phage DNA by
428 restriction-modification (R-M) systems and other related systems (BREX, DISARM, etc)
429 or by CRISPR-Cas systems; (iv) use of abortive infection systems that block phage
430 replication, transcription or translation; or (v) cyclic oligonucleotide-based anti-phage
431 signalling systems (Labrie, Samson and Moineau 2010; Bernheim and Sorek 2020).

432 A number of *in vitro* studies have reported the emergence of BIMs within a short period
433 of time after phage treatment (Fu *et al.* 2010; Le *et al.* 2014; Oechslin *et al.* 2016; Pires
434 *et al.* 2017b). In most of these studies, bacterial resistance to phages was caused by
435 mutations on genes encoding phage receptors, which include lipopolysaccharides, outer
436 membrane proteins, capsules, flagella, pili, among others. The emergence of phage-
437 resistant variants has also been noticed *in vivo* in several animal models as well as in
438 human pilot studies and case reports (Oechslin 2018; McCallin and Oechslin 2019).
439 However, some studies have highlighted the fact that the evolution of resistance observed
440 *in vitro* does not resemble what actually happens *in vivo*. For example, Oechslin *et al.*
441 studied the efficacy of a phage cocktail in the treatment of *P. aeruginosa* endocarditis and
442 observed that BIMs emerged *in vitro* but not *in vivo* (Oechslin *et al.* 2016). According to

443 the authors, this occurred probably because the bacterial mutations on phage receptors
444 rendering them resistant might incur fitness costs, with the bacteria becoming less virulent
445 and therefore easier to eliminate by the immune system. Other authors have also reported
446 the attenuated virulence of BIMs in consequence of modifications in cell surface receptors
447 for other bacterial species (Filippov *et al.* 2011; León and Bastías 2015; Sumrall *et al.*
448 2019).

449 Bacterial resistance to phages can be circumvented using different approaches (McCallin
450 and Oechslin 2019). The most common is the combination of multiple phages,
451 preferentially targeting different receptors and with complementary host ranges, in a
452 single preparation, which is usually known as a phage cocktail. In addition to displaying
453 a larger coverage against a particular bacterial species, such cocktails can also arrest the
454 emergence of BIMs. These are the main reasons behind the preferred use of phage
455 cocktails over single phage preparations in therapy. Phage cocktails might have a fixed
456 composition covering a broad host range (*prêt-à-porter*) or a customized formulation
457 designed for a particular patient (*sur-mesure*) (Pirnay *et al.* 2011). Another strategy
458 commonly used to deal with the problem of resistance during phage treatment is the
459 replacement of the phage against which the bacteria developed resistance by a phage that
460 is active against the resistant variant. While this is not easy for antibiotics, when it comes
461 to phages it can be quite simple given their abundance and diversity in nature as a result
462 of their constant co-evolution with bacteria (Rohde, Wittmann and Kutter 2018). Lastly,
463 the combination of phages with antibiotics or other antimicrobial agents can also be used
464 to avoid the development of bacterial resistance and to improve the therapeutic efficacy
465 (see below for more detail) (Torres-Barceló and Hochberg 2016; Tagliaferri, Jansen and
466 Horz 2019).

467

468 **Regulatory framework of phage therapy**

469 Regulatory authorities have classified phages as biological substances and, as such,
470 phages fall within the scope of the pharmaceutical legislation (Pelfrene *et al.* 2016;
471 Reindel and Fiore 2017). The regulatory framework in the European Union and in the
472 United States stipulates that a marketing authorization is required for medicinal products
473 prepared industrially or manufactured by a method involving an industrial process (Fig.
474 1F). As such, marketing a phage product requires proof of both safety and efficacy, and
475 also of quality by manufacture under GMP (Directive 2001/20/EC; Pelfrene, Sebris and
476 Cavaleri 2019). GMP compliance requires extensive financial resources (Pelfrene *et al.*
477 2016; Jault *et al.* 2019) and is therefore a critical obstacle for hospitals or non-for-profit
478 phage therapy centres. Current legislation calls also for predetermined qualitative and
479 quantitative evaluation of every constituent of the medicinal product. For phages,
480 recommended criteria (Parracho, Burrowes and Enright 2012; Pelfrene *et al.* 2016)
481 include the absence of prophages and antibiotic resistance in the bacteria used to produce
482 the phage(s), the lytic (non-temperate) and specific activity of individual phages on the
483 target bacteria, the control for impurities (e.g. endotoxins, residual reagents) in phage
484 preparations, and the test for potency and purity of the phages. This strict regulation is
485 somehow suitable for phage cocktails of fixed composition (*prêt-à-porter*) manufactured
486 at industrial scale, but is certainly inadequate for patient-specific, customized, phage
487 cocktails (*sur-mesure*) whose composition is variable and not intended for large-scale
488 distribution (Directive 2001/83/EC; Pelfrene, Sebris and Cavaleri 2019)(Pirnay *et al.*
489 2011).

490 Discussions between phage sponsors and regulatory agencies are ongoing to set more
491 satisfactory regulations for personalized phage therapy. The European Union currently

492 allows for a few exceptions on the requirement to obtain a product license, which apply
493 to the magistral formula (any medicinal product prepared in a pharmacy in accordance
494 with a prescription for an individual patient (Nahler 2009a)) and the officinal formula
495 (any medicinal product which is prepared in a pharmacy in accordance with the
496 prescriptions of a pharmacopoeia and is intended to be supplied directly to the patients
497 served by the same pharmacy (Nahler 2009b)), and for any advanced therapy medicinal
498 product (ATMP, medicinal product which is either a gene therapy medicinal product, a
499 somatic cell therapy medicinal product, or a tissue engineered product), if prepared on a
500 “non-routine basis according to specific quality standards, and used within the same
501 Member State in a hospital under the exclusive professional responsibility of a medical
502 practitioner, in order to comply with an individual medical prescription for a custom-
503 made product for an individual patient” (Directive 2001/83/EC; Pelfrene, Sebris and
504 Cavaleri 2019). An exemption is applied also for compassionate use, a treatment option
505 that allows an unauthorized (in development) medicine to be made available to groups of
506 patients who have a disease with no satisfactory authorized therapies and who cannot
507 enter clinical trials. However, compassionate use is only allowed for medicines
508 undergoing clinical trials or that have entered the marketing authorization application
509 process (Compassionate use | European Medicines Agency; Pelfrene, Sebris and Cavaleri
510 2019).

511 Due to the current unsatisfactory regulatory framework, Member States of the European
512 Union are finding national solutions for phage therapy regulation. The Belgian authorities
513 are pioneering phage therapy regulations in Western countries by establishing a national
514 regulation of magistral preparation of tailor-made phage medicines (Pirnay *et al.* 2018).
515 The regulation requires issuing of a monograph that judges in written form the quality of
516 the phage active pharmaceutical ingredient (API) to be used for the preparation of the

517 medicinal product. Every stock of the phage therapy medicinal product is then tested by
518 a Belgian approved laboratory to confirm the phage(s) comply with the phage API
519 monograph(s), issuing a certificate of analysis that approves its use. A pharmacist then
520 uses the certified phage stock for preparing a customized medicinal product based on the
521 prescription of a physician (Pirnay *et al.* 2018). This process has already allowed the
522 implementation of phage therapy in Belgium, but it is not yet ideal as all responsibility is
523 given to the prescriber and the pharmacist (Fauconnier 2017). Similar regulatory
524 principles were already in practice, for example, in Georgia and Russia. In Georgia,
525 ready-to-use phage medicines require a marketing authorization according to regular
526 regulation, while customized phage preparations may be prepared as magistral
527 preparation in an authorized pharmacy (Parfitt 2005). The Russian pharmacopeia includes
528 a monograph on phages for prophylactic and therapeutic use (Russian Pharmacopoeia
529 OFS.1.7.1.0002.15).

530 Other countries are also finding similar solutions. France has issued recommendations for
531 the use of phage medicinal products under the nominative Temporary Authorization for
532 Use (ATUn) (Phagothérapie). An ATUn can be issued by hospital pharmacies, for a
533 single patient who cannot participate in a clinical trial, at the request and under the
534 responsibility of the prescribing physician, allowing for the use of a medicinal product
535 without market approval if its efficacy and safety balance is presumed favourable for the
536 patient, in the absence of any approved treatment. In the United States, phages can be and
537 have been used following the Food and Drug Administration (FDA) emergency
538 investigational new drug (eIND) pathway (Schooley *et al.* 2017; LaVergne *et al.* 2018).

539 Further clinical evidence of the success of phage therapy in human trials conducted to
540 modern standards would help foster regulatory advance (Pelfrene *et al.* 2016), but current
541 regulatory issues affect also the conduct of clinical trials. A new provision in the

542 regulatory framework of the European Union may facilitate clinical trials with phage
543 medicinal products, by exempting GMP requirements in the preparation of investigational
544 medicine products (IMPs), “where this process is carried out in hospitals, health centers
545 or clinics legally authorized in the Member State concerned to carry out such process and
546 if the IMPs are intended to be used exclusively in hospitals, health centers or clinics taking
547 part in the same clinical trial in the same Member State” (Regulation (EU) No 536/2014).

548 In summary, current regulations will certainly undergo serious modifications before a
549 fully practicable regulation is implemented for phage therapy, as well as other customized
550 medicinal products meant to be tailored to an individual patient.

551

552 **THE FUTURE OF PHAGE THERAPY**

553 **Phages in One Health approach**

554 It is estimated that at least six out of ten known infectious diseases in humans are
555 originated in animals (Zoonotic Diseases | One Health | CDC). Moreover, the selective
556 pressure on phytobacteria drives evolution in a vast number of defence mechanisms,
557 which can result in increased virulence towards humans, especially those with advanced
558 age, immunodeficiency, or cancer (Erken, Lutz and McDougald 2013; Falkinham, Pruden
559 and Edwards 2015). The One Health concept recognizes that the health of humans and
560 animals as well as our environment are all intertwined. To improve the lives of all living
561 species, the One Health program proposes the integration of human medicine, veterinary
562 medicine and environmental science (<http://www.onehealthinitiative.com/>). Agriculture
563 and food safety are also included in this holistic and multi-sectoral approach to tackle
564 antimicrobial resistance (Baum *et al.* 2017; Hernando-Amado *et al.* 2019). Although

565 microorganisms will inevitably develop resistance towards antibiotics as a consequence
566 of genetic mutations or horizontal gene transfer, the problem of resistance is worsened by
567 the misuse of antibiotics since their discovery. A clear example is the use of antibiotics
568 as growth promoters at livestock farms, which impelled the European Union to create
569 stricter regulations to control their widespread usage (Kittler *et al.* 2017). To mitigate the
570 spread of antimicrobial resistance, new alternative therapeutics under the One Health
571 view are needed. Since their discovery, phages are being applied for the control of
572 bacterial proliferation in several microbiomes, such as humans (as reviewed above),
573 animals (Oliveira, Sereno and Azeredo 2010), several environmental settings (e.g.
574 wastewater treatments) (Withey *et al.* 2005), and on food industry (Abuladze *et al.* 2008).
575 A good example of the global use of phages are the diverse application opportunities in
576 food industry, where they can be used at all stages of food processing, from slathering
577 and crops to food transportation (reviewed by (Goodridge and Bisha 2011)), even
578 improving the shelf life of food products (Alves *et al.* 2019). In fact, several phage-based
579 products to be applied in food-stuff have already received the GRAS (generally
580 recognized as safe) classification by the Food and Drug Administration (FDA) in the
581 United States (Sarhan and Azzazy 2015). Therefore, the use of phages is consistent with
582 the One Health approach as they can be applied in different settings (e.g. food, animals
583 or crops) thus preventing the overuse of antibiotics and the dissemination of antibiotic
584 resistance to humans (Kittler *et al.* 2017).

585

586 **Emerging approaches**

587 The use of phages for the control of bacterial infections might be improved via
588 combination with other agents, especially when targeting the complex biofilm

589 communities (Koo *et al.* 2017). These combined therapies have often the advantage of
590 limited development of resistance towards agents with distinct modes of action due to the
591 fitness cost associated with resistance against multiple factors (Torres-Barceló and
592 Hochberg 2016; Chaudhry *et al.* 2017).

593 Probably the most obvious combination is that of phages and antibiotics (Fig. 1G). When
594 used simultaneously, phages and antibiotics have shown synergistic effects and
595 effectiveness against planktonic cells (Bedi, Verma and Chhibber 2009; Nouraldin *et al.*
596 2016; Jansen *et al.* 2018; Yazdi, Bouzari and Ghaemi 2018) and (especially old) biofilms
597 (Bedi, Verma and Chhibber 2009; Rahman *et al.* 2011; Chaudhry *et al.* 2017; Akturk *et*
598 *al.* 2019), where the individual treatments had restricted success. In cases where repeated
599 treatment with phages increased biofilm production, the combined use of phage and
600 antibiotics resulted in biofilm eradication (Henriksen *et al.* 2019). Structural changes in
601 the biofilm caused by one or both agents may be behind the enhanced efficacy. For
602 example, removal of peripheral cells by the phage may lead to increased resource
603 availability for inner cells and improve their metabolic state, making the cells more
604 susceptible towards phages and certain antibiotics (Chaudhry *et al.* 2017). Antibiotics
605 may also themselves cause changes in the biofilm architecture and thereby enable
606 increased invasion of biofilms by phages (Díaz-Pascual *et al.* 2019).

607 Synergism between antibiotics and phages does not happen for all phage-antibiotic
608 combinations (Knezevic *et al.* 2013; Kamal and Dennis 2015; Jansen *et al.* 2018) and
609 high doses of antibiotics can also antagonize phage propagation (Dickey and Perrot 2019).
610 This is particularly evident when using antibiotics that target cell protein synthesis
611 (Akturk *et al.* 2019). But in some cases, even though no synergism in antimicrobial
612 activity is observed, the combined use of phages and antibiotics significantly reduces or

613 even prevents the development of antibiotic- and phage-resistant bacteria (Coulter *et al.*
614 2014; Dickey and Perrot 2019).

615 While several studies have looked into the effect of phage-antibiotic therapies, few have
616 developed a rational approach to explore the bacterial response to these agents. An
617 example of such strategy is the isolation of phages targeting specific outer membrane
618 proteins that are used by bacteria as multidrug efflux pumps. Development of resistance
619 to this phage would require the bacteria to change the efflux pump and therefore increase
620 sensitivity against certain antibiotic classes (Chan *et al.* 2016). This approach was
621 successfully employed to save a patient suffering from a chronic prosthetic vascular graft
622 infection caused by *P. aeruginosa*, in which phage OMKO1 binding to efflux pump
623 proteins was used in combination with ceftazidime; evolution of phage resistance led to
624 increased antibiotic sensitivity and the infection was resolved (Chan *et al.* 2018).
625 Approaches like this are not only efficient but also extend the lifetime of our current
626 antibiotics.

627 Phages can also be co-administered with enzymes for improved activity. For example,
628 depolymerases can be used together with phages that do not naturally express them to
629 improve their activity against biofilms (Gutiérrez *et al.* 2015a). DNase enzymes can also
630 be used together with phages to degrade the DNA component of the biofilm matrix and
631 improve phage activity (Hughes *et al.* 2006). Other successful cases combined phages
632 with chlorine (Zhang and Hu 2013), triclosan, chlorhexidine, hydrogen peroxide (Agún
633 *et al.* 2018), cobalt (II) sulphate (Chhibber, Nag and Bansal 2013), xylitol (Chhibber,
634 Bansal and Kaur 2015), honey (Oliveira *et al.* 2017), and probiotics (Woo and Ahn 2014).

635 The modification of phage genomes is also being explored to improve phage therapy
636 outcomes (Fig. 1H). This approach is being fuelled by recent advances in the synthetic

637 biology field, with many techniques now available to engineer phage genomes (Martel
638 and Moineau 2014; Ando *et al.* 2015; Pires *et al.* 2016; Kilcher *et al.* 2018). The host
639 range of a phage is one of the main targets to engineer. While the high host specificity of
640 phages is advantageous by preventing targeting of beneficial bacteria, it also implies that
641 it is almost impossible to target all strains within a given species using a single phage.
642 Tailored control of a phage's host range is therefore a major goal in phage therapy.
643 Working towards this goal, several studies have swapped receptor-binding protein genes
644 between phages of different families, successfully exchanging the host range of the phage.
645 This has been possible between phages infecting the same (Yoichi *et al.* 2005; Mahichi
646 *et al.* 2009) or different species (Ando *et al.* 2015). Others had fused a heterologous
647 receptor binding domain to the receptor binding protein of a phage, thereby increasing
648 the phage host range (Marzari *et al.* 1997; Heilpern and Waldor 2003).

649 Phages can also be engineered to deliver specific cargo to enhance the phage
650 antimicrobial activity. For example, enzymes such as dispersin B and lactonase have been
651 engineered into phage T7 to increase the phage activity against biofilms (Lu and Collins
652 2007; Pei and Lamas-Samanamud 2014). Dispersin B, a glycoside hydrolase, is expressed
653 at high levels during T7 infection and released upon cell lysis into the biofilm
654 environment, where it degrades the matrix; by doing so, dispersin B increases the phage
655 efficacy on removing both bacteria and matrix from the biofilm (Lu and Collins 2007).
656 Lactonase was also engineered into phage T7, but to interfere with the bacterial quorum
657 sensing, making use of its ability to inactivate the quorum sensing acylated homoserine
658 lactones (Pei and Lamas-Samanamud 2014). Inactivation of the quorum sensing
659 molecules interferes with biofilm formation and leads to improved biofilm control by the
660 engineered phage. Curiously, this strategy was shown to work in multi-species biofilms,
661 where quorum sensing molecules of one species also increase biofilm formation of the

662 second species, and inhibition of the molecules by the lactonase reduces biofilm
663 formation in both species. This may therefore be an interesting alternative treatment
664 against multi-species biofilms in the future.

665 While most engineering efforts have centred on lytic phages, temperate phages have also
666 been the subject of a few engineering experiments for phage therapy purposes. The most
667 obvious approach consists on genetically modifying phages to become exclusively lytic.
668 This has been accomplished by deletion of the genomic module responsible for the
669 establishment of lysogeny (Dorscht *et al.* 2009; Zhang *et al.* 2013; Kilcher *et al.* 2018).
670 The creation of virulent mutants of otherwise temperate phages can easily extend the
671 number and diversity of phages available for therapeutic purposes. A great example of
672 the value of this approach is the recent use of a cocktail composed of one natural lytic
673 phage and two engineered temperate phages to successfully treat a 15-year-old patient
674 with cystic fibrosis with a disseminated *Mycobacterium abscessus* infection (Dedrick *et*
675 *al.* 2019). The temperate phages were engineered to become lytic via removal of the
676 repressor of the lytic cycle, and the cocktail was administered intravenously and was well
677 tolerated. Genetically engineered phages are not readily accepted for phage therapy due
678 to the inherent ethical issues of genetically modified organisms (GMOs) but this case
679 study clearly shows that engineering approaches are useful. The possibility of using
680 temperate phages engineered into lytic forms in phage therapy increases the number of
681 phages available for therapeutic use, by reducing/removing the risk of transduction of
682 bacterial genetic information (e.g. virulence-related genes) mediated by temperate phages
683 (Monteiro *et al.* 2019).

684 Temperate phages have also been engineered to deliver synthetic gene networks,
685 exploiting their natural capacity to integrate into the host bacterium chromosome, where
686 the phage expresses the molecule of interest. Phages have been modified as adjuvants to

687 antibiotics, by codifying dominant antibiotic sensitive genes (Edgar *et al.* 2012) or
688 CRISPR-Cas systems (Bikard *et al.* 2014; Yosef *et al.* 2015) that revert antibiotic
689 resistance in bacteria, or by codifying CRISPR-Cas systems designed to target bacterial
690 cells (Park *et al.* 2017).

691 Overall, engineering approaches can potentially improve the antimicrobial properties of
692 phages and create innovative strategies for fighting bacterial infections. The
693 consequences of genetic manipulation of phage genomes must be carefully addressed, but
694 phage engineering strategies should be effectively considered as a therapeutic option.
695 Additionally, engineered phages have easier patentability than natural phages, and may
696 therefore have more commercial interest.

697

698 **Can phage resistance become a global problem?**

699 Phage therapy frequently raises the question of whether the global use of phages could
700 lead to a widespread problem similar to antibiotic resistance. A definitive answer does
701 not exist.

702 First, phages will unlikely be used as a first line treatment against bacterial infections as
703 it happens with antibiotics. In a future perspective, phage therapy is expected to be applied
704 only in clinical cases of patients who experienced the failure of antibiotic treatments.
705 Additionally, contrary to antibiotic therapy, phage preparations for therapeutic
706 applications are expected to be developed in a personalized way by formulating phage
707 cocktails that might delay the emergence of bacterial resistance to phages.

708 In the scenario of phages being extensively used in the future both as therapeutic and as
709 environmental bio-control agents, it is possible that a strong selective pressure is imposed

710 towards the development of resistant bacteria. Still, it seems improbable that no phage
711 will be available in nature to infect a bacteria that has become resistant to a previous
712 phage. In fact, the long and continuous co-evolution of phages and bacteria (Dion,
713 Oechslin and Moineau 2020) have resulted in bacteria evolving a range of mechanisms
714 to avoid phage predation, and in phages developing effective counter-strategies to evade
715 the antiviral systems (Samson *et al.* 2013). This arms race between phages and their
716 bacterial hosts will not come to an end and, despite the emergence of resistant bacteria,
717 phages will certainly find a way to ensure their propagation. The use of strategies as
718 combined therapies and genome engineering may be an additional aid to prevent the
719 spread of phage resistance. Still, further studies are required to guarantee that the global
720 use of phages will not eventually compromise its efficacy.

721

722 **FINAL REMARKS**

723 In an era of global crisis for antibiotics, phage therapy has emerged as a potential
724 alternative with already proven cases of clinical success. The generic use of phages for
725 biocontrol meets the One Health Approach and is well aligned with the recently
726 established European Green Deal (European Commission 2019) that recommends
727 reducing significantly the use of antibiotics in food production. On the other hand,
728 scientific advances have contributed to a better knowledge of phage-bacteria interaction
729 enabling a safer and more efficient phage therapy. So, the conditions needed for the
730 reintroduction of phage therapy as a therapeutic practice are met. Nevertheless, the
731 widespread use of phage therapy creates additional challenges that go beyond the clinic
732 standpoint and carries extra demands. These include (i) the need of increasing phage
733 collections of reference phage banks; (ii) the development of efficient phage screening

734 methods for the fast identification of the therapeutic phage; (iii) the establishment of
735 efficient phage therapy strategies that tackle infectious biofilms; (iv) the set-up of phage
736 production protocols that assure quality and safety of phage preparations; and (v) the
737 guarantee of stability of phage preparation during storage and transport.

738 As infectious diseases have no borders, a global action plan to make phage therapy
739 worldwide available is needed. This obviously requires an active collaboration between
740 countries for overcoming logistic and regulatory challenges, and between clinicians and
741 scientists for filling current gap knowledges and fostering advances in the field.

742

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751

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753

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