COMPLEX BONE AND JOINT INFECTIONS: TREATMENT WITH BACTERIOPHAGES AS SALVAGE THERAPY

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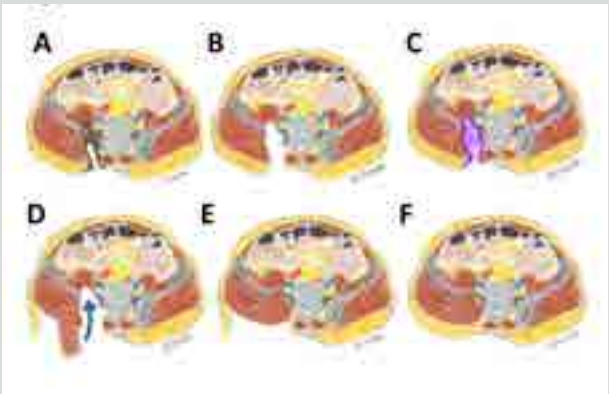
The treatment of bone and joint infection (BJI) is challenging, as the recurrence rate remains high despite conventional strategies based on surgery and prolonged antibiotherapy. We report on the use of bacteriophages produced in France (according to European good manufacturing practice) as salvage therapy in patients with complex BJI. A personalized bacteriophage cocktail was produced and applied locally during surgery. We think that this unique experience of innovative personalized medicine with bacteriophages is the first step to better identify eligibility criteria for clinical trials involving patients with more common BJI. Personalized phage therapy would be an excellent adjuvant treatment to improve the prognosis of BJI.

Background and burden of BJI

Different kinds of bone and joint infection (BJI) have been described and are associated with different therapeutic strategies and prognoses *(1)*. Some of them, such as uncomplicated childhood osteomyelitis, are easy to treat, requiring short-course antimicrobial therapy without surgery. Others, such as implant-associated BJI, which represent a very heterogeneous group, are more complex to treat and eradication of the pathogen is challenging. Indeed, pathogens develop various strategies to persist in *vivo* in such patients at the site of infection. Most bacteria, but especially staphylococci and *P. aeruginosa* are able to produce biofilm on the material surface or in a dead bone segment, so-called sequestrum *(1)*. Currently, the only way to eradicate biofilm is to remove it mechanically, i.e. to clean remove the implant and/or to resect all sequestrum. Biofilm is not the only way for bacteria to persist, they are able to invade bone cells then to persist by reducing aggressive virulent behaviour and form an intracellular sanctuary (especially *S. aureus*). Among implant-associated infections, it is important to distinguish prosthetic-joint infection from long-bone implant associated infection, also called osteomyelitis. Most prosthetic-joint infections are located at the hip or the knee in the elderly. These infections constantly need surgery and prolonged antibiotherapy. In the case of acute prosthetic joint infection (inoculation <1 month), the surgery consists in a debridement and a surgical lavage, with implant retention and the exchange of the mobile part only of the prosthesis (i.e., polyethylene articular inert element that allows mobility between the implants). In patients with chronic infections, one-stage (explantation and preimplantation during the same surgery) or two-stage (explantation, then reimplantation several weeks later) exchange procedure is mandatory, but these surgeries are significantly more invasive, with risk for peroperative hemorrhage and for more anesthetic complications, with a putative loss/reduction of motor function *(2)*. In patients with chronic long-bone implant associated infection, the surgical strategy depends on the local spread of the disease that may be limited to the cortical bone, or expanded to the medullary bone with instability (this is called septic pseudarthrosis), requiring

Figure1: **Patients with a right sacro-iliac osteomyelitis with bone exposition (panel A) requiring a two-step surgery with bone debridement (panel B), local application of negative pressure therapy (panel C), and then particular additional surgery with muscle and skin and soft tissue flap to cover the defect (panel D, E and F)**



large bone resection followed by complex reconstruction *(3)*. Some patients also have skin and soft tissue defects with bone exposition, that requires a particular additional surgery with a skin and soft tissue flap to cover the defect (Figure 1). In such patients, it is difficult to imagine a cure if the skin and soft tissue reconstruction is not considered. All these situations require a prolonged antimicrobial therapy from six weeks to three months targeting the pathogen(s) involved, using intravenous and/or oral antibiotics, depending on drug susceptibilities (antibiogram). The success rate reaches 60 – 80% in acute prosthetic joint infection, 80 – 90% in chronic prosthetic joint infection and varies from 30 to 90% in patients with chronic long bone implant associated infection, depending on the stage of the disease, and if a bo ne and/or skin and soft tissue reconstruction is required *(1-3)*. In all of these patients, especially those with the most complex disease form, team-work is required to personalize disease management, determine an optimal medico-surgical strategy, and limit treatment failure, motor disability and amputation risk. Concerning the burden of BJI, a national study in France based on the national health administration database demonstrated that BJIs have a major clinical and economic impact. The overall prevalence was 54 cases per 100,000 inhabitants, which agrees with other studies performed in Europe and the United States. BJI prevalence is age- and sex-dependent, with a six-fold increase in patients between 50 and 70 years old. Most patients have underlying diseases, especially diabetes, and related comorbidities, including ulcer sores and vascular disorders. In 2008, for France, only for the total direct cost of BJI-related care, the estimates reach €259 million (€7,178 per hospital stay); one of the main contributors to this cost being the rate of hospital readmission (19%) *(4)*. However, these cost estimates did not take into account indirect costs such as those associated with long-term care or rehabilitation. In fact, the long-term bone and joint infections-associated morbidity, which is estimated to involve 30 – 40% of bone and joint infection patients, mainly explains the massive individual and societal impact of bone and joint infections, including long-term or definitive incapacity for work, partial or total disability, amputation, reconstruction and the high inpatient and outpatient costs.

As a consequence, the French Health Ministry founded a network of hospital regional centres called CRIOAc (Centres de Référence des Infections Ostéoarticulaires complexes), with dedicated funding. Their mission was to facilitate the management of complex BJI, to provide an access for patients to experienced clinical teams, to benefit patients from adapted techniques for complex BJI and finally to promote clinical, translational and fundamental studies and researches. At the present time, nine CRIOAcs are approved in France, including the regional reference centre of the Auvergne-Rhône-Alpes Region: the CRIOAc Lyon (http://www.crioac-lyon.fr).

BJI is more and more associated with antimicrobial resistance

As BJI frequently occur after trauma and surgery, most of them are healthcare-associated infections. BJI are classically associated with staphylococci, streptococci, enterococci enterobacteriaceae, *Pseudomonas aeruginosa* and/or anaerobes. Staphylococci may be resistant to methicillin, and potentially to the most important drug combinations for the treatment of staphylococcal BJI: rifampin and fluoroquinolone. Some Enterococci are resistant to amoxicillin. Enterobacteriaceae occasionnaly produce extended spectrum betalactamases or carbapenemases and are frequently resistant to fluoroquinolone. *P. aeruginosa* are sometimes multi-resistant, with the emergence of pan drug-resistant strains. The impact of antibiotic resistance on the outcome of BJI is not well established, but is likely significant, as suboptimal antimicrobial therapy is associated with a higher risk of relapse. Furthermore, the bone penetration of most antibiotics is limited, especially for beta-lactams and glycopeptides, with only about 20% of the administered drug able to penetrate into bone. Finally, new antibiotics approved by FDA and/or EMA in the last five years (large spectrum beta-lactams such as ceftolozane/tazobactam and ceftazidime/avibactam; lipoglycopeptides such as dalbavancin; new oxazolidinones such as tedizolid) are not expected to be evaluated in patients with BJI.

In this context, therapeutic alternatives are much needed and very welcome to circumvent multi-resistance and therapeutic deadlocks because of clinical or physiological reasons.

Phage therapy and the Eastern European experience

Bacteriophages or phages are one of the most abundant organisms in the biosphere. A bacteriophage is a virus able to infect a bacterium. Using lytic bacteriophages as

antibacterial treatment is a very interesting approach to treat bacterial infections. Antibiotics need several intravenous or oral administrations in a day to reach significant concentrations and remain above the bacterial microbial inhibitory concentration (MIC) at the site of infection. Lytic phages act differently as they infect and rapidly kill the targeted bacteria by taking over its cellular machinery to produce new phagic components to ultimately assemble and release numerous new phage particles, that can infect gain bacteria from the same strain that are locally present. This latter phenomenon, in comparison with antibiotics, is exponential and self-sustained after a single or a few administrations. Lytic phages penetrate into tissues and remain present as long as multiplication in a susceptible bacterium is possible at the site of infection. Then, they are eliminated by the body when all susceptible bacteria are eradicated. No effect of phages on healthy tissue and cells has been reported because of their high specificity towards bacteria *(5)*.

The clinical practice of phage therapy is common in Eastern Europe, and in particular in the Republic of Georgia (Eliava Institute) and in Poland (Hirszfeld Institute of Immunology and Experimental Therapy) *(6-8)*. Historically, George Eliava was a collaborator with the French microbiologist Felix d'Hérelle from the Pasteur Institute, who discovered phage therapy in 1917. George Eliava exported the clinical practice of phages to Tbilisi in the early 1920s by starting to use a mix of phages (a "cocktail") named "Pyophage". That product targeted *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Proteus spp*., and *Streptococcus spp*. It was produced commercially in France until 1978. Following private investment, members of Eliava institute developed in the late 1990s a new phage company, Biochimpharm, that produces (but without following the European good manufacturing practice (GMP)) its own licensed versions of Pyophage. This "fixed" cocktail is currently available in public pharmacies throughout the country *(6, 7)*. In Poland, the approach is different, as it is based on selection of active phages from a bank against the individual bacteria that infects the patient, to adapt the treatment (personal medicine) and to ensure the antibacterial activity of phages used *(6, 8)*. Polish phages are also not produced according to European GMP standards.

In western Europe and the United States, a few patients have been occasionally treated with imported non-GMP phages, especially for patients with recurrent bacterial infectious diseases potentially associated with an extreme condition (Figure 2) *(6-10)*. In such countries, medical health authorities consider that it is of a crucial importance to respect GMP standards when producing phages for conducting clinical trials and targeting market authorizations, as manufacturing of bacteriophage drugs requires the elimination of bacterial components that are generated during the production process

Figure 2: Map of the Europe with the inventory of places where phagotherapy for BJI is used

such as toxins, in order to limit pyrogenicity and adverse events that may arise during phage administration/use.

Chronic osteomyelitis is currently one of the indications of phage therapy in Eastern Europe, especially in patients infected with multidrug-resistant isolates *(6-8)*. Indeed, there is no correlation between antibiotic resistance and phage efficacy as bacterial killing differs between antibiotics and phages. In this clinical situation, phages that are produced in a liquid form are used alone most of the time, without surgery, in patients with fistula or bone exposition. Phages are inoculated directly throughout the fistula or directly applied on an exposed bone using nebulization or direct local applications (Figure 3). In such patients, it is believed that phages go to and penetrate into infected bone in a step-by-step manner, by infecting the pathogen that liberates new phages that then penetrate themselves into bone and bacterial biofilm.

Manufacturing of bacteriophages by Pherecydes in France

Pherecydes owns a library with the ability to produce various bacteriophages targeting *P. aeruginosa* and *S. aureus*, belonging to Pherecydes Pharma library. Indeed, a specific bacteriophage targeting for instance *P. aeruginosa*, could be not always be fully active on all *P. aeruginosa* strains, that's why, as antibiotics, an in vitro evaluation of the phage activity, as it is currently performed for antibiotics (antibiogram) could be particularly relevant. The activity of phages are tested on the patient's strain by performing a phagogram (identification of the strain's susceptibility to the bacteriophage, on the model of antibiogram used for antibiotics) using two different in vitro methods to be able to prepare a cocktail of the most active bacteriophages on a particular clinical strain (Figure 4).

The CRIOAc network in France and the selection of patients for the use of bacteriophages in CRIOAc Lyon

The CRIOAc network aims to facilitate the management

Figure 3: **Patient with a tibia chronic osteomyelitis with bone exposition, for whom bone debridement with antibiotherapy followed by skin and soft tissue reconstruction is considered as essential to obtain a cure (panel A). Patient with femoral chronic osteomyelitis with purulent discharge from a fistula. Bone debridement is here also required, but not skin and soft tissue reconstruction (panel B). Chronic long bone osteomyelitis could be managed with only phagotherapy Eastern counties, by inoculated directly the phage in conta ct with the bone defect or throughout the fistula (panel C; from Kutateladze *M. Trends Biotechnol.* 2010)**

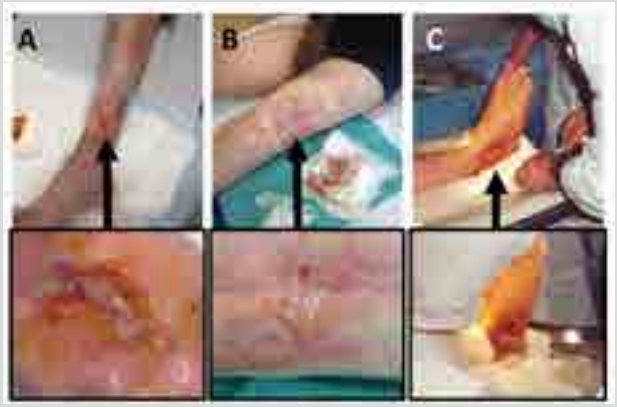


Figure 4: **Phagograms were based on a plaque assay (panel A) and a killing assay (panel B). Three out the four phages tested on the patient's strain led to the formation of plaque forming units (panel A, upper picture, red arrow heads) in the bacterial layer on the agar plate. The EOP score calculated with the phage titer derived from the dilution series on the patient strain (panel A, upper picture) and the dilution series on the reference strain (panel B, lower picture) was high for these three phages. These phages were considered as active and efficient. By contrast, although Phage D led to a partial lysis of the bacterial layer no PFU were visible, this phage was considered as inactive. In the killing assay (panel B), three out of the four phages showed a complete inhibition of the patient's strain growth (PN1777, PN1797, PN1658), while one phage (PN1658) had no impact on the growth PN1658**

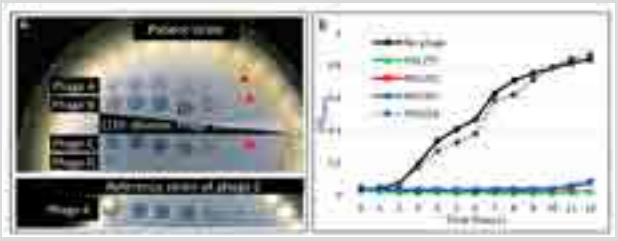
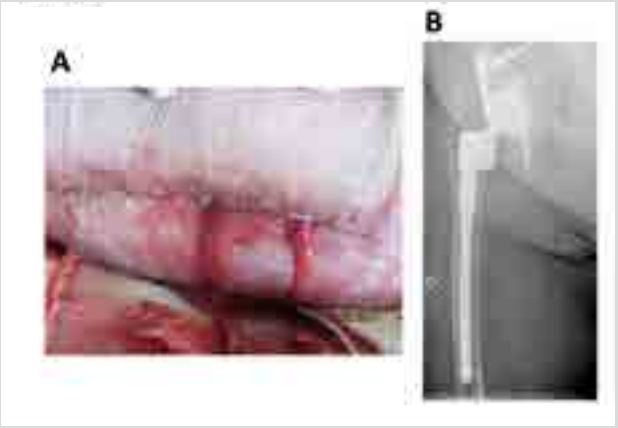


Figure 5: **Eighty-year-old patient with purulent discharge (panel A) during a relapsing polymicrobial prosthetic-joint infection. The prosthesis was previously already change four times in the past for infections. As there was no prosthesis loosening at X-Ray, it was difficult to imagine an explantation without serious bone damage (fracture) and peroperative risk of complications (bleeding) or death**



of complex BJI and to provide an access for patients to experienced clinical teams. Among the nine CRIOAc approved in France, the CRIOAc Lyon (http://www.crioac-lyon.fr) particularly aims to facilitate access to innovation for patients, from different approaches that include rapid and molecular diagnosis of BJI, use of local antibiotics, new devices such as silver-coated implants or bone substitutes with antimicrobial effects, and phagotherapy. The CRIOAc Lyon and Pherecydes are partners in a programme called PHOSA (http://www.phosa.eu) whose the final objective is to assemble and use cocktails of bacteriophages for patients with BJI.

CRIOAc Lyon recruits about 500 new patients each year, with heterogenous forms of BJI, including 100–150 prosthetic-joint infections, and 100–150 long-bone chronic osteomyelitis. Other forms of osteomyelitis, such as pelvic or mandibular osteomyelitis, are less prevalent. All cases are discussed in multidisciplinary meetings involving orthopedic surgeons, infectiologists and microbiologists, to personalize and optimize the complex management of the disease, taking into account the patient's general condition, medical history of BJI, antimicrobial susceptibilities, as well as the motor function and mechanical aspect of the bone and/or joint involved. Some patients present with complex BJI, defined by the presence of at least one specific criterion such as: (i) patient with severe comorbidity limiting treatment options and/or with severe allergy; (ii) patient infected with difficult-to-treat micro-organism(s) especially with multidrug resistance; (iii) BJI requiring bone resection and bone and/or soft-tissue reconstruction; (iv) relapsing BJI. Some patients present with particularly severe clinical situation with a poor prognosis, i.e. ageing patients with chronic infected large prosthetic joint for whom explantation is not feasible (Figure 5), or patients infected with pan drug-resistant pathogens. In 2017, among 1,132 cases discussed during multidisciplinary meetings (including 531 patients managed in our centre), we considered phagotherapy as salvage therapy in seven patients (case selection phase; Figure 6). In four patients, the phagotherapy was finally not performed: three had *S. epidermidis* chronic prosthetic joint, a pathogen for which no phage active on *S. epidermidis* was available in the Pherecydes library), and one had *P. aeruginosa* chronic prosthetic joint that finally required a debridement in emergency. Two other patients had *S. aureus* chronic prosthetic joint with productive fistula and for whom explantation was considered as impossible, and one patient with pelvic osteomyelitis who was infected with a pan drug-resistant *P. aeruginosa*. After the identification of each eligible patient, we discussed the indication with the ANSM (French National Agency for the Safety of Medicines and Health Products) and its dedicated committee called "Specialized Temporary Scientific

Committee in Phagotherapy".

Discussion with health authorities, performance of the phagogram, current process of preparation and administration of the bacteriophage cocktail

Finally, after discussion with health authorities and the specialized committee, we decided to propose personalized phagotherapy to the three latter patients as compassionate salvage therapy. The bacterial isolates were sent to pherecydes to perform the phagogram. Pherecydes has a library with the ability to produce various bacteriophages targeting *P. aeruginosa* and *S. aureus*, belonging to Pherecydes Pharma library. Phages could be tested on the patient's strain using two different in vitro methods i.e. plaque assay and killing assay (Figure 4). In the plaque assay 10µl of serial dilutions of each phage were spotted on the patient's strain as well as on their own reference strain. The appearance of plaque forming unit (PFU) on the bacterial layer indicated that the phage was active on the patient's strain. Moreover, the efficiency of plating (EOP) score, defined as the ratio of the phage titer on the patient's strain / phage titer on its reference strain, could be determined with the plaque assay and was informative about the active phage dose. In the killing assay (Figure 4), the patient's strain was inoculated at 107 CFU/ml in a 96-well plate in the presence or absence of one phage at three different doses. The bacterial concentration was recorded over time by optical density at 600 nm (Thermo Scientific Multiscan GC). The absence or decrease of bacterial growth in the presence of a phage compared to the culture without phage revealed the phage activity. The potentially selected phages were amplified on their own host in 1l of animal free Lysogeny broth culture medium. After centrifugation, the supernatant was vacuum filtered through 0.22 µm filters and then concentrated through a tangential flow filtration system to a volume of 100 ml in DPBS. Host DNA and endotoxins is eliminated through the purification process and their concentration measured to check they remained below the approved levels. Lastly, each phage type could be individually packaged at a concentration of 1.1010 PFU/ml in pharmaceutical grade glass vials containing 1 ml of each phage solution and then submitted to the following quality controls: sterility, phage identity, phage purity (level of residual bacterial DNA and proteins, level of residual reagents added during the purification process and level of residual endotoxins), phage titer and pH. Among these controls the level of endotoxins is critical: it is evaluated using the LAL assay (Thermo Scientific, 88282) according to the manufacturer. For each patient, three to six active bacteriophages were sent to our pharmacy. Our pharmacist (GL) prepared each cocktail in a volume of 30-50mL under sterile condition just before the administration. During each surgical procedure that consisted in arthrotomy-synovectomy in the two patients with *S. aureus*

Figure 6: Process in France to obtain the use of bacteriophages as compassionate use in patient presenting a bone and joint infection requiring a salvage therapy

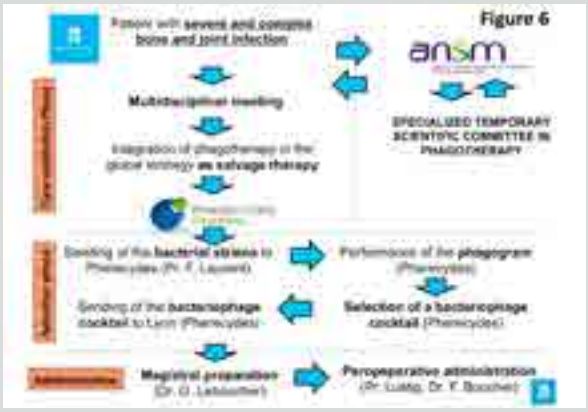
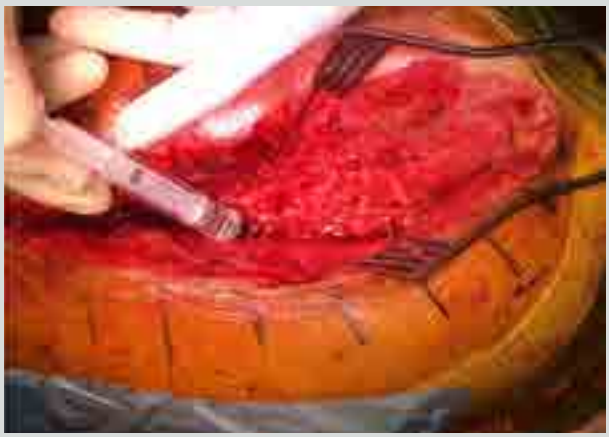


Figure 7: Peroperative administration of a cocktail of bacteriophages, after joint debridement and arthrotomy-lavage, just before joint closing in a patient with relapsing prosthetic joint infection



prosthetic joint and debridement and bone resection in the patient with pelvic osteomyelitis, the surgeon directly applied the phage solution at the site of infection. For patients with prosthetic joint infection, after arthrotomy-synovectomy and reduction of the bacterial inoculum, the joint was surgically closed tightly, just before the phage administration in the joint (Figure 7). For the patient with pelvic osteomyelitis, the phage solution was locally administered after bone debridement. In this latter patient, four local applications were performed before performing the skin and soft tissue reconstruction.

Future directions for phagotherapy in the field of BJI

There are a number of factors favourable to the use of bacteriophages in France: (i) the production of bacteriophages with a high level of purity, according to European GMP; (ii) agreement of the French National Agency for the Safety of Medicines and Health Products (ANSM) for the use of bacteriophages as compassionate therapy; (iii) motivation of infectiologists and orthopaedic surgeons from a reference centre that recruits a large cohort of patients, including more

complex cases that required salvage therapy and (iv) motivation of pharmacists that agree to take responsibility to combine the bacteriophages and to manufacture a magistral preparation (cocktail of bacteriophages) just before the peroperative administration. From our point of view, eligible patients for phagotherapy as salvage therapy are only patients evaluated in reference centre, and each case as to be discussed with the ANSM and its dedicated committee. It seems reasonable to limit this treatment to (i) patients with prosthetic joint infection at high risk of complications in the case of explantation, and for whom suppressive oral antimicrobial therapy is not an option and (ii) patients with chronic osteomyelitis due to multidrug-resistant pathogens (such as pan drug-resistant *P. aeruginosa/S. aureus/ Enterobacteriaceae*) with limited therapeutic options and for whom a skin and soft-tissue reconstruction is required. However, it is now time to consider phagotherapy in patients with less severe BJI, in adjunction to the conventional therapies (surgery and antimicrobials), to increase the success rate of this difficult-to-treat disease, especially in patients with *S. aureus* prosthetic joint infection, long bone osteomyelitis and diabetic foot osteomyelitis. Some previous data indicated that bacteriophages can penetrate biofilm, and could be nice candidates for such patients *(11-15)*. Crucial preclinical data as part of the PHOSA consortium (www.phosa.eu) will be available in 2018–2019. We will determine the bacteriophages activity in a large collection of *S. aureus* isolates responsible for BJI. We will also evaluate the in vitro activity of bacteriophages in bacteria embedded in biofilm, and in animal models of implant-associated osteomyelitis. Finally, clinical academic studies including patients with *S. aureus* prosthetic joint infection requiring prosthesis exchange and in patients with S. aureus diabetic foot will start at the end of 2018. Finally, it would be of interest to have available bacteriophages that are active on enterobacteriaceae and coagulase-negative staphylococci (such as *S. epidermidis*), as these pathogens are frequently involved in patients with BJI and are more and more resistant to conventional antibiotics.

Conclusion

Phagotherapy is an emerging option for patients with bone and joint infections. The compassionate use of bacteriophages manufactured in France according to European GMP has just been used as salvage therapy in selected patients with complex BJI due to *S. aureus* or *P. aeruginosa*. Preclinical data and data from clinical trials will help to expand the use of bacteriophages in this difficult-to-treat disease. ■

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Lyon Bone and Joint Infection Study Group:

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Dr Fabien Boucher is MD in Plastic Reconstructive and Aesthetic Surgery Department at the Croix Rousse Hospital and University of Lyon since 2012. He particularly specialized in reconstructive surgery and microsurgery. He is the author of scientific publications about microsurgical and reconstructive topics. He published a textbook atlas of perforators arteries, a surgical tool for new trends

in microsurgery. He is member of CRIOAc Lyon.

Dr Guy-Charles Fanneau de La Horie has over 20 years of experience in the pharmaceutical and biotechnology industries. Before joining Pherecydes Pharma, he held a number of international management roles, including posts at Schering-Plough, Biogen and IDM, in both Europe and the United States. More recently, he was managing director of PathoQuest, a company developing a NGS-based infectious disease diagnostic technology. Between 2006 and 2013 he was the CEO of Neovacs and coordinated the 2010 IPO. Prior to that, he spent eight years with Biogen, where he set up and ran the subsidiaries in France and Benelux. During his time with Biogen he managed the US$ 700 million salesforce in the United States and held Europe-wide responsibilities for marketing, regulatory and medical affairs. A graduate of the National Veterinary School in Lyon, France (1982), Dr Fanneau de La Horie also holds an MBA from INSEAD, awarded in 1988.

Dr Jérome Gabard joined Pherecydes Pharma in September 2009 to build the Company's expertise in preparing bacteriophages for therapeutic applications. He has been the architect of its first phage therapy clinical trial: PhagoBurn (2015–2017). Today he focuses on developing personalized medicinal treatments with phages. Since his research and development work in biotechnologies at DuPont de Nemours (1989–2000), Dr Gabard has been Life Sciences Director in The MarkeTech Group, where he developed sales and conducted strategic and operational marketing studies.

Professor Frédéric Laurent is full Professor in Clinical Microbiology at the University of Lyon. He is the chair of bacteriology department – Institut for Infectious Agents in Lyon North University Hospital, and co-director of the French National Reference Centre for Staphylococci. He is in charge of the diagnosis of Bone and Joint Infections in Hospices Civils de Lyon and is a member of the board of the CRIOAc Lyon (Regional Reference Centre for Complex Bone and Joint Infections). As co-PI of the team "Staphylococcal pathogenesis" in the International Centre for Infectiology Research (INSERM U1111 - CNRS UMR5308 – ENS Lyon – University of Lyon), he has a special interest in the understanding of the switch from acute to chronic BJI, in the prevention and treatment chronic forms and in the development and implantation of innovative technologies for BJI diagnosis.

Cindy Fevre, a microbiologist and expert in bacteriology, is the R&D manager at Pherecydes Pharma. She is in charge of leading the phage development effort of the Company. Her international experience (UMC Utrecht, the Netherlands) led her to manage R&D collaborations with an industrial perspective through multidisciplinary and multicultural research team. Her PhD carried out at Pasteur Institute (Paris) was on the antibiotic resistance, virulence and genetic diversity of Klebsiella. She has been working on other major human pathogens (listeria, Staphylococcus aureus, *etc.) and acquired a solid knowledge of the biology and genomics of bacteriophages.*

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