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PARTICIPANTS:

SESSION 6: Research Gaps:

Moderators:

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Bacteriophage Research Updates from NIH Preclinical Services:

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CRISPR-Assisted Engineering of *Staphylococcal* Phages to Combat Antibiotic-Resistant Infections:

ASMA HATOUM-ASLAN
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Respiratory Delivery of Bacteriophages:

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Optimizing Bacteriophage Regimens to Treat Pulmonary Infections:

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Panel Discussion:

JANE KNISELY, Moderator
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PARTICIPANTS (CONT'D):
Four-Dimensional Therapeutics:

ANTHONY MARESSO
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Opportunities and Challenges for Phage Therapy of *M. abscessus* Infections:

REBEKAH DEDRICK
University of Pittsburgh

Development of a Bacteriophage Host Immune Response in a Lung Transplant Recipient Receiving Phage Therapy for MDR *Pseudomonas aeruginosa* Pneumonia:

JENNIFER DAN
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Phage Selection for Reduced Virulence in Bacterial Pathogens:

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Panel Discussion:

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SESSION 7: Workshop Wrap-Up:

GRAHAM HATFULL
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Closing Remarks:

SCOTT STIBITZ
CBER

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P R O C E E D I N G S

(10:00 a.m.)

DR. KNISELY: Okay. So, I have 10:00, so we're going to go ahead and get started. Good morning and welcome to our third and final day of our bacteriophage workshop. I've been so excited to attend the talks the past couple of days and I hope today will continue in a similar vein. Today we're going to be focusing on state of the science and research gaps in phage therapy.

And without further ado, I'm going to turn it over to our first speaker, Dr. Erica Bizzell, who's an AAAS science and technology policy fellow in our branch. And I was also remiss not to acknowledge all the hard work that she had done as a member of our planning committee. So, Erica, please go ahead.

DR. BIZZELL: Thanks so much, Jane. And I will go ahead and start sharing my screen. I'll go ahead and get the presenter mode. Okay. Hopefully, everyone can see that all right?

DR. KINSELY: Looks good.

DR. BIZZELL: Okay, perfect. Thank you, everyone for joining me today for this presentation. My name's Erica Bizzell again, and I will be presenting to you some of NIH's Preclinical Services support of phage research. And I'd like to start by first thanking Dr. Erin Zeituni within the branch for the extensive work that she also has done to help develop this slide deck.

I'll start by giving a summary of what I'll be presenting today. I'll start with a background on NIH's Preclinical Services, or PCS. And then I'll give an overview of our *in vivo* phage therapy results through the PCS contracts available, including lung infection models and other infection models including UTI, thigh, and bacteremia models. And then I will give an overview of some of the future steps that we're taking in phage-related PCS contracts.

You may have seen this slide earlier in this workshop, but I'd just like to reiterate the mission of the National

Institute of Allergy and Infectious Diseases, NIAID, which is to provide leading research to understand, treat, and prevent infectious, immunologic, and allergic diseases. One of the mechanisms by which this mission is accomplished is through the Division of Microbiology and Infectious Diseases, or DMID's Preclinical Services. This is a suite of contracts that supports anti-infective product development, and this suite spans the entire product development pipeline from MIC testing all the way through GMP manufacturing.

This is intended to be a gap-filling service and not intended to take a product to licensure. And unlike our grants, this is actually not a cash award but a free service that is provided. It's meant to lower the risk and advance promising discoveries along the product development pathway.

Eligibility for these contracts and for these services is very broad, including researchers from academia, non-profit organizations, industry, and government, and domestic and foreign institutions. There's no

need to have any prior NIH funding. And the process to request these services is very simple and available year-round. It really just starts as an expression of interest email to our Preclinical Services contacts, two of which are listed here. Drs. Erin Zeituni and Erica Raterman. For more information on our Preclinical Services, I do encourage you to view Monday's focused breakout session on the PCS contracts when that recording becomes available.

So, today I'll be addressing how our Preclinical Services have been used to inform phage therapy development, specifically, our *in vivo* efficacy models. DMID's Preclinical Services has provided *in vivo* phage efficacy studies since 2015, including five phage product developers, 13 phages or phage cocktails that have been investigated, and eight different infection models that have been used to investigate these cocktails. The study results from these experiments have helped to support critical activities such as publications, FDA submissions, as well as

grant applications.

There are several considerations to—or parameters to take into consideration for *in vivo* efficacy models for phage studies, some of which we've even heard throughout this workshop in some of the human studies that we're presenting. The model parameters that have—some of the model parameters to be considered are the impact of the immune system, model immune system, the length of infection model, endpoints such as survival and CFU, and combination of phage therapy with antibiotics.

Some of the phage parameters that have been considered or that are necessary to be considered for phage studies are use of cocktails versus single phages, dosing routes and schedules, as well as phage kinetics and distribution. And since NIAID's Preclinical Services fit study designs specific to requestors based on needs, the phage efficacy studies have had diverse designs and parameters.

I will also note that these studies

in these models that we do use have been optimized primarily for small molecules. We don't have—in the past, we haven't had many contracts that have been specific to phage. But even with that, we have been able to find some very promising results, which I'll present now. I'd also like to note that if you ever do use our Preclinical Services, we will never present your study results without receiving your express permission, which we've done so for this presentation.

So, I'll start by highlighting some of the outcomes from our *in vivo* lung infection efficacy models, and I will—just to orient you to this table, each row corresponds to a requestor's Preclinical Services request, and these are all separate experiments. As you can see, there have been a number of different phage cocktails or formulations, including single phages, cocktails, combinations with or without antibiotics, different routes and intervals of delivery, including intratracheal, intraperitoneal. I hope you can see my cursor now. Intratracheal,

intraperitoneal, intravenous, and subcutaneous, bi-daily or twice a day, or three times a day.

And then the models that I will be highlighting today are focused on two bacterial strains, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. And as you can see, there are a wide range of outcomes. The most successful outcomes that we've seen through these studies that I'm highlighting have been through this 120-hour *Pseudomonas aeruginosa* lung infection model, whereas, we saw no significant improvement in the lung CFU, but trends towards improvement in both of the studies in the *Klebsiella pneumoniae* models. And I will highlight examples from both of these.

So, to start, in this first study, we saw that monophage therapy was able to enhance a suboptimal antibiotic dose in a multi-day lung infection model. In this model, neutropenic mice were infected intratracheally with *Pseudomonas aeruginosa* and then treated shortly after infection with a single phage

intratracheally with or without meropenem treatment. And then the mice were followed over the course of seven days when a final timepoint for survival would begin. And as you can see, when the mice were treated with just phage, we do see—we're able to see a significant increase in survival of the mice by one day, but all of the mice did succumb to infection by day two of infection.

However, when treated with an intermediate or lower dose of meropenem, you will see that mouse survival was able to be increased to 120 hours from 24 hours. However, when combined with phage therapy, this antibiotic treatment was significantly improved, and phage was able to enhance this antibiotic dose.

So, next, highlighting one of the 24-hour models or 26-hour models, we saw that treatment with a phage cocktail in this model in combination with an antibiotic provided no significant decrease in lung CFU after 24 hours. So, within this model, neutropenic mice were intranasally infected with *Klebsiella*

pneumoniae. And two hours post infection, phage were—at 2 hours and 14 hours post infection, phage were administered by bi-daily either subcutaneously, intraperitoneally, or intravenously with or without tigecycline. And then at 26 hours, the lung tissue was harvested and bacterial counts were enumerated.

Now, if you look at this central line here, this corresponds to the baseline level of bacteria at 2 hours post-infection, and we see with this orange line that when mice are treated with a placebo or saline with phage buffer, that significant bacterial outgrew. However, when treated with tigecycline at 50 mg/kg, there is a very significant decrease from baseline levels of bacteria. When that tigecycline dose is cut in half to a suboptimal dose, you see that there are baselines levels, there's no decrease in the baseline levels of bacteria.

So, looking at these three groups here, the three bars here, all of the following groups were phage treated with or

without antibiotic. So, subcutaneous intraperitoneal or intravenous treatment without antibiotic and with antibiotic. Now, as you can see, phage treatment alone was not able to improve—or was not able to decrease bacterial counts. We still see outgrowth of bacteria. However, there is a trend towards improvement when treated with the suboptimal dose of tigecycline in combination with phage therapy. However, not a significant improvement.

So, those are just a couple of the lung models that we have. And I'd like to highlight that the models that we do have, again, they haven't been optimized for phage therapy. Most of them have been used specifically or optimized for traditional small molecules and antibiotics. But we still are able to find some very interesting and promising results. And I'll go into some of what this has informed us a little bit later in this presentation.

Now, we'll look at some of the other infection models offered by our Preclinical

Services to investigate phage therapy, including our UTI, thigh infection, and peritonitis efficacy models. In this table, this top row corresponds to the UTI experiment. These two middle rows are using the thigh infection model, and the bottom is the peritonitis model. And again, several different phage formulations with or without antibiotics. Phage cocktails with or without antibiotics. Different routes of delivery and again, these different animal models. I don't know if you can see my cursor now, but UTI, thigh infection, peritonitis.

And while there are different outcomes, again, I would like to highlight these bottom two studies because both of them have been—were using the same phage cocktail as well as the same bacterial strain in the thigh infection and peritonitis models. However, we see very different outcomes.

I'll start with this peritonitis model. In this model, immunocompetent mice were used and infected intraperitoneally with *Klebsiella pneumoniae* and then treated with or

without tigecycline. And we see subcutaneous intraperitoneal, or IV treatment again. And then at eight days post-infection, blood and tissue were harvested, and survival of the mice was logged. And as you can see, when mice were treated with just saline, all of the mice succumbed to infection by day two. However, when treated with a suboptimal dose of tigecycline, there is a slight increase, but not significant increase in survival of the mice.

However, we see that all combination and monotherapy treatments resulted in significant protection of mice regardless of the route of phage delivery. And I'll also highlight that there was a tigecycline control here that also led to significant full survival, 100 percent survival of the mice.

So, now when we look at the same phage cocktail used in a different model with the same bacterial strain, we saw that the phage cocktail therapy with or without antibiotic treatment was actually unable to decrease bacterial counts in this 26-hour

thigh infection model. Here, neutropenic mice were infected intramuscularly in the thigh with *Klebsiella pneumoniae* and at two hours post-infection, much like the lung infection model I showed before, phage were administered as well as tigecycline, and this was a bi-daily dose, so, twice a day. And then tissue harvested—the thigh tissue was harvested, and I will go through the results here.

Once again, this base level line is the baseline level of bacteria. And when treated with saline, again, we see growth of bacteria. However, with treatment with a tigecycline dose, an effective—or significant decrease in bacteria levels. When that dose is lowered, again, we see growth of bacteria and much like before with phage treatment alone, which corresponds to each of these three high-bars, subcu, IP, or IV delivery, we still see growth, significant growth of the bacteria above baseline. And unlike before, we don't see a trend of protection or decreased growth of the bacteria when phage and antibiotics

were given in combination.

So, there are very different—we can't draw definitive conclusions as to why the therapy was not effective, as these are different models using neutropenic vs. immunocompetent mice and very different parameters. However, one of the things that we do want to do with our Preclinical Services is provide a means by which we can actually compare between our different models.

So, as I just highlighted, we did see differences in our phage efficacy *in vivo* despite comparable phage cocktail and bacterial strains used. And our PCS contracts to date can provide a means to investigate a variety of parameters including outcome measurements, immune state, dosing, etc. But it can make it challenging to identify optimal model parameters and compare across the different phage treatments.

Ultimately, DMID aims to provide predictive models, and therefore, we seek to understand why our treatments do or not work. And to date, as I mentioned before, most of

our PCS contracts have not been optimized for investigation of phage products. So, one of the gaps that we identified through all of these studies was that we were lacking PCS services that provided analysis of phage kinetics and distribution to support interpretation of efficacy study results.

And that's why we developed a task order that will soon be released and funded to evaluate phage kinetics and distribution. But as I said before, some of the lessons that we learned from these studies was that though we have endpoint data such as CFU and/or survival, this doesn't provide any significant insight into phage distribution and kinetics. And this task order plan is to investigate phage therapy efficacy, pharmacokinetics, and biodistribution in a multi-day *Pseudomonas aeruginosa* lung infection model with and without infection.

We hope that this will—we expect this to improve our understanding of the relationship between phage distribution and kinetics and phage efficacy. And we'll

evaluate implementation of this in our other models.

The structure of this task order is to characterize the phage dose and efficacy in this lung infection model, characterize phage biodistribution and kinetics, and there are also options within this task order to possibly characterize phage products in thigh and bacteremia models—the phage distribution in kinetics.

Our goal, as I said before, is to respond to the critical research gaps identified in our suite of services in order to provide more useful knowledge for our investigators. And we hope that through this task order we're able to do just that.

So, with that, I would like to thank you all for your attention and I'd like to encourage anyone who's interested in hearing more about how NIAID can help support your drug development program, to reach out to our Preclinical Services contacts, and they’re listed here again. Thank you very much.

DR. KNISELY: Thank you, Dr. Bizzell.

I think we have time for maybe one question and then we'll move on to the next speaker. So, we have a question from Sabrina Green. Are you correlating *in vitro* phage and antibiotic combination efficacy to the *in vivo* result? And could the phage and antibiotic combination not be an optimal combination, might you have antagonism instead of, you know, additive or synergistic effect?

DR. BIZZELL: So, for this contract for this task order, we're not looking at combination with antibiotic and phage. But that is something that we hope to do in the future where, yes, we hope to be able to provide that service for requestors in the future to look at that *in vitro* and *in vivo* as well.

DR. KNISELY: Right, and in some of the data that you presented in those models, we didn't do extensive analysis of that. And so, I think that is, as Dr. Bizzell described, some of the focus of maybe some future work. So, thanks.

So, our next speaker is Dr. Asma

Hatoum-Aslan from University of Illinois. And are you there, Dr. Hatoum-Aslan?

DR. HATOUM-ASLAN: I am here. Can you hear me okay?

DR. KNISELY: Wonderful, yes, we see you. Please, go ahead.

DR. HATOUM-ASLAN: Okay. So, I'm going to share my screen. Let me start the slide show. Hide the panel. Does that look okay?

DR. KNISELY: Yeah, looks good.

DR. HATOUM-ASLAN: Okay. Thank you. So, I'd like to start by thanking the organizers for putting together this fantastic workshop. I have learned a lot coming from the bench side learning about what goes on to get things off to the clinic. I'm also thrilled and excited to be here to tell you all about what we've been doing on the bench in regards to *Staphylococcal* phage engineering with the hope that others can potentially use CRISPR to engineer their phages if they wish to do so.

And so, we have been hearing so far about a lot of the challenges and limitations

to the, I guess, routine implementation of phage-based therapeutics. And so, they fall into at least these four categories that I've listed here, and I am sure I'm missing some categories. But some of the challenges that we are particularly interested in addressing have to do with effectiveness and potentially safety concerns. One issue that might impact the effectiveness of phage therapeutics, of course, is the ability of bacteria to either quickly evolve resistance or many bacteria are already armed with a great deal of immune systems, defenses to protect them against the phages, which will ultimately threaten to undermine phage-based therapeutics.

Under safety concerns, we've heard a little bit about how toxins can potentially be problematic in phage preps and dangerous. But also, I'd like to pull up another big research gap and a potential safety concern regarding the unknown genetic content that is found in any given phage. Really, about half of their genes have undefined functions yet. And so, those could potentially lead to unexpected and

potentially detrimental consequences down the line. And so, for us it's important to push forward phage biology to help shed light on the functions of those different uncharacterized genes.

So, research in my lab, we're really engaged in three related efforts that go towards helping to alleviate some of these concerns, particularly the first two that I've mentioned. So, we're constantly going into the wastewater and looking for new phages that can infect our study organisms. Obviously, every new phage is potentially a new weapon that can be used in a therapeutic application. But also, for us, we're interested in new phages because each one is a new tool that we can use to probe and understand the different immune systems that bacteria have.

And so, a lot of our effort is actually geared towards number two over here. We do a lot of basic research trying to understand the different mechanisms of immunity in bacteria, because as I mentioned earlier, these potentially can undermine phage

therapy.

And so, wherever it makes sense along the way, we are also interested in developing new technologies to help facilitate and promote the use of phage-based therapeutics. So, today, I'm going to focus on a technology that we developed in the lab based mainly on some basic findings along the way studying CRISPR systems, and so, we've learned how to use CRISPR systems in native *Staphylococci* to edit phages. And here just to give the credit to the people who did most of the work I'll talk about today, Naeem Bari, a graduate student in the lab who is very close to graduating and a former master's student, Forrest Walker.

And so, I guess I don't need to go into too many details about the problems associated with *Staphylococci* and these are our model organisms that we study, *Staphylococcus aureus* and *epidermidis*. The "S" in ESKAPE pathogens is *Staph aureus*. So, we all know about that one. But it was mentioned a little bit in at least one talk yesterday

about the problems associated with *Staph epidermidis* in causing implant- or device-related infections that are difficult, if not impossible to treat with conventional antibiotics because of the ability of *Staph epidermidis* to form biofilm. And so, *Staph epidermidis* in some is also very problematic. And so, these two present opportunities for us to utilize *Staphylococcal* phages either in place of or in combination with antibiotics as we've been hearing along the way.

Just a quick overview of the known *Staphylococcal* phages. They all belong to one of these three groups that I'm showing here. And I'm showing sort of the older names. I know that the ICTV is changing some of the names of these families, but I'll go with the conventional older names. So, they're either the smaller *Podoviridae* variety, *Siphoviridae* or *Myoviridae*, so the largest type. And so, this holds true for *Staphylococcal* phages and it might not be a generalization we can make with other phage groups. But for *Staph* phages, we do know that the *Siphoviridae*, those with

Siphoviridae morphology are all lysogenic.

And so, they have integrases in their genomes, so, they can integrate into the bacterial chromosome. They carry between one a five virulence factors in their genomes, and they’re also known to mobilize pathogenicity islands. So, they're definitely not suitable at least in their wild-type form for use in therapeutic applications. But we're left with the *Myoviridae* and *Podoviridae*, which are the strictly lytic varieties. They're great for therapeutics because they kill the host within minutes of infection. But that makes them also very difficult to study and genetically manipulate.

And so, that is what we have been interested in, and so, we, in studying CRISPR realized that this particular CRISPR type, native to *Staphylococcus epidermidis* is actually very useful in phage editing. And so, I wanted to say that if you're interested in using any CRISPR system to edit a phage, you must be aware a little bit about the mechanism of how the system works.

And so, I'll give you the quick rundown on how this CRISPR system works. It's a Type III-A. It's also called CRISPR-Cas10. And so, the one in *Staph epidermidis* has three spacers, which are basically small snippets of DNA that are derived from invading nucleic acids, and they're integrated in between these repeat sequences, the white boxes. And there are nine CRISPR-associated genes that are required for the system to work. And so, in the defense part of the mechanism, if spacers are already acquired, the first step is CRISPR RNA biogenesis where the repeat spacer array is transcribed into this long precursor, and then it goes through a couple of processing steps to generate the final mature CRISPR RNA species. And each of those dictate a single target for destruction.

And so, in the interference stage, there are five proteins encoded in the CRISPR locus that come together in different stoichiometries with the CRISPR RNA. And together they form a Cas10-Csm complex. And this complex can wage a two-pronged attack

against an invading phage. And for these systems, it's actually the RNA of the phage that is detected. And once that happens, Csm3 in the complex will introduce cuts into the phage RNA. Cas10 will cut the DNA. And there's another subunit that's not part of the complex, it's Csm6. It also acts on the phage RNA. And so, the end result is degradation of phage genetic material.

And there are additional layers of complexity to this system. But this is sort of the basic rundown. And, for example, in our basic research, we've been able to add another layer to the system. We found that it doesn't work on its own. It actually relies upon degradosome-associated nucleases to carry out different steps. So, for example, PNPase is important for CRISPR RNA processing. RNases J1 and J2 are essential, actually, for interference to work against phage.

But along the way in studying the system, we realized that it is special and it's kind of different from the other CRISPR types. There are currently six CRISPR types

that have been described. And what makes it special is its robust activity. And what I mean by that is if you a standard plating assay where you have an overlay of a lawn of cells and you spot some phage on top of the cells, if there's a CRISPR system that's a Type III targeting the phage, you will get no plaques. So, it's very difficult for phages to naturally escape the effects of this immune system. And so, we're able to harness this, you know, potent immunity as sort of a counterselection mechanism in a phage editing approach for these lytic viruses.

And so, I'll give you an example of how we, for example, can generate point mutations in a lytic phage using Andhra as an example. And I just wanted to give applaud to these little *Podoviridae* phage. I'm a big fan of them. In *Staphylococcal* phage cocktails, typically you'll find *Myoviridae* are being used. They're more common in collections. And they tend to have a broader host range. But the *Podoviridae* are also great. And I know there's increasing interest in kind of finding

more of those and adding those into the cocktails.

And I like these phages because definitely they're strictly lytic. They're pretty lethal. So, for example, Andhra infects the cell and within 30 minutes, makes 10 copies of itself and kills the cell. But it does all of this with just 20 genes and less than 20,000 nucleotides. So, they're minimal, and they have minimal uncharacterized genetic content that we need to sort of define.

And so, in the process of generating point mutations in Andhra, the first step using the system is to create and test what we call a targeting strain that directs—ideally, you want to target the region of interest that you want to introduce the edits. And in this example, we're using a targeting strain that uses the native CRISPR system in *Staph epidermidis* RP62a. It's in the chromosome. And all you need to do is just add a little plasmid with a single repeat and a spacer targeting the region of interest in the phage that you wish to introduce edits. And then you

do a simple plating assay. So, for this example, we're targeting *polA*, the polymerase gene.

And so, I show you an example where two spacers are designed against the exact same region, but they're reverse complements of each other. And only one of these is going to work because this system relies upon recognition of the mRNA. And so, basically this is just an example of the plating assay where in a negative control there's no CRISPR expressed or no CRISPR protection. You spot the dilutions of Andhra on top of the lawn of cells and you get a bunch of plaques.

Spacer A2, if that's present, it affords complete protection against the phage. So, that's designed correctly where the CRISPR RNA is complementary to the mRNA of the phage. Spacer A4 doesn't work at all and it's just the reverse complement of A2. And so, this is just a quantification of those results, but it illustrates how important it is to design the spacer properly in order for the system to work. And so, we've developed a short Python

script that takes into account all of the targeting constraints and requirements for Type III CRISPR systems that you're freely able to use, and you just input any gene of interest, and it'll find all the permissible targeted regions, basically. And so, you're free to use that if you'd like.

But once you've confirmed that you have, you know, created a targeting strain that works against your phage, the next step is to create the editing strain. It's exactly the same thing as the targeting strain except that you introduce also your donor DNA construct, which contains the region that you wish to edit. So, 250 nucleotides of homology flanking either side of the targeted region. And there's where you want to introduce your edits.

And so, for this example, we're just introducing as a proof-of-concept silent mutations across the targeted region. So, those are in magenta. And then also we introduce a cut site for screening purposes. And so, the idea here is when you co-culture

together the phage with the editing strain, all of the wild-type phage are just going to be eliminated by the CRISPR system. And if there is one phage in a million that happens to recombine with your donor construct, it'll be able to escape the effects of CRISPR, and it'll become a plaque on a plate.

And that's exactly what we see. And so, this is just data showing plaque forming units per milliliter where we co-culture together the phage with the editing strain for 2 minutes out to 12 hours. You get a bunch of plaques on the plate. If you do the same co-culturing with the targeting strain, importantly, you get no plaques on the plate at all. So, there's no plaque bar anywhere.

And so, having plaques at this stage is really encouraging because it means the phage have escaped CRISPR. And so, the next step is, of course, to purify the phages and then either sequence across the targeted locus, or here I'm showing a restriction digest where you just show that every plaque that we pick has acquired the mutations and

the digest site, the cut site.

So, we've been able to use this to edit early genes, late genes in *Podoviridae* phages and *Myoviridae* phages of *Staph aureus*. And this works beautifully every time. Every time we pick a plaque off an editing strain, it has the desired edit. So, it's a very powerful technique. We can also—because *Staphylococci* tend not to have native CRISPR systems, we've also been able to clone the entire CRISPR system into a plasmid, introduce that into CRISPR-less list *Staph aureus* and then use that system to edit *Staph aureus* phages. So, it's very versatile.

And I'll leave you to this publication here if you want to see some other sort of variations on that approach. Now I want to take a couple of minutes to talk about something that's not in that paper. We've also been able to generate deletions in nonessential loci in a single step, and that's with the targeting strain. So, the idea here is if you are targeting a region that is not essential for phage replication, it's feasible

that some random mutant phages might have lost that region and then you can select for those.

And so, pretty much every time we've tried targeting a gene, we never get any plaques on the plate. And so, we realized, okay, we have to find something that's nonessential, and the most obvious, I guess, region when you're looking across this genome for a nonessential piece is this little intergenic region here about 100 nucleotides. And so, Nayeem went ahead and designed a spacer that targets that region, and then you do indeed find plaques on a plate when we stopped the phage. Many orders of magnitude fewer than if there's no CRISPR protection, but that was very encouraging.

And so, we went ahead, and this is a blowup of that intergenic region. And we sequenced a handful of those phages that grew on the plate. And in fact, they were able to acquire random deletions in the targeted region. They went from two nucleotides out to 100 nucleotides. But all of them overlapped partially the region that was targeted by

CRISPR. And so, you can create random deletions in nonessential loci without even having a donor construct in a single step.

And so, then we started to ask the most basic question about some of these uncharacterized genes here in gray. Are these essential for phage replication? And to answer it, Nayeem went ahead and created these targeting constructs against each of the genes that are in gray and then looked for plaques on a plate. And we could not find any. So, that allows us to hypothesize that probably these genes are essential for phage replication.

So, this is a very versatile system. And I just want to highlight some of the things that we're interested in doing with it. We're definitely interested in characterizing genes with unknown functions. And also, getting more insight into the requirements for phage host range. We are also interested in minimizing phages using this system. You can tighten intergenic regions or delete genes that are not essential for its lytic activity.

And the reason for minimization is to remove sort of some of the unknown genetic content, but also to be able to add more capabilities. So, we've heard about this. This is not something new. Where you can basically add a CRISPR system into a phage if you have a lot of space. Or if you have a little bit of space, enough for another gene, you might consider adding an enzyme that degrades biofilm. You might consider if you want a phage reporter as well as a therapeutic, you can add a reporter gene, or there are a bunch of other things that you can potentially add into a phage to give it additional capabilities and make it more lethal.

And then finally, I was happy to hear a few people kind of touch on this idea. I know that specificity of phages is definitely one of the benefits of phage therapy. But that's a double-edged sword because then it causes a huge time lag between the time that a patient comes with a strain that needs to be treated to the time where you can identify phages that can be used to treat

it. And so, ultimately, I can imagine like this idea having a lot of merit where if you develop these predefined phage cocktails where we can fine-tune the host range to basically target a panel of pathogenic species that typically occur together and have those ready. And also, they would have more defined genetic content, so less unknown material to worry about. And those could be utilized right away to treat a patient who is in need.

And then I just want to take a last minute or two to walk you through my advice if you wanted to edit phages that infect your favorite organism. The first question I would ask is does it have a CRISPR system in it already? And if the answer is yes, the next question is, immediately, is the system active? So, there are a number of reasons why there may be a system present, but it may be inactive. One possibility is it's a remnant of a system and part of it got lost. Another possibility it's increasingly—we understand now that there are these anti-CRISPR proteins that are everywhere. They're in prophages and

lytic phages and plasmids that basically block CRISPR's activity. And so, even if you have a system, you should not assume that it's active. So, you would either need evidence from the literature or your own evidence to show that it is active to use it for editing.

And if the answer is yes that it's active, then you can do the two-step process that I just talked about where you create a targeting strain and test it. In order to test it, you must know what CRISPR type you're dealing with. Types I, II, and III are preferred because I would say together they're the most widely distributed, but also they have all been used in the literature to edit phages. So, we know that they work.

So, I talked about a Type III system, which targets RNA in the phage. And so, there are specific targeting requirements there. But Types I and II target DNA. So, there's more relaxed requirements. But also, they have a protospacer adjacent motif requirement. So, you must be familiar with the targeting requirements in order to create the

targeting strain. But once you have done this and tested it, and you show at least an order of magnitude reduction in phage plaques, then you can definitely go ahead, introduce your donor DNA construct, and see if you can screen for your desired edited phage.

So, the other possibility in answering the first two questions is the answer is no, there is no native CRISPR system or there's a system, but it is not active. In which case, I would recommend that you go to the database that I have cited here. It's my go-to database to screen for the types of CRISPR systems that are present in organisms whose genomes have been published. It's the CRISPR Cas database. And so, if you can identify a related organism with a CRISPR system, preferably Type I, II, or III, then you can move forward with that one. You'd have to put it into a plasmid and introduce that into your organism and then go through these steps. Okay.

So, real quick. I'd just like to wrap up to acknowledge again, this is my lab

group at Illinois. I've already acknowledged Nayeem and Forrest, a former master's student who contributed to this work. Also, thank you so much to NIAID for funding, as well as NSF and Burroughs Wellcome Fund. Thank you all for your attention, and I'm happy to take questions now or at the panel.

DR. KNISELY: Thank you so much. That was a great talk. We do have a number of questions that we'll get to during the panel discussion. Right now, we're going take a short break. So, everybody be back by 10:50 Eastern time. Thank you.

(Recess)

DR. KNISELY: Okay. Welcome back. I see that our next speaker, Dr. Reinhard Vehring from the University of Alberta is at the ready. He'll speak to us about pulmonary delivery of bacteriophages. Please go ahead.

DR. VEHRING: Thank you very much. Just checking, can you hear me well and see my slides?

DR. KNISELY: Yes, we can.

DR. VEHRING: Okay, very good. So,

thanks again for the invitation. This is perhaps a little bit of a topic that is outside of the core interests of many phage biologists and physicians. But I think it should actually be considered a little bit more.

This is about respiratory delivery of bacteriophages, and I will address three different areas. Here's a quick outline of my talk. The first part, I will talk about respiratory phage delivery via nebulizers and focus on the question of can we just use any nebulizer or is there a little bit more to it? The next topic is about delivery to animals. We need that, of course, in toxicology and efficacy studies. And there, I would like to focus on the question of how to get a sufficient dose in particular into small rodent airways because that's often what we do first, and it's more tricky than often thought. The last topic is about dry powder delivery. And there I would like to answer the question of whether there is a suitable dosage form perhaps for developing countries here.

Okay. Let's start with the nebulizers. So, phage delivery via nebulizer is routinely used now, it's a familiar tool in most hospital settings. We do have nebulizers around there. And the advantage, of course, is that it requires comparatively little specific formulation development. So, it can be kept in the liquid formulation, and other than perhaps some buffering and so forth, there is not that much to do compared to say converting it into a dry dosage form. And we have also many studies in the literature that we can successfully deliver phages with nebulizers into the lung. And there, nebulized phages have advantage in clinical trials, as you can see here.

Nevertheless, I think it is very important to consider that there are losses during nebulization that can be substantial. So, what are the typical losses, and is the type of nebulizer important here? And for this, I would like to point out a study that I find quite important done by Nicholas Carrigy in 2017, where he compared various types of

nebulizers, and I'm just showing you two here. Here's a vibrating mesh nebulizer and a jet nebulizer.

The phage that was used in this study was phage D29, which is an anti-tuberculosis phage. And you can see here for the jet nebulizer, we have upon nebulization, very substantial inactivation. More than 3-1/2 logs of titer loss. So, that's 99.98 percent inactivation, whereas for a mesh nebulizer here, the inactivation was still present but at a much lower level. So, that, of course, has quite drastic effects on the actual delivery rate, right? So, here if you look at the jet nebulizer, there are only a few 10,000 phages delivered per minute here. Which in most cases, I would think is not enough to do any—make any difference.

So, whereas for the vibrating mesh generator, you get a quite substantial delivery rate here. So, yes, I think the first question we can also already answer here. It makes a difference what kind of nebulizer you're using because there's a difference here

of a factor of 6,000 in the rate of delivery here.

Now, of course, we would like to find out why that is so. Why does it make such a big difference to use one nebulizer versus the other? And we looked into a likely reason here for the titer reduction with the jet nebulizer. And what we came up with is that it most likely is caused by repeated baffle impaction and re-nebulization.

So, these jet nebulizers work by taking fluid out of a reservoir. So, here is your phage suspension in blue. You provide a high-pressure air jet and a venturi effect would suck up the liquid and then atomize the liquid. However, this type of atomizer produces droplets that are far too large generally for inhalation purposes. And most of these will be separated off in this baffle system that you have here. And only the small ones that can make that kind of tortuous path through the nebulizer and then will be inhaled. Or here in this setup, they were

captured on a filter for analysis.

What happens to the other larger droplets is that they are impacted on these baffles and then they drip off back into the reservoir. And then the cycle starts again. So, they will be re-nebulized or re-atomized as you can imagine.

Now, that leads for the emptying of a typical dose through a nebulizer to a very large number of re-nebulization cycles. So, Nicholas Carrigy here did a little mathematical model where he came up with an average number of nebulization cycles that phages would undergo in this type of atomizer or nebulizer, however you want to see it. And he saw that they will be on average nebulized about 100 times. Of course, that puts a huge cumulative stress on the phage. And that, I think, is most likely the reason for this very drastic inactivation that we have seen there.

Now, why is the vibrating mesh nebulizer in this case putting less stress on the phages? The droplet production mechanism is different here. So, here is the nebulizer

here on the right. There's a reservoir here and it just puts a liquid volume onto a mesh plate. So, here is the channel that goes onto this mesh plate that you see here, which is just a plate with a hole—with a large number of orifices. Here's an SEM image of how these orifices look like so they're tapered. It's a very small orifice, so people will think, okay, that puts a lot of stress on phages if by shear stress in that case if I put that through.

But it turns out that the big advantage is, of course, is that I only do that once instead of 100 times. And that most likely will be advantageous for phage delivery. So, the type of mechanism that you have in a nebulizer plays a role, of course.

Okay. So, now if you look into the literature, there is more work on jet nebulization losses. And you can see that there is a huge difference here. So, the D29 phage that I have shown you have more than 3 log loss. Whereas, other phages were less susceptible. So, what we can learn from that

is that phage inactivation in nebulizers is phage-specific. So, that's nice if you work with a robust phage, but in a way, you cannot know that. So, you would have to test it up front.

Further factors of importance that can be derived from the literature is that the composition of the liquid feed suspension is important. If you have, for example, different levels of purification, that can lead to phages latching onto bacterial debris like is shown in this TEM on the side here. And also, the salt content and particularly ionic strength may make a difference. Level of agitation, temperature, and humidity during the administration. And in my opinion, what is very important is the presence of protein binding surfaces, so the kind of vials and tubes that you're using is important, and exposure to air liquid interfaces. Because all of that can lead to aggregation. So, you have something like this where you get a clump of phages in the end that is not very effective anymore.

So, let's go to the next topic, and that would be respiratory phage delivery to animals. Often we do that to rodents, and the first thing I'd like to point out it's far more difficult to deliver aerosols to rodents than to humans because of their very small airway dimensions, and they are obligate nose breathers. So, that means you cannot go through the mouth, you have to go through the nose of the animal.

I would advise against using intratracheal insufflation. That is really not a good way of evenly distributing an aerosol in a rodent lung. We have run some studies that are unpublished because they were done in industry, where we have shown that this isn't very—where we have seen that this isn't a very repeatable way of delivering to the lungs of rodents.

So, the way to go is nose-only inhalation systems, in my opinion. But there the downside is that they are relatively slow in delivering the dose to the animal. And you're constrained by oxygen requirements, so

there has to be enough airflow so that the animals do not suffocate, and you have a limited exposure time because even after training, these animals will get very likely agitated after a while. And so, you cannot go beyond a certain limit of time.

If a nose-only inhalation system is coupled with a nebulizer, then you have to live with the fact that most of the aerosol droplets generated by a nebulizer are simply far too large for mice. So, that means careful optimization of animal dosing systems is essential. This is not something that is done easily. So, here I would certainly suggest that it's a good idea to consult with experts in the field and have done that for a long time.

So, I'm going to show you a bit of an example of how this was successfully done again for D29 here. This was a study where we were trying to do prophylaxis of a *Mycobacterium* infection via inhalation of bacteriophage D29 again. And for that, we needed quite a bit of phage into the lungs of

the animals. So, here is the system. So, the vibrating mesh nebulizer was put into an antechamber to separate out the large droplets. And then the aerosol was fed into a plenum, and then mice can freely inhale from this plenum through the nose into their mouth, of course.

There was a mathematical model developed that optimized all the various parameters of the system, various liquid and gas flow rates. And in the end, we were able to achieve an improvement of four orders magnitude for the lung dose, but it took quite a while to get it right.

This is how the system looks on a bench, a bit organized, and I apologize for the picture here. But what I wanted to point out is that we got to the point where the resulting dose to the mice was so large that we got on average one phage per alveolus. And that was the whole point, because we were thinking that that would be necessary to achieve a prophylactic effect.

Another thing I'd like to point out

is that one of these dosing tubes here was replaced with an actual filter. The filter was hooked up to a breathing simulator, which is— in this case can be accomplished with a syringe pump. And this, I think, is necessary to verify the amount that was actually given. So, that's another thing that I would like to suggest is that when you deliver via respiratory routes, you have to find a way to verify what the actual dose was that was given to the animal, because otherwise, there are just far too many unknowns here that could mess up the dose.

Okay. This is just to show that this actually worked kind of nicely. So, we've seen a prophylactic effect of first giving phage D29 and then challenging with tuberculosis afterwards. But I think this was only possible because we got a significant amount of phages delivered to the lung.

The other thing I'd like to show here or point out again is that it's easier to deliver to humans. So, if we think about nebulization to humans, we can easily get like

200 times the number of phages into a single alveolus compared to the mouse. And that looks kind of promising here for bacterial eradication.

Now, we have done some work in vaccine delivery lately where we have switched from nebulization in the nose-only inhalation system to dry powder delivery. And we found that that is far easier. The reason for that is that you can actually deliver particles that are specifically tailored to the needs of the small rodent. So, we can make particles that are close to nanoparticles, so around one micron or so. So, we don't waste most of the dose on droplets that are far too large anyways for the mice. So, this system is described in the publication here, if you're interested.

And that brings me to the last topic. That is about dry powder delivery. Why would we even do that? So, the idea is that for global health applications, we need thermal stability because of the absence of a reliable cold chain. And it needs to be robust

on delivery, suitable for resource-poor settings. And also, we would like inexpensive simple devices that are ideally usable by untrained folk.

So, I've just shown you two possible options here. There's a passive dry power inhaler here, that’s a DPI. All of these are marketed already, so, there's little regulatory concern here. And this is a nasal delivery device here that could be easily adopted for phage delivery to the nose.

Now, of course, you need to first make a dry, respirable dosage form. And what we have looked into is can we use spray-drying to do that? That's actually already has been—it has been demonstrated for over 10 years now that that is possible. So, here's the seminal study cited here. This was the type of dryer that was used, but afterwards we have used different spray dryers, so, it's possible with a whole range of different spray dryers. The idea is to have a glass stabilizer. In this case, mostly trehalose and perhaps a few other excipients here that provides the phage

stabilization similar to what is happening in desert plants pretty much.

And then you make it more dispersible by putting something onto the surface of the particle that decreases the cohesive forces between particles. So, that is all well-known how to do that. And then you have to select a process that doesn't put too much thermal stress on the phages, and select an outlet condition that provides low water content in the powder, because that is correlated with a high glass transition temperature. And these kind of systems have been developed over the last 10 years I would say, and we are now at the point where they provide stability at up to 40 degrees Celsius for a year.

Now, here just back to that first study. These are the phages that were used here, individual *Burkholderia* phages and some cocktails. This is the powder morphology that we have seen. So, separate particles—point out this scale bar here so they're fairly small. So, easy to inhale. And processing losses were

less than one log, so, that seems to be acceptable. But again, I'd like to point out that the process loss depends on the phage species. It's not generally possible to come up with a standard formulation platform, a standard process, and a standard delivery device for all phages. So, this has to be done fairly early on in the development process for the specific phages or cocktails that you are considering.

DR. KNISELY: You have three minutes.

DR. VEHRING: And here is the…

DR. KNISELY: Three minutes, Dr. Vehring. Thank you.

DR. VEHRING: Yeah. Yeah, I'm getting to the end of it. So, here's the result of lung delivery from a simple dry powder inhaler. So, this is a capsule-based device. You put the phage into the capsule, the phage powder and pierce the capsule with these little push buttons here and inhale.

Generally, commercialized products have total lung masses, or total lung doses in percent of the fill mass here that are around

the 30 percent range, perhaps even less. So, this was an outstanding result where we had very good delivery to the lung. However, we are not interested primarily in how much mass of powder we're delivering. We would like to know what the actual titer is that we put into the lung so this is the total lung dose now in plaque forming units. From a single capsule inhaled, you get into the range of 10 to 7th to 10 to the 8th, which should be probably in the range of efficacious doses for many applications.

So, that looks very feasible. And that already brings me to the conclusion. So, for the three different topics. Respiratory phage delivery via nebulizer, that's actually quite well developed. However, what perhaps is a bit underappreciated sometimes is that phage nebulization can cause substantial titer loss, especially if you use the wrong nebulizer, and to the point where actually your therapy or study fails, because you're not delivering anything anymore of substance. The titer loss is phage specific, so, there's no general

recommendation that can be given. And, of course, from that it follows that before using phage therapy, the loss in the nebulizer should be characterized because otherwise just flying blind.

Respiratory phage delivery in animal models is more difficult. So, that's the moment where you would pick up the phone and ask the specialists. Animal dosing systems should be carefully designed and optimized for this purpose, and the actual delivered lung dose should be assessed experimentally and verified. And the take home message from our work here that I can convey is that dry particles are far easier to administer than liquid droplets, which gives us some additional motivation to develop them.

Dry powder, spray-dried phage delivery is feasible. It hasn't been—it hasn't made its way into product development yet. But we know now bacteriophages can be spray-dried into a thermal stable dry powder. And we can deliver it efficiently, and dry dosage forms are obviously far better suited for developing

countries where most of the infectious disease burden is that we're actually targeting.

And that brings me to the end. Thank you very much. I'd be happy to answer any questions.

DR. KNISELY: Thank you so much. I learned a lot. Very enlightening talk. I think we'll move on to our last talk before our panel discussion. And definitely we'll have some questions for you, Dr. Vehring, at that time.

So, our next speaker is Laurent Debarbieux from Institut Pasteur. He will speak to us about optimizing bacteriophage regimens to treat pulmonary infections.

DR. DEBARBIEUX: Hello, thank you, for the invitation. And thank you for having me giving this talk about what we've been doing the past few years on optimizing the bacteriophage regimen to treat pulmonary infections.

So, the basic principle of phage therapy is very simple. I think everyone understand that.

DR. KNISELY: We see—I'm sorry to interrupt. We see you and we hear you. We don't see slides, if you are trying to share slides.

DR. DEBARBIEUX: I apologize. I apologize.

DR. KNISELY: Okay. We can see them, but not yet in presentation mode. There we go.

DR. DEBARBIEUX: There we go. All right.

DR. KNISELY: Okay.

DR. DEBARBIEUX: So, let's go back to this basic principle of phage therapy with phage targeting the bacteria, and hopefully you get a treatment out of it. So, the challenges in this field, and amongst many questions, you know, which phage amongst many phages you can use? And once you have identified the phage you want to use, then the next question will be the dose, the administration, and the frequency. And all of these questions are important to answer to make it a real treatment for our patients.

So, in all, we are developing animal

models to try to answer some of these questions. And over the years, we've been using different pathogens, and in particular, *Pseudomonas aeruginosa* and *E. coli* with their specific phage. And we have been working with different mice models including wild-type and MyD88. So, immunocompetent and deficient mice.

So, one thing that you have to clearly perceive when we are talking about pulmonary infection and in particular acute infection is these models are extremely acute, in terms of you have a very narrow window of infections. So, I can report here the dose of the bacteria we used to initiate these infections, so, here it's for the case of *Pseudomonas aeruginosa*. And clearly, one thing to discern is the dose we are using in the lab on a regular basis that allow 75 percent survival in 24 hours, and 10 percent in 48. But I'd like to point out that if you reduce this initial dose by only two times, then most of the mice will survive.

As you increase this dose twice as much, then most of the mice will die within 24

hours. So, the window of using this type of model is very narrow. And so, if you do not infect enough the mice, the mice will survive, so they are basically not infected. So, the window to test the efficacy of the phage treatment is pretty narrow.

So, with this scheme here you have the survival of the animals that have been untreated in red here. And those that have been treated by the phage PAK_P1, so a single phage, and both the bacteria and the phage are being introduced, being administered to mice by intranasal administration. And you see the result that there is a dose-dependent efficacy of the treatment here, the more you increase the dose of the phage the higher is the efficacy of the treatment.

But it was a starting point, and we wonder after getting those first results whether this will be also true for any phage and bacteria pair. So, we moved to *E. coli* as a second pathogen. And we observed exactly the same limitation with the acute infection, meaning that the window of observation is also

very narrow with this particular pathogen. And so, despite this short window, we could also demonstrate that a single phage treatment was as efficient as antibiotic treatment. So, both being able to rescue the deadly infected mice.

So, with that we wanted to move with the more dynamics aspect of the treatment and tried to dig into the different parameters that are actually limiting, eventually limiting the efficacy of the treatment. But with the dynamics, we used the bioluminescent bacteria to record over time the infections. And you can on this picture here on the left column the group of mice that are being infected and non-treated with phage. So, the progression of the infection is quite rapid. And on the right column, you have another group of mice that have been treated by the phage at two hours after the bacteria. So, we don't see any difference between the groups at two hours, neither at four hours, but at six hours you can already see the difference between the two groups, meaning four hours after the phage administration.

So, the good thing about the bioluminescence, that data, is that you can plot them on a time scale. And you can follow the same animal over the course of the experiment. So, here you have the group of untreated animals that will ultimately die from these experiments. While the group of animals that have been phage-treated control the infection and survive the challenge.

So, another thing that you can do with this bioluminescence data is that you can exploit them further by making a correlation between the luminescence and the CFUs. So, if you sacrifice mice at several time points, count the CFU and make a correlation to the luminescence that you have recorded from the live animals, you can make this correspondence between luminescence and CFU.

So, we exploit this tool to ask and question the efficacy of the treatments in different conditions. And so, here we go back to the *E. coli* infection model, with strain 536 and the phage 536_P1. Still a single administration of the phage, but here we have

been looking at different treatments, meaning looking at intratracheal and intravenous phage administration, and two different phage have been also tested. So, we did frequent luminescence recording and at a couple of time points we sacrificed the mice to get the PFU and CFU counts.

So, a lot of the data has been, you know, published in this bioRxiv, you can have access to it. And beyond this quite huge experiment, we can see the number of mice that we've been using is quite important here. We have been providing this very recent data to a team of colleagues here working in another unit, in biomodelling, trying to modelize the treatment and appreciate what would be the difference of efficacy between the different groups of administration of the phage in the different doses.

So, here is the snapshot of all the mice that have been used for this work, with the different groups from untreated to IV with the low dose of phage, IV medium, intratracheal low, medium, and high. And the

one thing that we have been clearly identifying from this set of experiments, is the dotted line here in the middle. That's actually is right at the value of the amount of bacteria that is the threshold above which the immune response will be saturated. So, any amount of bacteria that will be higher than 6.8 log 10, then will lead to the death of the bacteria while any other amount of bacteria then the immune system in these immunocompetent mice, will take care of the infection by itself.

So, using this set of data and our colleague from the (inaudible) models developed this type of model with different compartments with what we call susceptible bacteria that are susceptible to the phage, of course. We define a refractory population of bacteria that combined both the bacteria that become resistant to the phage with also that are inaccessible to the phage. And we define also compartments of bacteria that are infected but not producing phage, and all the others that are infected and producing phage.

On top of it, we needed also information about the phage itself and its distribution to the animal that is not infected and also infected. So, we did this experiment of distribution looking at the lung distributions of phage. From following intratracheal administration, you can see that the amount of phage in the lungs is quite stable over time, while after intravenous here in red, rapidly the phage reached the lung, but then quickly disappeared from these uninfected mice.

If we look at the infected mice, since there are already bacteria in the lungs, so there is no surprise with the fact that the values here are much higher than the values that you have in uninfected mice. Of course, the phage amplifies, and it's there at a lot higher level following the intratracheal administration, while in the intravenous administration, the phage still is pretty high but has a lower amount of phages recovered over time in the lungs of these mice.

So, with all of these data, our

colleagues developed a mathematical model, and here is a snapshot of the match between the actual experimental data, you see represented here in circles, and the solid lines that are presenting the prediction by the mathematical model. And so, we have the color code that correspond to the different groups.

And so, I show you here only 18 profiles, but we have the profiles for all the mice that we were using in this treatment. And this is to show you that the modeling is actually fitting pretty well the experimental data, with the line that is crossing almost the best fit with the dots.

And on top of this model that takes into account the different compartments and different treatments, we also developed part of the model that is dedicated to the survival prediction. And this is the green line that you have on all of these graphs. And this was derived from the mathematical model and provides information on the chance of survival from the mice that have been treated with these different conditions and administration

route.

So, once we have the model set up, then we can perform simulations. That's the whole value of this model. So, here we have a simulation that is focusing on the variation in the inoculum. So, 10 to the 6th, 10 to the 7th, and 10 to the 8th. And I remind you that the 10 to the 6th mice will most likely survive the infection by themselves without any impact of the treatment. And with 10 to the 8th, mice will be rapidly infected and may be much more challenge for a treatment.

And if we look at the different population of bacteria, susceptible, refractory, and etc., what you can see here is that the largest difference is on this population of refractory bacteria between the three simulations. Where basically with 10 to the 6th CFU that this refractory population doesn't really count for anything. And at 10 to the 8th, this refractory population is actually preventing the success of the treatment. And while at 10 to the 7th you can see that the different routes of

administration can actually succeed in treating the infection.

So, the bottom line here, the message is that by increasing the bacterial inoculum, you decrease the treatment success because of the growth of this refractory population of bacteria. So, here we focused on this condition, which would be that this (inaudible) condition and from that (inaudible) condition, we ask whether the model will predict what will be the variation if we change the burst size of the phage. So, the very same data here is still in the middle, and they have been altered with the phage that is producing about 500 new particles per cycle.

So, if we introduce into the model variation such that the burst size is decreased by 10 or is increased by 10, what would be the consequence for the prediction? And what we observed from these different graphs here, is that lowering the burst size is actually increasing the differences between the treatments. I'll remind you here the

different color coding is corresponding to the different treatments, either IV, IT, and low or high dose of phages. So, you have a bigger discrepancy, bigger differences between the treatment regimes in these conditions with your lower burst size compared to your higher burst size, where basically all the treatments that you're using will produce the same effect.

Now we are starting from this condition with a low burst size and from which we are going to ask what would be the consequence of changing the lysis rate? And so, this is the regular lysis rate of these phages, having been estimated from the data obtained *in vivo*. Here is the lysis rate in the middle that has been increased by three-fold. And here at the bottom, is the lysis rate that has been calculated from *in vitro* experiments. And what you can see with this variation of the lysis rate is that more you increase the lysis rate, more you actually compensate for the low burst size, which is expected if you think about where the phage

infected the bacteria. So, this is one of the values of using a mathematical model to predict what would be the variation of the treatment, the efficacy of the treatment by using different phage.

So, now I'm switching to the last topic that I wanted to mention during this talk is the role of the host during the phage treatments. And as mentioned before, the acute model means that if you have a low infectious dose as a starting point, then the mice will defend themselves without the help of the phage. So, to question how much of the immune system will take part of the efficacy of the phage treatment, we use the MyD88 mice, which is a mutant that is a knockout for that gene that is an intermediate protein that is central to the signaling of pathogens in mice. So, MyD88.

And when we perform the phage therapy treatment in these MyD88 mice, we observe that the efficacy of the treatment was pretty moderate even and pretty low compared to the wild-type mice. But looking closely at

the data, at the bioluminescence data here, you can see that, of course the untreated control has an uncontrolled increase of bacteria. But for the phage-treated group of mice, you can see that between 8 and 24 hours the amount of light has been reduced. So, this reduction means that the phage has been doing its job, the phage has been killing the bacteria. But unfortunately, after 48 and 72 hours, the bacteria took over.

And so, we looked at the bacteria here at 24 hours and found that these bacteria were actually becoming resistant to the phage. And the failure of the treatment in MyD88 mice was caused by growth of phage-resistant bacteria in this particular (inaudible). And from this data, we actually defined what we have called immuno-phage synergy that is taking place in the immunocompetent animals, where both phage and the immune system are taking care of the bacteria, meaning that the phage rapidly decrease the load of the pathogenic bacteria. And whether the bacteria defend themselves by growing phage-resistant

variants doesn't really matter because the immune system has already been primed, and then this immune system can take over the whole residual bacteria and then proceed with the full treatment. So, we…

DR. KNISELY: Three minutes.

DR. DEBARBIEUX: Yeah, I'm almost done.

DR. KNISELY: Thank you.

DR. DEBARBIEUX: So, we also collaborated with our colleague, Joshua Weitz in Georgia Tech that modelized this phage therapy efficacy in immunocompetent and immunodeficient mice, and here you see on the left side the simulation in the MyD88 mice where the immune system is completely flat, so it was not stimulated. And you can form the three populations, the phage population that is increasing because it is killing the susceptible bacterial population here. And at the same time, there is a growth that is uncontrolled, the growth of the resistant bacterial population over time.

And once again, once you build the

mathematical model on the system, you can make predictions, and the prediction here is about the level of immune differentiation that you could have in the animal to succeed or fail for the treatment. And the prediction simulates that with 50 percent reduction in the immune activation then this treatment will still work, while with higher deficiency, then the phage treatment will fail. And currently in the laboratory, we actually are working on toward ways to prevent the growth of phage-resistant bacteria by tweaking phage or immune systems.

So, the conclusions of this brief talk would be the single administration of phage is more efficient by intratracheal than by intravenous. But we haven't yet tested the multiple administrations. So, maybe multiple administrations by intravenous may compensate the lower efficacy.

The simulations by our mathematical model suggest that the characteristics, the phage characteristics have a low or to moderate impact of a treatment success. But we

need to confirm this by experiments, those are predictions, simulations. We need now to go back to the bench and prove or disprove this prediction. Clearly, in immunocompetent animal you need or you—the efficacy of the phage treatment, you're relying on the synergy with the immune system.

And we are now investigating what will happen in intermediate immune responses. And, of course, another benefit of the mathematical modeling is that this reduces the need for experiments, on mice experiments, to evaluate different phage-bacteria pairs. So, our current challenge is to question whether or not the current mathematical model will be adaptable to other phage-bacteria pairs and further model infection sites when targeting other diseases.

And with that, I would like to thank the people that have been doing the work and in particular Raphaelle Delattre who has been working a lot with this project, as well as fundings and collaborators. And I thank you for your attention.

DR. KNISELY: Thank you, Dr. Debarbieux. So, with that, I would like to invite all of this morning's speakers to join the panel. Turn your cameras back on. We have a number of questions in the chat and those of you in the audience, please feel free to enter additional questions if you have them for the panelists.

So, I'll start with a question that came in for Dr. Bizzell. How do you control for phage lysis of bacteria after sampling in the animal models? Does the phage titer from samples in the compared *Klebsiella* experiments that you showed differ?

DR. BIZZELL: So—oh, okay. I'm unmuted. So, for those two experiments, so those two model systems, at least the beginning phage titers are comparable, 10 to the 9 phage. We don't test for phage titers after sampling. But they are homogenized and diluted and plated soon after harvest and held on ice during that processing. But there could be still some lysis we don't control for that for this—for those two experiments. But the

initial titers are comparable.

DR. KNISELY: It reminds me of the question that came up during yesterday's clinical panel, right? With the same question kind of coming up there is what happens in your samples after you've collected them? And I think it's tricky. Certainly, there are ways like Dr. Schooley mentioned yesterday. You could use a neutralizing antibody to your phages. But that seems a bit laborious, and of course, you'd have to have them for each of the phages that you're using, so.

Okay. So, let's move on to a question for Dr. Hatoum-Aslan. So, do you know if CRISPR/Cas systems are considered more robust in *Staph aureus* and other Gram-positive organisms compared to Gram-negatives? The person asking the question has had much more trouble finding *Staph* phages compared to phages for many Gram-negative bacteria.

DR. HATOUM-ASLAN: Yeah, so I commiserate with that observation that it is challenging to find *Staph aureus* phages. Also, for us relying on, you know, looking for

plaques. And we repeatedly go to the wastewater to look for phages. *Staph epidermidis*, it's pretty easy for us to find phages, but *aureus* is more challenging. It's probably not because of CRISPR, because if you look in the databases for sequenced *Staph aureus* isolates, most will not have a CRISPR system. And so, but I do believe that in part, at least, it could be due to other immune defenses that *Staph aureus* has. And so, that's the other thing that we are interested in the lab is identifying and characterizing new immune systems. And we discovered one that seems to be pretty prevalent in *Staph aureus*. I have a bioRxiv preprint out on it, Bari, *et al.*, if you want to look at that. But it's basically a single gene that provides very robust immunity to a diverse array of phages. And so, we're calling it Nhi for a nuclease-helicase-mediated immunity because those are the activities of it.

But there's that in *Staph aureus* strains. You can find many Nhi homologues, but I'm sure there are plenty of other immune

systems that we have yet to discover. And so that could explain why it's been difficult. And I don't know if other people have had those challenges. I don’t want to say I'm happy to hear that at least somebody else is struggling with this, but it's good to know that it's not just us.

DR. KNISELY: Thank you. A question for Dr. Vehring, have you compared different commercially available mesh nebulizers? And did they behave equally?

DR. VEHRING: The answer to that is no. We have not compared different commercially available mesh nebulizers. In this study, we were primarily interested in picking nebulizers that have different operating principles.

DR. KNISELY: Mm-hmm.

DR. VEHRING: So, we picked one as a typical representative of that group of nebulizers. But and also we—what I haven't shown in this talk is that we also looked into the Boehringer Ingelheim system where you have two impinging jets pretty much. So, that's

again a different type of nebulizer. But no, we haven't done that. But I would say it's probably a good idea because of the presence of different surfaces, right? We know that phages can be inactivated in contact with surfaces, and not all of these mesh nebulizers will have the same materials in their contact surfaces, right? So, it's probably still a good idea to run a test, whether your particular nebulizer that you're interested in using is actually inactivating the phage.

DR. KNISELY: Thanks. And kind of an extension of that question. Have you done a direct comparison between a nebulized phage and versus phages that are delivered intratracheally and potentially even intranasally?

DR. VEHRING: No. We have not used the insufflation anymore. First of all, the device is no longer available. I think the Penn-Century device that was used in the past for intratracheal insufflation is no longer available now. And my experiences in the past in my industry time with intratracheal

insufflation was negative enough that I didn't even consider it.

You know, to me, because we're interested in, for example, evenly distributing aerosol into the lungs, right? Into all areas of the lungs. So, intratracheal insufflation in my opinion is a little bit like throwing a bucket of paint into a room on the floor and expecting that all the walls are perfectly painted afterwards. There is no real inhalation in that system, right? So, you're actually putting a little spray into one particular spot in the trachea of the rodent or wherever. And then you pretty much hope that that will somehow evenly distribute in the lung. And I don't think that that is a particularly good approach.

DR. KNISELY: Dr. Debarbieux, have you had experience in those different delivery methods intranasal, intratracheal? I assume you have not used nebulizers in your animal experiments, but have you observed any differences, or do you have recommendations on sort of optimal delivery mode looking at

pulmonary infection?

DR. DEBARBIEUX: Well, what we have been looking at, indeed, is intratracheal and intranasal, we haven't used any nebulizers. That's because that expertise is not so easily available. And what we found is that given the acute infection systems that we're using because the bacteria are so pathogenic, that if you use the intratracheal, the infection will start extremely rapidly. And so, that would be extremely severe for the infection.

When you do compare the intratracheal administration of phage to intranasal, we haven't found any major difference in the efficacy. As long as we use a dose that correspond to—you know, we couldn't identify even one log difference between intratracheal and intranasal. You would imagine that if you do intratracheal you will have more phage in proximity to bacteria, but, this is not actually exactly true. As, you know, mentioned by Reinhart, if you dump locally a lot of phage, that doesn't mean that the phage will be evenly distributed. And so,

that means that the efficacy of the treatment might be not so important compared to intranasal, where the distribution is a little but upper and where the repartition of the different particles will be more even to the lungs. So, we couldn't find any major difference between the two intranasal or intratracheal. And for ease of use, intranasal is much more recommended.

DR. KNISELY: Yes. That's been our experience as well. We've done a little bit of work not on phages, but on the bacterial delivery in our Preclinical Service contracts and have found very little difference, actually.

Okay. So, let's see. Oh, sort of a related question for maybe Dr. Debarbieux and Dr. Vehring. Have you looked for systemic presence of the bacteriophage after delivery via these different routes of pulmonary delivery?

DR. DEBARBIEUX: Yes. We looked and we couldn't detect any significant amount of phage in kidney, livers, and other organs that

we were looking at. So, meaning that out of 10 mice maybe we could have detected phage for 3 or 4, but nothing that is really significant. So, maybe this is, you know, related to the dose that we use, which is not extremely high. The highest dose we used was 10 to the 8th. So, maybe if people are using more, a higher dose, they may recover some phages in the other organs. But we basically didn't detect much in the other organs.

DR. VEHRING: Yeah, that doesn't sound surprising to me because of the size of the phage, right? So, based on the size, there should be virtually no transport from the lung to the systemic spaces, unless there is some active transport, which cannot ruled out. There is a paper, a 2014 paper by Semler and Dennis that showed that if you develop a phage intraperitoneally, so, you would get—you will get some phages into the lung, right? So, they make it into the lung, but they may have replicated there. So, you may get a very small amount of phage into the lung that then replicate when they find bacteria into the

lung.

But what they also found is that IP delivery was not sufficient in actually suppressing in this case, it was a *Burkholderia* infection. Whereas, inhalation delivery did. So, I think this area isn't fully researched yet. So, the transport in between the different compartments in both directions is not fully clear to me.

DR. KNISELY: Okay, thank you. A question for Dr. Hatoum-Aslan. How common are phage recombinases encoded by *Staph* phages? And do you know of examples of integration of lytic phage sequences into genomes beyond CRISPR?

DR. HATOUM-ASLAN: Yeah, that's a great question and something you definitely want to consider before using a phage for therapeutics. And so, to my knowledge, the type of phage that infects *Staph*, which typically have the recombinases have also *Siphoviridae* morphology. So, all *Siphophages* to my knowledge do have recombinases, which indicate that they're probably going to be

able to integrate into the genome. And so, those are typically considered not suitable for phage therapeutics at least in the whole phage kind of type therapeutic.

In terms of the lytic varieties, the *Podoviridae* and *Myoviridae*, to my knowledge, there are no reports in the literature of either in part or in whole integrating into a genome. I know that doesn't mean that it doesn't ever happen, but I'm not aware of any reports on that. But you also still need to consider the possibility that these phages might mediate generalized transduction by mispackaging a plasmid or some portion of the genome of the bacteria. And that's kind of a danger with all phages.

I did want to mention one of the other things that makes the *Podoviridae* very attractive in my opinion at least for the *Staphylococcal Podoviridae* phages is that they rely on a protein priming mechanism for their replication. So, their genome is linear and it has a terminal protein on either end on the five-prime ends. And not a whole lot is known

about how packaging works in those phage. But I'm assuming that that terminal protein is required for DNA that does get packaged into the phage capsid. And so, I think it is much less likely for the *Podoviridae* to mediate generalized transduction. And there are examples of the *Myoviridae* allowing for generalized transduction.

And so, I kind of went away from the question there. But I guess the main point is that I think the lytic variety are generally considered safe.

DR. KNISELY: Thank you. So, Dr. Debarbieux, you talk a bit about the role of the immune system in clearing the infection, you know, cooperating with the phages to clear infection. So, what is your opinion on the use of neutropenic mice to study phage infection, to model phage infection—I'm sorry, phage treatment of infections?

DR. DEBARBIEUX: The thing is the neutropenic mice have been like a standard model for evaluating antibiotics, for example. And so, that's why people have been using

these models so far for evaluating phage. And it's only part of the answer. You cannot build full information on neutropenic animals because then you're missing part of the system that will take place in infected humans, okay?

So, I think the lesson to learn from this is that the sequential intervention of the different actors in the process of the infection. So, in the limit of the animal infections, you initiate the infection with the bacteria and rapidly you treat with the phage. Because in this, you know, lung infection, the infection is very acute. If you delay the treatment, then there is no chance—or almost no chance that the treatment will succeed, because the damage that the bacteria is causing to the lungs is too severe and so the mice cannot recover.

So, once you start the treatment with the phage very early, you will rapidly decrease the load of the pathogenic bacteria. And then whatever happens, whether active bacteria become resistant to the phage or stay susceptible, doesn't really matter because the

immune system will take over the rest of the bacteria. So, that's where the two systems actually act together. So, they act one after the other.

Now, I don't recall clear data from other studies where people have been showing that on a much delayed infection, meaning that you infect let’s say, day one with the bacteria and then you wait for two or three days before treating anymore with the phage, what would be the value, the role of the immune system in this animal where the immune system has three days to increase its capacity to manage the bacteria.

So, it depends also on the pathogenesis of the bacteria. The bacteria has some ways to escape the immune response, etc., etc. So, it's very limiting to concentrate on only neutropenic mice. I understand that sometimes it's necessary to just demonstrate the proof of concept that the system works, let’s say. But it won't be very helpful for translation to the patient if you keep ignoring what part of the immune system can

actually do in the treatment. Because if we just ignore that, then we could have simply concluded from our own experiment that the phage treatment never worked, while it does work in an immunocompetent animal.

So, that's something that people should be aware, that sometimes I understand it's necessary to use neutropenic mice, but you should not just stick to that and go back to a more realistic situation where the immune system is also solicitated by the infection itself.

DR. KNISELY: Thanks. And we have a related question for you. Do you consider—did you consider to estimate the removal of phage by the host immune system, and is it important for calculation of phage dosing?

DR. DEBARBIEUX: So, in the model, what we appreciated from the biodistribution of the phage following intratracheal or intravenous administration, we could calculate the decay of the phage and rely on this very short time window. Now, these experiments are only for 48 to 72 hours, so we have been

looking for longer time period. But from this experiment, we actually indeed introduce in the model parameters that will take into account the decay of the phage from the system. And but we haven't yet appreciated in the simulation whether an improved decay rate will affect the output of the treatment. That's actually a good question. We could look at that.

DR. KNISELY: Thank you. And Dr. Vehring, someone has asked a question wondering what the value of conducting a nebulized phage study in an animal model is given the difference in—that you noted, the difference in anatomy and physiology between them and humans. So, how does it sort of translate to how you might apply it in a human infection?

DR. VEHRING: Yeah, that's a little bit outside of my core area of expertise, but I can give you my thoughts here. So, the route of administration is obviously important in this case. So, it makes a difference in how we deliver phages. And we are not going to go

into non-human primates right away or something like that. So, we will have to go into animal models that are less characteristic of the human airways than we would like to. But it's still, it's obviously better than nothing, right? So, my take on this is that we should be as characteristic or as close to the human system and as close to the actual mode of delivery as possible without doing some really heroic efforts, let's put it that way.

And in that sense, I think rodent models have their place. I mean, that is, it's a good first start. But I'd like to add something, because I saw in the Q&A here, is that people are pointing out that these micro sprayers are still available. That is, it fits into the topic here because these micro sprayers do not really simulate the mode of delivery very well. The spray that is produced at the end of this little syringe pretty much has a particle size or a droplet size that is far greater than what you ever would inhale normally.

So, that is not simulating as well as you could respiratory delivery into the lungs. So, that's perhaps one more reason to consider a more representative system. So, the point is, to summarize this again, is that I think because we have seen in the literature that the mode of delivery is important, and it's also, of course, important for toxicology concerns, we should run animal models that use the same mode of delivery as much as possible as what is intended later on in the patient, of course, right? And yeah, some of this is a bit tedious. But I think it's well worth the effort because the mode of delivery in this case can make or break the therapy.

DR. KNISELY: Thank you. In the last few minutes, before we break for lunch, I would just like to open it up to the panel members. If there are any concluding remarks you'd like to make or any questions that you see in the chat that you would like to answer live. I'd also like to invite you to answer the questions that we haven't gotten to in the panel in the chat during our lunch break. So,

anything that anybody would like to add or address at this point?

DR. BIZZELL: I can just address the one question about whether nebulization is encouraged. It just says, someone mentioned that sub-cu, IP, and IV phage administration seemed to be inadequate for murine models of intratracheal and intranasal bacterial infections, and whether we've encouraged collaborators to explore nebulized phage for pulmonary infection. And we don't—our Preclinical Services don't currently include nebulization as an option, although we do have IT as a proxy, or intratracheal. Through this discussion, of course, we've learned that, who knows whether it's the exact proxy, but we also would hesitate to make any judgments as to whether one route is superior based on this limited experimental data, so.

DR. VEHRING: Yeah, if I can add a little bit to this. In the end, I think we have to go back to where the global need is, right? So, nearly all of the infectious disease burden is in developing countries. And

good luck with a nebulizer there or with dosage forms that have no thermal stability. So, I think we see that in the current COVID crisis, that even if there is a vaccine available, it cannot be delivered sometimes because the internal structures in these countries is not possible to accept it, right?

So, we should aim in particular if we are interested in fighting infectious diseases, we should aim for dosage forms that are actually suitable for global delivery. And I think that will be—will have to be dry dosage forms, and there should be a little bit more emphasis, perhaps, on developing these things early on. Because you cannot switch to a dry dosage form at phase three or something like that. That's not going to happen.

DR. KNISELY: Yeah, a very good point. And Dr. Yen addressed that yesterday in her talk about the cholera phages she's working on.

DR. VEHRING: Right.

DR. KNISELY: Okay. Well, I see that it is noon here on the east coast of the

United States. And so, I think with that we'll send you to lunch. And again, thank you for great talks and a great discussion. And thanks to the audience for all your wonderful questions.

When we come back at 12:30, my colleague Dr. Joe Campbell also from NIAID will continue moderation of our research gap session. Thank you.

(Recess)

DR. CAMPBELL: So, hi, this is Joe Campbell again. The recording just started. I'm about to introduce Anthony Maresso from the Baylor/TAILOR Group. Anthony has been mentioned several times, and his group has been mentioned several times in the clinical sessions as a sources of the phages, and today Anthony will be giving a talk on—entitled "Four-Dimensional Therapeutics." So, Anthony, are you ready?

DR. MARESSO: Yes, let me share my screen. Are you able to see that, Joe?

SPEAKER: Yes. If you want to put it on presentation mode?

DR. MARESSO: Okay.

SPEAKER: There we go.

DR. MARESSO: Okay, and you're able to effectively hear me?

SPEAKER: Yes, sir.

DR. MARESSO: So, thanks, Joe. The title of my presentation is "Four-Dimensional Therapeutics." My name is Anthony Maresso, and I am a professor at Baylor College of Medicine and a faculty founder of TAILOR Labs. Hopefully, by the end of this talk, you'll understand why I titled it this way.

I'd first like to start by thanking all the members in my group. A good number of these people are responsible for the work you're going to see today, all of our phage friends and sources of funding for the work, as well as all these institutions that we work with. And I would especially like to thank the organizers of the workshop, in particular Erica and the NIH and our great people at the FDA. We work with both of them on a regular basis, both in the basic science and clinical arm.

I'd also like to point out that we have two openings for phage biologists in our group, so if you know anybody that needs a job and likes the topics we're working on, please send them our way.

So, it was difficult for me to figure out what to talk about with you today. I was somewhat confused because there are so many topics that have been covered, and I didn't want to be redundant. "Research Gaps" is the title of this session, so I think I just went to what I think is the most important single concept that everybody should be considering in all their work regardless of what you're really doing, and that can really be illustrated with two main problems.

So, the first problem is nature is complex and cares little about certainty, but certainty is how humans structure drug discovery. And so, what I mean by that is this picture where essentially we, under very idealized conditions, screen for a target compound that acts on a specific enzymatic target under the most ideal conditions, and

then as it goes through this system, it's expected that somehow this is also going to be able to contend with the complexity of the host, right? So, you go from an ideal situation with limited three- or four-dimensional impact on the drug to a non-ideal situation and hope it works. And I think this is the reason why the bench to bedside translation is full of failed drugs that fall through the gaps to the chasm of death, right?

The second problem is we don't think like bacteria. And you're going to see a little bit of that today. And because of that, I don't think we think like phage. And so, what do I really mean? What I really mean is what impacts the therapeutic's efficacy? What impacts your phage's ability to actually work? And if you don't know what you're working with, I think you're setting everything downstream up for a big problem, and so that's what we're going to consider the concept of space and the concept of time.

So, space, first dimension, Y axis,

this is where all drugs fall, most existing therapeutics. They have a target, pure target, you have a dose, duration. Those are the parameters that you study.

But you could consider a second dimension, a planar view here, right, 1- and 2D, where you might have another target, another bioactive compound that's addressing your disease, and, of course, you're going to consider dose and duration there, too. So, these two will work together hopefully, right, and that's two-dimensional.

The third dimension is the 3D environment around—or where those two drugs are expected to act, right? So that's the host, how does the environment impact therapy, and then the fourth is how your disease changes with time. That's the fourth dimension. So, can your drug actually adapt and address if the disease changes as well?

So let's talk about the first three dimensions, space. This picture shows an intestinal enteroid taken from my—I had a colonoscopy and we drew organoids from it, and

this is *E. coli* growing on those intestinal enteroids, and you can see right away just from the simple EM micrograph that they're forming biofilms, right? So your drug has to contend with that. That is a three-dimensional effect that you have to address when you're thinking about your medicine.

But that's just a limited part of the three-dimensional environment. Think of all the complexity and all these devices bacteria love to grow on. And then think about the complexity that the chemical environment of blood brings, or the chemical environment of urine. And that is where you're expected to have your drug be efficacious in.

But bacteria are a different beast, so you also have to consider time. They change with time, right? So, in the microbiome, we know you generate 9 million new mutants of bacteria every day, so if you take the number of people on planet Earth, count the number of days, that's like 10 to the 18th novel mutations per year. You only need one to destroy antibiotics. You have to consider

time. You can't ignore time. And that's just *de novo* mutation. We know that bacteria change in very unique ways, right? The mutagenic tetrasect, four ways they mutate.

So, what out there can contend with space and time? Well, you guys all know the answer, it's phage, but I want you to know the answer for two reasons. Number one is a numerical reason, there are 10 to the 31 of these on planet Earth. And number two, they've been around for 3 billion years and have learned how to address space and time. And we call that genetic potential, which is completely untapped, the cryptic genosphere. So, we wish to probe that to get around space and time.

Here's an example. This is a couple hundred liters of fresh- and seawater from in and around our institution. We found 10,000 unique viruses just in this sample, 10,000, the vast majority of which have never been discovered or characterized. And in that data set we found 821,612 new genes, 70 percent of which cannot be annotated. So, that is an

unlimited genetic potential to tap into that has evolved to directly deal with killing bacteria.

And that's the basis for the formation behind TAILOR Labs, tailored antibacterials, innovative laboratories for phage research. Some of it was talked about in some of the talks. There's two objectives. The first is we wish to understand the basic science of phage therapy, and the second is we wish to make personalized phage cocktails for troubling patient cases. Okay.

So, for the second objective, I'm just going to mention that we've developed a complete end-to-end phage finding evolution library-making, safe-making, phage-testing paradigm that will allow you to have potentially a really unique cocktail for your patient's troubling case. So, if you're interested in that, you can contact me. We work with the FDA to have these things approved and we have treated a number of patients, 10 of which we're going to report very soon, where we have pretty good clinical

and microbial cures.

So, we have a large library as well that you have access to for all ESKAPE pathogens, we're up to almost 200 phages now for each of these. And a lot of them have been tailored to address the problems these pathogens cause. So, send us a note if you're interested in that.

But we're really in here to talk about objective one, which is addressing space and time, but that's how we make our cocktails for these tailored cases. So, let's talk about the first two dimensions. And we heard a little bit of this already. We call this synography, where we simply on one dimension have either an antibiotic or a phage and on the other have either another phage or another antibiotic. And we just simply test all possible concentrations or PFUs across 10 orders of magnitude in a simple *in vitro* plate assay. And we get all types of results when we do this, right? We can mathematically model this to determine whether it's additivism, synergism, antagonism, or no effect. But I

just want to show you a few highlights.

So, here's one where when we use ceftazidime with a phage that we really like, HP3, we see clear synergy, right, so this is a very effective two-dimensional effect, right? So that's good, synergy. But when we use a folic acid synthesis inhibitor, trimethoprim, with the same phage, we see no effect. In fact, the only effect is with the phage. When we use a DNA topo inhibitor, ciprofloxacin, with phage HP3, we see complete antagonism amongst many of the concentrations that you can possibly interact with in two dimensions, so that wouldn't work. You certainly wouldn't want to engineer that into a patient.

With some protein synthesis inhibitors, we see a mixed effect, both synergy and antagonism. It doesn't really depend on the type we're using, so you have kanamycin or chloramphenicol, it's the same type of thing, mixed effect. And when we used colistin, a cell membrane disruptor, we also see mixed synergy and antagonism.

The point is when you probe two

dimensions, you learn so much about what's going to happen when the two are interacting. And we have found essentially that across all major classes of antibiotics and even antibiotics within a class, it's really difficult to predict what's going to happen.

So, I mentioned that example of synergy that we really liked. Here's an example of it, right? But when we do the exact same experiment in human blood, it fails. And when we do the same experiment in human urine, it also fails. And so that's the effect of the third dimension, the host, on the treatment paradigm.

So, the key points are these results hold true for other phages and other antibiotics, 1D and 2D, and then they break down when the third dimension is added. And so, you really have to interrogate those points. You need to consider these issues when you design your banks, cocktails, and treatment paradigms.

Now let's consider the host, the third dimension, so three interacting parts in

one, which we call the space part. So, we have a really good phage HP3 that works at killing *E. coli* in a bacteremia model. This is a sepsis model, a murine model. You can see that here, right, about a five-log drop in CFU numbers. They get healthy, all the organs have reduced bacteria, so this is a successful outcome. That's great.

But if we put that same strain of bacteria in the intestine of the same strain of mice, deliver the phage in two or three different ways, and ask if it'll work, it absolutely does not. So that's unsuccessful, right? So, this is an effect again of the space. It's not working in the context of the intestine. Why not?

Well, we figured that out. It turns out mucus was inhibiting the phage. This is a whole long series of biochemical studies to prove this. You can read about it in the paper, but mucus was essentially inhibitory. So, we just simply said, well, why not strain to see if phages have evolved to kill bacteria in mucus-like environments? You would imagine

that in mucosal surfaces that had to have developed in 3 billion years of evolution. And, in fact, we found a phage that worked really well in mucus relative to all others. That's shown here it has a 1.5-log effect compared to these other phages.

But look at this phage in pure broth culture. It's actually quite poor at killing, right? Whereas these other phages were very good, but they didn't work in the mucus. So, this is a new phage that seems to work really well in mucus, but doesn't work under optimal conditions. This is what it looks like just to show you a picture, it's a little *Podovirus*.

So, to test this hypothesis, we used the organoid technology where we make these little intestines in culture and then we put bacteria and phage in them. So these bacteria, they grow on this mucus layer. It's really a neat model system. And what we found was essentially looking here by microscopy, is that this phage, this mucus-loving phage binds to your epithelial surface, it binds to the intestinal epithelial surface at the exact

site where the bacteria like to bind. In fact, it uses the same receptor. But other phages don't have this ability, and they don't bind at all.

And so, when we deliver this phage into the intestine in that model where the other phages weren't working, it works. It clears the *E. coli* from the intestinal tract. And we were able to show it through this localization study, where this mucus-loving phage binds to the exact niche where the bacteria are binding in mucus and works in space. So, it's an example of a phage dealing with a third dimension, right, that untapped cryptic genosphere. This is just a model to show that.

It's not just mucus. We did this for biofilms in urine, too, or any other system really. This is just showing you an example of that. We grow biofilms all the time, and in urine we have phage that work really well in urine on biofilms and other phage that do not. And some of these phage are very poor in perfect idealized conditions. And so if we put

E. coli in a human catheter and make a biofilm, and we make a cocktail of these biofilm-seeking phages, it works very well compared to the untreated situation here where you have a six-log drop in CFU numbers.

So, the point is your phage should address the third dimension, the infection environment, and screen using real world conditions that are simulate the infection environment.

So, let's now finish the talk by talking about the fourth dimension, time, my favorite one actually. I talked about resistance and how resistance is a major problem, right? But that's one that works in time.

And so, we just simply created model systems to select for bacteria that overcome phage either *in vitro* or during an infection assay. And then we studied why the bacteria overcame resistance and if we could develop phage that would overcome that resistance. And so here's a great example of the phage working on the—this is a parental strain of *E. coli*,

the non-evolved *E. coli*, the non-resistor. Nine-log drop, wonderful, right, but the resistors that come out of our assays? Completely resistant to the phage. You cannot kill these bacteria with this phage, this original phage. All resistant, right? So we made a machine we call Modi-phi, where you simply train or evolve phages to slowly adapt to resistor strains of bacteria, in this case *E. coli*, over time. And this machine can run for hours or days and it will report to us when we have a lytic effect, an adaptation that has occurred. So that's addressing time.

So, simply in chamber one, you have a permissive host. It grows a lot of phage. Some of those phage have mutations, you might even induce these mutations with mutagens. The phage will cross the barrier, the bacteria will not. It will jump to a host. Most phage will not adapt, but some will, and then when it does adapt, you can grow it and isolate it, right?

And in this particular case, we found a phage in five hours. And that new

phage, that adapted phage killed every resistor from those previous screens. That's shown in green here. Phage 3.1, right? Whereas phage 3.0 didn't do anything on those resistors. And when we deliver it into an animal, it's effective at clearing the bacteria. You could play this game, right, until you find phages that address all resistors.

DR. CAMPBELL: Anthony, three minutes.

DR. MARESSO: Okay. Thank you, Joe. So, phage 3.1 adapts to the resistors that overcome phage 3, and phage 3.2 adapts to resistors that overcome phage 3.1. And we found they have simple mutations in their tail fibers, which meant they were adapting at the surface of the bacterium. So, you develop this model whereby you continuously evolve these phages to cut off trajectories. So, you want to create cocktails that see the future and are decisive at preventing resistance, and then combine them with your other phage that broaden the host range. And this is a perfect

example. Here's the parent cocktail, resistance arises within 12 hours, but our predictive cocktail, the evolved one, completely shuts off resistance. The bacteria cannot develop resistance in this scenario.

So, I'll close with the points that I would like all of you to substantially consider developing your phage that address space and time. Those are going to be the best medicines. And you have an unlimited potential from which to tap into, and you want to, I think, turn this paradigm on its head and flip it over so that you're addressing the most complex conditions first to identify your phage and then put them through the ringer to make them a medicine, because, after all, we're not a culture of LB.

So, thank you for your time and I'll take questions in the workshop panel.

DR: CAMPBELL: Great. Thank you, Anthony, that was a wonderful start to this session.

Our next speaker is Bekah Dedrick from Pittsburgh and—University of Pittsburgh

and she is going to be telling us about opportunities and challenges for phage therapy of *M. abscessus* infections.

And are you ready, Bekah? Take us away.

DR. DEDRICK: Okay. Hopefully you can hear me and see my presentation okay?

DR. CAMPBELL: Yep.

DR. DEDRICK: Okay. So, thanks, Joe, for that introduction, and thanks for having me speak today. I will be focusing on phage therapy for *M. abscessus* infections as this is the organism of interest in the Hatfull Lab. First, I will summarize our first case study published in 2019 where we show the effective use of phage for *M. abscessus* infections. This was a landmark study, first of all because it was the first time mycobacteria phages were used in a human with an *M. abscessus* infection, and also the first use of engineered phages for an infection in a human. Then I'll talk a little bit about our more recent case study that really highlights the challenges of phage therapy, and these two

cases really represent opposite ends of the spectrum concerning phage therapy success. Finally, I'll take you through some challenges and some solutions that we're hard at work on in the Hatfull Lab, and you can read our most recent Annual Review of Medicine review there at the bottom for further details.

So, for our first case, this was a case study published in 2019. This was a patient from the Great Ormond Street Hospital in London. The patient had cystic fibrosis and had undergone a double lung transplant. She had a difficult-to-treat disseminated MDR *M. abscessus* infection, and the patient had exhausted all antibiotic options available to her and she had nothing else that she could take. We received the strain in Pittsburgh to test for phage susceptibility, and within about three months we were able to find a three-phage cocktail that was efficient at killing this particular drug.

After several more months of regulatory hurdles that were jumped through, the patient began phage therapy treatment in

June 2018. The cocktail was administered by IV twice daily at 10 to the 9th PFU per dose.

And just to give you a brief summary of the outcomes of that study, most importantly there were no adverse reactions to the phage therapy. We did see some phage recovered from the patient samples, consistent with some *in vivo* replication. She had improved lung function and she gained weight. And she had an infected liver node that resolved after six weeks. So, you can see up here on the right, this is a PET scan here before treatment and then here after treatment. The large liver node was completely gone. This is a cross-section CT scan, and you can see the same thing here. So, this is the enlarged liver node, and then it's gone after six weeks of treatment with IV phage therapy.

This patient also presented with several skin nodules, and when cultured, these also grew *M. abscessus,* and you can see that they were a little bit slower to resolve, but they did look better over time, and she had closure of her sternal wound from the

bilateral lung transplant. Most importantly, the patient was able to return to a normal routine, she attended school and got her driver's license. So, that was exciting.

We tested the patient's serum over time for phage-neutralizing antibodies, for an immune response to the phages. We did not see that. We also looked for phage resistance over time in clinical isolates obtained from the patient during therapy, and we did not see that either.

After this case was published, we received now over 200 *M. abscessus* clinical isolates that were sent to us from around the world. And we often get asked the question, you know, now that you've published this case, can we just have this broad phage use for all *Mycobacterium abscessus* infections? And the answer is no. And hopefully, you'll learn more about that today in my talk.

But some of the challenges with *M. abscessus* that we face is that the bug is intrinsically resistant to antibiotics. Also, the growth of *M. abscessus* is unlike most of—

well, I think all of the ESKAPE pathogens, right? It has a four-hour doubling time, which is a fast-growing mycobacteria compared to tuberculosis, but it is quite longer than *E. coli*, which is a 30-minute doubling time.

We also have issues in liquid culture. When you grow mycobacterium *M. abscessus* it often clumps. And so one way to sort of prevent this is to use Tween or some other detergent in the culture when you're growing it, but we can't do this because we noticed that our phages can be affected by the Tween in the culture. And so, we typically use sonication to disperse those clumps.

And lastly, colony morphology has really determined phage susceptibility for us, and I'll talk about that a little bit later in the talk. And so, there's two types of colony morphology for *M. abscessus*, smooth and rough. And what we found is that for rough strains, we can often find at least one therapeutically useful phage, but for smooth strains that's definitely not the case.

And as you all know, there's many challenges with phage therapy. So, the first is finding a suitable phage for the clinical isolate, also what dose of the phage to use, the length of treatment given to the patient. And we're always looking for phage resistance and, of course, immune reactions. And so, that sort of takes us into the second case in which we did see an immune response.

And so, this case was with Dr. Keira Cohen at Johns Hopkins University and her team, where she was treating an 81-year-old male patient with bronchiectasis, who was immunocompetent. He had refractory *M. abscessus* and *M. avium* lung disease. He was on antibiotics for five years for these infections, but he was still acid-fast bacteria smear positive. So, Dr. Cohen sent the strains, both strains, to us to test for phage susceptibility. Unfortunately, we didn't have any phage that were able to infect the *Mycobacterium avium* isolate. But we were able to find three phages that infected the *M. abscessus* clinical isolate. And so, we

administered phage therapy to this patient the same way as I told you about in the first case, as a three-phage cocktail by IV, twice daily, at 10 to the 9th PFU per dose.

And the great thing about this case was that the patient was able to provide sputum samples over time, so each month during treatment we were able to collect sputum samples and then determine the log CFU per mL in the sputum. And so that's what you'll see here on the Y axis. In red is the pre-phage therapy of *M. abscessus* level, so there was about a two log CFU per mL in the sputum pre-phage therapy, and the blue represents the *Mycobacterium avium*. At the bottom you'll see his antibiotic regimen and then when the mycobacterium phage cocktail was started.

And so, after one month of treatment, we saw that the log CFU per mL actually was reduced for *M. abscessus,* and so we were excited about that and thought, okay, you know, maybe this is working, maybe we'll get a great outcome. Unfortunately, after two months, we realized it wasn't going to be that

simple. And, unfortunately, his *M. abscessus* rebounded back up to pre-treatment levels after two months of therapy. And this trend continued, and you actually see that after six months of treatment, the patient had more CFU per mL of *M. abscessus* in his sputum than he had pre-phage therapy. You can also see that the *M. avium* isolate went down over time, and this was due to antibiotics that were started specifically for this isolate.

And so, you know, we got this data and we thought to ourselves, okay, how—why would this be happening? And of course, your first, I think, gut reaction would be, you know, maybe there is phage resistance occurring in the bacteria. And so, we tested, we were able to isolate and test post-IV isolates, IV treatment isolates. And all of them were still susceptible to all three phages used in the cocktail. And so, we knew it wasn't phage resistance.

So, the other option is an immune reaction to the phage and that the phage were being neutralized. And so, we set up a phage

neutralization assay where basically, we just take the serum, and we take the phage that we're treating with, we mix them together, allow that to incubate, and then we serially dilute them and plate on a top agar overlay and look at the titers. So that's what I'm going to show you here.

So, these are three top agar overlays. Here on the left you have pre-phage serum that was incubated with phage Muddy, month one through month six. And then the phage-only control, so, like I said before, this is just Muddy alone, this is the titer. This is BPs alone and then this is the ZoeJ alone. These were the three phages that were used in the cocktail.

And what you can see is that the pre-phage serum sample does not show phage neutralization, but you can see quite clearly that months two through six show complete neutralization of phage Muddy that was in the cocktail. There is also neutralization of BPs and ZoeJ, although not to quite the extent that Muddy was neutralized. And so, this

pretty much informed us that the patient had an immune response to these phages, and that's why we're seeing this rebound of *Mycobacterium abscessus* in the sputum.

So, we wanted to take this one step further and complete ELISA assays and look at IgG, IgM, and IgA responses. On the top row here are each of those incubated with Muddy and then in the middle is BPs and at the bottom is ZoeJ. And what you can see here, this black line is pre-phage serum, and then these colored lines to the left show the shift after month one through month six of phage therapy. And so, we see a strong IgG and IgM response to all three phages. We don't see this for IgA, which I think makes sense because it's more of a secretory antibody. So, we were able to confirm this phage neutralization with the ELISA assay. And we also saw that there was no improvement in the chest CT scans, so on the left you'll see pre-treatment, in the middle is two months post-phage therapy, and six months post-phage therapy on the right, and essentially there

was no change.

So, after six months of IV treatment, Dr. Cohen put an amendment in for the IND that the patient was treated under, and the patient was switched to nebulized phage therapy. We used the same three phages that we used for IV, and so far, we've tested months one, two, and three months post-neb *M. abscessus* samples, and all of these are still sensitive to the phages in the cocktail. We are currently testing both sputum and serum for phage neutralization post-nebulized phage.

And I'm sure most of you are thinking, well, gee, you know, why can't you just give the patient new phages? And, unfortunately, for *Mycobacterium abscessus,* it's not always that easy. And so, we published a paper this year about the determinants of phage susceptibility in *M. abscessus* strains, and one of the strong indicators is colony morphology, and I hinted to this earlier.

So, on the top here you can see two examples of *Mycobacterium abscessus* with a

rough colony morphology, so, these colonies look a bit more dry. And for 80 percent of rough isolates, we can find at least one therapeutically useful phage.

Unfortunately, for smooth isolates, you can see here, these look a little bit more mucoid. We have no therapeutically useful phage for smooth strains yet. We're hard at work hard on that.

In this paper, we also sequenced 82 different clinical isolates, and we analyzed them, their genomic data for both prophages and plasmids. And we found that most strains have between one and six prophages, and actually over half of the strains contained between one and three plasmas. From previous work in the Hatfull Lab, we know that prophages often provide phage defense systems that protect against infection from other phage. And so, we predict that this is a determinant of phage susceptibility for *M. abscessus*.

Just to give you a little bit of a broader picture of that, this is going to be a

little bit busy, but hopefully you'll take home the message that these strains are very, very diverse. I'm showing you phylogenetic tree of these 82 *M. abscessus* clinical isolates. In blue here are *M. abscessus* subspecies *abscessus*, in green is *M. bolletii*, and in pink here is *M. massiliense*. And what we did is we screened each of these against our favorite therapeutically useful phage. And if—each of these columns here represent a different phage, and if you see the circle colored in, that means that that phage infects and kills that strain well.

And basically, what I want you to take away from this is that there's no pattern. We don't see that within all *abscessus* subspecies *abscessus*, that there are filled in green dots, and that maybe for *massiliense* there's filled in orange dots. So, there's lots of strain variation.

And in addition to that, like I said before, we see a lot of prophages in these genomes. We're able to cluster those based on sequence of similarity, and if a specific type

of prophage is present in a strain, then we colored in a square here. And so, here's all the different types of clusters of prophages that we found in *M. abscessus* clinical isolates, and you can see just a smattering of color here to show which strains have which prophages, so they're super diverse.

And lastly, we did the same thing for plasmids. So up at the top, all of the different plasmid clusters --

DR. CAMPBELL: Rebekah, three minutes.

DR. DEDRICK: Thank you, Joe.

DR. CAMPBELL: Sorry.

DE. DERICK: That's okay. All the different plasmid clusters that you can see here. And again, if the plasmid was present in the clinical isolate, then we colored in the square. So, hopefully this gives you a little quick snippet as, into the variation of these *Mycobacterium abscessus* clinical isolates and the difficulty that we have in finding phage to treat them.

Okay. So, in summary, phage therapy

for *Mycobacterium abscessus* infections can be useful, as I showed you with the first case. We do see lots of variation in phage susceptibility among these *M. abscessus* strains. It's difficult to predict, and currently every patient that we treat requires personalized phage therapy. Of course, additional case studies will provide further insights into efficacy, but a lot of these patients are compassionate use cases, and they're extremely sick.

Immunocompetent patients, we need to make sure that we're checking for phage-neutralizing antibodies and looking for those immune responses. And that's a lesson that we definitely learned from the second case study.

Okay. So I'd like to acknowledge Dr. Graham Hatfull, my mentor at the University of Pittsburgh; Carlos, Bailey, and Krista, who were very important members of the team for both the case studies; and other members of the Hatfull Lab there. At the Great Ormond Street Hospital, the first patient that we

treated was under the care of Dr. Helen Spencer and her team. The second case with Dr. Keira Cohen and her team at Johns Hopkins. And of course, our continuous collaborators Dr. Benson, Dr. Schooley, and Dr. Strathdee. And I'm happy to take questions during the time that we have.

DR. CAMPBELL: Okay. Thank you, Bekah. That was wonderful. I think we'll save the questions for the panel discussion, and we'll move on to the next talk.

The next speaker is Jennifer Dan from UCSD, and she's going to be telling us about the development of bacteriophage host immune response in a lung transplant recipient receiving phage therapy for MDR *Pseudomonas* pneumonia.

DR. DAN: All right. So, I'm going to tell you a case story about a lung transplant recipient we had seen in May 2017. I'll give you a little about the clinical case to set up, basically, the patient's environment for the bacteriophage. This is a 67-year-old man. He had undergone a bilateral lung transplant

in October 2016 for hypersensitivity pneumonitis and pulmonary fibrosis. Unfortunately, his transplant course was complicated by primary graft dysfunction. He required prolonged mechanical ventilation. He developed stenosis of his right mainstem bronchus, which is like the epi-anastomotic site, and that required bronchial dilation and stenting. He also developed multiple episodes of *Pseudomonas aeruginosa* pneumonia while in the ICU. He developed acquired hypogammaglobulinemia, which required monthly intravenous needle globulin infusions a month after his transplantation. He also developed acute cellular rejection two months after his transplantation, and that required additional immunosuppression with Basiliximab, which is an anti-IL-2R receptor blocker, monoclonal. He also developed chronic lung allograft dysfunction requiring photopheresis, and chronic kidney injury requiring hemodialysis. So, this is the protoplasm of the patient, basically.

We saw him in May 2017, when I was

on the transplant service, and at this point, he had developed a multidrug-resistant *Pseudomonas aeruginosa* pneumonia. It was resistant to many antibiotics including zeocin, meropenem, and the newer—at the time, the newer antibiotics, ceftazidime-avibactam. The transplant patient was maintained on immunosuppression, and his immunosuppression at that time was Prednisone, about 5 to 10 milligrams daily, along with Sirolimus, and then around when we were starting to treat him, he received a dose of IVIG and also photopheresis. We then obtained an emergency IND to use bacteriophage therapy, in conjunction with systemic antibiotics. We contacted, at the time, AmpliPhi, and four phages were found to be susceptible. So, these were used for the patient. The patient started IV phage treatment for two weeks, and also nebulized inhaled phage therapy for about three weeks. So, this is our patient, and this was published in Time magazine in December 2017. And he's there with Dr. Strathdee and her husband, Tom Patterson. I wanted to set up

the timeline of treatment for the patient because we are going to be discussing the development of an immune response to this phage therapy. So, there are two cycles of treatment in late May 2017, to about June. This is cycle one. He received a cocktail of four phages, which we label AB-PA01. Unfortunately, he developed another pneumonia after this treatment and had to be treated again with phage therapy. This time AmpliPhi provided us with another cocktail, we denote this as m1. AmpliPhi provided us with a cocktail that contained the same four phages of the cycle one, but with an additional phage, so for the future slides, this will be denoted as m1. We also were in contact with the Navy, and they were able to provide us with different cocktails as well. So, cycle two for this patient had—consisted of the five-phage cocktail, follow by a three-phage cocktail, and then again with the five-phage cocktail. And after he completed his cycle two of therapy, he was continued on suppressive phage therapy, and at the same time, listed on

the bottom, are the antibiotics he had received.

And this is just a table going over the phages, so cycle consisted of these four phages, which were provided by AmpliPhi at the time. Cycle two consisted of five phages, this is, I think it was Pa176. And then the Navy Phage Cocktail: cocktail one of three phages, and cocktail two of two phages.

So, our overall goal was to determine if the patient developed an immune response to phage therapy. So, in May 2017, this was an interesting—I thought this was an interesting idea because this was, basically, a novel antigen that very few people have seen, and it would be interesting to see if you're giving something through continuous infusion, are you going to develop an immune response? Normally, with vaccine, we just give an injection, an intramuscular injection and wait to see if someone develops an immune response. But this is an interesting case because he is getting IV infusion of a foreign antigen. So, to do this, we collected blood at

day 0, 7, 14, 21, and 28 for cycle 1, and we also collected blood for cycle 2, and we analyzed the antibody response by ELISA, and we also looked at his cell-mediated immune response by an assay we developed, the Activation Induced Marker Assay, to look for antigen-specific T cells.

So, the easiest thing to look at first is, does the patient develop an antibody response? So, we did an ELISA, basically, and we are looking here on the left graph at an ELISA to the four-phage cocktail, and on the right graph, an ELISA to the five-phage cocktail. So basically, we coated the plates with either the four-phage cocktail or the five-phage cocktail and then looked to see if the patient developed a response, an IgG response to the phage therapy. So, the patient's data is plotted here with the open circles. Because the patient did receive IVIG, I was able to obtain some IVIG that was going to be discarded, and it was from the same vendor that we used to provide the IVIG on the in-patient for the patient. And interestingly,

the IVIG also had some detectable at least phage-specific immunoglobulin responses, and the way I was able to quantify this was I normalized this to a standardized pool of normal healthy donors of pooled plasma, basically, and I was able to find that—even in pooled plasma, I was able to detect a low-level response, an IgG response to phage. So, I don't know what they're actually responding to, what part of the phage they are responding to, but even normal healthy donors, when I was able to normalize it had a very low response to phage therapy. So, as you can see with cycle one, the patient had a little response here, but by cycle two, he was making a much greater response, so it's almost a two-log difference here. And then for the five-phage therapy, the patient also had a little bit of a response. The way to see if the patient is actually making any—if these IgGs are active is to do a neutralizing assay. So, this neutralizing assay was done by AmpliPhi. I think Dr. Lehman did this. So, we tested the patient's serum for its ability to

neutralize two different hosts. So, the control host one is a *Pseudomonas aeruginosa* that plaqued well with two out of the four phages that were in this four-phage cocktail. And control host two is a different *Pseudomonas aeruginosa* that plaqued better with the other two phages in the four-phage cocktail. And again, you can see that after day 15, by day 21, the patient is making neutralizing antibodies on both hosts. So, he's making neutralizing antibodies to the four-phage cocktail, and this increases with time, even into the cycle two. And for the five-phage cocktail, again control host two plaques better with the two of the five phages, whereas control host one plaques better with three of the five phages. So, control host one is that extra phage, and the five-phage cocktail is able to plaque better on control host one. So, control host two for the five-phage cocktail basically looks like the graph for the four-phage cocktail, whereas control host one it seems to—the trend is still there, but then, there doesn't appear to

be any neutralization towards the end of the therapy, I'm not exactly sure why.

Additionally, we wanted to look at the cell-mediated immune response. So, for us, we look at T follicular helper cells. So, T follicular helper cells are a type of CD4 cell, and we usually think of these in the germinal center here. So, when we get a vaccine or a foreign antigen, your dendritic cell, your antigen presenting cell picks up that foreign antigen, processes it, and then presents it to a T cell. The T cell then decides, do I become a Tfh cell? Do I become a Th1 cell, etc? If it does decide to become a Tfh cell, the Tfh cell then goes to the TD border. The Tfh cell upregulates certain markers, which we will refer to later on like CXCR5, PD1, and ICOS, which helps it migrate into the germinal center. And in the germinal center, the Tfh cell then instructs the germinal center B cells to undergo somatic hypermutation and affinity maturation, and then these B cells can later exit the germinal center as either memory B cells or plasma

cells that make these antibodies. So, these are the plasma cells that are making these antigen-specific antibodies or phage-specific antibodies that we have seen before. So, in circulation, because we didn't get any lymph nodes from this patient, we were able to look at circulating Tfh cells, which we identify here. So, by flow cytometry, we take the patient's blood, we gate on the CD4 cells, and then we gate on CXCR5 expression, because that's the marker that the Tfh cells need to traffic to the germinal center and CD45RA-negative. So, our circulating Tfh cells are defined as CXCR5-positive and CD45RA-negative. We can then further look at activated circulating Tfh cells, which we defined as co-expression of PD-1-positive and ICOS-positive of the subset. So, for cycle one at day zero, the patient had a baseline of 1.02 percent of activated circulating Tfh cells. By day 7 and 14, with a continuous infusion—well, not continuous infusion, with infusion of IV phage therapy, we see that his activated cells increased, 7.11 percent and 15.5 percent. By

day 14, we stopped the IV therapy and he's on nebulized therapy only, and his activated Tfh cells are at 6.62 percent and 8.09 percent.

And here's a graph looking at the kinetic, looking at the activated Tfh cells as well as the plasmablasts. So, plasmablasts are the B cells that are helping to make immunoglobulin, and we tracked this over time of his cycle one and cycle two treatment. So, his activated circulating Tfh cells are at 1.00 percent, and it peaks at day 14, at the end of his infusion therapy. It declines a little bit, but it’s still present by the time he starts cycle two of infusion therapy. They again peak, and then it starts to decline a little bit, particularly when he's changed, not that much, but when he's changed to the Navy cocktail, and again when he resumes the five-phage cocktail. We can see also with the plasmablasts, there is a subtle increase, but not as drastic as with what we see with the activated Tfh cells.

We then wanted to look at phage-specific CD4 T cell responses, and to do this

we used our Activation Induced Marker Assay or the AIM Assay. Basically, we take PBMCs from the patient, and we stimulate the cells with peptides or proteins for 24 hours. In this case we stimulated the cells with the phage—with the phage cocktail he got. Then after 24 hours, we stain the cells and then assess the cells with flow cytometry.

So, this is a FACS plot looking at phage-specific CD4 T cells. On the top we have the patient, and on the bottom, we have a normal healthy donor. So, here we have unstimulated—so, just basically no stimulation, the baseline expression of phage-specific CD4 T cells. So, this our just baseline activation for the T cells, is 0.0627 percent, and this is based on co-expression of OX40 and PD-L1 as our activation markers. We also used a negative phage cocktail that consisted of *Staph aureus,* and for this patient, we got a frequency of 0.152 percent. And then when we used the phage cocktail consisting of five phages, we got a frequency of 1.11 percent. And as our positive control,

we used a potent T cell mitogen, Staphylococcal enterotoxin B, and the patient was able to respond to that, and it was 15.8 percent. And in our normal, healthy donor, there's no response to the negative phage, there's no response to the five-phage cocktail, but the normal, healthy donor does make a response to SEB.

Here we are looking at the kinetics of the phage-specific memory CD4 T cell response. So, here we're looking—instead of total CD4s, we gate out the naive population here which we based on CCR7 and CD45RA-positive. Everything else is memory cells. So, we are looking at phage-specific memory CD4 T cells based on co-expression of OX40 and PD-L1. So, here on the left, we are looking at the total memory CD4 T cells for the four-phage cocktail, and we see that at day 21, we start to see that the patient begins to make phage-specific CD4 T cells, and then this increases at day 52, when he's starting cycle two. And we see a peak here at day 66 and 73. This is when he's actually on the Navy phage

cocktail. And I'm not sure why it goes away at day 80. I know around day 79 is when we transitioned him from the Navy phage cocktail to the five-phage cocktail from AmpliPhi. He also received a round of IVIG and photopheresis at that time. And as a comparison, this is what we see in normal healthy donors. We also looked at the five-phage cocktail, and again, we see this increase around day 66 and 73, and then this is—oh, sorry, and this is what we see for normal healthy donors. Then finally we looked at—does this patient develop a phage-specific circulating Tfh cell response? We want to know that because we see an antibody response. We saw the development of phage-specific IgG. We saw the development of those IgGs are able to neutralize phage. And we also saw the development of activated circulating Tfh cells with IV infusion. We saw the development of phage-specific CD4s. So, we should see the development of phage-specific circulating Tfh cells as well. So, this patient—our baseline stimulation here for this day of collection

was 0.568, and then with the four-phage cocktail, we see that the patient had a 5.15 percent circulating phage-specific Tfh frequency. And with the five-phage cocktail, we see that the patient had a 5.82 percent circulating Tfh frequency. And again, the patient did respond to SEB, our positive control.

So, if we look at the kinetics of the phage-specific circulating Tfh cells, we see that again, like the CD4 T cells, the patient—we begin to see the development of phage-specific circulating Tfh cells at day 21, and this gradually increases and peaks at day 73. Again, I'm not sure what happened clinically, other than he did receive photopheresis and IVIG, for the reduction in the frequency of the circulating Tfh cells here. At day 80, this is the kinetics of what we see and again, we see minimal amount of expression by—

DR. CAMPBELL: Jennifer.

DR. DAN: —normal healthy donors—yes?

DR. CAMPBELL: Three minutes.

DR. DAN: Okay. And then for the five-phage cocktail, we also see similar kinetics for the circulating Tfh cells.

So, in conclusion for cycle one, the patient received an IV infusion of the phage for 14 days straight. He responded to the treatment and developed minimal secretions and was actually able to ambulate in the room, which is nice. And if you look at the kinetics of the response, he actually developed an activated circulating Tfh response at day 14, if you remember, after the infusion. And this was followed by phage-specific circulating Tfh cells and CD4 T cells, which we saw at day 21 as well as neutralizing antibodies at day 21. Interestingly, day 21 marks the peak of any germinal center activity for vaccination. It's the peak time if you were to give someone a vaccination, of when you want to look to see if someone makes an immune response. So, it was actually nice to see the kinetics of this following what we would anticipate. And then for cycle two, the patient had a delayed clinical response to the five-phage therapy

and the Navy phage cocktail, but this may have been due to pre-existing four-phage cocktail-specific circulating Tfh cells and the neutralizing antibodies the patient developed.

So, I want to thank everyone at UCSD and Shane at La Jolla Institute, Shane Crotty at La Jolla Institute for helping me with this. This was done in conjunction with Dr. Aslam, Dr. Schooley, and Dr. Strathdee, but also on the research side with Dr. Shane Crotty, who's my mentor. Thank you.

DR. CAMPBELL: Thanks, Jennifer, that was very nice. I think we'll save the interesting questions for the chat. And I will now introduce our last speaker for this section, Dr. Paul Turner from Yale. Paul is going to talk about phage selection for reduced virulence in bacterial pathogens. Start when you're ready, Paul.

DR. TURNER: All right. Thank you. All right. Well, it's wonderful to be with you, virtually. Thank you for attending. This has been a fabulous workshop. I appreciate the invitation, and I have a lot to say in a short

period of time. So, I'm just going to launch right in. These three pictures give away a lot of what we're trying to do, and you've heard about during this workshop, as we can do phage hunting in the wild. We can find and characterize phages that are excellent candidates for therapy as shown in that middle picture. And on the right, we can use this an emergency patient treatment, and we're starting to do clinical trials.

So, the disclosure at the beginning though is that I'm a co-founder of Felix Biotechnology, and that's a company that seeks to commercially develop phages for therapy.

So, phage therapy has a tremendous amount of promise, but it also has some costs to it, and the one that we've been focusing on a lot is the evolution of phage resistance in bacteria. Lytic phages on the left, these are, in my view, prime candidates to do the therapy. On the right, you have so many examples of the kind of innate defenses, as well as adaptive defenses that bacteria can have against phage attack. So, my point here

is that phage therapy, in my opinion, is inevitably going to lose out in terms of the evolution of phage resistance in the target bacteria, and the question is, how do you build in something that works effectively even though that inevitability probably exists.

So, I've been studying evolutionary tradeoffs for a long time, as an evolutionary biologist, and evolution by natural selection is primarily the mechanism, the process by which variation on this planet exists in populations and across species. And what you see time and again, is that evolution involves compromises. So, if you just don't have to look very far beyond humans and find that when humans do better than other animals as hurl objects with accuracy and a high amount of speed, and this does not happen in our closest relatives, the chimps. But focusing on humans as a species, we can do a lot of our activities because we walk around all the time on two feet, unlike the other great apes. But this places our lower backs under a lot of stress, and therefore we suffer a lot of back

pain as humans. The point here is that the evolved traits in any species, including in humans, have some liabilities.

What we try to do is take this approach to phage therapy. So, our innovation, so to speak, is to find lytic phages that will do the work of phage therapy, but we want to find specific ones that bind to virulence factors or antibiotic resistance mechanisms. For the purpose of this talk, I'm just going to lump both of those together into a term, virulence. So, the goal here is the classic one. You want to kill the bacteria. But the overarching goal beyond that is shown on the right. You want to compromise bacterial evolution to go in a particular direction on average. So, a couple of hypothetical examples shown from this review paper in 2020, sorry, 2019, is that if you have efflux pumps that are removing antibiotics, that make it into the cell—that certainly happens in a lot of bacteria that we worry about for infections. But if you find a phage specific to proteins of efflux pumps, then it will kill the

bacteria in the classic way and also steer the evolution of phage resistance down a better path, I would argue, for biomedicine, where here you would often find that the bacterial mutants that avoid a phage attack have compromised ability to remove antibiotics from the cell and therefore, they become antibiotic sensitive. On the right, I will be faster in describing this capsule can shield the bacteria from detection, from the immune system. If you have a phage specific to that, then the bacteria again have a problem. They'll evolve phage resistance, and they'll avoid a phage attack, but they could change or even remove that capsule, making them easier to detect by the immune system and therefore they become less virulent.

So, some evidence of this, I'll go through the next few slides fairly quickly, because it's already published, and you can go look at the papers for details. One of the best examples we've got for a phage that targets a bacterium and causes the evolution of phage resistance to coincide with re-

sensitivity to antibiotics, and this is a phage called U136B. We're using this in the laboratory as a model, and its host is *E. coli*. So, here in the middle is really what I want you to key in on. If you look at various strains of *E. coli* with knocked out genes, in this case for this particular phage, it's showing that below the limit of detection, you can't find the phage's ability to replicate on this knockout if TolC is missing, whereas a variety of other outer membrane protein knockouts, the phage does just fine. And this tells us that TolC is something very crucial for this phage to bind to and initiate infection of those bacteria. Here on the right is the consequence. So, under control conditions or benign environments, the wild-type bacteria, a TolC engineered knockout, and the spontaneous phage resistant mutant by example, they all grow normally. But here in the presence of antibiotics at a particular concentration—in this case, it's tetracycline at a particular level—the TolC knockout, as expected, has a

problem growing. And indeed, you find that to be the case for the phage-resistant mutant as well.

For this particular phage, it has two co-receptors, it has two receptors that it uses on the cell surface. And the other one is a portion of the lipopolysaccharide, or LPS. And we find this in a lot of the phages that we study. Here is a side view of LPS on the left, and I've colorized here in these rectangles the portions of LPS where we find that this particular phage binds. Again, in the middle, we can knock out various genes that contribute to formation of LPS, and only these in this colored portion here are the ones that are critical, that if you remove them again, the phage cannot grow, and you see below the limit of detection. On the right is the consequence. And in this case, if the bacteria undergo a change in LPS to avoid a phage attack, they become more sensitive to colistin, a different antibiotic than tetracycline, and therefore, for this one phage, it actually has two interactions with

the target bacteria, two ways the bacteria can evolve resistance to phage attack, but it leaves them more vulnerable to antibiotics.

So, we've done a lot of work with this particular phage. And I'll just go through a few more results from this 2020 paper. For example, we've isolated 20 phage-resistant mutants, spontaneous mutants. Six of them had changes of TolC, 14 of them had changes in LPS for this particular analysis. And what we have is a perfect prediction of a pleiotropic consequences or the tradeoffs that are due to phage resistance. Here on the left, in all cases, these efflux pump mutants, they have a lower MIC in the presence of tetracycline compared to the ancestral MIC of the original strain. Here on the right, all 14 LPS mutants had a lower colistin MIC compared to the ancestor. But interestingly here, this bar is showing those bacteria that are resistant to LPS, and they actually have a better ability to grow in the presence of tetracycline. That is a complication that one would like to avoid, if you're using phages

and antibiotics in synergy, do you have a problem if you find that this is not consistent across antibiotics, and that had been talked about in at least one of the earlier talks in this workshop. Fortunately, in this one case, we sort of find a tradeoff within a tradeoff. In the case that the bacterial mutants that resist the phage attack and have debilitated—well, basically, if they have some increased ability to grow in the presence of tetracycline, they actually grow worse in the presence of colistin compared to the other bacterial mutants. So, these are the kinds of tradeoffs within tradeoffs that we've been looking at as we've heard in this workshop. It can be very complex to figure out what's going on with phage, bacteria and an antibiotic synergy tossed in.

One more bit of set of data for this particular phage and *E. coli* model is basically we can't control whether any of those mutations will spontaneously occur when a patient is undergoing therapy. But it is important to think about—not all of those

mutations are equal. They have to compete with one another in order to fix in the bacterial population. One way to start to explore this is look at the growth consequences of the different mutations. And that's what's shown here. If you see here in the dark black line, the solid line, this is the growth of the wild-type bacteria, *E. coli* on an automated spectrophotometer over 24 hours. And in relation to that, in purple, these are the TolC mutants. They seem to show no cost of the resistance to phage attack in terms of their ability to grow under normal conditions, whereas those LPS mutants have a big problem. They're growing much worse than the wild-type bacteria. We would therefore predict that if we don't control any of that and the spontaneous mutations simply arise in a bacterial population, probably TolC is how these bacteria will solve the phage problem, because they don't have as many fitness consequences for ordinary growth when they change TolC compared to LPS, at least under the conditions that we studied here.

And indeed, when we look at experimental co-evolution, this is what we observe. Starting first here on the right. If you just let the bacteria evolve on their own over the course of 10 days, essentially, they can show some fluctuation in that phenotypic trait, the resistance to tetracycline, but it more or less stays at that wild-type level. Whereas here in blue, if you co-evolve the phage and the bacteria over the same time period, you see a tendency of the tetracycline resistance to drift downwards as a trait, meaning that these bacteria are becoming more sensitive to tetracycline over time as they evolve resistance to the phage, and that is consistent with our prediction that the TolC mutations are less debilitating for fitness of the bacteria, and those are the ones that will probably win out.

Now, I've talked so far about tradeoffs and how we're trying to pick particular phages that interact with the bacteria, kill them in the classic phage therapy sense, as well as select for

tradeoffs, but we are not so naive to think that that's the only outcome. The possibility is also for something called trade-ups to evolve, and that's where bacteria change in response to selection, and then they improve in some traits that they're not being selected to change. Now, the first three columns in this rather busy cartoon—this is from a paper that we published in 2020 in Current Biology—there are basically three different examples here of how we could choose a phage to toggle antibiotic resistance to change it to sensitivity if you use that phage in therapy. But here on the right is what I want to show you, is the unfortunate consequence if you choose the wrong phage, then it could be interacting with the bacteria in a way that the bacteria gain phage resistance, and at the same time, they'll gain antibiotic resistance. That is not what we would want to achieve, but this could easily happen if the phage and the antibiotic are entering the bacterium through the same portal. And that's what we observed and recently published on, for example, in two

phages, T6 and U115 that are lytic phages that infect *E. coli*, and they happen to enter through the TSX porin, and that is the same way that albicidin antibiotic can enter the cell. So, what you find is a perfect cross-resistance to all three agents, no matter which one you use in selection for these bacteria to gain resistance to one. And it achieves resistance to all three. And we localize this as expected to the TSX gene in *E. coli,* and we basically published on the various ways in which that mutation—different sets of mutations could arise from the same effect.

So, nevertheless, despite all those complexities, a lot of what we've had is indeed some success when we try and develop this and use this in emergency patient treatment, trying to use phages that select for such tradeoffs, by and large, this is successful, despite all the complexities that I just mentioned. So, this table is not showing you all of the 20 chronic infections, most of them pulmonary, that we've treated to

date. They've all happened with zero safety problems. Here on the left, it's a reminder for me to say that a lot of the patients in this chart have lung problems. They have either cystic fibrosis or non-CF bronchiectasis or COPD. But essentially, as you see in the chart here, there's a lot of success, but what we don't know are the gory details of what is happening inside of these patients, and that's what you've seen in the workshop so far, and that's what I will key in for a moment. So, for example, are the bacteria actually evolving in the way that we would predict? Are the phages staying in these patients and evolving alongside, and is there any interaction that would lead to co-evolution? We don't know that, and we are still looking into this.

I'll go quickly to a published first case. And this was a man who had an aortic arch replacement, and unfortunately, he came to develop a multidrug-resistant chronic infection with *Pseudomonas aeruginosa*. But fortunately, we were able to deploy a phage,

OMKO1, and it synergizes with an antibiotic. And in this case, we deployed the phage and the antibiotic. There was this synergy, and it cleared the infection in a single dose. You see here some of the details of his case, and I refer you to those published studies there, if you want more details.

So, within his case, it was a bit of a mystery. You know, we're hearing in this workshop that I'll talk about in a moment, when we deploy phages multiple times in a day and over the course of multiple days. In this man's case, we actually were able to solve this problem with a single dose of the phage and an antibiotic that synergized to become effective. And why is this? We've explored this with Joshua Weitz's group at Georgia Tech and had a paper published in 2020 that predicts that if you create a model here that appreciates that you can have phage, antibiotic, as well as innate immune system synergy, here on the lower right, it's when all three are acting together that you predict that you get this very large white space of an

outcome of the cleared infection. So, the point here is that antibiotic and phage can work in synergy to reduce the bacterial population size to a level where the immune system can actually kick in and help the patient.

So, I'll end by talking about some unpublished data that, if we now go back to one of these phages that we've placed the people, OMKO1, and we look *in vitro* at co-evolution between that phage and its bacterial target, multidrug-resistant *Pseudomonas aeruginosa*, do we see the kind of co-evolutionary outcome that also leads to the tradeoff that we hope to have occur within the patients? So, I'll quickly say that we've tried this in three experimental treatments, and here in the lower right is a control. So essentially, the first thing that we wanted to know is if you put the phage and the bacteria together and do a serial transfer experiment or an experimental co-evolution study, do they even stay in the system together with one another? And indeed, that is the case. So, in

all three of the treatments, you find that the phages and the bacteria are still there by the end. Interestingly, though, the phages in these treatments are suppressing the size of the population of the bacteria to a lower level than if the bacteria were there in the system on their own. That's not very surprising, other studies show this, but we would like to see this in the case of this particular phage, and indeed, it has that property of reducing the host population size.

So, we did a massive screening within that evolution experiment that I just mentioned, and imagine that over the course of every single day from each one of those experimental treatments, 96 clones were taken and they were screened in three different environments: ordinary growth medium, the ability to grow in the presence of co-evolving phages, and the ability to grow in the unselected environment of tetracycline. So, because nearly 100 were taken each day, essentially, you've got about 3,000 different phenotypic measurements in this one study

which is quite unusual.

And here are the outcomes real quickly. As expected, it was only in these three experimental treatments that you get a trending upwards, in the evolution of phage resistance in the bacteria, whereas here in the control, that did not change over time. That is satisfying, and it tells us that the co-presence of the phage and the bacteria together leads to greater selection for phage resistance in the bacterial population. Do we then see the tradeoff that we've observed *in vitro* and that we hope occurs within the patients and seemingly occurred in the patient I just mentioned?

And the answer is, not always. In only one of the cases, this one here in blue, treatment one we have this trending downwards, whereas in the other two treatments here, as well as in the control, whatever these bacteria are doing could just simply evolve within the laboratory environment. It tends to a slight trend upwards in their tetracycline resistance rather than a tetracycline

sensitivity. So, that's very interesting, and the question is, why is this happening?

What we think is occurring is that there's different types of co-evolution that can occur between a phage and a bacterium and I don't have too much time to get into this. But one can do something called a time-shift assay, where you take the bacteria or the phage, and because we have all the power to place the samples in the freezer, we can resurrect these and look at how to, for example, the bacteria, how do they grow in the presence of phages of the future that have undergone co-evolution, but we now challenge the bacteria of the past to grow in the presence of those pages. So essentially, these time-shift assays are very powerful for suggesting coevolution and how it occurs. And it can occur in two different ways: an arms race co-evolutionary dynamic, or what's called a fluctuating-selection co-evolutionary dynamic. Here on the right are what we've observed for both bacterial fitness and phage fitness is that the blue data here to the left

and the right of this zero line, you can see that these values are hugging either the one value here or the zero value below. And there's not that much variation in those values across time. And that suggests that this is arms race co-evolutionary dynamic, whereas in the other two instances, we get a fluctuation, fluctuating-selection co-evolutionary dynamic, a different type of an interaction has occurred between the phage and bacteria through co-evolution. And we think that this difference is what is driving the evolution of the tradeoff in one case, whereas the absence of the evolution and tradeoff in the other two cases.

So, next steps for us are very many. One is that we have this FDA-approved clinical trial. It's actually not upcoming, it's ongoing at Yale New Haven Hospital. It's happening right now. It's abbreviated as CY-PHY. Essentially, we're looking at the ability of the phages to protect the lungs of healthy volunteers who happen to have cystic fibrosis over a multi-month period compared to those

who received a placebo. So, the goal here is to see whether the exacerbations and just the success of bacteria to colonize the lung is less, if you deploy these phages on a prophylactic manner of a multi-month period, compared to individuals who only receive a placebo.

So, another step would be some of what you heard about in this workshop. We want to take samples from our emergent cases, pre and post therapy, as well as those from receiving the clinical treatment, the clinical trial treatment. And just a little teaser for what we tend to see. These are unpublished data, but imagine in the case here, we have a phage that we've used in people that target *Pseudomonas aeruginosa* that selects against a virulence factor called pyocyanin. Here on this graph on the left, you can see if you take clones at different time points from a treated patient, then over time, you find on average the ability of those *Pseudomonas aeruginosa* that are still in the lung, but they are debilitated in their ability to make

pyocyanin. And this is because we've deployed the phage and tried to use that in therapy. And here on the right is the consequence, if you take those clones of bacteria, grow them in the lab, take their supernatant only and put that on airway epithelial cells, you would expect that over time there would be a reduction in the ability of that supernatant to trigger inflammation in those cells of human origin. In other words, you should see a reduction in the virulence of those bacteria over time, and indeed, that's what we see. Two indications here, IL-8 and IL-6. So, last, I'll say that our next steps also involve a center for phage biology and therapy at Yale. That was just, we just got an investment from the university to do this, and the goal here is to invest in more phage biology research and phage therapy development at our university, and the mission is to advance and support this research, translate those advancements into new clinical therapies, and all the while, train and educate students, scientists, health care professionals, as well

as the community. A lot of the key players are shown here on the slide, and I'm running probably short on time, and I don't want to mention everybody by name, but, essentially, we're excited to finally launch this. The website is not live yet. You'll see that pretty soon, but the point is, we've already staffed this out with a large number of researchers and support research folks, as well as some visitors that are already starting to come to our laboratory to work with us, and we would like to do more of that in the future, and certainly, happy for that to include you if you're interested.

So, I'll end by acknowledging my lab group past and present. They've really been the ones who have done the work that I've talked about today. Especially today, I did mention the work of Ben Chan, a research scientist in my group; Katie Kortright, a postdoc; our two physician partners, Jon Koff and Gail Stanley; Barbara Kazmierczak is a great provider of resources for us, as well as others. I really am very thankful to Barbara

and others for providing reagents and resources to us. Alita Burmeister is a postdoc in my group, actually a research scientist. And I'll end by thanking Deepak Narayan who was the surgeon on our first patient case. Unfortunately, we lost him to cancer a few years ago, but without his ability to believe in what we've done, we never would have been able to treat that first patient as well as others subsequently. So, thank you very much for your attention. I hope I ended more or less on time because my timer stopped on me, but I'll stop sharing and take any questions during the Q&A if there are any.

DR. CAMPBELL: Thanks, Paul. If we could have the other panelists join, and maybe while they're doing that, I will take the liberty of asking Paul a question. Paul, in the last vignette that you told us, you were selecting for strains that were less-that were pyo-minus. I was just wondering if you removed—if you look, what happens if you remove the phage selection? Do they revert?

DR. TURNER: Yeah, that is a good

question. A lot of what we're still studying now is, you know, what are the consequences of past selection for future performance in evolution? I mean, we have always studied that. That's an evolvability question, and we find a lot of differing scenarios depending on what the initial mutation that comes in. This is some of the details I did not have time to go into for that co-evolution study. If you have some mutation coming in and solving the problem, it's going to be impactful for further co-evolution downstream, whether that would be arms race or fluctuating-selection. We cannot control any of that, of course. And essentially what we are trying to find out are the boundaries by which we can be comfortable deploying a phage or phages together and understanding the consequences for the selection that that causes on the bacteria, and are those bacteria really painted into a corner? Or is it a pretty big parameter space where something unexpected could occur next? So, in other words, as Dr. Maresso was talking about, we really want to know what is

happening in the complexities of these systems from a prediction standpoint, which is terrifically difficult, but that's, in my opinion, where this science has to go. I hope that was a good enough answer. I'm not sure I had a good one for it.

DR. CAMPBELL: Oh, no, thanks. That was good. One question which came up in the chat, for Bekah. and I will also pose it to Jennifer, one of the questions, Bekah, you may have already answered this privately, but in writing. But one of them was, why would—do you have any ideas why the response to Muddy was stronger than the other two phages? And I guess for Jennifer, after Bekah's answer, do you have any evidence of some of the phages being more immunogenic than others?

DR. DEDRICK: Yes. So—

DR. CAMPBELL: Sorry. Go ahead.

DR. DEDRICK: Go ahead Joe. I am sorry.

DR. CAMPBELL: No, I just want to say you go ahead first Bekah, and then you, Jennifer.

DR. DEDRICK: Okay, yeah. So, the question was, why do we see such a great immune response to Muddy in the second case that I presented? And, you know, our bottom line is, we don't know. But you could imagine a situation where Muddy is just actively replicating more than the other two phages in the host, and, you know, there is just more of them around. And so, you get an immune response to Muddy that's stronger because of that. That's a hypothesis that we have.

DR. DAN: And then to answer the question, did I find any phage to be more immunogenic? I have only tested the negative phage, which is a *Staph aureus* phage on the patient and normal healthy donors, and I didn't see any response. And I only tested the phage cocktail, they weren't separated. So, I only had the four-phage cocktail and the five-phage cocktail to test. And I haven't tested any other phages.

DR. CAMPBELL: Thanks. Let's see, I'm going to pose a question to Anthony and Paul, and I just sort of broadly—Paul mentioned a

clinical trial that he is doing, and I know Anthony's been involved in so many different clinical studies. And I am wondering to use a term that Paul's fond of, how are the results, and how do you envision the results of these studies evolving your thinking on phage selection? And maybe Anthony, you can go first and then we will let Paul chime in.

DR. MARESSO: Joe, you are asking specifically whether or not we're learning to better make cocktails based on results from the trials or…?

DR. CAMPBELL: Yeah, that and I guess sort of can you comment on how the successes and failures, inform your later, you know, inform or help evolve your process of selecting phages?

DR. MARESSO: Yeah, so, for example, with UTIs, there is many examples. So, for like UTIs, one of the underappreciated elements with a chronic UTI is that, mostly for *E. coli*, which I think is around 80 percent of all UTI's, they will form intracellular immunities inside the bladder

epithelium, right? And this will also be a way for them to avoid not only the immune system, but antibiotic treatment. And so, what you might imagine is that once you've gone through—we've seen this in our own animal models, and we think this may also be potentially true in people. When you've observed the ineffectiveness of your phages in this model often enough, you then begin to wonder whether or not you should start selecting for phages that somehow get in the bladder epithelium. And so, there is some evidence from our own group that there are uptake mechanisms that probably the epithelium naturally has, where some phages seem to be able to enter. And so, this might be a selection mechanism, you might start to tailor the treatment paradigm to where you can actually have a group of phages in your cocktail that perhaps get inside and meet the bacteria in the intracellular bodies, as well as a group of phages that work really well in a urine environment and are more luminal, right? This is the type of, we call them

medical simulators. They are really just preclinical models that we build in the laboratory to answer these questions and find phages that work in those contexts. I think for the group at large, that, from what I have seen over the last three days, you know, there's resistance—like the top microenvironments you have to consider all, right, resistance, biofilms, and it looks like possibly interactions with the immune system. So, if you can build models that address, that—specifically find phage that overcome these elements, then I think your cocktails, your treatments are going to follow, too, the success of them will follow. That's why we start with the difficulty first and try to find the phages that have evolved essentially to work in those environments to begin with. Is that a good answer, Joe?

DR. CAMPBELL: Sure, and let’s hear what Paul's thoughts are.

DR. TURNER: Sure, two things briefly, just merely the matching of the phages with the patient sample. If you

understand or if you’re measuring the full genomes of the phages and characterizing them or something about the genome sequences of the target bacteria, a lot of things suddenly become more illuminating, there's presence of prophage, you know, what are the consequence on the bacterial side that says that immediately there is resistance to one phage, but sensitivity to another one? Right? So, simply the screening process helps us understand these systems. Now, we tend to—I should say I, it is not like I am averse to using cocktails, but they're very complex. So, I would rather find a very broad host-range phage or evolve one. But I think everybody knows that. No, I am not at all against cocktails. But the point is, you know, there is, I am trying to keep things as simple as possible. But kind of echoing what Anthony just said is that we have also seen that the phages we've already deployed in people differ in their ability to enter cells of human origin. So, this tells us already that there’s a differing degree of interaction of some

phages with human cells and should we expect—and I don't know that it's necessarily true—that those that are interacting more intimately are going to be eliciting a greater immune response. So, this work is being done in collaboration with Jen Bomberger at Pitt, along with her postdoc, Paula Zamora. And the jury's out on what I just said, but definitely we get a differing ability of phages that we've deployed in emergency cases to enter human cells, and I think that's fascinating. So, it definitely should help us understand.

DR. CAMPBELL: Great, thanks. I'm going to move on to some of the questions for Bekah and Jennifer that were in the chat, unless you guys answered them and they disappeared on me. Hold on. Maybe I just have to look at the "and answered." Did you guys just answer some questions?

DR. DEDRICK: I did. Yes. Sorry.

DR. CAMPBELL: All right.

DR. TURNER: Bekah's very efficient. I didn't even look at them.

DR. CAMPBELL: Okay. The question—one

of the questions you just answered is, but maybe you can answer for all of us, is that phage is a good adjuvant, do you think a phage attached with a minute amount of host proteins activated the cell-mediated response *M. abscessus*?

DR. DEDRICK: Yeah, so I think Dr. Biswajit was talking about the first case study. And so, you know, when we prepare our phages, we do double cesium-band them, and then they undergo extensive dialysis before we use them therapeutically. And of course, we do, you know, sterility testing, endotoxin assay, all that. But you are right, in that we don't necessarily test those phage preps for the bacterial host proteins, right? And could they actually elicit an immune response that enables the patient's immune system to actually effectively, you know, target the bacteria? And I suppose that that is a possibility. But for the first patient, I would argue that she had undergone a double lung transplant, and she was on immunosuppressants. So, I kind of think that

that might not have occurred. And also, for the first patient, we did see evidence of phage replication *in vivo*. But that is something definitely to keep in mind.

DR. CAMPBELL: And for Jennifer, there is a question saying, did you get a chance to study CD4-plus T cell response against *Pseudomonas aeruginosa* after phage treatment?

DR. DAN: No, unfortunately, we weren't able—we didn’t get any blood after cycle two, so we weren’t able to test for CD4 T cell responses. You could look at the beginning of cycle two as, you know, there is a gap between cycle one and cycle two, and we did get blood before starting cycle two. It was only a gap of probably like 30 days, but the patient maintained some phage-specific CD4 T cell response. So, his recall response would vary depending on what immunosuppression he was on. So, Sirolimus is a potent T cell blocker in terms of, probably, proliferation and things like that. So, that probably did have some effect on it. I'm not sure what

photopheresis—how photopheresis would affect his T cell response, but he was getting photopheresis during the entire two cycles, so it didn't seem to impact it as much, as we were able to phage-specific CD4 T cells. So, the only evidence I had that he'd had some lingering CD4 T cells after phage therapy was at day zero when he was receiving cycle two.

DR. CAMPBELL: Thank you. And maybe I'll just ask another question to Bekah and Jennifer. Do you—based on what both of you, learned from your studies, and I know you have not done that many studies, but are there some lessons learned? So, Bekah, maybe I will start with you, are there things you guys would have done differently with this patient to hopefully avoid the immune response from causing problems?

DR. DEDRICK: Yeah, I mean, one of the things that we, I think we are considering now is that maybe going in with a three-phage cocktail initially with an immunocompetent patient isn't the way to go. Perhaps going in one phage at a time and just enabling one

phage to go in. If the patient has an immune response, at least you can put in another phage after that. So, sort of a sequential phage therapy. That being said, you know, I heard Dr. Aslam speak yesterday, and I just want to bring up that we had similar situations in that she talks about, she had patients with immune response to phage therapy, but it didn't seem to affect the outcome of the phage therapy. So, it seemed like the patient still did well. And although it is not—we do not have all of this published yet, but we do have a few cases that also show that as well. And so, I think each case is very, very complicated. But these are all things to keep in mind, yeah.

DR. DAN: Yeah, I agree. So, like, with our patient, I think the patient ended up doing well at least for the first cycle, he has an (inaudible) and was able to walk around (inaudible), whereas after the second cycle it was more of a prolonged pneumonia treatment. But he was able to get over at least that period of having to be—having to get over that

clinical period for the pneumonia. But he did require some suppressive therapy. And what we were dealing with is someone who had graft dysfunction basically. It was requiring photopheresis and a lot of immunosuppression. In terms of whether his immune—and even though he went through all that, he was still able to make an immune response to the phage. So, with all the things that I've shown, I think it was phage-specific, because I was able to test the *Staph aureus* phage and compare it to the phage cocktail. But I do not quite know that, since that's my only example, I don't know if they were to test a different phage, would the patient also develop a response. And in terms of the ELISA, what part of, or what are we making—what was the patient making the immunoglobulin to initiate a reaction to? What part of the phage is he making a reaction to? And why am I able to find it in IVIG with normal, healthy donors at a much lower concentration? Obviously, because I was testing that particular phage, I am not sure, but I don't know. I don't think the immune

response made much of a difference, at least for cycle one. For cycle two, when he did have some immune response to the four-phage cocktail, maybe that's what prompted us switching to Navy, the Navy three-phage cocktail, which was completely different. And that might have made an impact, I'm not sure, though. It's all hand waving.

DR. CAMPBELL: Thanks. Anthony, I have a question. This is my own question, I was just wondering if you used your two chamber system and instead of putting a resistant mutant, put in phage with mucin or something and try to select for the ability of phages to gain the ability to work in the presence of mucin, like you suggest, select for the ability of phages to act against resistant mutants?

DR. MARESSO: We haven't done that yet, but that's where we would like to go with this. I think you have to have the right phage. I think Paul would agree that if you cannot sort of *de novo* assemble a system that isn't really there to begin with, but you

might be able to pull out a rare variant from a heterogeneous population that is successful in that environment, that may not have been able to be pulled out over time in some static system. But the key with the system is that it’s continuous co-culture. So, we essentially keep the bacteria in log phase indefinitely, they don't, they don't peter off. They are always dividing, there is always a burst going on, and so you’re amplifying phage per unit time at a greater rate. And so, some of them acquire some mutations or already have mutations that are very rare in the population. And then if the selection is right, they’ll grow over time, and we'll get them. So, where we’ve done this, when we've been successful with this, is adapting or training to resisters, like you saw in the presentation, as well as pulling very rare phages out of a system that couldn't be captured by the traditional methods, that were in very low abundance. We've done it for *Pseudomonas* and *E. coli*. The next step in this process is to essentially create like a blood-

like or a urine-like environment or, as you mentioned, a mucus-like environment, and see if we can pigeonhole phages to be selected that are better in those environments versus just a broth culture.

DR. CAMPBELL: Thanks, and I will ask a question sort of to you and Bekah. Do you think—and I know I've talked to Bekah a little bit about this privately, but whether, if she was able to provide you with enough of her serum, which neutralized her phages, that you can use that system? That there is, I think, there is a limitation in how much serum you can get out of a patient, unfortunately? Well, probably fortunately. But down the line, that would be something that could be done?

DR. MARESSO: Yeah. Bekah, you probably don't have a lot of that material. These flasks that we do these evolution experiments, are, you know, 10 to 30 ml, I know that you do not have at that level. But certainly if we can, like, simulate it by generating neutralizing responses, say, from a rabbit, where we have hundreds of milliliters

of serum, that does negate a phage's ability to hit its target, right? We perhaps can engineer the evolution experiment to learn the limits of that. I think, but I would preface it, I think Paul would agree. I think though, you'll have to have some type of—you have to start with, I think, phage that, perhaps challenge this or have these challenges at, like, the environment already and might have systems that make them have a sort of stealth-like to the immune system to begin with. And then perhaps you'll build an appendage that is a little bit better. Because I could totally envision that the mucosal surfaces, you know, every organism has a mucosal surface where there's IgA and IgG, that phages would have simply adapted to be able to kind of get around that to hit their targets. And so, we want to find those phages, because the prediction would be that those would be the ones that maybe have less neutralization during a treatment paradigm. Of course, I do like the idea of succession, where you know, you start with one phage, you maybe get a

response, it cuts it off, and you use other phages down the line, and you continue this. But I think that it's possible to actually find phages that don't stimulate these neutralization responses as well. And then there might be phages that induce, you know, the inflammatory response that—you've already seen this, right—that are really synergistic. You might actually want those phages in your treatment, even though many of the microbial elimination effects, might be indirect and not related to the target killings.

DR. TURNER: Can I jump in there? Totally agree that, yeah, if you have phages that are interacting with human cells and stimulating an interferon response, I am not so sure that's a bad thing, right? That's kind of getting a bonus, and the question is, are we often getting that bonus in phage therapy and only recently are we measuring what's going on? So, a bit of a related answer, also a thought is, I think it is echoing a little bit with what Anthony said. Look, there is a reason submarines look like sharks and orcas,

it is because we engineer things all the time based on the history of natural selection on this planet, where things have evolved traits that are useful for human applications. So, I just, I really feel like if you don't do the deep basic research on phage-bacteria interactions, you will necessarily miss out on what phages are doing, interacting with cells that might be of huge benefit and all kinds of applications like phage therapy. So, I guess this is always where I stand on my soapbox and say, don't forget the basic research that has to go on alongside what we have been doing.

DR. CAMPBELL: Yeah, and related to that, Bekah, can you remind me is Muddy the one that started out as temperate and you got rid of the repressor, or is that one of the other ones?

DR. DEDRICK: No, Muddy is a lytic phage. But both BPs and ZoeJ, which were used for both of the patients I presented today, were genetically engineered to remove the repressor and integrase, yes.

DR. CAMPBELL: Darn it. I was going

to argue that the lysogenic phage was less likely to have undergone selection, for you know, avoiding the immune system, because maybe it spent so much time as a lysogen.

DR. DEDRICK: Hmm, sorry.

DR. CAMPBELL: Damn, a perfectly good theory shot down by data.

DR. DEDRICK: But, if you do not mind, I will just add in that, yeah, I totally agree with what Anthony and Paul just said and that, you know, we did. And I think you and I had talked about this, Joe, that we did look for neutralization escape mutants from phage Muddy in the patient's serum. But you are right, Anthony. I mean, I don't have 30 ml of it. I have a lot less. But we could talk about doing something else, yeah.

DR. CAMPBELL: Okay. So, I guess the panel is about to wrap up, and then we're going to have some closing remarks from the workshop from Graham. But I'll give each one of the panelists—I will thank them once again for a stimulating discussion and wonderful talks and give them each a chance to give us

one more pearl of wisdom before they go off. Or not?

DR. TURNER: I can never—if you let me speak, I will. So basically, I am really intrigued a lot by deploying phages as a cocktail, or alone, or in sequence. And I feel like what you are going to see from our group and others are massive data sets that examine what are the consequences genetically as well as phenotypically for the target bacteria. In my opinion, I think that would be hugely valuable information.

DR. DEDRICK: I would just say I got a couple of questions about nebulizing phage, and if we expect to see an immune response to that, and I think that, you know, that's really important and that's something that we should consider. You know, various routes of administration, what kind of immune response is seen.

DR. MARESSO: My word of advice for the community would be: There is an old biochemical saying, we talk about it in the lab all the time, don't waste clean thinking

on dirty enzymes. And we would modify that to basically be, don't waste a clinical trial or an experiment or a case on phage that don't address space and time. So, try to learn what you're working with at the most mechanistic level and try to select the phage that have evolved properties that will enhance their activity in the bacterial microenvironment. We think they are likely to have better outcomes.

DR. CAMPBELL: Thank you all, and I guess I will ask you guys to turn off your videos and ask Graham to turn on his, and without further ado, if Graham can... I know you have some slides to show. Graham has the challenging—the challenge of telling us what we all learned in this. So, but I'm sure he is up to the challenge. So, are you ready to go, Graham? We can't hear you Graham. You want to try again, Graham?

MR. PINSON: Still can't hear you.

DR. TURNER: Maybe you didn't log in through the webinar?

DR. HATFULL: Can you hear me now?

MR. PINSON: Yep.

DR. HATFULL: Oh, just one of those headphone problems. All right, let's try showing the screen again. Is this okay?

MR. PINSON : Yes.

DR. HATFULL: Well, thank you very much, Joe. Yes, I think a most unenviable task. I'm not quite sure how I'm going to do this. Let me just start by acknowledging the disclosures here: I do consulting for Janssen Pharmaceuticals and for Tessera, Inc. And then, I just want to start by saying what a fantastic workshop I think this has been, and a huge thank you to all of you at NIAID and NIH and FDA for what you've done to put this together. Thanks obviously, go to all of the moderators and speakers, and everybody who has attended all of these sessions and all the excellent technical support.

I thought the meeting was really terrific for lots of reasons, but two specifically. So, one is the breadth of topics, perhaps there were some aspects of the therapeutic use of phages that were not included, but it was a fantastic

cross-section, if that's the right term, of the basic biology and the clinical experiences, the plans, the trials, the support, and the various aspects of the dynamics which will influence the successes and failures of the field going forward. And so, I really appreciated that. I thought it was exceptionally a well put together program, and I'd just like to say thank you. And if I may, on behalf of all the participants, for putting that together. Secondly, it was a real delight to see such a diversity and variation of the presenters and the scientists, regulators and others, where there was a really great representation, I thought, of people who I would think of as in the relatively early stages of their career, showing great progress in this field. And I think that infusion of the relatively junior investigators into this field is a really good sign of the healthiness of this entire area and topic. The world of phage biology investigation and utilities hasn't always been like that. I think we've been through at least

a couple of decades where recruitment of new exciting investigators was relatively meager, and so I see that as a particularly important aspect of the field, and it reflects upon what I think is probably quite a bright future.

So, Joe is correct that I can't cover all of the items, and I don't want to simply repeat all of the items that we've heard about over the past couple of days—two and a half days, that I think were nonetheless really terrific. And I sort of made a list of things that we've gone through here, which I'm not going to go through in detail, but this is a very broad coverage of the topics and I really liked it a lot.

If there's one, I think, really central message that we all need to have a firm grasp of, it's that there is no one therapy. We are—the field is dominated by issues of specificity, and as I think Dr. Hatoum-Aslan commented on this double-edged sword of specificity, that the specificity of phages for the host is very attractive in terms of using these, sort of, guided missiles

to take out bad bugs, but the specificity constrains them to particular subsets of strains and makes the broader coverage of the bacterial pathogens more complicated. And I think today we've also learned that we need to think about specificity, not just in those dimensions, but also in some aspects of space, in terms of where the infections and the interactions happen during an infection, and also as a matter of time during the course of treatment. And I think these aspects of specificity are what have made phage therapy and the therapeutic use of phages such a challenging field over the years, and it's why progress has been, kind of, spotty, let's say.

We're not talking about one therapy, but many, many different types of therapies with different infections, different strains, and different types of phages. And so, there are certain, I would call them assumptions or declarations or comments that we've heard through the course of the meeting that I want to, perhaps, talk about. And I don't want to challenge those assumptions. I think for the

most part that they're perfectly fair and perfectly right. It's just that there's exceptions, and the exceptions occur simply because of the different circumstances that we are encountering with different types of infections and different types of bacteria. And so, I'll go through these very briefly here, but then I want to go through and focus on some of them in a bit more detail, and really use some of our experiences with the phages of the *Mycobacteria* to provide some illustrations, not so much of the details, but of the principles that we have to navigate these exceptions for particular pathogens in order to understand how to move forward productively.

So, we heard about how phages are attractive because there's always phages that can be identified with pretty much any bacterial pathogen. We've heard that strain specificity can often be predominantly determined by receptor availability, and resistance appearing through loss of the receptors, for example. We heard that

temperate phages are really not useful therapeutically, which is true as such. Phage resistance is always encountered *in vivo* and *in vitro*, yes, but maybe differently in different bugs. And that bacteria are easy to grow and do all of these types of *in vitro* assays, which makes it very—which simplifies the way to account for phage growth and phage behavior. These are all generally true, I think, but we'll look at the exceptions.

I also just wanted to comment that if we have time at the end before we go to the final comments from Scott, I will answer questions. So, if there are topics and questions that come up and we have time at the end, put them in the chat or the Q&A, and I'll address them.

Okay, so, my lab's been interested in Mycobacterial infections, and we've been working on Mycobacterial phages for a very long time. We saw early on in the workshop, when hearing about the number of IND or eIND applications coming through the FDA that *Mycobacterium* infections were not maybe at the

top of the list, but they were close to the top of the list, behind *Pseudomonas* and *Staph*; but I think that illustrates how serious some of these Mycobacterial infections are to treat with antibiotics; and therefore, there is a strong need for phages, because there are often no other ways to respond to the infections. When we think about Mycobacterial infections, they can be divided into those that are the so called non-tuberculosis *Mycobacteria*, or NTMs, of which there's a number of important pathogens, but I'm going to focus on *Mycobacterium abscessus*, which is certainly one of the more prevalent ones; and then there's tuberculosis itself, which is caused by *Mycobacterium tuberculosis*.
M. abscessus and MTB are obviously relatively closely related pathogens, although they have a number of distinctive differences. So, the infections with *Mycobacterium abscessus* tend to be constrained to mostly CF patients and some non-CF bronchiectasis, but are serious; so, a relatively substantial population, but relatively small if you compare it to TB.

We've heard very little about TB over the course of the workshop; however, it really dwarfs the clinical issues and health issues of many of the other pathogens we've heard about. Ten million new cases a year. A quarter of the world is infected, although often without symptoms of TB. It kills about one-and-a-half million people a year.

Mycobacterium abscessus tends to be intrinsically resistant to many antibiotics. It's often unresponsive to antibiotics when given to the patient, and those antibiotics are often administered for long periods of time, many months or years, and the toxicity is a very severe problem. We hear a lot of patients who are saying "I've got this infection, but it's the antibiotics that are killing me."

With TB, most TB infections are actually drug-sensitive, but in response to the use of antibiotics, there's just a large number of cases now caused by multiple drug- or extensively or totally drug-resistant TB; and clearly there's a need for alternative

therapies there.

Amongst *M. abscessus,* as we've heard, but it's an important issue, there's enormous strain variation in phage sensitivity. So, a phage that infects one particular strain doesn't necessarily infect other strains, or even any other strain, and it's a critical and a key limitation to the broad use of phages for *M. abscessus* infections.

TB is different. TB is much more like the cholera case that we also heard, which is more clonal, much less variation, and therefore a relatively small cocktail of phages appears to be capable of infecting, what we think is most clinical isolates of *Mycobacterium tuberculosis*. The cause of those changes and variations in sensitivity, we don't yet fully understand; although, we just heard the comment that the prophages, which are abundant in *Mycobacterium abscessus*, may actually be playing roles in that; although we now know that prophages are essentially absent from MTB. So, maybe that's also reflecting the

more clonal aspects of the strains.

Because of these parameters, the treatment has to be personalized, phage treatment for *M. abscessus* has to be personalized, where we think that personalization to TB is not only not required, but because of the extremely slow growth of TB, 24-hour doubling time, personalization is not really a simple thing to do. And so, for *Mycobacterium abscessus*, there's actually quite a lot of instances where compassionate use is warranted. Although for TB, in part because of the development of new drugs and because of the various parameters that we heard about earlier from folks at the FDA, TB is really hard to find compassionate use cases for as well, and so they differ there.

We've heard several really nice talks about the use of nebulization, the giving of phages by aerosolization to the patients. You could do this to CF patients, but it's really challenging because of the congestion of the airways and the difficulties

of confidence in getting the phages to where they can go, although clearly, as we just heard from Paul Turner, it can be done and has been done with some success. That's much less of a problem for TB, even though both of these are really often lung infections. Aerosols are likely to be more plausible for TB, as we heard from Reinhard Vehring as well. But, both of these strains—there's been very few phages isolated on these strains alone; so yes, you can isolate phages from the environment on many different strains of bacteria. It turns out to be really tough to find them if you use these strains directly for their isolation. And then, contrary to the notion that you can always just pick the lytic phages, we actually have relatively few lytic phages for either of these. Most of the phages that do infect either of these two strains are actually temperate phages, and so it's either that or nothing, and that has influenced how we've moved forward with some of these therapies, and I'll mention that briefly as well.

Okay so, if you can't find phages,

then where do you get them from? And I want to briefly describe a couple of approaches that I think are likely to be generally useful. This difficulty is not only for *Mycobacterium abscessus*, but we also heard, I think Dr. Hatoum-Aslan was talking about, some challenges in even *Staph* strains where it's not always easy to get phages.

So, there's two solutions. One solution is to use a surrogate host, and we've made substantial use of phages that were isolated on a strain of *Mycobacterium smegmatis*, which doesn't have the same pathogenic profiles and is relatively fast growing, and because of the PHIRE and SEA-PHAGES integrated research education programs, we have large numbers of students on a national basis involved in phage discovery and genomics. It's led to a large collection of ten thousand individual and archived phages isolated on *Mycobacterium smegmatis* of which over two thousand of those have been sequenced, and so we have substantial and detailed genomic data as to how they relate to

each other. The diversity is great. So, in only two instances has the same phage with the same sequence been isolated twice from independent occasions, but they differ in every possible imaginable way; and so, we can think of them as being many different types, depending on how you determine a type, and great diversity within those types of related phages. Lots and lots of temperate, as I mentioned, and only subsets of these types, but a significant subset, do infect either *Mycobacterium abscessus* or *Mycobacterium tuberculosis*. So, those are the phages that we've taken advantage of.

The variation we see in *M. abscessus* is essentially unpredictable from a genomic point of view, and it's true for these and the other phages that we've looked at, and as I mentioned, relatively minor variation, when you look at *M. tuberculosis,* using these phages. We've heard about evolution of phage specificity, and this is something that we've done with these phages, in our case, really just looking for host range mutants that can

expand the types of strains that a phage can infect. So, an *M. smegmatis* phage we may find does not efficiently infect a particular strain of *Mycobacterium abscessus*, but we can relatively easily isolate and recover from plaque assays host range mutants, which have usually single point mutations, sometimes in tail fiber genes, sometimes elsewhere in the genome, that now can efficiently infect other strains, and we've managed to use those therapeutically as well.

My final point is that having phages that grow on *Mycobacterium smegmatis* is actually a huge advantage in terms of production. Growing large amounts of phage on TB is definitely a problem, and growing them on the pathogenic *Mycobacterium abscessus* strains is really challenging as well; and so, that's a real key advantage of using a surrogate, that it's useful for preparation as well as a source of phages.

And so, just very briefly, to illustrate this point about these *smegmatis* phages. This is showing a phylogeny of

clinical isolates of *Mycobacterium abscessus*—that's actually a phylogenetic representation here, three different subspecies. These strains at the top here are part of a clade of very closely related strains, and yet nonetheless, these *smegmatis* phages have these unpredictable and sort of characteristic variation in the ability to infect these strains. It's not predictable from the genomic point of view as far as we can tell at this point, and so, each one—each strain has to be tested. Nonetheless, this set of relatively small numbers of phages gives us pretty good coverage of these strains.

That's one source of phages, then. Use a surrogate host and then screen amongst those that have the appropriate host range properties that might make them useful. I'd like to argue that strains—and this may extend to *Staph*, *Pseudomonas* and other strains, that carry prophages—that those prophages may be useful too. So, prophages are abundant in *Mycobacterium abscessus* genomes, and they vary enormously from one strain to another. As I

said, that's very different than TB, and we have developed—or are developing new bioinformatic tools for identifying and extracting prophage sequences. So, we can be confident of those assertions, I think. The phages that we can see bioinformatically are clearly distinct from the *Mycobacterium smegmatis* phages, so in some sense this is a bit of a surprise, because you'd think you would be able to isolate them from the environment, having been released from those naturally lysogenic strains of *Mycobacterium abscessus*, even though that appears to not be the case as far as we can tell. However, by looking at spontaneous induction, and by careful choice of hosts, we can—we've managed to get a number of those prophages to now grow lytically. So, the trick is finding a strain that will release a phage and a strain that is sensitive to that phage. And if you have a large collection of strains, which we now do for clinical isolates, pairwise—large numbers of pairwise comparisons can provide you now the opportunity to grow these phages

lytically. I'm just showing six here that all have these *Siphoviral* morphologies. These have all been introduced as resident prophages. This is what the genomes look like, as an example. We have about twenty of these so far, but we think we have a large number more that we can get out, and have the capacity to use those therapeutically. And so, we can use those, we can check the tropisms of those back on these same similar types of strains, and we can see some of these now ex-prophages can expand the tropisms of the overall phages that we have, potentially for therapy.

However, those are temperate phages, and as we've learned, we don't want to use those therapeutically, because they form lysogens in the infected bacteria at frequencies of maybe 10 percent or higher, sometimes much higher. And therefore, that inefficient killing isn't going to be good for therapeutic purposes. However, I'd argue that if you only have temperate phages available to you, it doesn't mean they're completely useless as you can engineer them—and we've

heard about this from a couple of talks—you can engineer them so that they are now capable of growing lytically and only lytically. And Dr. Hatoum-Aslan talked about the CRISPR selection systems that they've developed, which I think is really nice. And I think that we'll probably hear more in the future of other different types of phage engineering strategies. I just would put in a comment on this recent paper on the use of what we call CRISPY-BRED, which is using CRISPR as a counterselection system exactly as was described for the *Staph* phages, but here we combine it with a recombineering system which promotes efficient recombination between a phage genome and a synthetic substrate. And then, so, the recombineering gives you essentially the recombinants, and then CRISPR selection gives you the counterselection against the wild-type, and this is a useful way of making phage mutants that are defective in repressor and integrase, both of which have to be taken out for using one of these phages therapeutically, as well as of course any

other phages—any other genes which may be implicated in virulence.

Receptors and receptor availability is clearly a key parameter in specificity, and—we've heard from a number of talks, but I think it's going to be an ongoing lesson for us all—there are many, many ways in which bacteria can become resistant to a particular phage, and in some cases it may be receptor-mediated, in other cases much less so. So, what I told you is that these phages—these strains of *M. abscessus* have abundant prophages, and they also have abundant plasmids. And even strains here which are extremely closely related vary in their phage receptibility profiles, and they also vary greatly in their prophage content and in their plasmid content. So, we would surmise here that the prophage and the plasmid content is largely responsible for influencing the phage infection profiles, not because of things like repressor-mediated superinfection immunity, but because prophages express genes which generally defend, although with specificity,

against other types of phages. And there's been several papers out on this, including one that's relatively recent here, where we can show specifically that prophage-encoded genes defend against phage attack with specificity and therefore confer and likely play roles in these overall specificity profiles.

Phage resistance, we've heard quite a lot about over the last three days or so, and it's clearly a critical parameter. It can happen at relatively high frequencies and certainly frequencies which are expected to give you failure during therapy if you were to use one phage alone. However, the frequencies and mechanisms of resistance are almost certainly really different in different types of bacteria and different strains. We think that resistance is plausibly a substantial concern in *Mycobacterium tuberculosis* and would really drive you towards the use of cocktails for TB treatments. For *Mycobacterium abscessus,* that appears not to be true, and resistance appears to be rare *in vitro*, and for us, not encountered for the most part *in*

vivo, either.

So, in the interest of time, I'll just say that we've looked at a number of combinations—at least we've reported, we've actually done more than this now—of *Mycobacterium abscessus* strains with different phages, and using at least moderate amounts of bacteria, this number or more, and only twelve of these strains did we get any survivors at all. We get very efficient killing when the phage infects. And then, when we do get resistant mutants, we sometimes get—sometimes can be fully resistant, but often get these partial resistant phenotypes as well. And then when we sequence some of these, we don't know what the role of the mutations are, but it's not simply loss of some receptor, although there may be indirect effects on the surface which influence receptors. So, for example, we have mutations in the *uvrD* gene or in *rpoZ*. It's unclear what the role of these is, but resistance, when it does occur, is by mechanisms that we don't fully understand. And overall resistance is at such a low level that

we think that monotherapy with a single phage for *M. abscessus* is actually a viable option, and for all of the monotherapy experiments that we've done—or, not really experiments, but treatments that we've done, we've never observed resistance *in vivo*.

Finally, just a quick comment: This is unpublished for the most part, but we've actually been involved in about twenty-three patient treatments. We've had a lot of requests. These requests come about one very three days, and it's a substantial workload just responding and keeping up to date with these, but we've been involved in these twenty-three or so treatments on a global scale, mostly CF. And as we've heard—and I'm not going to dwell on this as we've heard from others—these are compassionate use cases, every one is different, and they're not a controlled experiment. So, we learned one thing, and one important thing, which is safety. We've seen no adverse reactions here, as we've heard from other speakers, and I think that's the good news. And then we have a

lot of other information that we can glean from these, which we hope will influence the kinds of clinical trials that will follow. So, we see lots of encouragement, from improvement—there were different endpoints to measure which may be making them transplant eligible, or clearing a disseminated infection, or completely cure of infections. And so, we think that there's good news there and encouraging news to move forward with. We've heard about antibody responses, I'm not going to say too much more about it, except, clearly it can and will influence how we move forward with these types of therapies.

So, finally, let me say just a thank you to the people that contributed to these aspects that we've talked about today, especially those in the therapeutic aspects, Chip Schooley and Connie Benson at UCSD, Helen Spencer in London, Keira Cohen at Johns Hopkins, and the people mostly from my lab. A shout-out to the folks for funding for something like forty thousand undergraduate students as phage hunters who have contributed

to the phage collections that we have, and the newly formed Pittsburgh phage project, or P3 program, which is helping to coordinate all of the phage therapy initiatives at the University of Pittsburgh.

So, if we have time for a question or two, I'll answer that; and just say a big thank you for your attention and for an absolutely inspiring and fantastic workshop.

DR. CAMPBELL: We have a question from Dr. Stibitz at the FDA. From Scott: "Have you considered making lysogenic phages virulent by substituting repressible phage promoters with constitutive ones? Do you see any possible advantages to this approach?"

DR. HATFULL: We haven't done that specifically. Mostly we try to go in and remove the repressor and the integrase, and it is challenging for some of the reasons that Dr. Hatoum-Aslan talked about, that we can—so, the problem is for the lytically growing prophages, we can't engineer them in *Mycobacterium smegmatis*. And this may sound like a subtlety, but they have to be done in

the *Mycobacterium abscessus* strains, and we are only just really coming up to speed with transferring all of the genetic tools that we've developed that work in TB and *Mycobacterium smegmatis* to have them work in *Mycobacterium abscessus*. So, we haven't done anything quite as fancy as trying to juggle the expression systems, but I think the power of the engineering, and indeed of doing synthetic genomics, is really going to open up the field to many more types of these types of modifications that Scott's talking about.

DR. CAMPBELL: Great. Thank you, Graham. Thank you for the nice summary of the meeting, and thanks once again to all of the speakers and everyone that has participated. I think it's been a great workshop and I will let—Scott Stibitz has a few closing remarks, because no FDA workshop is complete without an FDA person saying "These aren't my views," or, "These are my views, these aren't the views of the FDA." Thanks, Graham. Scott, do you want to take over? Scott?

DR. LEHMAN: Hi Joe, Scott had a

technical problem. Can you put up his slides from the storage box? He'll be here in a moment.

DR. CAMPBELL: I don't know how to put them up from the storage box. I don't know if…

MR. PINSON: I'll bring them up.

DR. CAMPBELL: Thank you.

DR. STIBITZ: Thank you everybody and I'm very sorry for that. Of course, it happened exactly as Joe said my name. My Zoom just completely froze and grayed out, I have no idea what happened.

Thank you everybody. My name is Scott Stibitz. I work at the FDA in CBER, and one of my responsibilities is overseeing CMC review of phage therapy products, bacteriophage to be used for therapy. I had to leave my notes on my other computer, so it may be a little while I collect my thoughts. Okay. So, please note these housekeeping notes, and I believe we will send out an email to this effect to all registrants as well. So, could you give me the next slide, please?

Great. Okay. So, I've introduced myself. I think this is a point at which this will date myself and part of the audience, but I ask myself "Well, how did I get here?" And in other words, why am I the one giving concluding remarks? And all I can say is that I was in a meeting of the phage planning committee and had to go away for a minute, came back and my name was penciled in. So, I would be more upset, I would object more, but I think Dr. Turner put it very well, "If you give me a chance to talk, I will." And the other is that I'm actually very honored to make these closing remarks.

I think one of my primary tasks today is to give a lot of shout-outs and thanks to people who've made this possible. I also wanted—I thought it was interesting that Joe characterized Graham's job as kind of a difficult one. It appeared to me that, as I listened to his opening slides: Well, perhaps the only one that's more difficult is to follow him and try to say some of the same things. So, forgive me for the clear harmony

between some of the things that I'm going to say and that Graham said.

But first, let's start with the—next slide please. With the planning committee. I debated about whether to put my own face on this slide, although I did participate in the planning of this, but, I made my peace with it, because the other people on this committee did the majority of the hard work. And so, I feel comfortable giving shout-outs to everybody. I think it's worth mentioning that this process was years in the making. At least part of that's due to COVID, the fact that we were originally shooting for 2020. But, we've had meetings, we've had a lot of meetings, as we've tried to wrestle with the program. It's undergone major changes, minor changes, as we tried to get the overall structure right and to get some of the details right. And so, it's very nice to hear the kind words from Graham. But, it also brings me to the following slide which is that we are very interested in your feedback. I think Graham is kind of—so, I should say, we did not set up, we did not

institute a specific questionnaire, but we rather—it'll be more informal. If you have comments you'd like to share, be they positive or negative, please just send them to this email address. I promise you we will look at them carefully and consider your opinions. Although, I think that Graham has really provided a blueprint, I think I remember words such as fantastic and inspiring, so, feel free to use those as well.

Let's see, what's next? Great. So, one of the reasons that we at the FDA have really enjoyed working with the folks at NIAID over the years, on workshops and other things, is just the fantastic team of people they have for putting on meetings. Up till now, they've all been in-person meetings, and so we've been still amazed at how smoothly things have run; but, you know, COVID came along, and AV also means IT; and I think you will all agree with me that the support in this regard has just been fantastic. I've listed some of the folks here, some with whom we've had the most direct contact are bolded, but everybody contributed

to this effort, and a great big thanks goes out to you guys. Next slide, please.

So, this is the meat of the workshop, these are the people who presented all the great science that you've heard. I have to say that, I mean, I'm just so impressed with the level of science that I've seen. I've got so many ideas, so many questions. It's just really been exciting, I think, in that regard. It really exceeded expectations, but the other thing is I was just so impressed with the consistency of the conciseness and clarity with which these 20-minute talks have been given, and have just given really the maximum amount of high level scientific information in a fairly short period of time. And even more amazing to me is the fact that essentially everybody kept to time, and if you've ever been involved in running a meeting, you know how important that can be. I don't think we were ever more than 5 minutes behind schedule, so, that's just great. So, next slide, please.

And then of course, again I'm very

aware that these slides are pretty much paralleling exactly Graham's slides, but so be it. I think this is all good. I just wanted to give some brief statistics on the participants for this meeting. This is based on the registration data. We had a total of more than 970, last time I looked, maybe we topped 1000, I'm not sure. Six continents are covered. No one from Antarctica came, sadly. A total of 46 countries, and of course there were multiple sectors of academia, industry, healthcare, and government, which we, of course, fully expected and sought. But, if I could have the last slide?

You know, I prefer to break down or to think about the participants in this meeting in this way. What I've done is to, just off the top of my head, kind of break down what we've been talking about in terms of different, I guess disciplines for lack of a better word.

We've got Basic Science, we've got Translational Science, and we have Clinical Science, we have product development, and

also, of course, Regulatory Science represented by the FDA, and research funding represented by our colleagues at NIH. And, you know, I thought about: well, how do these interact? And basically, what you see is the set of all possible arrows.

I don't think this is gratuitous, I think if one stops and one picks any two disciplines, and thinks about interactions between them, it's obvious, and you can think of many examples. So, I think this just really shows the interconnectedness and the ways in which we interact. And when I say "interact," I mean things like communication, collaboration, appreciation for the job that other people and other disciplines do and the difficulties they encounter, as well as mutual respect.

And so, I just wanted to say that this is the whole point of the workshop, this is what we want to foster, catalyze, whatever word you would like to use. But, it seems to me that, while there are many moving parts here, what unites is that we're all trying to

find a way to use the amazing biology of phage to address issues related to infectious disease.

And we at the FDA, and I think I can speak for my NIH colleagues, are proud to be members of this community. So, I will end there. Thank you very much. Thank you everybody. I think the meeting has been a success and we anxiously await your thoughts on the matter.

(Whereupon, at 3:06 p.m., the PROCEEDINGS were adjourned.)

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CERTIFICATE OF NOTARY PUBLIC

DISTRICT OF COLUMBIA

I, Mark Mahoney, notary public in and for the District of Columbia, do hereby certify that the forgoing PROCEEDING was duly recorded and thereafter reduced to print under my direction; that the witnesses were sworn to tell the truth under penalty of perjury; that said transcript is a true record of the testimony given by witnesses; that I am neither counsel for, related to, nor employed by any of the parties to the action in which this proceeding was called; and, furthermore, that I am not a relative or employee of any attorney or counsel employed by the parties hereto, nor financially or otherwise interested in the outcome of this action.

(Signature and Seal on File)

Notary Public, in and for the District of Columbia

My Commission Expires: May 31, 2022