>> Standing by, at this time all participants in a listen-only mode. If you have any questions, please direct them to the Q&A feature on the toolbar. Today's conference is being recorded, if you have any objections, please disconnect at this time. Now I'd like to turn the meeting over to Dr. Ben Beard. You may begin.
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>> Thank you! Good afternoon and welcome to our webinar today that's sponsored by the HHS Working Group on Lyme and other tickborne disease. My name is Dr. Ben Beard, I'm Chief of the Bacterial Diseases Branch in CDC's Division of Vector-borne Diseases. Today's webinar will focus on the state of the science and our understanding of Borrelia persistence in animal models and in humans. And I'd like to emphasize that the views expressed in this webinar today, as you can see in this slide, are those of the participants and do not reflect the official policy or position of the Department of Health and Human Services or the U.S. Government. As an introduction to the topic, most of you will know that Lyme disease is caused by the spirochete Borrelia burgdorferi here in the United States. It's harbored in small animals and is transmitted by ticks that are on deer. Currently, there are over 30,000 cases that were reported to, to us at CDC, that was in 2012, and published studies, as well as recent CDC estimates suggest that this number is likely a ten-fold underestimate of the actual numbers of diagnosed cases per year. Lyme disease is the 7th most common reportable disease here in the U.S. and cases have been increasing steadily both in numbers and in distribution. The symptoms of Lyme disease range from an erythema migrans rash that's seen early in the course of infection to neuritis, carditis, and arthritis in later disseminated stages of illness. Prompt treatment with 2 to 4 weeks of oral doxycycline results in symptomatic cure of the great majority of these patients. A subset of patients, however, especially those who are diagnosed and treated in later stages of illness, may have persistent fatigue, muscle aches, short-term memory problems, and other non-specific symptoms. One of the highest priority research needs in the field of Lyme disease is to elucidate the specific cause or causes of symptoms in these patients, and to determine the safest and most effective treatment options. And now I'm going to turn over the webinar to Dr. Joseph Breen. Joe's my colleague at NIH, so, Joe?
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>> Thank you Ben. What I'd like to review here is briefly talk about the objectives of the webinar. My name is Joe Breen, I'm the Program Officer for Lyme disease at the NIH and the National Institute of Allergy and Infectious Diseases. So basically I manage all the grants funded by our institute for Lyme disease, which is really the majority of, of Lyme disease grants throughout NIH. Our objectives for the webinar is really to discuss the state of the science of persistence of infection by Borrelia burgdorferi. And what we hope is this leads to a better understanding of the topic and lead, therefore, to improved diagnostics, safer and more durable therapeutics, and improved prevention options, which are really the mission of NIAID and also how Ben and I work together to achieve those goals through the HHS Working Group, along with other partners as well. Ben, can you advance please? So, here's the speakers and the topics that we're going to discuss today. We're going to be led by Dr. Stephen Barthold who's going to talk about comparative biology of Borrelia burgdorferi persistence, primarily in a mouse model system. And Dr. Linda Bockenstedt from Yale is going to talk about

animal studies to assess Borrelia burgdorferi persistence, also primarily in a mouse-animal model system. And then Dr. Monica Embers from Tulane is going to lead us into studies done in non-human primates, studying, again, persistence of Borrelia burgdorferi. And, and then we'll have Dr. Adriana Marques, also from NIAID and NIH, talking about some very recent studies for searching for persistence of infection in Lyme disease. And each speaker will have 10 minutes to present and will have 5 minutes for questions. I'll go over that in the next slide. And then we have some time scheduled at the end for a little bit of a roundtable of questions that can be entered by the presenters or the audience, people listening today. Dr. Linden Hu has a presentation that will really talk about Borrelia burgdorferi persistence, the science, the consensus, what do we know, and the controversy, and really, where do we go from here? What kind of things do we want to continue to look at experimentally to try and better understand persistence in Borrelia burgdorferi, which is really why we're all participating and here today. Can you go to the next slide Ben? So questions can be, can be submitted online through the webinar interface, so that would be using the Q&A feature on the toolbar, and if you can, please identify yourself and particularly who the speaker that you're addressing, so we can try and direct them to the person with the appropriate expertise. Next slide please. And this will be archived, so this is available in the future if you need more information or would like more information about Lyme disease. There's a website from the CDC and also from the NIAID where you can find more information, and these presentations will be archived here once they're available. It does take a little bit of time to transcribe, but they will be available, and you can check these websites to look for that update. At this point, I'm going to introduce Dr. Stephen Barthold from University of California, Davis, who's going to talk to us about comparative biology of Borrelia burgdorferi persistence. Dr. Barthold?
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>> Well thank you Joe. This is a disclaimer, I'm a veterinarian, not a physician. So I see the world in a slightly different perspective. Eighty percent of human infectious diseases are of animal origin, and Borrelia is among those zoonotic diseases. Second slide. I want to start my presentation with a couple of fundamental issues of, or features of Borrelia infections. Borrelia burgdorferi’s life cycle in the wild is rather complex. It requires multiple stages of ticks and multiple reservoir hosts including rodents and so on, and it would not work if reservoir hosts were not persistently infected. Furthermore, persistent infection must not to do harm the host, so that the primary host. As we see here in this image, Peromyscus leucopus is an important host in the east and Midwest, and these animals are not clinically affected by infection and they’re persistently infected basically for life. This is an essential component of the evolved biology of Borrelia burgdorferi. Next slide. So, if we look in the skin, and this is a histologic section of skin in which you can see little, brown, squiggly lines that are Borrelia burgdorferi. Borrelia does not live intracellularly, does not form biofilms in people, and a lot of speculation gone forward in terms of how Borrelia persists and evades host immunity. It's actually extracellular and it likes collagenous connective tissue and here we see in the skin. It’s right there are at the interface where a tick is going to come along and pick it up. The remarkable thing is that during persistent infection--that is weeks or months into the infection of

whatever host it may be infecting--there's virtually no inflammatory change in response to its presence. That's an important feature as well. As I said, it's evolved not to harm its host, and it has very complex mechanisms by which it evades host immune clearance. Next slide. So, all of this has been confirmed in laboratory animal models, and here we see Peromyscus in the left, middle panel, there are laboratory Peromyscus in which we've confirmed persistent infections, but also in, in laboratory mice, rats, hamsters, guinea pigs, gerbils, and two species of non-human primates. Thus, persistence is a universal behavior. Persistent infection lasts for many months, if not the entire life of these various hosts. Next slide. So, the question and the focus of this webinar is persistence in humans of course, and without treatment it's certainly been well-confirmed that persistent infections can occur in humans, and the question of the day is following antibiotic treatment, does persistence take place? Next slide. So, a couple of concepts here when we get back to persistence. It's probably unrealistic to expect that antimicrobial therapy, per se, will eliminate every single microorganism from the infected host, and the role of antimicrobial therapy in vivo can be thought of in terms of tipping the balance in favor of the host's own defenses against a particular pathogen. This is a universal concept of antimicrobial therapy towards bacterial infections. However, the normal biology of Borrelia burgdorferi is immune evasion and persistence, and so the question at hand is, can antimicrobial therapy be expected to be completely effective and eliminate all organisms? Next slide. I'm not going to delve into specific studies, but rather take a 30,000 foot view of studies that have taken place in various labs throughout the United States and Europe using different animal models, the mouse, the dog, and the Rhesus macaque which Dr. Embers will be talking about, with a variety of different classes of antimicrobial agents: doxycycline, amoxicillin, azithromycin, ceftriaxone, and tigecycline. In all of these labs, in all of these studies, there are common themes or comparative results that have confirmed that Borrelia burgdorferi DNA can continue to be detected in tissue of these animals months after completion of antibiotic treatment, including in our most recent study, literally 12 months after antibiotic treatment has been completed. Also a consistent finding is that the tissues from these animals are consistently culture negative. We find Borrelia DNA, but we can't culture the spirochetes out of the tissues. Next slide. So the question before us then is does this mean that there are viable spirochetes surviving after antibiotic treatment, or is this simply DNA debris that's persisting in these hosts? And, again, looking at these various collective findings, we can see spirochetes in the connective tissue of the ligament or tendon of a mouse following treatment with antibiotics, we can also use xenodiagnosis studies feeding ticks upon these treated animals and find that the ticks acquire the DNA, and we can see spirochetes within these xenodiagnostic ticks by immunofluorescent antibody detection. We can also transmit the DNA from treated animals by tissue transplants. If we take tissues that are PCR DNA positive, we can transplant those tissues into naive SCID mice and cause transfer of the DNA but also dissemination of the DNA to key target organs. And we can also transmit DNA from the xenodiagnostic ticks back into naive hosts. Recently, we can also demonstrate with more sensitive techniques, RNA transcription of multiple Borrelia burgdorferi genes, which suggests that these organisms are transcribing RNA and are functionally viable in some way. Our most recent study has shown actual

resurgence of DNA levels in these animals at 12 months following completion of antibiotics to levels equivalent to untreated, infected animals at the same age and environmental conditions. Next slide. So we get back to key questions. Previous animal studies have not shown evidence of recrudescence, but by holding animals for 12 months, we have found that recrudescence actually does happen. Studies have not shown persistence of clinical or histologic findings of an inflammatory response, but this is consistent with Borrelia behavior. Therefore, even if a few residual B. burgdorferi spirochetes or their DNA debris persist after antibiotic treatment in animal systems, they no longer appear to be capable of causing disease. If we go back to that basic principle that during persistent infection, whether or not the animals have been treated with antibiotics, we see no inflammation or disease. So, next slide. The question arises then, are the hosts responding to the presence of these non-cultivable, apparently viable spirochetes? And our most recent study has looked at various cytokines. Cytokines are chemicals that the body uses to transfer information from one immune cell to another, and what we see in comparison to uninfected age-matched animals is that the relative RNA transcription of multiple cytokines was taking place. Is this specific to live Borrelia burgdorferi or the presence of antigen or DNA? Who knows? But certainly there's a pro-inflammatory cytokine response going on in these persistently-infected animals. Question is-- Do these relate to the human condition? We have no way of directly answering that with animal model systems. But these animal models are very useful in testing specific hypotheses, treatment regimens, and so on, and so, they've been very useful in understanding the comparative biology of this organism, and from those studies, I think we can extrapolate at least some information to the human condition. Next slide. And so, I've listed the specific references that I've been referring to, and you can read them at your leisure by looking them up on the web. Thank you very much!
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>> Thank you Dr. Barthold. At this point, I would like to address some of the questions. I'll start out with one, how does the Borrelia burgdorferi evade immune clearance during persistent infection?
>> This is the million dollar question. I think the scientific community has picked away at this a little bit, and found some mechanisms of immune modulation and, or antigenic modulation, complement evasion systems and so on, but we really do not fully understand how Borrelia can persist. I think that the persistence stage of the infection is the last great frontier or area that we really need to understand Borrelia biology. We have very little information on that.
>> Another question that came is with regards to, how do your findings relate to possible, well, the findings of Borrelia burgdorferi, but also potentially Borrelia miyamotoi in the Eastern U.S.? The question is too long to read, but basically they're asking about how your results may compare between those two Borrelia species or, or do they?
>> I cannot answer that. I assume miyamotoi is also a persistent infection. Its biology is no doubt similar, but I don't know that much about that organism.
>> Okay. Another question, do studies in animals show that persistence after antibiotic treatment lends credence to long-term antibiotic therapy?

>> This was speculation because we really haven't tested that, but animal model systems allow us to test those questions. I suspect that long-term antibiotics would simply keep the organisms in a sequestered mode. But our most recent study in which we see resurgence of spirochetes out at 12 months suggests that maybe an alternate approach would be intermittent treatment with short courses of antibiotics, and this would be based on clinical symptomology and other things. Again, that can be tested quite accurately in these animal models.
>> Another question from a listener is how can you explain the immune-reactive debris, such as the DNA that you mentioned, and how that might correlate with the myriad of neurological symptoms that can occur in people?
>> Boy, that's a million dollar question. There's no direct way we can detect that, but, you know, the inflammatory state, whether it be prostaglandins or cytokines or, or whatever during the course of infection, leads to fever, aches, pains, lots of nonspecific symptoms in humans, which we can't measure in animals. So, it's only speculation on my part, but perhaps those are the mechanisms by which neurologic signs or symptoms may be occurring in humans. The deficiency of animal models is that we don't get neurologic infection in rodent models, and that's because Borrelia likes connective tissue and rodents don't have much connective tissue in their brain whereas people have lots in their meninges and perivascular spaces. We see neurologic disease in larger animals like horses, and it's likely to be related to the direct presence of Borrelia and the inflammatory response that they're inducing.
>> Great. And I think one last question, one of the listeners asked about, how do you know that there weren't other reasons that cytokines were raised or lowered, whether, could there be other infections, for example, that would be responsible for your result?
>> Well, you know, that's certainly a good question, and I think it's always wise to look at such results very critically, but what we did in that study is we maintained the animals in a pathogen-free environment. We monitored for mouse infectious diseases, particularly viruses over the course of that experiment, and the control animals, the uninfected, normal controls, were maintained side-by-side at the same time, same age, same environment, and so we're comparing cytokine levels between the post-treatment infected animals and uninfected animals, and that's the best we can do in scientific designs, is try to control those variables.
>> Great. Thank you very much Dr. Barthold. At this point, I'd like to move to our next speaker, and I might remind Dr. Barthold, we may have time at the end to address more questions. The next speaker is Dr. Linda Bockenstedt from the Yale School of Medicine. Dr. Bockenstedt?
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>> Thank you Joe. Next slide. The reason we're here today--to remind people--is that we're trying to understand the persistence of symptoms that can be disabling after some people are treated for Lyme disease. We don't really know why that occurs, but several possibilities exist, and there really is a lot of evidence supporting various aspects of each of the theories that are listed on this particular slide. The one though that is of most concern is whether these symptoms are due to persistence of spirochetes that can multiply and cause recurrent disease. Next slide. When we examine the animal studies that evaluate persistent infection after antibiotics, there are many factors that are inherent in the experimental design that can influence the outcome. Some of these are

listed here on the slide. For example, the Borrelia strain, because we know that some are more infectious than others and some actually won't even infect certain hosts, such as a dog, and will, infect—-preferentially--other hosts. The way the infection is introduced into the animal is important, whether it mimics the way people might acquire infection, for example by a single tick bite; by the mammalian species that's been studied and whether it has a normal immune system; whether the animal has been genetically modified or whether the immune system has been suppressed by drugs; the choice of antibiotics and whether the dosing of the drug has been optimized to kill the bacteria; and finally, the methods that were used to detect persistence of the organism. Of course, for most bacterial infections, the gold standard is culture, but in the case of Borrelia we know that even with persistent infection, it's difficult to culture the organism from humans. In animal models, we can sample much larger areas so it's easier. There are other methods that we can use as surrogates, and those include PCR-based methods, microscopy, looking at immune responses, but once you see those items, those traces or footprints of the Borrelia, you have to then determine whether they actually represent a live organism that can reproduce what Borrelia do, which are replicate, infect other hosts, and actually cause disease. Next slide. Over a decade ago, we tried to address this issue of persistence in a study in mice in which we infected them using ticks. These were laboratory-reared ticks. We put five ticks per mouse, and then we used the feeding of uninfected ticks, this is something called xenodiagnosis, to assess whether spirochetes could be persist after antibiotics, and I have to tell you that after significant searching, this was like a needle in a haystack, we found rare spirochetes in ticks that fed on some of the antibiotic-treated mice for a short period, up to three months after antibiotic treatment, but not thereafter. We even tried suppressing the animal's immune system to see if we could make these spirochetes expand in numbers. At the end of the experiment though, we couldn't culture Borrelia from any of the antibiotic-treated mice, but we did detect trace amounts of DNA in some of the tissues. Now as Dr. Barthold mentioned, antibiotics are not necessarily meant to kill every last bacteria. They kill the majority, and the immune system mops up the rest, presumably, if the immune system cares enough that they're there. I think that's an important point to make. We considered that what we detected were basically attenuated spirochetes that were the residua of infection and would eventually die or be eliminated by the immune system. To try to develop a system in which we could get more of these attenuated forms to study, we turned to a mouse that has a deficient immune system that allows Borrelia to achieve much higher numbers in the tissues, more than 100 times that of what you would see in a normal mouse that's been infected. Using those mice, we found that with antibiotic treatment, only one of the antibiotic-treated mice was clearly infected, and this was determined by culture and a multitude of other methods. We could only detect DNA in the tissues of the rest of the antibiotic-treated mice, but not in the ticks that we used for xenodiagnosis. At the same time we were doing that study, we studied normal mice on the same mouse background and found only trace DNA in a single antibiotic-treated mouse. Now those results differed from what we had published in our 2002 study, and when we looked at the differences between the studies, there were a couple of things, one had to do with the pharmacokinetics of the antibiotics we had administered, but the second reason was that we were using a completely

different mouse background. This was a B6 mouse which is more resistant to infection and disease than a C3H mouse, which we had used in our previous study. So we went back and used C3H mice in which we introduced this genetic mutation on and that caused the immune deficiency the led to elevate pathogen burden and got basically the same results that we obtained with the B6 mouse background. Our imaging techniques had been improved during that time and we actually were able to do live imaging so that we could look in the tissues of live anesthetized mice and see spirochetes moving around. In the antibiotic-treated mice after infection that was after treated, we could not find any more moving spirochetes, but we did find abundant remnants of the spirochetes near cartilage, which you can see there in green, the remnants in the picture there, which contained DNA, but we couldn't detect live Borrelia by various methods. Next slide. Dr. Alan Barbour wrote an editorial in the same issue of the JCI that we published this paper, describing that what we were detecting after antibiotics was likely the remains of infection. In other words, antibiotics had crippled the bacteria to the point where they could no longer replicate and they may be breaking up into remnants that contain DNA, or DNA and protein that eventually must be cleared by the immune system, and that may take some time. Next slide. Dr. Barthold mentioned that infection can reappear in mice if one waits a long time. I'm showing you here in this slide some unpublished work of ours, which we conducted for different reasons in both C3H and the immunodeficient C3H mouse strain, to see whether duration of infection prior to treatment affects the outcome. We followed these mice for quite a long time afterwards. The answer to our particular experimental question here--Does duration of infection prior to antibiotics make a difference?--Was “no” in this study. And, in particular, I want to point out that in the C3H mice that had the immune deficiency, those remnants of the spirochete that we had seen earlier had become less abundant and, in some mice, undetectable by 9 months after completion of the antibiotic therapy. Importantly though, in this study, the normal C3H mice were examined more than a year after treatment, and at that late time point, no detectable DNA could be found in the tissues, and we could not introduce infection into new mice by tissue transplant. So on the surface, these results seem to contradict those that Dr. Barthold has published and recently told you about. But this result challenges us to think about other possibilities we might not have considered previously in our experimental design. Next slide. In looking at the animal studies that have been conducted, looking for Borrelia persistence after antibiotics, the majority have introduced infections using cultured spirochetes. And we know that bacteria grown in culture -- and this is common to all bacteria that have been studied --are essentially a mixed population in terms of growth characteristics. In the case of Borrelia, in particular, when they're grown in culture, they may also be genetically different, because Borrelia has a hard time maintaining a complete repertoire of its genetic material during replication. These slower-growing bacteria may be more resistant to antibiotic treatment. It's not that they have a true drug resistance, but they may not be as sensitive to the effects of the antibiotic. When you start growing these bacteria in culture, and particularly when the culture becomes more dense, these persister types, as some people call them, can become a significant proportion of the population. I believe that the simple explanation for the differences in the studies that I've conducted and those that Dr. Barthold had conducted

most recently is in the spirochetes we use to introduce infection in these animals. We both used cultured spirochetes, and treated the mice with the same antibiotics, but the spirochetes I introduced were probably more in the earlier growth phase, and had fewer of these persisters than those that Barthold used in his study. But I think the real question we're asking here is whether persisters arise with tick transmission. Although we know that spirochetes start multiplying greatly when ticks start to feed on a host--in fact, their numbers increase exponentially--only a very few can make it through the, the tick immune system, to the salivary glands and enter the skin at the tick bite site. Tick saliva helps those few spirochetes survive. Next slide. If I were to consider how to best design animal studies to gain insight into human disease, I think we have to carefully consider the question we're asking, if it's whether antibiotics eliminate live spirochetes that can cause recrudescent infections when antibiotics are stopped, then we need to introduce an infection in a way that most closely resembles the way people acquire Lyme disease, through the bite of a single infected tick that's been infected the way ticks become infected in nature, that is, by a larvae feeding on an infected mouse, and allowing the larvae to molt to the nymph, and then the nymph can infect your experimental mouse or animal. If, on the other hand, you want to examine features of persisters, you need to stack the deck to improve your chances of detecting them. We tried that with our immunodeficient mice, but there are other ways that this might now be improved to study these organisms. I think that can be done if you used the high dose inocula of these late-phase cultures; if you use ticks instead that have been artificially-infected with cultured Borrelia as opposed to allowing them to acquire infection from the animal itself, or if you use a large number of ticks to transmit an infection. That was the case in dog studies years ago, which are listed in Dr. Barthold's last slide, by Dr. Straubinger, where he used up to 40 adult ticks to transmit an infection. And then lastly, by using animals that have immune deficiencies that may allow for survival of some of these forms. That, I think, brings me to the host itself. Next slide. There are subtle genetic differences that may play a significant role in expression of disease. Recent research has actually uncovered an explanation for why the B6 mouse, on the left below, is more resistant to the inflammatory manifestations of infection with Borrelia than the C3H mouse on the right. The C3H mouse has a genetic polymorphism, a difference in a single gene that leads to low expression of an enzyme that normally clears inflammatory proteins that are produced normally by our cells during an everyday cell life. Infection results in an increase in the production of this particular group of proteins, which in the absence of appropriate clearance can cause more inflammation independent of the inflammation that's driven by the bacteria itself. Next slide, In concluding, I think we have to understand that human biology is very complex and I believe that persistence of Borrelia after antibiotics, and other possible explanations for persistence of symptoms after Lyme disease needs to be studied in the human system. I think this for a variety of reasons: we are learning more about factors that influence our health in many ways which are not just in our genetic makeup, but they're influenced by the bacteria that we have in and on us—this is something called our microbiome. Our health is influenced by how our immune systems have been shaped over the course of our lives by our experiences with other pathogens, and also because of the types of

microbes that we harbor within ourselves. Ongoing studies that are funded by the NIH and other groups are gathering information about how natural variations in your genes, immunity and the microbiome among people contributes to disease, and this is disease that's not just infectious disease, but entities like obesity and susceptibility to these other problems. These types of broad research approaches undoubtedly are going to raise new questions we haven't yet considered that may help us explain the sequela of Lyme disease, and I think that by turning to the human system to try to understand this disorder, we'll be learning a lot more. And I'll end there.
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>> Great. Thank you Dr. Bockenstedt. There's a couple of questions that come in and we have time for. The first one is: Are there differences in persistence between burgdorferi strains? You may have mentioned it at the beginning of your talk?
>> Well, you know, what's interesting is that when this has been studied in people, the strains of Borrelia that can be isolated from the skin and those that you find in the blood are slightly different. So there are many more types of strains that might be isolated from an erythema migrans lesion than what you can isolate from other sites in the human body, so those that disseminate are just a subgroup of those that seem to be detected in an erythema migrans lesions, which suggests that there's difference in virulence and infectivity of these strains. And that has actually been reproduced in an animal model. So I think that, yes, there could potentially be differences in the ability of certain strains that persist and others might be less able to do so.
>> Thank you. And another question actually relates to an earlier question, do you have any thoughts about how the immune reactive material that you did find might possibly be responsible for, you know, obviously the, trying to draw the connection between long-term physical disability in people is difficult, but given the immune response, can you give your thoughts about that?
>> I think this is a really important question. We have to think, when you think about the human body, we have acquired infections during our lives that are still with us. I can give an example: if you had chickenpox as a child, you still have the chickenpox virus in you, and your immune system is working all, day to control that virus and keep it from giving you symptoms. If your immune system is suppressed or you’re physically stressed you can actually have recrudescence of infection with the chickenpox virus and that comes out in the form of shingles. That’s an example of a persistent infection that you're not paying attention to, but your immune system is. So in terms of these particular remnants that we're finding, certainly, they may be eliciting some kind of response from the host. Whether the person feels that response is unclear. And it’s going to very difficult to figure that out in a human system. We can detect in an animal system immune responses to the debris that may be ongoing in the tissues because the immune system may be trying to clear it. But whether that's also translating into symptoms people have, is something that needs to be explored, and I think, again, looking at how people's immune responses are different in somebody who's cleared the infection, or feeling well, I should say, after antibiotics versus somebody who's not feeling well after antibiotics might help us understand why they're feeling the way they do.

>> Okay, thank you. I think we have time for one more question. There, one of the listeners asks, they, they didn't understand—why the method of inoculation might matter if the bacteria are growing inside the host anyway?
>> When you use a cultured inoculum, you can introduce a wider variety off organisms that the immune system may care more or less about. And so the ones that are slower growing may be ignored to some extent compared to the ones that are faster growing and may be raising more red flags to the immune system. That's one possibility to explain those results. So I think that if we want to study in that detail, we have to kind of stack the deck, as I mentioned, to see if you really put in many of these organisms that are so-called persisters, would you actually have a greater expansion of those and what would the immune system do then in that situation?
>> Thanks. Actually we have time for one more [question], and I think it's a good question for you because it's your imaging data, and that is: you observed different morphological forms of Borrelia in your studies and how might these different forms affect the immune response, potentially therapy and persistence?
>> I don't see different morphological forms. The organisms we see are usual spirochetes. Depending upon the orientation, they may look more linear or some may be wavier, but the, the forms themselves are the same. We have seen them, and this was published in our paper, that when we watch over time, we have seen a spirochete traveling through tissue and then suddenly, abruptly stop, and it seems to ball up and then, poof, disappears. The kinetics of that reaction is similar to the kinetics of how a phagocyte or immune cell can take up an organism, and how it pulls in the organism inside and degrades the organism. So in that study, in our mice, we haven't labeled the immune cells to show that if we see that phenomenon, whether that's actually a spirochete that's being engulfed by an immune cell. I think those are something. We have not seen anything that might be resembling a cyst form that's sitting in tissues forever.
>> Okay. Thank you. Thank you very much for your presentation, for answering all those questions. So at this point I'd, I'd like to turn over to Dr. Monica Embers from Tulane University, who's going to tell us about studies about Borrelia burgdorferi persistence in the non-human primate. Monica?
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>> Thank you Joe. Today I'll be talking specifically about the non-human primate model of Lyme disease and studies of persistence. First of all, post-treatment Lyme disease syndrome can be explained by a number of different, in a number of different ways. Potential causes of post-treatment Lyme disease include the induction of inflammatory responses by lingering dead spirochetes or remnants that Dr. Bockenstedt just talked about. It can also be caused by the continuation of active spirochetal infection or as an autoimmune response, and this would constitute irreversible sequelae from previous active infections. Most likely, it's a combination of these three and can vary from patient to patient. There are a couple of considerations when it comes to antibiotic treatment. Doxycycline is by far the most prescribed antibiotic for Lyme disease. And doxycycline is a microbiostatic antibiotic. What that means is that the antibiotic actually acts on actively-dividing cells that expose the growth of the bacteria. So efficacy essentially relies on the immune clearance of these static bacteria. Borrelia burgdorferi evades the

immune response, as we know, in many different ways. So we have to question how effective the immune response actually is. We also have evidence that dormant bacteria, or slow-growing bacteria, are more tolerant of microbiostatic antibiotics. In addition, Borrelia burgdorferi survives for many months inside ticks without nutrient replenishment or replication. So dormancy is actually a part of their phenotypic repertoire. And finally, Borrelia burgdorferi can be found in deep connective tissues and in joints, so we have to think about what the tissue penetration of an antibiotic is. I'm not aware of studies measuring antibiotic levels in tissues versus blood. So in, in terms of the rhesus macaque model of Lyme disease, we know that rhesus macaques very closely mimic the multi-organ character of human Lyme disease. This includes disease hallmarks, such as erythema migrans, carditis, arthritis, and neuropathy of the central and peripheral nervous systems. The spirochete burden also in tissues following dissemination is likely very small, as it would be in humans. This is a low-level infection, and it's difficult to culture the spirochetes from humans or monkeys, whether they've been treated or not after a disseminated infection. Some advantages of this model are that compared to mice, the disease course, including the duration and quantity of Borrelia in the blood, and the immune response, is more similar to that of humans. Also in comparison to human samples, the infection history of a rhesus macaque can be known. We know the exact point of infection, the exposure duration, and the previous exposure history. Also, tissues can be examined post-necropsy for the presence of Borrelia, which cannot be done in humans. Post-treatment Lyme disease syndrome is primarily comprised of objective symptoms, so it can only be conveyed by humans and not by animals. For example, we cannot ask our monkeys if they have persistent fatigue or myalgia. However, we can inspect potentially-infected tissues, such as muscles, joints, and nervous system, to uncover signs of inflammation. This could be inferred to contribute to post-treatment Lyme disease syndrome if we find the presence of Borrelia in those tissues. In 2012, we published a paper on persistence of Borrelia in rhesus macaques. Here we show that intact spirochetes recovered by xenodiagnosis from treated monkeys. We also showed that Borrelia RNA was detected in tissues of infected animals, whether they were treated or not. This showed that spirochetes could persist post-treatment in a representative animal model. This left some caveats and open questions. First, there, at the time that we did the study, there was a lack of pharmacokinetic data in rhesus macaques for doxycycline. We have since performed these studies and found that the level of antibiotic that we used for our studies was far, far exceeded that recommended for humans. Also, as Dr. Bockenstedt mentioned, we did not use tick-mediated infection. So the question becomes whether or not the initial inoculum can affect treatment efficacy months later. Important questions that resulted from these studies are what is the phenotype of persistent spirochetes and are they viable, or are they attenuated or perhaps in a state of dormancy? Also, can spirochetes persist long-term or are they eventually just cleared from the hosts? So we designed the following study to repeat our previous study, but this time using tick-mediated infection. So we've essentially repeated the experiment. We start with infection of the animals, 10 animals, with nymphal ticks. And at four months post-infection, five animals were treated with a 28-day regimen of doxy, and five animals were untreated. Three months after antibiotic treatments, the animals were

subjected to xenodiagnosis. Also, at five months post-infection, the animals were again subjected to xenodiagnosis, and the necropsy was performed. So if we look at the antibody responses of 5 of these 10 animals, we see different patterns. I'd like to draw your attention to C6 here, which is one of the antigens. Each figure represents one antigen, each line represents one animal, and this is the antibody titer over time for the course of the infection for that animal. If you look at C6, you can see that the dark blue and red lines correspond to two animals that were treated. At 28 weeks of infection, you can see that the antibody, or that the C6 titers declined significantly following antibiotic treatment for those that were treated, and the levels remained elevated for those animals that were untreated. One animal, shown in purple, which is actually an untreated animal, did not have a C6 response at all. So there was some variation, so we know that our animals were productively infected. Shown here is the tick-mediated infection in Panel A. In Panel B, we show 1 animal out of 10 that developed a bona fide erythema migrans lesion. Others exhibited some diffuse erythema. We also showed that cultures in biopsy tissue resulted in positive infection in 5 of 10 monkeys, and detection by DNA PCR was, was positive for 8 of 10 monkeys. When we looked at the pathology, we found small pockets of inflammation in various tissues, whether the animals were treated or untreated. For example, shown here is perineural inflammation of the right ulnar nerve. We also saw axillary node hyperplasia, and we saw hyperplasia in the lungs of two animals. In a treated animal, we also saw cervical spinal nerve inflammation and focal inflammation in the skeletal muscle. Now, this may or may not be related to infection. So next we need to look for Borrelia in these sites to determine if Borrelia plays a role in the induction of inflammation. In terms of the xenodiagnostic tick results, we found that few of our ticks were positive. So we noticed that after our second feeding of ticks, we found these erythematous papules at the site of the tick bite. And this indicated to us that anti-tick immunity could be playing a role. This was confirmed by histology. In Panel B, you see the normal skin tissue, and in Panel C, you see inflammation at the site of the tick bite. We knew from previous studies that, that anti-tick immunity would not affect transmission, but we know now that perhaps it could affect xenodiagnosis, because these are very different processes. In conclusion, in order to examine the infectivity of persistent spirochetes, we designed the following experiment. We took six naive rhesus macaques, infected them by needle inoculation, and at four months of infection, treated half of them with doxycycline for 28 days, and left three untreated. Those monkeys were then fed upon by naive ticks, and tick contents were pooled from each group of animals, and inoculated into naive monkeys, and into, into severe combined immune deficient mice. After the injection of tick content, the monkeys and mice were subjected to xenodiagnosis, various skin biopsy, tissue biopsies, and serology and eventually necropsy. Currently, the experimental protocol is complete, and we're looking inside ticks and recipient animal tissues for Borrelia burgdorferi. And with that, I'd like to thank the members of my lab and of the primate center in general. Thank you.
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>> Thank Dr. Embers. There's time for just a few questions. The first one is from a listener who wants to better understand how xenodiagnosis

works. How does pre-Borrelia DNA get attracted to the tick in the first place?
>> That's a very good question. There...one possibility is that we're not using the right reagents to try to detect the spirochetes in the ticks. They may be of these different forms, and we're not seeing them as spirochetes. But I find it difficult to explain how DNA can migrate without a spirochete to a feeding tick.
>> Related to that is how does just finding DNA itself indicate viable Borrelia, or does it mean something else? That's relate, that's actually, that question relates to many speakers, but you're up.
>> [Laughing] I would say that DNA itself does not indicate a live spirochete. More valuable would be RNA or an intact organism. So, I think you need to, to use multiple methods in order to determine if those spirochetes exist, and if they're in a metabolically-active state.
>> Okay. And I think the last question for this section, someone asked about co-infections, which it doesn't sound like you looked at other, but have you done this with other bacteria?
>> We have not looked at co-infections. But that's something very important to consider when we think about the proportion of people who generate rashes at the site of the tick bite. Previous exposure, co-infection, those sorts of things could contribute to whether or not patients develop rashes or anti-tick immunity, which is moderate in humans.
>> One more question, sorry, one listener asks, only one monkey developed bull’s-eye, although all the same strain was used and they were all theoretically treated the same, can you explain?
>> Sure. We did use the exact same strain, which is not indicative of what would happen in humans. Also monkeys are not humans, so it's difficult to infer that just because only 1 of 10 monkeys developed an EM rash, that only 10 percent of humans develop an EM rash. The only way to determine, truly, how, what proportion of humans develop a rash would be to take a mixture of different isolates in the, in the environment, and let them feed on 100 people and see how many actually develop a rash. So, I think, for our studies, it's related to the, the animal species, and the Borrelia species, and I, I hesitate to, to infer those results to, to human disease.
>> Okay, alright, great. Thank you very much Dr. Embers. And I'd like to move now to Dr. Adriana Marques, who's actually here at NIAID. She's going to discuss Searching for Persistence of Infection in Lyme Disease. Dr. Marques?
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>> Thank you Joe. Good afternoon. I'm Adriana Marques. I work at the NIAID, part of National Institute of Health. I'm a physician. My work is in clinical research on Lyme disease. And I'll be talking today about our study using ticks as a medical device for xenodiagnoses of Lyme disease in humans.
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As it already has been discussed, the pathogenesis of post-Lyme disease symptoms is an area of great controversy. And it's likely that different factors will play a role in an individual case. For example, in one patient, it may be just the natural resolution of the disease, where in another patient it may be due to another condition that develops after

Lyme disease. There have been four placebo-controlled antibiotic treatment trials for post-treatment Lyme disease syndrome. These trials have showed that in general re-treatment provides little, if any, benefit and carries significant risk. But one of the questions that physicians face is whether persistent infection could be the cause of the symptoms in a particular patient. This can be challenging mainly because there's no simple test that can easily differentiate patients who may still have the infection and could potentially benefit from further treatment. The available antibody-based assays cannot be used to determine successful eradication of the organism; and current direct tests for the presence of B. burgdorferi, which are culture and PCR, have low sensitivity outside the skin and blood samples from untreated patients with early Lyme disease--mostly erythema migrans or PCR in synovial fluid of patients with Lyme arthritis. As has been discussed animal studies have shown that spirochetes or their DNA may persist after therapy in dogs, mice, and monkeys and could be acquired by xenodiagnosis. So xenodiagnosis could provide researchers with a new tool with which to study the mechanism of disease in humans. I want to point out that ticks are not simply “crawling needles and syringes”. Tick saliva has been shown to be a chemo-attractant for the organism, and feeding ticks have the potential to aggregate and concentrate bacteria from a wide area, improving sensitivity. Now nothing was known about the parameters of the xenodiagnosis of Lyme disease in humans. So we set up a Phase I study to develop the technique and to assess the safety of the procedure. The results of the Phase I study are now published in the journal *Clinical Infectious Diseases*. This is the first human study of the use of xenodiagnosis to detect B. burgdorferi infection. What is not in the publication is a description of the enormous amount of work that went into actually doing this study. This was a collaborative study and participants were enrolled at three sites. The study was approved by the IRB at each center and written informed consent was obtained from all participants. The ticks are considered a diagnostic device and the study was conducted under IDE, an investigational device exemption, approved by the FDA. An independent medical monitor reviewed interim data for safety. These were the study groups. One group included patients who had erythema migrans, 1 to 4 months after antibiotic therapy. The aim of including this group was to reproduce the early treatment group in the mice studies. Other groups included patients with post-treatment Lyme disease syndrome, and patients with persistently high levels of C6 antibodies after antibiotic therapy. One of the difficulties was who would be the positive control group. While the best control group would be patients with untreated erythema migrans, we thought that that would be too risky to not treat these patients for the time needed for the ticks to complete their feeding, due to the possibility of dissemination. So we decided to have patients with erythema migrans, who were just starting antibiotic therapy, as possible positive controls. And I say possible because it's known that culture and PCR of skin biopsies becomes negative very quickly after starting antibiotics. We also planned another possible positive control group, which were patients with Lyme arthritis who had not been treated. These patients had had the infection for months and a few days without antibiotic therapy would not influence the disease. Now, unfortunately, we were not able to enroll in this group doing this study, because patients who came to us already had started antibiotic therapy or had received steroids. For negative controls, we

enrolled healthy volunteers who had no history of Lyme disease and were seronegative. Here's how the xenodiagnostic ticks were prepared. These were pathogen-free Ixodes scapularis larval ticks that come from a laboratory-maintained colony at Sam Telford's laboratory at the Cummings School of Veterinary Medicine. One-third of the larval ticks from each batch was tested for B. burgdorferi, Babesia, Anaplasma, Borrelia miyamotoi, Bartonella, Rickettsia, deer tick virus and orbivirus by PCR. SCID mice were infected with subsets of larvae from each batch and monitored for one month of illness. Also a subset of the ticks was also tested by the PCR electrospray ionization mass spectroscopy at IBIS for Francisella, Babesia, Borrelia, spirochetes, Rickettsia, and alphaproteobacteria. This is how the tick placement procedure was done. The first day, about 25 to 30 larval ticks were placed under a retention dressing. If possible, the area of placement was close to an area where disease was observed, like the erythema migrans site or close to an affected joint. Participants then returned to the clinic for tick removal, starting at day 3 or 4, and at the day when all the ticks were removed, a skin punch biopsy was performed at a feeding site. Participants were then followed at 7 to 10 days, 4 to 6 weeks, and 3 months after tick's removal. They kept a diary card of symptoms for the first month. Now remember there was no previous protocol for xenodiagnosis with Ixodes scapularis larva in humans. So, it took us a lot of work, but thanks to the amazing work of the research nurses, we developed a retention dressing using the Le Flap dressing, which is used for maggot therapy of wounds. We modified this dressing by adding a foam ring to create a barrier between the ticks and adhesive. With this dressing, we were able to get between 30 to 50 percent of ticks to feed successfully. A very important part of this study was, of course, how we were going to test the ticks for acquisition of infection. Our initial protocol followed the animal studies. The live fed ticks were allowed to molt to nymphs, and the nymphs were placed on SCID mice and allowed to feed. After feeding, the nymphs were then tested by culture in PCR, and the SCID mice was checked at 2 weeks by culture and PCR of ear punch biopsy, and at 4 weeks, by culture and PCR of skin, ankle joint, heart, and bladder tissues. Now, a few months after we had the placement procedure working, we reviewed the results and we realized that we were losing too many ticks during molting and recovering of the nymphs after feeding on the SCID mice. Because we could not afford to lose even one of the diagnostic ticks, we changed the way the ticks were handled. The protocol was amended to remove these steps and perform direct analysis of the fed larva. So in protocol 2, ticks were crushed and tested directly by culture and PCR and injection of lysates into SCID mice with subsequent culture and PCR. We also started a collaboration with IBIS, and a portion of the ticks were tested directly by using their assay. This assay used 8 PCR primer pairs that target 7 Borrelia genes and can be used to distinguish genotypic variation. An important point is that larval ticks are very, very small, and each tick was tested individually and was tested by only one method.
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Here are the characteristics of the participants in the study. We had 36 individuals who underwent xenodiagnosis, 21 men and 15 women with a median age of 55 years. 7 patients underwent more than one procedure. Participants, including 10 patients with high C6 antibody levels, 10 patients with post-treatment Lyme disease syndrome, 5 patients with

erythema migrans after they had received antibiotic therapy, and 1 patient with erythema migrans early in treatment, our positive control, and 10 healthy volunteers. About the high C6 group, these participants enrolled in a median of 4.5 years after the original diagnosis, and had received a median of 2.5 courses of antibiotics. The most common presenting manifestation was Lyme arthritis. In the post-treatment Lyme disease syndrome group, these patients enrolled a median of 3.8 years after the original diagnosis, and they had received a median of 2 courses of antibiotics. The most common initial presenting manifestation of Lyme disease was erythema migrans, and the most common symptoms at enrollment were fatigue, difficulty concentrating, memory complaints, and arthralgias. Here are the results of our study. We learned that xenodiagnosis was well-tolerated. All participants successfully completed the tick placement, and there were no withdrawals during the study. The most common adverse event was mild itching at the site, which was seen in 58 percent of the participants, with a median duration of three days. There were no serious adverse events associated with the procedures, and larval ticks required 4 to 5 days to feed to repletion. Of the 23 participants with Lyme disease who had at least one tick tested, either by protocol 1 or 2, 19 were negative, 2 were indeterminate, because we could not rule out laboratory contamination. The ticks from all the healthy volunteers were negative, and all tissues from the SCID mice tested negative by PCR and culture. All the skin biopsies were negative by culture and culture PCR, and 6 biopsies were tested directly by the IBIS assay were negative.
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We had two participants who we considered that they had positive xenodiagnostic results. One was our positive control, the patient with erythema migrans who had started therapy for doxycycline at the same time that the ticks were placed. This participant was completing the fourth day of antibiotic therapy when the ticks were collected. Ticks from this individual tested positive by the IBIS assay on two separate specimens. One from a single tick and one from a pool of three ticks. The single tick was positive for two B. burgdorferi genotypes. Six other ticks on this participant tested negative by culture and PCR. The skin biopsy was negative. This individual repeated xenodiagnostic procedures 7 months after completing the antibiotic therapy, and 10 ticks were tested using the IBIS assay and were negative. One patient with post-treatment Lyme disease syndrome was considered positive in two separate xenodiagnostic procedures, performed 8 months apart. From the first xenodiagnostic procedure, one nymph was found to be positive by PCR of the nymph lysate culture. The positive PCR of this culture was confirmed by additional PCRs, and the DNA extracted was then tested by IBIS and identified it as a novel genotype of B. burgdorferi. Four other nymphs were negative in all testing, and all tissues from the SCID mice, on which the nymphs were fed were negative. Xenodiagnosis was repeated 8 months later. At that time, only two fed ticks were recovered. Direct testing by IBIS revealed that one tick was positive for B. burgdorferi, and the results were consistent with the previously-found genotype. The other tick was tested by PCR and culture and was negative. So in summary, we have developed a protocol for xenodiagnosis with Ixodes scapularis larva in humans that is well-tolerated. Adverse events were minimal, limited predominately to

itching at the tick bite sites. We have shown that up to 30 larval ticks can be applied and 30 to 50 percent of the ticks feed successfully. We also showed that larval ticks required 4 to 5 days to feed to repletion in humans. Our initial result showed that the majority of the patients with Lyme disease treated with antibiotic therapy are negative by xenodiagnosis. Caveats include that, in general, we tested only a small number of ticks per participant, particularly early in the study, and in animal studies, the number of fed ticks tested were important for sensitivity of the xenodiagnosis. The more ticks tested, the higher the power. Another important point is that we found DNA only, what may be not sufficient evidence in regard to presence of viable spirochetes; and that there's no gold standard for comparisons of these results. So what is in the future? Xenodiagnosis may be used as a tool to develop better tools and to test hypotheses and new strategies for therapy. Next we hope we will be able to perform studies to identify whether persistence of the bacteria, or bacterial products, as shown by xenodiagnosis, can be used to predict persistence of symptoms. And here's the most important slide, with the people that made this study possible. They include the study teams at NIH, Tufts, Yale, and Mansfield Clinic, as well as the collaborators at IBIS. Also I want to thank Fred Gill and Judith Starling at the NIH Clinical Center, and the staff of RCHSPB/NIAID who helped with this study. And principally, we thank the the study participants for their enthusiastic involvement with this study. And I stop here. Thank you!
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>> Great, thank you Dr. Marques. I have one question that came in and says how did you determine that the two instances of laboratory contamination and establish that your other results arose from DNA within the tick and not from contamination?
>> Well for, when could not rule out laboratory contamination with further study, to confirm the results, therefore, we put it as inconclusive. The positive results, on the post-treatment Lyme patient, we have shown, we've shown that there was another genotype of Borrelia that we didn't have any in the lab that would match that strain.
>> Okay. Thank you. One more brief question, if I could, isn’t finding a positive Borrelia in one participant indicative that persistent bacteria is a possible hypothesis?
>> Can you repeat the question please? I couldn't hear you well.
>> Sorry. Isn't finding a positive Borrelia in one participant indicative that persistent bacteria is a possible hypothesis? That came in from a listener.
>> I think it is a possible hypothesis, but we did not prove that, that we found Borrelia, we found DNA from Borrelia, so that goes back to the discussion that the other speakers we have been discussing about, viability. And how to prove it.
>> Perfect, thank you. There are a couple other questions, but I think we better move to our next participant, and then, if there's time, we can revisit, the, the next two [questions] are related. Thank you for your presentation Dr. Marques. I'd like to introduce Dr. Linden Hu from Tufts, who's going to talk about the consensus and controversy of Borrelia burgdorferi persistence. Dr. Hu?
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>> Thank you Joe. So when Joe and Ben asked me to talk about, to kind of sum up the, the different studies and talk about consensus and

controversy, I actually thought the consensus part was going to be a really short part of this talk, but actually when I went back over the studies, if you ignore the, , the discussions for the studies and the editorials that were written, if you looked at the actual data, a lot of the findings are very, very consistent across different laboratories, across the antibiotic regimens and the delivery systems and the host, and, although a lot of time has been spent today talking about the differences between the studies, I actually think that the fact that so many different people are seeing the same results in different systems, really speaks to the robustness of the findings. When I hear findings that are only done, replicable by one lab in, certain media, when the wind is blowing from the northwest at 10 miles an hour, I think "artifact", and when I see things that are robust like this, you know, you feel much more confident in the, in the results. So, what are the things that I think almost of all of the studies have seen? And one is that, antibiotics greatly decrease the number of bacteria or the amount of DNA in treated animals, and I think everybody would agree with that. Many of the studies, I think most of the studies, looked at the C6 antibody titers and everybody saw a decrease after antibiotic therapy, and I think that just speaks to the fact that the numbers of bacteria are going down. More interestingly, I think all of the studies, at least in some proportion of their animals, have seen DNA and/or RNA persist, and it can be detected either by xenodiagnosis or by DNA or RNA amplification techniques. Multiple studies have also seen that the protein antigens persist and can be detected by any number of different immunofluorescent techniques. And the last thing that I think that, you know, holds true across all these studies, is nobody has been able to culture these bacteria after antibiotic therapy. So, where, where have the most controversial aspects been about all these studies? I think one thing that has gotten hit on in multiple editorials is that the antibiotic regimens have differed and there have been questions raised about the appropriateness of the doses that are used in the different animals. The other part that, I think is confusing and it goes back to the non-cultivate-ability of these bacteria is that transmission from a xenodiagnostic kick to an uninfected animal or transplantation of tissue from one animal to the other has only been seen by one group, and this would be additional evidence that, the bacteria are alive and the, the particles are, are movable. So, what are the implications for human Lyme disease? And I think I skipped this slide here. Sorry. So, and I think this has been mentioned by multiple speakers, and that is that, you know, there is, unfortunately, no good animal models for post-treatment Lyme disease syndrome, and, therefore, it's very tough to take any of these studies and link the persistence of whatever it is we want to call it--bacteria, DNA, RNA proteins--to symptoms of post-treatment Lyme disease. And I say yes here, because, obviously, if you know that there is something foreign that's detectable there, it's a legitimate question to raise, whether it's related to these symptoms. But unfortunately, the animal studies that we have now, while they're very interesting, can't answer this question directly. So let's take on some of these issues that have been raised with the, the animal studies, because I think they're still instructive and they still have potential to inform our future human studies. So, what about the issues of antibiotic dosing? And here, Monica addressed it a little bit and I think Steve addressed and Linda addressed it, about whether the doses of antibiotics in the

animals mimic what’s actually seen for these antibiotics when they're used in humans. And I guess I'm going to take the tact, that in some sense, in my mind, that matters less. Because if you're talking about clearance being related to a very narrow therapeutic window of antibiotics, I think then you would have to say that there's going to be the likelihood that many, many humans would have persistence of these bacteria, because, as a physician, I can tell you that most of, most people can't take the antibiotics as prescribed, and that includes me, when I get sick. You know, I can't take my antibiotics three times a day on an every 8-hour schedule, and the physicians, also for many of these antibiotics, we don't dose adjust for size or weight. So we give the exact same dosage to a 350-pound person as we would to a 99-pound person, and I can tell you that the pharmacokinetics are likely to be very, very different. And then you have all the other genetic differences in renal excretion and metabolism that are different from person to person, so, if you have to be in a very narrow range to manifest clearance of these organisms for success of the antibiotics, then I think we're in trouble. But I'm going to put that issue aside and deal with kind of the more interesting issue, I think, which is what about this inability to culture after antibiotic therapy?
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So, there are a number of different possible explanations, and these aren’t all of them. These are the ones that I think have been most discussed, and, in some sense, they're all unlikely and they all break established rules, but, something’s got to be true. So, initially when the reports of persistence were coming out, the first concern, because most of the detection was done by PCR amplification or other non-culture techniques, was whether this could be laboratory contamination. I think as more and more laboratories have been finding the same results, I think that becomes much less of a likelihood. The other two main hypotheses: one is that the DNA, RNA and proteins from bacteria can persist for many months after the organisms are dead; and two, that the bacteria persist, but are somehow altered by antibiotic exposure to no longer be cultivatable. And let's take a look at data we might have support either of these hypotheses, and I'll tell you right up front that, for both of them, there's not that much to support either of them. So, if you want to look at data to support persistence of bacterial products without bacteria, when you look at DNA from killed bacteria that are injected into mice, it pretty quickly becomes undetectable. I think those were studies that were done by Mark Wooten’s lab. If you look similarly at DNA or RNA from other sources, like CPG DNA or fetal DNA which you can detect in the mother up until birth, and then it's very rapidly cleared, within hours from the maternal blood. So, other sources of foreign DNA and RNA get cleared very, very quickly. And then if you look, there are multiple studies of foreign proteins, fluorescent proteins from different sources that have been injected into animals or humans, and the clearance is very, very quick. So what about survival of non-cultivatable bacteria? What evidence is there for that? So, you've heard from a couple of the different presenters about persisters. And just to go through what persisters are, persisters are actually seen in many different types of bacterial strains, after antibiotic treatment. Persisters are defined as phenotypic variants that form during normal bacteria growth that are not, so there's no genetic alteration in these bacteria. And theoretically, they can return to the same state as all

the other wild-type bacteria. What's been found is that there are likely to be multiple pathways to forming these persister cells, but many of them revolve around slowed cell division. And persisters are thought to possibly be a reservoir for reactivation in other bacteria, and are usually cultivatable after removal of antibiotics. So that's something that's different than what we're seeing here with the Borrelia story. And persistence in these other bacteria often occur in the absence of symptoms, so you can find them in patients who were treated for these particular bacteria and they are now completely healthy. So it's unclear whether they might be related to persistence of symptoms at all. So, really, there's not really a good explanation for why Borrelia would become non-cultivatable after antibiotics. Some studies have suggested that plasmid loss might be a reason for non-cultivability. Borrelia lose plasmids very quickly when grown in culture, but when you look at the studies, the reliability of detection of plasmids in the settings is a very, very low number that bacteria and bacterial DNA, is unclear, and the results have been pretty inconsistent in terms of which plasmids get lost. Such that I don't think you can make a coherent story out of it. There has been some evidence for antibiotic selection of non-replicating bacterial persisters in other bacteria. For example, there was a recent paper about salmonella in Science, however, even there, those eventually resume growth, and if you think about it, if the reasons for having persisters is to protect the bacteria against clearance from naturally-occurring antibiotics, in that sense, the bacteria would have to regrow at some point for this to be a useful mechanism. So, this is a slide I added at the last second based on information that Steve Barthold gave me, so I'm going to kick any questions about this over to Steve, but Steve pointed out that there's actually a literature in Coxiella burnetii, it's a similar story to Borrelia, where it's a long-term disease that requires long-term antibiotic therapy to cure, there is a post-Coxiella fatigue syndrome in a subset of patients, and there have been studies where there's long-term persistence of antigens and/or DNA without cultivatable bacteria. And, in fact, there was one study that showed that patients 12 years after treatment, when they took samples from patients 12 years after treatment and injected them into mice, it resulted in detectable antigens recovered from the spleens of these mice, but negative cultures and negative PCR, and the authors of that paper described it as a potential antigenic immunomodulatory complex. So, I don't know what to make of these studies. It's interesting, and it may be that rather than Borrelia breaking the rules, it's that there are rules that we don't understand about how these bacteria persist and what it actually causes. So, in summary, what do we need to do next and where do we go from here? Well I think one thing is that we certainly need a better understanding of what happens to these Borrelia proteins from killed organisms. So, to better understand Linda's data, as to whether you can see long-term persistence of antigens and, and proteins after injection of these proteins. We also could use new strategies for trying to cultivate bacteria after antibiotic therapy, if they stop growing, why do they stop growing if they're alive? Are they waiting for a signal to regrow? Or are they really dead and we're just, you know, just trying to jumpstart the dead? We also need better tests for detecting presence of small amounts of Borrelia products, Borrelia or their products in humans. Adriana talked about the use of xenodiagnosis, and xenodiagnosis is I think an important tool for detecting Borrelia, but it's obviously not

something that can be done on a wide scale, and if we could find something that works better that's able to detect it, then we'd have a tool for really determining if there's any correlation between the presence of these products and the persistence of symptoms. And then, finally, sorry, I'm having computer problems, really I think what everybody is getting at is that the only way to get at whether any of these really interesting findings have meaning for persistent symptoms in patients with post-treatment Lyme disease is to really do the human studies, and to try and determine whether we can find a test that can be used to predict persistence of symptoms, and with that I'll stop.
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>> Okay, thank you Dr. Hu. We have just a few, very short minutes left. But I, there's one short question that came in for Dr. Marques. Will you be continuing your xenodiagnosis study?
^M01:27:38
>> Hello? We, we hope we can. At this point, we are trying to, finding ways to continue those studies. [Inaudible], as Linda has mentioned, we think it can be an important tool to develop new tools that might be easier to use. And we hope we will be able to perform the studies that will correlate, that will try to, to see the prevalence of symptoms in the, xenodiagnosis results.
>> Great. Thank you. I just wanted to remind the listeners, and the participants, that this presentation, it will be transcribed and archived with the presentations included, and it will be available both through the CDC website and the NIAID website after the transcription occurs. So there is a delay, but it will be available, at a later time, and will be publicly available for anyone who wasn't able to participate here today. And, and there's a, a minute or so left, I believe Dr. Bockenstedt had a comment about the DNA in ticks?
>> Yes, thank you Joe. I wanted to answer the question about how does DNA get into ticks and if, if it's free DNA, and I think there's a presumption that it's free. This DNA may be sequestered in other cell types that might be long-lived resident cells in your skin. They're, for example, ticks, when they take up a blood meal, are taking up anything that's coming into their environments attracted by the, by the tick feeding, that will include cells such as macrophages in the skin that may harbor something that is Borrelia related. We don't know how long Borrelia might be retained in certain subtypes of macrophages. This is something that's a, a new area of exploration for people, just in general, it, it's how different subtypes of macrophages respond to different stimuli, and so I think that, that, the presumption is that its free, but I don't necessarily think it's free. It could be traveling in a, in a mammalian cell, it could be traveling in a Borrelia cell. And I think that's an important question to answer.
>> Great. Thank you. Actually, we're out of time, and I want to thank all the participants and the listeners for calling in and for entering questions. And thank you Dr. Beard, as well, for organizing on the CDC end. And at that, I think we'll have to close. Thank you all.
>> This concludes today conference. Thank you for your participation. You may disconnect at this time.
^E01:30:22