Xenodiagnosis after Antibiotic Treatment for Lyme Disease – Phase 2 Study

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Lead Principal Investigators (PIs):
Adriana Marques, MD
Linden Hu, MD

Site PIs:
Adriana Marques, MD (Laboratory of Clinical Immunology and Microbiology [LCIM]/NIAID)
Linden Hu, MD (Tufts University)
Gary Wormser, MD (New York Medical College [NYMC])
Kenneth Dardick, MD (Mansfield Family Practice)
Luis Marcos Raymundo, MD (Stony Brook University)

Study Sites:
NIH Clinical Center
Tufts Medical Center
NYMC
Mansfield Family Practice
Stony Brook University (State University of New York)

Sponsor Medical Monitor (SMM):
Shirley Jankelevich, MD
Medical Monitor
Clinical Monitoring Research Program
Leidos Biomedical Research, Inc.
5705 Industry Lane, Room 248
Frederick, MD 21704
Phone 301-846-7322
Email: shirley.jankelevich@nih.gov

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ADE	adverse device effects
AE	adverse event
BTPV	blacklegged tick phlebovirus
CDC	US Centers for Disease Control and Prevention
CRFs	case report forms
CFR	Code of Federal Regulations
CSO	Clinical Safety Office
ELISA	enzyme-linked immunosorbent assay
EM	erythema migrans
FDA	US Food and Drug Administration
FNLCR	Frederick National Laboratory for Cancer Research
GCP	Good Clinical Practice
HRPP	Human Research Protections Program
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IDE	Investigational Device Exemption
IRB	Institutional Review Board
LCIM	Laboratory of Clinical Immunology and Microbiology
MM	Medical Monitor
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NYMC	New York Medical College
OCRPRO	Office of Clinical Research Policy and Regulatory Operations
OHRP	Office for Human Research Protections
PCR	polymerase chain reaction
PI	Principal Investigator
PLDS	post-Lyme disease syndromes
PTLDS	post-treatment Lyme disease syndrome
SAEs	serious adverse events
SBV	South Bay virus
SCID	Severe Combined Immunodeficiency
SMM	Sponsor Medical Monitor
SRCP	Safety Review and Communication Plan
UADEs	unanticipated adverse device effects
UPs	unanticipated problems
UPnonAE	unanticipated problem that is not an adverse event
US	United States

STATEMENT OF COMPLIANCE

The trial will be carried out in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Good Clinical Practice (GCP) and the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR Part 312, and/or 21 CFR Part 812)

NIH-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; an IRB determination will be made regarding whether a new consent needs to be obtained from participants who provided consent using a previously approved consent form.

PRÉCIS

Lyme disease is the most common vector borne disease in the United States. Although antibiotic therapy is clinically effective in treating the symptoms of Lyme disease for most patients early in the course of disease, a significant number of patients who receive therapy report persistent symptoms. The cause of persistent symptoms after antibiotic therapy for Lyme disease is an area of great controversy. Recent studies have shown that the organism (*Borrelia burgdorferi*) may persist in animals after antibiotic therapy and can be detected by using the natural tick vector (*Ixodes scapularis*) to acquire the organism through feeding (xenodiagnosis). Whether this occurs in humans is unknown. Currently available tests for human Lyme disease do not allow determination of persistent infection after antibiotic therapy.

We performed the first study of the use of *I. scapularis* larva for the xenodiagnosis of *B. burgdorferi* infection in humans. Our pilot study showed that xenodiagnosis was well tolerated with no severe adverse events (AEs). The most common AE was mild itching at the site. In this small pilot study, xenodiagnosis for *B. burgdorferi* was positive in 2 participants and indeterminate in 2 participants. Further studies are needed to determine the sensitivity of xenodiagnosis in evaluating the infection status of Lyme disease patients.

In this proposal, we want to further investigate the utility of xenodiagnosis for identifying persistence of infection with *B. burgdorferi* in treated human Lyme disease. Our objectives include assessing the link between detection of *B. burgdorferi* by xenodiagnosis and persistence of symptoms in patients diagnosed with Lyme disease, within 1 year, post therapy; compare the rate of detection of *B. burgdorferi* by xenodiagnosis after therapy in participants with post-treatment Lyme disease symptoms; identify subject characteristics related to the likelihood of detecting *B. burgdorferi* by xenodiagnosis including: time from infection, time between infection and therapy, time from therapy; and continue to assess the safety of xenodiagnosis in humans.

The results of study have the potential to resolve this long-standing controversy in Lyme disease pathogenesis. While xenodiagnosis is unlikely to be widely used in clinical practice due to the labor intensity and speed of testing, if our study shows a linkage between positive xenodiagnostic testing and persistence of symptoms after *B. burgdorferi* infection, it may prove to be a useful tool for testing new strategies for treatment and for correlation with more generally applicable diagnostic markers. Understanding the pathogenesis of persistent symptoms following Lyme disease, and identifying reliable diagnostic tests for determining the success of antibiotic therapy, is critical to the medical management of these patients.

1 BACKGROUND

Lyme disease is the most common vector borne disease in the United States [1]. It is transmitted to humans via the bite of infected *Ixodes* ticks. At the tick bite site, *Borrelia burgdorferi*, the causative organism of Lyme disease, enters the skin, often resulting in the characteristic erythema migrans (EM) rash. From the original entry site, the organism spreads to distant locations affecting the heart, joints and central nervous system. Many studies have shown that Lyme disease is treated successfully with antibiotics in the majority of cases, and patients with objective evidence of treatment failure are rare with currently recommended regimens [2-5]. Patients with late manifestations can have a slower response to therapy, sometimes taking weeks or months to recover [6-14].

A portion of patients treated for Lyme disease will have persistent or relapsing non-specific symptoms (such as fatigue, musculoskeletal pain, and cognitive complaints) after receiving an adequate course of antibiotic therapy, a condition that is called post-Lyme disease syndrome. The best estimates of the prevalence of post-Lyme disease syndrome come from studies of patients with erythema migrans who received appropriate antibiotic treatment. 10-40% of such patients have persistent or intermittent subjective symptoms of mild to moderate intensity 12 months after completion of therapy [3,4,15-19]. The most common post-Lyme disease symptoms are fatigue, arthralgias, myalgias, headache, neck stiffness, paresthesias, sleeplessness, irritability, and difficulty with memory, word finding, and concentration [3,4,15-18]. The appearance of post-Lyme disease symptoms seems to correlate with disseminated disease, a greater severity of illness at presentation, and delayed antibiotic therapy [3,20-24]. The pathogenesis of these symptoms (whether due to persistent infection, immunologic dysfunction, or other mechanism) remains an area of great controversy.

A major concern has been that the symptoms of post-Lyme disease syndrome may represent persistent infection with *B. burgdorferi*. Hunfeld *et al*. [25] reported a 50% recovery rate of *B. burgdorferi* from erythema migrans skin biopsies done before therapy in 3,421 patients, and 1.7% of skin biopsies of patients (19 out of 1148) remained *B. burgdorferi* positive by culture 2 months (range 1.3-3 months) after antibiotic therapy. Spirochete persistence was not accounted for by the development of resistance to the antibiotic. Objective evidence of *Borrelia* infection in patients with post-Lyme disease syndrome has not been found using polymerase chain reaction (PCR) [26,27] or culture [26,27]. It should be noted, however, that *B. burgdorferi* culture and PCR have low sensitivity in most body fluids from patients with Lyme disease[28,29]. Other tests that have not been helpful in evaluating patients with post-Lyme disease syndrome include changes in C6 antibody levels [30] and antibodies in immune complexes [31]. Current serologic testing for antibodies to *B. burgdorferi* remains positive for years after therapy and declines very slowly making it essentially useless for confirmation of successful therapy. There are many anecdotal reports of patients with different persistent symptoms improving with prolonged antibiotic therapy (and worsening with cessation), but randomized controlled trials to date have not shown significant, sustained benefit from antibiotic therapy [26,27,32]. The question of whether patients continue to harbor *B. burgdorferi* after antibiotic therapy has become a very contentious issue[33].

Although the prevailing belief in the scientific and medical establishments is that patients do not continue to harbor *B. burgdorferi* and that long-term antibiotics are of no value, recent animal

data suggest that eradication of *B. burgdorferi* by antibiotics may not be complete. Studies in dogs, mice and non-human primate have shown that *B. burgdorferi* DNA can be detected in tissues for extended periods after antibiotic therapy[34-40]. Though positive testing for *B. burgdorferi* DNA by PCR has been used for diagnosis of the infection [41 42 43,44], it must be noted that PCR has the potential to recognize DNA from dead or dying bacteria [45].

Obtaining tissue samples of heart, joint, or bladder for testing would not be feasible as a diagnostic test in humans. *Ixodes* ticks feeding on antibiotic treated animals were able to acquire *B. burgdorferi* during their blood meal [34]. The organism could be detected by PCR of the tick and could also be transmitted to immunodeficient SCID mice by the ticks during their next blood meal. These studies have now been replicated by other groups both in mice and in monkeys. [38-40,46,47]

The use of a natural vector (i.e., *I. scapularis)* to detect the presence of an organism is called xenodiagnosis. Xenodiagnosis has been used in the diagnosis of human infections in instances where other diagnostic testing is difficult either due to difficulties/risks of obtaining proper specimens for testing or due to difficulty in cultivating the organism. Xenodiagnosis has long been used in Lyme disease research to provide definitive evidence of a host's infection status. For Lyme disease, tick saliva has been shown to be a chemoattractant for the organism and thus feeding ticks have the potential to aggregate and concentrate bacteria from a wide area, improving sensitivity [48].

Xenodiagnosis by feeding uninfected ticks on patients after antibiotic therapy could offer a minimally invasive method for determining the persistence of *B. burgdorferi* after antibiotic therapy. We have performed the first study of the use of *I. scapularis* larva for the xenodiagnosis of *B. burgdorferi* infection in humans. The primary goals of this study were to develop procedures that could be used in xenodiagnostic testing of patients after antibiotic therapy with Lyme disease and to determine the safety of tick xenodiagnosis in humans.

We have performed xenodiagnosis in 43 individuals. Xenodiagnosis has been well tolerated with no severe AEs. The most common AE was mild itching at the tick attachment site. Results from the initial 36 participants are described in our manuscript “Xenodiagnosis to detect *Borrelia burgdorferi* infection: A first-in-human study”, which has been published in the *Clinical Infectious Diseases* journal[49].

Participants who underwent xenodiagnosis included 10 patients with high C6 antibody levels, 10 patients with post-treatment Lyme disease syndrome (PTLDS), 5 patients with acute erythema migrans after completion of antibiotic therapy, one patient with erythema migrans on therapy (who also underwent xenodiagnosis after antibiotic therapy), and 10 healthy volunteers. Seven patients underwent more than one xenodiagnosis procedure.

As no previous protocol for xenodiagnosis with *I. scapularis* larva in humans existed, we developed a retention dressing using the LeFlap™ (Monarch Labs, Irvine, CA) dressing, which was modified for use with ticks. Participants enrolled early in the trial had fewer ticks feed successfully due to issues with the tick retention dressing that resulted in entrapment of the ticks in the adhesive.

After modifying the dressing by adding a foam ring to create a barrier between ticks and adhesive, we were more consistently able to get between 30 and 50% of ticks to feed successfully.

Xenodiagnosis was well tolerated. All participants successfully completed the tick placement and there were no withdrawals during the study. The most common AE was mild itching at the site, which was seen in 58% of participants, with a median duration of 3 days. Repeat of the xenodiagnosis procedure was similarly well tolerated, with mild itching at the site as the most common complaint. There were no serious AEs associated with the procedure. Although not a primary goal of this pilot study, we did find 2 participants that tested positive and 2 participants who tested indeterminate for the presence of *B. burgdorferi* in the xenodiagnostic ticks.

In this Phase 2 study, we plan to expand our study of xenodiagnosis for Lyme disease in humans. Xenodiagnostic evidence that *B. burgdorferi* survives in humans after antibiotic therapy may fundamentally change the approach to patients with persistent symptoms and provide physicians and researchers with a new tool with which to study the mechanisms of disease.

2 STUDY OBJECTIVES

1. Objective #1:
   Assess the link between detection of *B. burgdorferi* by xenodiagnosis and persistence of symptoms in patients diagnosed with Lyme disease, within 1 year, post therapy.

2. Objective #2:
   Compare the rate of detection of *B. burgdorferi* by xenodiagnosis after therapy for Lyme disease in participants with symptoms of post-treatment Lyme disease.

3. Objective #3:
   Identify subject characteristics related to the likelihood of detecting *B. burgdorferi* by xenodiagnosis including: time from infection, time between infection and therapy, time from therapy.

4. Objective #4:
   Continue to assess the safety of xenodiagnosis in humans.

3 STUDY DESIGN AND METHODS

3.1 Experimental Design

This will be a prospective non-randomized multi-site study conducted at 5 sites: 1) Tufts Medical Center, Boston, MA (Dr. Linden Hu, co-PI and Tufts site PI); 2) NIH Clinical Center, Bethesda, MD (Dr. Adriana Marques, co-PI and NIH site PI); 3) New York Medical College, Valhalla, NY (Dr. Gary Wormser, site PI) and 4) Mansfield Family Practice, Storrs, CT (Dr. Kenneth Dardick, site PI), and 5) Stony Brook University, Stony Brook, NY (Dr. Luis Marcos Raymundo, site PI).

All investigators are well-recognized experts in the field of Lyme disease and have prior experience in research. Up to 240 participants will be enrolled across all sites.

3.2 Inclusion Criteria

Criteria for the diagnosis and therapy for Lyme disease can be found at "The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America" [5].

Patients with Lyme disease, post-therapy (N=100)

1. Age 18 or older.
2. Lyme disease diagnosed in the previous 13 months, fulfilling the case definition of confirmed or probable Lyme disease by the US Centers for Disease Control and Prevention (CDC) https://wwwn.cdc.gov/nndss/conditions/lyme-disease/case-definition/2017/ .
3. Completion of 1 course of antibiotics at least 3 months and up to 12 months between the end of the therapy and the xenodiagnostic procedure.
4. Antibiotic treatment fulfills the Infectious Diseases Society of America guidelines for the recommended therapy for Lyme disease [5].

Patients with post-Lyme disease complaints at least 12 months from initial treatment (N=40)

1. Age 18 or older.
2. Diagnosed with confirmed or probable Lyme disease fulfilling the case definition of Lyme disease by the CDC https://wwwn.cdc.gov/nndss/conditions/lyme-disease/case-definition/2017/ .
3. Received recommended antibiotic therapy for Lyme disease [5] at least 12 months between the end of the initial antibiotic therapy and the xenodiagnostic procedure.
4. Persistent or recurrent symptoms that began or worsened within 6 months of the diagnosis and treatment for Lyme disease.

Acute EM (N=40)

1. Age 18 or older.
2. EM diagnosed by the study physician.
3. Receiving antibiotic therapy for Lyme disease for less than 48 hours.

Lyme Arthritis (N=40)

1. Age 18 or older.
2. Lyme arthritis[50] and have not received antibiotic therapy for the disease.

Healthy volunteers (N=20)

1. Age 18 or older.
2. No prior history of Lyme disease.
3. Negative whole cell enzyme-linked immunosorbent assay (ELISA) or C6-based antibody test for Lyme disease.

Patients with recently diagnosed (acute) EM (within 48 hours of starting antibiotic therapy) and patients with untreated Lyme arthritis will be recruited in an attempt to increase the chances of finding a positive result by xenodiagnosis (an attempt of a "positive control"). While patients with acute untreated EM would be the best positive control group, it would be unethical to withhold therapy in these patients for the few days required for tick feeding, due to the risk of dissemination of the organism and possible morbidity. Patients with untreated Lyme arthritis will

be recruited to establish whether xenodiagnosis can be used to identify infection in late stage Lyme patients where the bacterium is known to be present. These patients have been infected for months and will not be harmed for delaying therapy for a few days. Lyme arthritis is a late manifestation of *B. burgdorferi* infection, and hematogenous dissemination already occurred at this late stage. Studies have shown that the presence or absence of previous antibiotic treatment is more predictive than the duration of untreated arthritis for the success of antibiotic therapy in Lyme arthritis. [6,8,51-53] Similarly, patients who just started therapy for EM may still have live *Borrelia* in the skin and xenodiagnosis may be able to recover the bacteria (but culture of skin biopsies from patients with EM become negative very quickly - within one dose - on antibiotic therapy).

While treatment for Lyme disease will not be offered under this protocol, it may be available via different clinical research protocols or regular medical care at the study site. If not, treatment will be prescribed by the patient's primary care. For patients with untreated early Lyme disease (erythema migrans), antibiotics can be started at the same day of tick placement. For patients with untreated Lyme arthritis, antibiotics can be started after collection of xenodiagnostic ticks (usually 4-5 days, up to 7 days). For patients with Lyme arthritis, if less than 14 ticks fed successfully and if the participant agrees, antibiotic treatment can be delayed until after the repeat procedure (see Section 4.7).

Patients with acute EM and untreated Lyme arthritis will be able to re-enroll as Patients with Lyme disease, post-therapy. Therefore, in case of positive results, we will be able to compare between the procedures.

Negative control patients will include healthy volunteers from Lyme endemic areas who have never been diagnosed with Lyme disease and have a negative *B. burgdorferi* ELISA and C6 antibody titer.

3.3 Exclusion Criteria

1. No antibiotic therapy active against Lyme disease in the previous 3 months (except patients with acute EM). Prophylaxis with a single dose of doxycycline 200 mg is not an exclusion.
2. History of allergy to surgical tape or dressing.
3. History of severe reactions to tick bites (granuloma or systemic reactions).
4. Inability to maintain the dressing for any reason.
5. Pregnancy or lactation.
6. Unwillingness to use an effective method of birth control for the duration of participation in the study (women of child-bearing potential only) and for at least 3 months following the last tick placement.
7. Use of investigational therapy and devices during the time of the study and/or in the month prior to signing the informed consent.
8. Active severe skin disease, uncontrolled diabetes, cancer other than non-melanoma skin cancers, autoimmune disease requiring immunosuppressive therapy, or history of HIV, chronic viral hepatitis, or syphilis.
9. Oral or IV steroids in the previous 2 weeks (topical, nasal, inhaled, intra-articular, and replacement doses of steroids are not exclusions).

10. Any other condition that, in the opinion of the investigator, would make the patient unsuitable for enrollment or could interfere with the patient participating in and completing the study.
11. Refusal to participate in specimen collection and storage for future study related use.

3.4 Exclusion from Skin Biopsy Part of the Protocol

1. History of forming large thick scars (keloids) after skin injuries or surgery.
2. History of excessive bleeding after cuts or procedures.
3. Currently taking anticoagulants.
4. History of allergy to lidocaine.

3.5 Special Populations

Children: Children are excluded from this protocol as there is no direct benefit to the participants, the risk from the skin biopsies is small but above minimal, the procedure is invasive and can be stressful for children, and there is concern over their ability to maintain the LeFlap dressing.

Women: Pregnant and breastfeeding women are excluded from trial participation as there is no direct benefit to the participants.

Adults who lack capacity to consent: Adults lacking decision-making capacity to provide informed consent are excluded at screening as there is no direct benefit to the participants. Enrolled participants who permanently lose the ability to consent during participation will be monitored for safety but will have no additional research procedures performed.

NIH staff: NIH staff and family members of study team members may be enrolled in this study as this population meets the study entry criteria. Neither participation nor refusal to participate as a subject in the research will have an effect, either beneficial or adverse, on the participant's employment or position at NIH.

Every effort will be made to protect participant information, but such information may be available in medical records and may be available to authorized users outside of the study team in both an identifiable an unidentifiable manner.

The NIH Information Sheet on NIH Staff Research Participation will be made available. Please see Section 16 for consent of NIH Staff.

4 STUDY PROCEDURES

4.1 Initial Visit

After written informed consent is obtained, participants will have a history and a physical examination performed. Blood will be drawn and tested for Lyme serologies (approximately 8 mL of blood). Patients with acute EM will also have blood collected for culture, PCR, and other direct tests for *B. burgdorferi* (approximately 50 mL). These tests may be performed at NYMC, NIH, and Tufts laboratories. This initial visit may or may not be combined with the tick placement visit (see below).

4.2 Tick Placement Visit

This visit may occur together with the initial visit, or may be a separate visit, up to 1 month apart. If a separate visit, eligibility criteria will be reviewed again to ensure patient remains eligible. At this visit, approximately 40 mL of blood will be drawn and a urine sample may be collected and stored for future study related research. Stored specimens will be useful for future development of new diagnostic tests or to test for as yet to be identified emerging infectious agents. Female patients of childbearing potential will have a urine or blood pregnancy test performed at this visit. The results of the pregnancy test will be reviewed before the placement of the ticks. Self-reported symptoms will be characterized using symptom scales, the SF-36v2 questionnaire, and fatigue scales.

Except for patients with acute EM, ticks will not be placed at this visit if the individual is found to have abnormal vital signs (temperature >38.0°C, a pulse greater than 105 beats per minute, systolic blood pressure >160 mm/Hg or diastolic blood pressure >95 mm/Hg, in at least 2 measurements) and these individuals will be asked to follow up with their personal physician. They may be re-evaluated for tick placement at a subsequent time when the vital signs are within acceptable limits.

Skin Biopsy

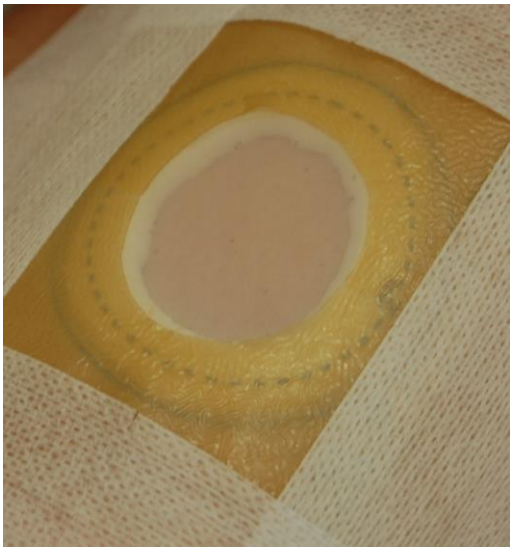
Eligible participants (acute EM, untreated Lyme arthritis and PTLDS) who agree to undergo the optional skin biopsy will have two 2 or 3 mm skin punch biopsies performed outside the site of the tick placement. The skin will be cleansed and anesthetized with 2 percent lidocaine with epinephrine. Each biopsy will be performed using a "punch"- a small disposable tubular knife. The wound will be closed, if necessary, with steri-strips or (rarely) with 1 or 2 interrupted nylon sutures. A bandage will be applied and participants will receive the instruction sheet regarding follow up procedures (Appendix 1). If suture(s) are placed, patients will be asked to return in 7 to 10 days to have the suture(s) removed. Biopsies will not be done on face, neck, scalp, or over the tibia. One biopsy will be used for *B. burgdorferi* culture and/or PCR. A second biopsy will be stored for future study related research.

If the Initial Visit and the Placement Visit do not occur together, the initial skin biopsy may be performed at either visit, accordingly to the preference of the participant and study personnel.

Xenodiagnosis

Between 25-30 larval ticks that have been aged and are known to be ready to attach will be placed (Figure 1). The area chosen for the placement will be as close as possible to the site of the previous EM, if possible. Ticks will not be attached to head, neck or genitalia. If necessary, body hair will be shaved at the site. The ticks will be placed inside the Creature Comforts II LeFlap™ dressing which has been modified for tick use with the addition of a foam inner layer. The LeFlap is a pre-assembled confinement dressing that is used with medical maggots. Participants will be requested to keep the dressing dry for the duration of the attachment. Participants will receive an instruction sheet with instructions regarding care of the dressing and a Diary Card. The subject will be asked to complete a diary card daily starting the date of placement of the ticks through 30 days after tick removal.

Figure 1: Feeding of *Ixodes* Larvae under LeFlap Dressing



4.3 Tick Removal (4-6 days after tick placement)

Larval ticks typically feed for 4 to 5 days but it is possible that some might feed as long as 10 days. Participants will return to the study clinic for removal of the dressing and recovery of the ticks at 4-6 days. Pictures may be taken of the site. Approximately 40 mL of blood will be drawn for future study related research. Participants will be asked during the visit whether there were any complications during the feeding of the ticks, including adverse reactions and tick escape, and the diary card will be reviewed at this visit.

If there are a significant number of ticks still attached, the individual may be asked to return over the next subsequent days for evaluation until the majority of the ticks have detached. If this occurs, the ticks that dropped off will be collected, the dressing will be changed, and the individual will be seen within 24-72 hours for removal of the other ticks and completion of the visit. These visits may need to be repeated up to day 10 from the time of placement.

Eligible participants who agree to undergo the optional skin biopsy will have two 2 or 3 mm skin punch biopsies performed outside the site of the tick placement. The skin will be cleansed and anesthetized with 2 percent lidocaine with epinephrine. Each biopsy will be performed using a "punch"- a small disposable tubular knife. The wound will be closed, if necessary, with steri-strips or (rarely) with 1 or 2 interrupted nylon sutures. A bandage will be applied and participants will receive the instruction sheet regarding follow up procedures (Appendix 1). If suture(s) are placed, patients will be asked to return in 7 to 10 days to have the suture(s) removed. Biopsies will not be done on face, neck, scalp, or over the tibia. One biopsy will be used for *B. burgdorferi* culture and/or PCR. A second biopsy will be stored for future study related research.

The study team will contact each subject 7-10 days post-tick removal to determine if they are experiencing any AEs. The participants will also be instructed to call the study staff if any new symptoms occur during the period when the ticks are attached and for 30 days after tick removal. A 30-day window was chosen for the following reasons: 1) Adverse reactions to tape or the tick bites are expected to resolve within 2 weeks after removal of the ticks; 2) in the very unlikely event that there is an as yet unidentified infection that is transmitted by the ticks, all of the known

tick transmitted diseases have an onset of symptoms within 30 days of tick detachment. Participants will be specifically told to contact the study personnel for any fever, chills, joint pains, muscle pains, neck stiffness, or severe headache, as these are symptoms of known tick-borne diseases. For the first month after removal of the ticks, the participants will be asked to maintain a daily diary, which will be collected at the 1-month visit.

4.4 1-Month and 3-Month Follow-up

In addition to the contact with the individual at 7-10 days post-tick removal, participants will be followed at 4 to 6 weeks after the removal of ticks to ensure that no unanticipated events have occurred. This visit may be in person or by phone. Participants will be specifically questioned about fever, rash or infection at the site of tick attachment. They will be questioned about any intercurrent illnesses. Self-reported symptoms will be characterized using symptom scales, the SF-36v2 questionnaire and fatigue scales. Forms are self-administered and can be administered in paper form or computer, including online. If the visit is in person, approximately 40 mL of blood will be drawn and a urine sample may be collected for storage for future study related research. The diary card will be reviewed at this visit.

Participants will also be followed at 3 months (±2 weeks) after the mesh is removed. This visit may be in person or by phone. Participants will be questioned about any intercurrent illnesses. They will complete the symptom scales, the SF-36v2 questionnaire and fatigue scales. Forms are self-administered and can be administered in paper form or computer, including online. If the visit is in person, approximately 40 mL of blood will be drawn and a urine sample may be collected for storage for future study related research.

Participants will be contacted to discuss their individual results from xenodiagnostic studies when these become finalized. The results are expected to be available within one year of the procedure.

4.5 12 Months after Start of Antibiotic Therapy

Participants enrolled under the Lyme disease, post treatment group will also be contacted at 12 months (±4 weeks) after the start of their antibiotic therapy for Lyme disease. This visit may be in person or by phone. If the visit is in person, approximately 40 mL of blood will be drawn and a urine sample may be collected for storage for future study related research. Self-reported symptoms will be characterized using symptom scales, the SF-36v2 questionnaire and fatigue scales. Forms are self-administered and can be administered in paper form or computer, including online.

4.6 Early Discontinuation/Withdrawn from the Study

If a participant decides to withdraw from the study, the investigator may ask the participant who is withdrawing whether the participant wishes to provide continued follow-up and further data collection subsequent to their withdrawal from the interventional portion of the study. Participants that undergo tick placement and discontinue study participation by the PI's wishes will continue to come to visits for subject safety assessments or may be asked to provide vital safety information over the phone. Attempts will be made to contact participants who are lost to follow up.

4.7 Repeat of Xenodiagnosis Procedure

All repeats of the xenodiagnosis procedure are optional.

Repeat of the xenodiagnosis procedure can be offered to participants in 3 situations:

a. Participants in whom the xenodiagnosis procedure failed to recover at least 14 intact engorged ticks.
b. Participants in whom the xenodiagnosis procedure is positive.
c. Participants who have completed the study as acute EM or Lyme arthritis patients and are eligible to participate as *Patients with Lyme disease, post-therapy*.

For participants in whom at least 14 intact engorged ticks are not recovered, 1 additional xenodiagnosis procedure (placement of ticks) will be offered. This does not require re-enrollment. Based on our experience with the phase 1 study, we expect that this may occur in up to 50% of the placements. Xenodiagnostic procedure will not be offered to participants with acute erythema migrans, as they will have started antibiotic therapy at the time of the initial tick placement.

For participants in whom the xenodiagnosis procedure is positive, re-enrollment will likely be required, due to the time necessary for results from the testing of the xenodiagnostic ticks to be finalized, as participants will likely have completed the follow up phase of the study. The study procedures will be repeated as described above, including the skin biopsy (optional) and follow up visits.

Participants who completed the study as *Acute Erythema Migrans* or *Lyme Arthritis patients*, and are eligible to participate as *Patients with Lyme disease, post-therapy*, will be enrolled as part of the new patient group and a new study number will be assigned. The study procedures will be repeated as described above, including the skin biopsies (optional) and all follow up visits.

Therefore, the maximum number of xenodiagnosis procedures offered to a participant for each group and situation, and the expected time period for the procedures are described in the Table 1 below.

Table 1: Maximum Number of Xenodiagnosis Procedures to be Offered

Group	Description	Xenodiagnosis, initial	If xeno positive	Enroll as a new participant?	Total	Time period between first and last possible tick placement
1	Patients with Lyme disease, post-therapy	Up to 2	Up to 2	No	Up to 4	3-6 months
2	Patients with post-Lyme disease	Up to 2	Up to 2	No	Up to 4	3-6 months

	complaints at least 12 months from initial treatment					
3	Acute EM	1*	*	Yes, as Group 1	Up to 5 (1 as EM, up to 4 as post-therapy)	7-18 months
4	Lyme Arthritis	Up to 2	*	Yes, as Group 1	Up to 6 (2 as LA, up to 4 as post-therapy)	7-18 months
5	Healthy volunteers	Up to 2		No	Up to 2	2 weeks

* Repeat will not be offered, as patients will have received antibiotic therapy at this point and results would not be comparable.

The date of each visit will be determined by the date from each procedure. In case there is overlap of visits, visits may be combined. Participants will be followed until completion of the protocol from the time of the last xenodiagnosis procedure. Lyme serologies will be repeated if performed more than 2 months from the placement. Approximately 40 mL of blood will be drawn and a urine sample may be collected for storage for future study related research at the repeat Tick Placement visit if the repeat xenodiagnosis procedure is more than 21 days from the initial tick placement.

Research bloods (approximately 40 mL of blood) will be drawn at the repeat tick removal visit for future study related research.

- The tick placement site may be the same or a different site, depending on individual preferences.
- For participants repeating because of less than 14 engorged ticks recovered, the period between the tick placement procedures can be from the time of tick removal up to 3 months post tick removal, and up to 1 repeat may be offered for each planned round of xenodiagnosis to ensure adequate collection of ticks for testing.

Participants who are offered the procedure because of a previous positive xenodiagnosis do not need to meet entry criteria at the time of the repeat procedure. All participants must fail to meet any exclusion criteria at the time of tick placement.

4.8 Ticks

Larval ticks will be obtained from Dr. Sam Telford from a laboratory maintained tick colony at Tufts Veterinary School. These ticks are hatched from eggs laid by ticks that have fed only on specific pathogen free laboratory animals that were purchased from established vendors. Larval and nymphal ticks will be maintained on CB17 SCID mice, which are highly susceptible to many infections since they are immunodeficient. Mice that serve as hosts to tick colony material are screened monthly by blood smear and PCR for the main deer tick-transmitted agents.

B. burgdorferi, *Babesia microti,* and *Anaplasma phagocytophilum* (human pathogens known to be transmitted by *I. scapularis* ticks) are not transmitted transovarially (from mother to larvae). Deer tick virus, a flavivirus closely related to Powassan virus, has been linked to rare cases of human encephalitis [54,55]. Deer tick virus is inherited; however, its presence is readily detected in

a laboratory colony fed upon mice because all mice die with paresis within 4-14 days. There are some bacteria in ticks that can be transmitted transovarially including *B. miyamotoi* [56,57]. There have been reports of *Bartonella spp*. in *Ixodes* ticks, but transmission of any *Bartonella* spp. by ticks to humans has not been established [58,59].

Each batch of ticks prepared for use in human xenodiagnosis will be tested for all known *Ixodes* transmitted human pathogens. Approximately one-third (⅓) of the larvae from each egg mass will be crushed and tested by PCR for the presence of the known human pathogens *B. burgdorferi*, *B. microti, A. phagocytophilum, B. miyamotoi, Bartonella* spp*.,* deer tick virus and orbiviruses. In addition, a representative number of larvae from each batch (not less than 25) will be tested at Dr. Linden Hu's laboratory using a panel of broad-range primers targeting a wide range of genera including Spirochetes (all *Borrelia* species), *Francisella*, *Bartonella*, *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Babesia*. Any egg mass that tests positive for any of these organisms (but for the *Rickettsia* endosymbiont of *I. scapularis*, see below) will be discarded. Production of additional ticks will be halted until a root-cause analysis of the source of the positive samples has been performed and the data presented to the Medical Monitor (MM) and the site PIs.

Although our ticks are tested to be free of all known human pathogens, the ticks are not sterile and do carry commensal organisms. Almost all *I. scapularis* ticks carry a rickettsial symbiont [60,61]. At this point, there is no known disease associated with this symbiont. Anecdotally, batches of larvae have fed on one individual 3 separate times (as many as 40 at a time). The individual has remained seronegative for antibodies to spotted fever group and typhus group rickettsiae, and has not experienced any illness associated with feeding larvae (Dr. Sam Telford, personal communication). Screens of mice serving as hosts for larvae, in the past, have never demonstrated antibody to spotted fever group rickettsiae, suggesting that the endosymbiont does not exit the tick by feeding. We test the xenodiagnostic ticks for *Rickettsia* sp. in order to have baseline information about their infection in case future issues arise.

Recently, Tokarz et al. performed high-throughput sequencing to identify viral sequences in the *I. scapularis* genome.[62] In addition to deer tick virus described above, they identified sequences that partially aligned with viruses of the genus *Nairovirus* (named South Bay virus (SBV) and the genus *Phlebovirus* (named blacklegged tick phlebovirus, BTPV). For both, the assembled sequences showed low similarity to other members of the genus. Moreover, they were unable to identify any sequences representative of potential M segments (the glycoprotein-coding segments). Attempts at virus isolation by inoculation of *I. scapularis* SBV PCR-positive pools in Vero, Cos7, C6/36, and 297 cell lines were unsuccessful. They also found partial sequences with weak homology to viruses within the order *Mononegavirales*, with the greatest similarity (17%) observed with the Nyamanini and Midway viruses.

We have extended our testing panel to include additional assays for these potential viral entities that currently are known only from cognate nucleic acid sequences (BTPV, SBV, and deer tick *Mononegavirales*). We have screened 5 different samples of ticks (2 different lineages) that were used for the previous study from 2010-2013 and found that 5/5 were positive for cognate sequences matching SBV, 4 of 5 for BTPV, and 0/5 for deer tick *mononegavirales*. We conclude that inasmuch as these batches of ticks were used for the Phase 1 safety trial even though they contained cognate sequences that matched putative viral sequences, like the rickettsial

endosymbiont of *I. scapularis* (REIS, now known as *Rickettsia buchneri*)[63], there is no evidence of ill health resulting from the feeding of these ticks on study subjects. SCID mice have remained uniformly healthy after feeding ticks and we do not suspect any adventitious agent in our tick colonies that would compromise safety, regardless of the possibility of putative agents that have recently been or remain to be described. We will follow this general strategy as new agents that are not known human pathogens are identified. If a new pathogen is identified or a previously non-pathogenic organism is identified as a pathogen, use of ticks in xenodiagnosis will be halted until we develop a test to identify the presence of that pathogen or its products.

4.9 Testing of the Xenodiagnostic Ticks

Recovered larval ticks will be tested for the presence of *B. burgdorferi* by PCR, culture, and/or direct immunofluorescence. Other techniques with high sensitivity and specificity to detect *B. burgdorferi* and/or other tick-borne pathogens may be used as they become available.

4.10 Optional Skin Biopsy

Up to 4 optional skin biopsies will be collected to test for evidence of *B. burgdorferi* in the skin. The test results will be compared with the xenodiagnostic test results. Skin biopsies will be offered before the placement of ticks (either during Initial Visit or Tick Placement Visit) and after tick removal in patients with acute EM, untreated Lyme arthritis and PTLDS, and only after tick removal for participants in the Lyme disease, post-therapy group and for healthy volunteers. The reason for offering before and after placement for patients with acute EM, untreated Lyme arthritis and PTLDS is to be able to compare the results of the biopsy before and after and the results from the xenodiagnostic ticks, as tick feeding may increase the chance of finding *B. burgdorferi* in the skin site. Study results have shown that spirochetes were easily seen at the site of tick attachment after 4 days of feeding, but not on samples taken earlier [64]. For participants in the Lyme disease, post-therapy group, recent unpublished data has shown that skin biopsies are negative in EM patients after 6 months of therapy. Therefore, only biopsies after tick removal will be done.

4.11 Discussion of Results

Participants will be informed that xenodiagnosis is an experimental test and therefore there are no guidelines to govern treatment of participants who test positive or negative by xenodiagnosis for the presence of *B. burgdorferi*. Participants will be notified of their test results by phone or in person at the completion of all testing of their ticks. The study doctor will discuss the implications of the test results with the subject.

5 RISKS

5.1 Xenodiagnosis

Anticipated risks to the participants include:
1) The development of allergic dermatitis from surgical tape used for sealing the mesh; or
2) The development of a mild or moderately pruritic or painful local reaction to the tick bite, which does not require medical attention.

From our experience with the 43 participants who underwent the xenodiagnosis procedure, the procedure is very well tolerated. All participants successfully completed the tick placement and

there were no withdrawals during the study. The most common AE was mild itching at the site, which was seen in 58% of participants, with a median duration of 3 days. Repeat of the xenodiagnosis procedure (performed in 7 participants), was similarly well tolerated with mild itching at the site being the most common complaint. There were no SAEs associated with the procedure. All these AEs resolved and did not require any intervention. No ticks escaped from the dressing.

Problems which may also occur and be serious but are not anticipated (and are therefore reportable as Unanticipated Adverse Device Effects [UADEs]) include:

A) the development of a skin infection (cellulitis) from scratching or skin breaks secondary to 1 or 2 (above) or
B) the remote possibility of developing tick paralysis
C) the remote possibility of infection with a yet unknown pathogen
D) the remote possibility of developing an allergy to red meat

Allergic dermatitis is typically self-limited with removal of the allergen (tape). Recovery may be aided by the application of topical steroids.

A minority of patients who have been bitten frequently by ticks will develop antibody responses to tick saliva and may develop a localized reaction with a granuloma. These reactions are self-limited.

Breaks in the skin caused by allergic dermatitis or scratching of tick bites can result in the development of cellulitis. Development of cellulitis secondary to allergic dermatitis or scratching is a very rare event, but could require either oral or intravenous antibiotic therapy.

Although the ticks are tested for all known human diseases that are transmitted by *I. scapularis*, the ticks are not sterile and do carry commensal organisms, and there is a possibility that a new, unknown pathogen may be carried by the ticks. If a new pathogen is identified or a previously non-pathogenic organism is identified as a pathogen, use of ticks in xenodiagnosis will be halted until we develop a test to identify the presence of that pathogen or its products.

Tick paralysis is a rare complication of tick bites. Patients present with acute symmetric ascending flaccid paralysis that generally begins in the lower extremities. Paralysis can ascend to the upper extremities, followed by cranial nerve involvement and respiratory muscle involvement, if the tick is not removed. Occasional patients may present with focal weakness or a cerebellar presentation. Removal of the tick is the definitive therapy, and patients will usually improve quickly after removal; however, symptoms can progress after tick removal (most commonly in Australian cases, associated with *I. holocyclus*). Most cases in North America are associated with the bites of *Dermacentor andersoni* (the wood tick) and *D. variabilis* (the dog tick), occur in children under the age of 10, particularly girls (probably because long hair make difficult to notice the feeding tick), and the tick is attached to the head or neck. *I. holocyclus* is the predominant species causing tick paralysis in Australia [65]. *I. scapularis* is not associated with this disease, although a case associated with *I. scapularis* was reported in Mississippi [66]. Assuming the tick identification was correct, it seems to be a highly unusual complication when

compared with the very high number of deer tick bites that occur yearly. A neurotoxin produced in the saliva of female ticks causes the paralysis. The *I. holocyclus* toxin has been identified and is named holocyclotoxin. The transcriptome analysis of over 8000 expressed sequence tags from 6 different salivary gland cDNA libraries from *I. scapularis* showed only a poor match with holocyclotoxin, suggesting the toxin is not found in *I. scapularis* [67]. Therefore, it is highly unlikely that patients in this study will develop tick paralysis as a complication of tick placement, as *I. scapularis* is not associated with this disease, ticks being used are in the larval stage (adult female ticks are usually the stage associated with tick paralysis), and patients in the study are adults.

There has been an association between serious allergic reactions to red meat (including delayed anaphylaxis) and tick bites in some susceptible individuals [68,69]. It is caused by an IgE antibody that binds to galactose-α-1,3-galactose (alpha-gal). Individuals with red meat allergy often reported a more severe local reaction to tick bites, with significant itching and redness around the bite. It is unknown if this allergy can develop after *I. scapularis* bites. The association has been described in Australia with *I. holocyclus* [68]. It has also been described in patients in the southern US [70]. A study suggested that as many as 20% of the population in Virginia, North Carolina, and Tennessee states, regions where *Amblyomma americanum* is common, have serum IgE antibodies to alpha-gal[69]. Individuals in the southeastern US (where *Amblyomma* is more prevalent) are more likely to have antibodies against alpha-gal than individuals from Boston and Scandinavia [69]. Patients with a history of severe reactions to tick bites are excluded from participation in the protocol, but it is possible that exposure to ticks may trigger a future allergic reaction in susceptible individuals. No reports of meat allergy were seen in the Phase I trial of Xenodiagnosis.

Anaphylaxis following a tick bite has rarely been reported [71 72-74]. These reports include the *Argus reflexus*, *Rhipicephalus sanguineus*, *I. holocyclus* and *I. ricinus*. However, to our knowledge, it has not been reported in *I. scapularis* ticks. Recurrent anaphylaxis has been reported in *I. pacificus*, a tick that is closely related to *I. scapularis* [75]. Allergic reactions may follow the bite of any of the different tick life cycle forms and is related to the release of salivary protein. Because these reactions are rare, it is uncertain whether repeated tick bites increase the risk of anaphylaxis.

Repeat of the xenodiagnosis procedure may increase the risk of sensitization and local reactions. It is known that people can become sensitized to tick bites over time and after having had many tick bites. A study of *I. scapularis* bite reactions and their protective effect against acquisition of Lyme disease on Block Island, Rhode Island, showed that among 610 participants, 52% reported at least 1 tick bite (mean 2.2, 95% CI 2.0–2.4), and 32% reported itch with any tick bite. None reported anaphylaxis associated with tick bite. The probability of itch doubled as the number of reported tick bites increased from 1 to 2 (21% to 46%, respectively) and doubled again from 2 to 4 reported bites (46% to 97%, respectively; linear trend $p < 0.001$). There was an inverse relationship between tick itch reaction and development of Lyme disease. The likelihood of Lyme disease infection decreased with >3 reports of tick-associated itch (odds ratio 0.14, 95% CI 0.94–0.03, $p = 0.01$). The possible mechanisms include a heightened awareness by a person of an attached tick with removal of the potentially infecting tick before pathogen transmission can occur, premature detachment of ticks before pathogen transmission can occur, or neutralization

of some components of tick saliva that ensure successful feeding and facilitate pathogen transmission. [76,77]

There is a risk that ticks, either fed or unfed, may escape from the dressing. Loss of ticks should not pose a risk to either the subject or other people in contact with the subject. *Ixodes* ticks require high moisture content and would not survive in an indoor or urban setting for more than 1 or 2 days. Unfed ticks would be uninfected and at most, would attach at a site other than the forearm on the subject or, possibly, on a family member. Fed ticks are not expected to feed again for 3-6 months. Because the ticks are tested and carry no known disease, an unfed tick that escaped into a suitable environment in the subject's yard or surrounding area, would not pose a threat to other humans, wildlife or add a new species into the environment. *Ixodes* ticks are highly prevalent in rural and suburban Massachusetts, Rhode Island, Connecticut, New York, Maryland, and Virginia.

5.2 Blood Drawing

The two hazards associated with blood drawing are those of venipuncture and those associated with blood loss. Risks of venipuncture are pain at the site of the needle stick, the potential formation of hematoma at the site of the needle stick, and a small potential for infection and/or inflammation at the site of the needle stick. Occasionally, individuals may faint as a result of vasovagal reactions to the procedure. The major risk of repeated blood drawing is anemia. Current guidelines for maximum amount of blood drawn for research will be followed.

5.3 Punch Skin Biopsy

Punch biopsies have a low incidence of infection, bleeding, non-healing, or significant scarring [78,60]. Rarely, there may be injuries or damage to the structures beneath the skin site (such as an artery or a nerve). Patients rarely may have local allergic reactions to the tape. Severe allergic reactions (anaphylaxis) to lidocaine are very rare.

6 BENEFITS

There are no direct benefits to the study participants. If the xenodiagnostic test is positive, they will know that they still harbor *B. burgdorferi* in their body. It is currently unknown whether treatment with more or different antibiotics will be beneficial to these patients

The benefit to society is that if it is shown that patients continue to harbor bacteria after antibiotic therapy, this may result in a paradigm shift that will lead to new controlled therapeutic trials. In addition, xenodiagnosis may become a new method for diagnosing persistent infection after antibiotic therapy.

7 STUDY ANALYSIS

7.1 Sample Size Justification

This study is powered to detect difference in the positivity rate between symptomatic and asymptomatic individuals. Self-reported symptoms will be characterized using symptom scales, the SF-36v2 questionnaire and fatigue scales. We will use a composite score derived from the measurement to assign symptomatic and asymptomatic status to the participants.[79]

While the majority of the patients with Lyme disease who were treated with antibiotic therapy were negative by xenodiagnosis in our Phase 1 study, the number of patients tested in each group was small and the upper limit of the 95% confidence interval of each proportion is relatively high (up to 46%). Also, an important caveat is that the number of tested xenodiagnostic ticks per participant in general was also small, and the number of engorged ticks that are tested for each individual is an important variable, as the more engorged ticks tested, the larger the probability of detecting a positive. For example, if the true sensitivity for a single engorged tick to detect the bacteria is 1/10 (based on results of the mice studies), then one would need to recover 14 engorged ticks to have 75% sensitivity and 22 fed ticks to have 90% sensitivity.

Based in our very preliminary data, the upper limit of the exact 95% confidence interval for the proportion of positive xenodiagnosis in symptomatic patients (10%) was 0.445. Table 2 shows the sample size needed for 80% power, with a Type I error rate of 0.05, assuming varying assumptions for the true prevalence rates for asymptomatics (p_1) and symptomatics (p_2). For example, with a total sample size of 86 individuals, there would be 80% power to detect a between-group difference in the positivity rate if the true prevalence is 5% or less in the non-symptomatics and at least 30% in the symptomatics, assuming equal numbers between the 2 groups, (43 non-symptomatics and 43 symptomatics).

Table 2: Sample Size Needed for 80% Power with a Type I Error Rate of 0.05, and Varying Assumptions for the True Prevalence Rates for Asymptomatics (p1) and Symptomatics (p2)

	$p_2 = 0.40$	$p_2 = 0.30$
$p_1 = 0.05$	54	86
$p_1 = 0.025$	46	68
$p_1 = 0.01$	42	60
$p_1 = 0$	40	56

Assuming a 10 to 20% rate of missing data due to failed screenings, assays or participant drop-out, we plan to recruit up to 100 individuals.

For the other groups post-Lyme disease syndromes (PLDS), acute EM, and untreated Lyme arthritis, the precision with which the rates can be estimated from the observed data depends on the true underlying response rate and the sample size. Exact Clopper-Pearson 2-sided 95% CIs for the observed positivity rate the different proposed group sizes and a range of numbers of positive individuals are shown in Table 3. Healthy volunteers (negative controls) results will be used for specificity of the testing.

Table 3: Exact 95% Confidence Interval for the Observed Positivity Rate, for Varying Group Size (N) and Observed Numbers of Positive Individuals

# of observed positives samples	N=20	N=40
0	(0.000, 0.161)	(0.000, 0.09)
1	(0.009, 0.236)	(0.004, 0.13)

2	(0.0279, 0.301)	(0.014, 0.165)
5	(0.112, 0.468)	(0.054, 0.261)
10	(0.299,0.701)	(0.142, 0.402)
15	(0.531, 0.888)	(0.242, 0.529)
20	(0.838, 1)	(0.352, 0.648)

Data will be collected for any positive tests by xenodiagnosis. Because there is no gold standard test among those we will employ, comparative positivity rates between the tests will be determined. Summaries of patient characteristics will be assessed by study group. For categorical variables, the numbers and proportions will be tabulated; for continuous variables, the mean, median, SD, and interquartile range will be reported. Fisher's exact text will be used to examine the association of 2 categorical variables. For continuous variables, t-tests and nonparametric methods, such as the Wilcoxon or Spearman's correlation coefficient, may be used to assess associations between subgroups of patients.

AEs related to xenodiagnosis will be tabulated and summaries with numbers and proportions will be given.

7.2 Anticipated Difficulties

One anticipated difficulty is the recruitment of participants and the details of the xenodiagnostic protocol. In the Phase I pilot study, we did not have major issues with patient acceptance in either the *B. burgdorferi* infected or healthy control participants. One major barrier to recruitment in the first phase of the study was study physician availability. In the pilot phase, we attempted to recruit patients with EM at the time of diagnosis for immediate tick placement. We found that it was difficult to enroll these patients as the timing of when these participants would present is unpredictable and patients are understandably more concerned with their new diagnosis than traveling to see a study physician. We will now focus on recruiting patients in the interim between diagnosis and the completion of therapy, which will allow predictable scheduling of the participants. Also, in the past, we included private practice physicians who were to enroll participants. In the absence of a study infrastructure at the private practice sites, we found that enrollment from these sites was minimal. We are now changing our procedure so that the study coordinator will review the chief complaints of patients scheduled in clinic and be responsible for alerting the physician and then making informational calls to the patient. Patients who agree to enroll will be scheduled in groups and the study coordinator will travel to the physician's office to assist the physician with entry and enrollment.

An anticipated problem is the possibility of re-infection with *B. burgdorferi* after antibiotic therapy. Because these participants live in endemic areas with repeated exposures to ticks, it is possible that they may become re-infected through a new tick bite after the antibiotic treatment. Re-infection is a relatively uncommon event, especially in the first year after the original infection. Our questionnaire will specifically ask participants about occurrence of EM lesions or other signs of Lyme disease in the interval between antibiotic therapy and evaluation for enrollment in the study. It is also worth noting that, even in the unlikely event a reinfection occurs for one of our study participants, a positive xenodiagnostic test in this group would be an important finding as these would be missed by standard serological testing and these patients would go untreated.

7.3 Interpretation and Impact

At the completion of this trial, we will have a better idea of what the rate of positivity for *B. burgdorferi* by xenodiagnosis is in patients with Lyme disease after treatment. We will have assessed whether patients with persistent symptoms of Lyme disease are more or less likely to have persistently detectable *B. burgdorferi* by xenodiagnosis. Understanding the linkage between persistent infection and persistent symptoms is critically important as it would affect the way that persistent disease is thought to develop mechanistically and would open the door to many new studies of both mechanism and for attempts to eradicate residual infection.

8 ASSESSMENT OF SAFETY

8.1 Documenting, Recording, and Reporting Adverse Events

This trial is being conducted in compliance with 21 CFR 812 [FDA/IDE] regulations. Safety Reporting for this study includes additional events definitions and requirements set forth by the Sponsor and the IRB.

At each contact with the subject, information regarding adverse events will be elicited by appropriate questioning and examinations and will be:

- immediately documented in the subject's medical record/source document,
- recorded on the Adverse Event Case Report Form (AE CRF), and reported as outlined below (e.g., Sponsor, IRB, FDA).

8.2 Definitions

The NIAID Clinical Safety Office (CSO) is responsible for sponsor safety oversight of this study, and the definitions below comply with CSO requirements.

ADVERSE EVENT (AE) - An adverse event is any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (e.g., abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to the research.

ADVERSE DEVICE EFFECT (ADE): A subset of AEs, as defined above, that are specifically related to the use of an investigational medical device.

SERIOUS ADVERSE EVENT - an AE resulting in any of the following outcomes:

- death
- is life threatening (i.e., an immediate threat to life)
- inpatient hospitalization or prolongation of an existing hospitalization
- a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- congenital anomaly /birth defect
- is Medically Important*

* Medical and scientific judgment should be exercised when deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be

immediately life threatening or result in death or result in hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed above. These should also usually be considered serious.

UNEXPECTED ADVERSE EVENT - any AE (including SAEs and ADEs, as defined in this protocol), that occurs at a level of specificity or severity that is not consistent with the protocol. Expected means that the event has previously been observed with the study procedure and is identified and/or described in the protocol.

Unanticipated Problem (UP)
An Unanticipated Problem is any event, incident, experience, or outcome that:

1. is unexpected in terms of nature, severity, or frequency in relation to:
   a. the research risks that are described in the IRB-approved research protocol and informed consent document; Investigator's Brochure or other study documents; and
   b. the characteristics of the subject population being studied; and
2. possibly, probably, or definitely related to participation in the research; and
3. places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized. (Per the IDE Sponsor, an AE with a serious outcome will be considered increased risk.)

Serious Unanticipated Problem (UP): A UP that meets the definition of a Serious Adverse Event or compromises the safety, welfare or rights of subjects or others.

Unanticipated problem that is not an Adverse Event (UPnonAE): An unanticipated problem that does not fit the definition of an adverse event, but which may, in the opinion of the investigator, involve risk to the subject, affect others in the research study, or significantly impact the integrity of research data. These events may involve a greater risk of social or economic harm to subjects or others rather than physical/psychological harm. Such events would be considered a non-serious UP. Examples of a UPnonAE include a breach of confidentiality, accidental destruction of study records, or unaccounted-for study drug.

UNANTICIPATED ADVERSE DEVICE EFFECT (UADE) – "Any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with, a device, if that effect, problem, or death was not previously identified in nature, severity, or degree of incidence in the investigational plan or application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects." (21 CFR 812.3) In summary, UADEs are serious, unanticipated, and related (possibly, probably or definitely) to the device.

Anticipated AEs related to the study device (xenodiagnosis) include the following:

- allergic dermatitis from surgical tape,
- mild or moderate pruritic local reactions and mild or moderate painful local reactions to the tick bite that do not require medical intervention.

All AEs and ADEs that are identified from the time the xenodiagnostic ticks are placed through

30 days after the last use of the device (ticks) will be documented in the subject's medical record and will be recorded in the study database.

NOTE: AEs and ADEs of any severity could be considered serious (e.g., medically important) based on the medical judgment of the investigator. For example, cellulitis of any severity grade should be considered serious.

Protocol Deviation: Any change, divergence, or departure from the IRB-approved research protocol.

1. Major Deviation – Deviation from the IRB-approved protocol that have, or may have the potential to, negatively impact, the rights, welfare or safety of the subject, or to substantially negatively impact the scientific integrity or validity of the study.
2. Minor Deviation – A Deviation that does not have the potential to negatively impact the rights, safety, or welfare of subjects or others, or the scientific integrity or validity of the study.

Non-compliance: Failure of an investigator to follow the applicable laws, regulations, or institutional policies governing the protection of human subjects in research, or the requirements or determinations of the IRB, whether the failure is intentional or not.

- Continuing Non-compliance – A pattern of recurring non-compliance that either has resulted, or, if continued, may result in harm to subjects or otherwise materially compromise the rights, welfare and/or safety of subjects, affect the scientific integrity of the study or validity of the results. The pattern may comprise repetition of the same non-compliant action(s), or different non-compliant events. Such non-compliance may be unintentional (e.g. due to lack of understanding, knowledge, or commitment), or intentional (e.g. due to deliberate choice to ignore or compromise the requirements of any applicable regulation, organizational policy, or determination of the IRB).
- Serious Non-compliance – Non-compliance, whether intentional or not, that results in harm or otherwise materially compromises the rights, welfare and/or safety of the subject. Non-compliance that materially affects the scientific integrity or validity of the research may be considered serious non-compliance, even if it does not result in direct harm to research subjects.

8.3 Investigator Evaluation Adverse Events and Adverse Device Effects

The Investigator will evaluate all potential AEs and ADEs with respect to:

Seriousness (criteria listed in Section 8.2. above),
Anticipated vs. Unanticipated (for devices as defined in Section 8.2 above),
Expected vs. Unexpected (for study procedures as defined in Section 8.2 above),
Severity (intensity or grade – Section 8.3.1 below),
Causality (relationship to the study device or to a study procedure) according to the guidelines in Section 8.3.2

8.3.1 Severity

Grading of AEs will conform to the toxicity scale in Appendix 3. For items not described in Appendix 3, the Division of AIDS Table for Grading Adult and Pediatric Adverse Experiences

Version 1.0 - December 2004 (Clarification dated August 2009) will be used (https://rsc.tech-res.com/docs/default-source/safety/table_for_grading_severity_of_adult_pediatric_adverse_events.pdf?sfvrsn=6).

All clinical AEs that are not listed in the toxicity scale will be assessed for severity and classified into one the categories below:

- **Grade 1 (Mild)**: Symptoms causing no or minimal interference with usual social & functional activities or represent mild increase over baseline
- **Grade 2 (Moderate)**: Symptoms causing greater than minimal interference with usual social & functional activities or represent moderate increase over baseline
- **Grade 3 (Severe)**: Symptoms causing inability to perform usual social & functional activities or represent severe increase over baseline.
- **Grade 4 (Life threatening)**: Symptoms causing inability to perform basic self-care functions OR Medical or operative intervention indicated to prevent permanent impairment, persistent disability, or death
- **Grade 5 (Death)**

8.3.2 Causality

All AEs will be assessed by the investigator for relationship to study procedures and the study device. A causal relationship (possible, probable, or definite) means that a research procedure or the study device caused or is reasonably likely to have caused the AE.

The best estimate of the causal relationship between the study device and an AE will be made by the investigator using the following causality categories:

Definitely Related
- reasonable temporal relationship
- follows a known response pattern
- clear evidence to suggest a causal relationship
- there is no alternative etiology

Probably Related
- reasonable temporal relationship
- follows a suspected response pattern for the procedure or the device
- no evidence of a more likely alternative etiology

Possibly Related
- reasonable temporal relationship
- little evidence for a more likely alternative etiology

Unlikely Related
- does not have a reasonable temporal relationship OR
- good evidence for a more likely alternative etiology

Not Related

- does not have a temporal relationship OR
- definitely due to an alternative etiology

8.4 Reporting Serious Adverse Events, Unanticipated Adverse Device Effects, and Unanticipated Problems to the IDE Sponsor

8.4.1 Adverse Events

Line listings, frequency tables, and other summary AE data will be submitted to the Sponsor when needed for periodic safety assessments, review of IDE annual reports, review of IDE safety reports, and preparation of final study reports.

8.4.2 Serious Adverse Events

All SAEs (regardless of relationship and whether or not they are also UPs) must be reported on the Safety Expedited Report Form (SERF) and sent to the Sponsor CSO by fax or e-mail attachment. Deaths and immediately life threatening SAEs must be reported to the CSO within 1 business day after the site becomes aware of the event. All other SAEs must be reported within 3 business days of site awareness.

SPONSOR CLINICAL SAFETY OFFICE CONTACT INFORMATION:

Clinical Safety Office
5705 Industry Lane
Frederick, MD 21704
Phone 301-846-5301
Fax 301-846-6224
E-mail: rchspsafety@mail.nih.gov

8.4.3 Unanticipated Problems

Unanticipated problems that are also AEs must be reported to the CSO and sent by fax or e-mail attachment no later than 7 calendar days of site awareness of the event. UPnonAEs are not reported to the Sponsor CSO.

Report all UPs that are also AEs to the CSO on the NIH Problem Report Form.

8.4.4 Unanticipated Adverse Device Effects (UADEs)

All ADEs that are identified from the time the xenodiagnostic ticks are placed through 30 days after the last use of the device will be documented in the subject's medical record and will be recorded in the study database.

UADEs (unanticipated, serious, and possibly, probably, or definitely related to the device) must be recorded on a SERF, clearly identified as a UADE, and sent to the CSO (addressed in Section 8.3.1) within 10 working days.

21CFR812.46 requires the Sponsor to conduct an investigation of all UADE reports. The results of the investigation will be reported to the FDA, all reviewing IRBs, and all participating investigators within 10 working days after the Sponsor becomes aware of the UADE.

If the Sponsor determines that a UADE presents an unreasonable risk to participants, the Sponsor shall terminate all investigations, or parts of investigations, presenting that risk as soon as possible but no later than 5 working days after the Sponsor makes this determination and no later than 15 working days after the Sponsor first received notice of the effect. The investigation may not resume without subsequent IRB and FDA approval (21CFR812.150)

8.5 Follow-up of Adverse Events and Adverse Device Effects

AEs temporally associated with study procedures and ADEs that occur from the time the informed consent is signed are followed until final outcome is known. AEs temporally associated with a study procedure that have not resolved by the end of the study follow-up period are recorded on the AE CRF as ONGOING.

ADEs that have not resolved by the end of the study follow-up period are followed until final outcome is known. If it is not possible to obtain a final outcome for an ADE (e.g., the subject is lost to follow-up), the reason a final outcome could not be obtained will be documented by the investigator.

8.6 Pausing Rules for the Protocol

The PI will closely monitor and analyze study data as it becomes available and will make determinations regarding the occurrence and grading of AEs. AEs will be evaluated with regard to the known complications associated with the study procedure (xenodiagnosis) and the presence of concurrent medications or concurrent illnesses. Upon determination of any Grade 4 AE or any 2 Grade 3 AEs (in different participants) thought to be definitely, probably, or possibly related to the study procedure, the study will be halted (no new enrollments and no further use of the study procedure by the investigators) and a UADE report will be submitted to the local IRB and to the OCRPRO Safety Office. Approval by each IRB and the FDA is required before the study can resume.

The IRBs, the NIAID, the FDA, or other government agencies, as part of their duties to ensure that research participants are protected may discontinue the study at any time. Subsequent review of UADEs by the IRB, the Sponsor, the FDA, and other regulatory authorities may also result in suspension of further trial interventions/administration of the study procedure at a site. The FDA, other regulatory authorities, and the Sponsor retain the authority to suspend additional enrollment and Study Agent/Intervention administration for the entire study as applicable.

9 DATA AND SAFETY MONITORING PLAN

The PI will monitor protocol data (through regular communication with the study teams at the other sites) that could affect subject safety or confidentiality by communicating at pre-arranged intervals with the Site PIs. Information to be shared includes AEs, documentation of subject evaluation, treatment and disposition, and coding of subject samples and data. Serious and other AEs will be reported to the site’s IRB and the CSO as outlined above.

9.1.1 Safety Review and Communications Plan

A Safety Review and Communications Plan (SRCP) has been developed for the protocol. The SRCP is an internal communications document between the PI and the CSO, which delineates the safety oversight responsibilities of the PI, the CSO, and other stakeholders. The SRCP also includes the overall plan for conducting periodic safety surveillance assessments.

9.1.2 Sponsor Medical Monitor

A SMM, representing the Sponsor (OCRPRO), has been appointed for oversight of safety in this clinical study. The SMM will be responsible for performing safety assessments as outlined in an SRCP.

A monthly (or other specified frequency) conference call with the SMM, the PI, and members of the study teams from all sites will review all AE safety data (Grade 1 or higher) as specified in the SRCP. A safety report will be prepared for distribution to the safety team, and will include data elements specified in the SRCP.

10 REPORTING PROCEDURES

10.1 Investigator Reporting Responsibilities to the IRB

Each site will report AEs to their local IRB according to their IRB's policy.

10.1.1 Reporting Procedures at the NIH

Reportable events will be tracked and submitted to the NIH IRB as outlined in Policy 801.

10.1.2 Investigator Reporting Responsibilities to Local IRB(s)

Investigators are responsible for submitting UADE Reports and UP summaries that are received from the Sponsor to their local IRB/Ethics Committee. Investigators must also comply with all local IRB/Ethics Committee reporting requirements.

10.2 Reporting to the NIAID Clinical Director

At the NIH site, the PI will report UPs, major protocol deviations, and deaths to the NIAID clinical director according to institutional timelines.

11 FINANCIAL COMPENSATION

Participants will be paid the established NIH daily rate for participation in particular aspects of the protocol. As of 2015, outpatient visits rates are $20 for the first hour and $10 for each additional hour. Separate additional payments will be provided for the following:

Table 4: Financial Compensation

TEST	PAYMENT
Skin Biopsies	$25 for each skin biopsy
Xenodiagnosis	$100

At the NIH site, payment will be provided through the NIH Clinical Center Payment Office as a check, direct deposit, or automated clearing house payment after the completion of each study visit. Payment or reimbursement for travel-related expenses (e.g., transportation, hotel, meals) will be provided according to the NIAID Travel Policy.

12 PLAN FOR MAINTAINING THE PRIVACY AND CONFIDENTIALITY OF SUBJECT RECORDS

Subject records will be kept at Medical Records Center for each site. Research records will be kept in a locked file cabinet, and only the site PI or associate investigators will have access to the research records, unless required by law. The investigator (and/or designee) will make study documents (including source medical records) readily available for inspection by the local IRB, the FDA, the site monitors, and the Sponsor for confirmation of the study data.

13 INTENDED USE OF THE SAMPLES/SPECIMENS/DATA

Blood, urine, and skin samples collected under this study may be used to test for known and yet unknown pathogens, immunological and inflammatory markers, and host reactivity to tick salivary protein. Patients may choose not to have their specimens stored or used for research on unrelated diseases and conditions.

Samples and data collected under this protocol will be used to study Lyme disease and related disorders. No human genetic testing will be done on these samples.

How samples/specimen/data will be stored
Access to research samples will be limited using either a locked room or a locked freezer. Samples and data will be stored using codes assigned by the investigators or their designee(s). Data will be kept in password-protected computers. The key linking the study code to identifiers will be maintained at each site and will only be accessible to investigators or designees at that site.

How samples/specimen/data will be tracked
Samples will be tracked using Excel or BSI databases. Samples may also be stored and tracked utilizing the Frederick National Laboratory for Cancer Research (FNLCR) repository operated by Leidos Biomedical Research, Inc.

What will happen to the samples/specimen/data at the completion of the protocol?
In the future, other investigators (both at NIH and outside) may wish to use these samples and/or data for research purposes. If the planned research falls within the category of "human subjects research" on the part of the NIH researchers, NIH IRB review and approval will be obtained.

This includes the NIH researchers sending out coded and linked samples or data and getting results that they can link back to their subjects..

What circumstances would prompt the PI to report to the IRB loss or destruction of samples/specimen/data?
Any loss or unanticipated destruction of samples or data (for example, due to freezer malfunction) that meets the definition of a reportable event will be reported to the NIH IRB according to NIH HRPP Policy 801.

Any loss or unanticipated destruction of more than 30% of samples or data that compromises the scientific integrity of the study will be reported to the IRB.

14 DATA MANAGEMENT PLAN

Study data will be collected at the study site(s) and maintained on standardized electronic or paper case report forms (CRFs), or an electronic data system. Research data may also be kept using Excel (with appropriate controls to protect data integrity and subject confidentiality) as well as multiple programs for statistical analysis.

These forms or systems are to be completed on an ongoing basis during the study. The CRFs and instructions will be distributed to the site(s) by the Sponsor or printed by the sites themselves after Sponsor approval. Data entries on paper CRFs must be completed legibly with black ballpoint pen. Corrections must be made by striking through the incorrect entry with a single line (taking care not to obliterate or render the original entry illegible) and entering the correct information adjacent to the incorrect entry. Corrections to paper CRFs must be initialed and dated by the person making the correction. Data entered into electronic CRFs or data systems shall be performed by authorized individuals. Corrections to electronic CRFs or data systems shall be tracked electronically (password protected) with time, date, individual making the correction, and what was changed.

The Investigator is responsible for assuring that the data collected is complete, accurate, and recorded in a timely manner. Source documentation (the point of initial recording of information) should support the data collected on the CRF, and must be signed and dated by the person recording and/or reviewing the data. All CRFs should be reviewed by the Investigator and signed as required with written or electronic signature, as appropriate. Source documents include all recordings of observations or notations of clinical activities, and all reports and records necessary for the evaluation and reconstruction of the clinical trial. Source documents include, but are not limited to, the subject's medical records, laboratory reports, ECG tracings, x-rays, radiologist's reports, subject's diaries, biopsy reports, ultrasound photographs, progress notes, pharmacy records, and any other similar reports or records of procedures performed during the subject's participation in the study. Data from CRFs, CRIMSON Data System, or other electronic database will be collected directly from participants during study visits and telephone calls, or will be abstracted from participants' diaries and medical records. The subject's medical record must record his/her participation in the clinical trial and, what medications (with doses and frequency) or other medical interventions or treatments were administered, as well as any adverse reactions experienced during the trial.

Study results: Each subject will receive a letter at the completion of the study to discuss the findings of the study. The letter will be prepared and submitted to the IRB for approval before distribution.

15 SUBJECT RECRUITMENT

Lyme disease affects persons in all age groups, with peaks occurring for children aged 5-9 years and adults 55-59 years. Males and females are nearly equally affected in all age groups. The racial breakdown of persons with Lyme disease, according to the national surveillance data from 1992 to 2006 [54], shows a preponderance of white (94.1%). This reflects the demographic profile of the population at risk, both by geographic location and behavioral risk factors. Current scientific evidence does not indicate that the natural history of Lyme disease differs based on gender or racial minority status. All efforts will be made to recruit patients whose gender or minority represents the general population. Only adult participants will be recruited for this study. Patients may self-refer or be referred by a physician to the protocol. At this point, no advertisement is planned, but may be used in the future. If that is the case, it will be submitted to each site's IRB for approval.

Participants will be recruited at 5 study sites, 3 of which (NIH, Tufts and Mansfield) participated in the Phase 1 xenodiagnostic study. The current study sites include the NIH Clinical Center, Tufts Medical Center, Mansfield Family Practice, Stony Brook University (State University of New York), and NYMC.

The NIAID Lyme disease program at the NIH Clinical Center has evaluated more than 400 patients. For this study, we have established collaboration with Dr. John Aucott at Johns Hopkins University, who has a large referral network for a longitudinal study of patients with EM that has already enrolled over 100 participants. Dr. Aucott will be referring patients to the NIH site.

Tufts Medical Center is a tertiary referral center for Lyme disease seeing over 100 cases per year. The majority of these cases are patients experiencing persistent symptoms including arthritis. Tufts will also oversee recruitment of participants by Dr. Kenneth Dardick, who directs a large Family Medicine Practice in Mansfield, Connecticut. Dr. Dardick has been involved in Lyme disease research for more than 20 years. His clinic cares for about 100 patients with acute Lyme disease and 25 patients with persistent symptoms per year.

Dr. Wormser has enrolled over 1200 patients with Lyme disease in various studies on pathogenesis, diagnosis, clinical manifestations and treatment. Lyme disease patients were mostly recruited from the walk-in Lyme Disease Diagnostic Center at New York Medical College, now in its 25th year of existence.

Dr. Marcos is an infectious diseases physician at Stony Brook University Department of Medicine. He is the current director for the outpatient clinic for tick borne diseases overseeing more than 400 patients with any tick borne disease in Suffolk County.

16 CONSENT PROCEDURES

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continuing throughout the individual's study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the participants and their families. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the subject and written documentation of informed consent is required prior to starting intervention/administering study product. Consent forms will be IRB-approved and the subject will be given the opportunity to read and review the document. Upon reviewing the document, the investigator or designee will explain the research study to the subject and answer any questions that may arise. The participants will sign the informed consent document prior to any procedures being done specifically for the study. The participants should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The participants may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the participants for their records.

Consent for NIH staff will be obtained as detailed above with following additional protections:

Consent from staff members will be obtained by an individual independent of the staff member's team whenever possible. Otherwise, the consent procedure will be independently monitored by the CC Department of Bioethics Consultation Service in order to minimize the risk of undue pressure on the staff member.

17 STUDY RECORDS RETENTION

The investigator is responsible for retaining all essential documents listed in the ICH GCP Guideline. All essential documentation for all study participants is to be maintained by the investigators in a secure storage facility for a minimum of 3 years. The FDA requires study records to be retained for up to 2 years after marketing approval or disapproval (21 CFR 812.140), or until at least 2 years have elapsed since the formal discontinuation (terminated or completed) of clinical development of the investigational agent for a specific indication. These records are also to be maintained in compliance with IRB/EC, state, and federal medical records retention requirements, whichever is longest. All stored records are to be kept confidential to the extent provided by federal, state, and local law. It is the investigator's responsibility to retain copies of source documents until receipt of written notification to the contrary from the OCRPRO of the NIAID. No study document should be destroyed without prior written agreement between OCRPRO/NIAID and the PIs. Should the investigator wish to assign the study records to another party and/or move them to another location, the investigator must provide written notification of such intent to OCRPRO/NIAID with the name of the person who will accept responsibility for the transferred records and/or their new location. NIAID must be notified in writing and written NIAID permission must be received by the site prior to destruction or relocation of research records.

18 PROTOCOL MONITORING

As per ICH-GCP 5.18 and FDA 21 CFR 812.43(d) clinical protocols are required to be adequately monitored by the study Sponsor. This study monitoring will be conducted according

to the "NIAID Intramural Clinical Monitoring Guidelines." Monitors under contract to the NIAID/OCRPRO will visit the clinical research sites to monitor all aspects of the study in accordance with the appropriate regulations and the approved protocol. The objectives of a monitoring visit will be: 1) to verify the existence of signed informed consent documents for each monitored subject; 2) to verify the prompt and accurate recording of all monitored data points and prompt reporting of all SAEs; 3) to compare abstracted information with individual participants' records and source documents (participants' charts, laboratory analyses and test results, physicians' progress notes, nurses' notes, and any other relevant original subject information); and 4) to ensure protection of study participants, investigators' compliance with the protocol, and completeness and accuracy of study records. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements (Office for Human Research Protections [OHRP] as applicable) and applicable guidelines (ICH-GCP and FDA 21 CFR 812) are being followed. During the monitoring visits, the investigator (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit.

The investigator (and/or designee) will make study documents (e.g., consent forms, and pertinent hospital or clinical records) readily available for inspection by the local IRB, the site monitors, and the NIAID staff for confirmation of the study data.

A specific protocol monitoring plan will be discussed with the PI and study staff prior to enrollment. The plan will outline the frequency of monitoring visits based on such factors as study enrollment, data collection status and regulatory obligations.

19 CERTIFICATE OF CONFIDENTIALITY

To further protect the privacy of study participants, a Certificate of Confidentiality has been issued by the NIH. This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

REFERENCES

1 Bacon, R. M., Kugeler, K. J., Mead, P. S., Centers for Disease, C. & Prevention. Surveillance for Lyme disease--United States, 1992-2006. *MMWR Surveill Summ* **57**, 1-9 (2008).

2 Smith, R. P. *et al.* Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. *Ann Intern Med* **136**, 421-428 (2002).

3 Nowakowski, J. *et al.* Long-term follow-up of patients with culture-confirmed Lyme disease. *Am J Med* **115**, 91-96 (2003).

4 Wormser, G. P. *et al.* Duration of antibiotic therapy for early Lyme disease. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **138**, 697-704 (2003).

5 Wormser, G. P. *et al.* The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis* **43**, 1089-1134, doi:10.1086/508667 (2006).

6 Dattwyler, R. J., Halperin, J. J., Volkman, D. J. & Luft, B. J. Treatment of late Lyme borreliosis--randomised comparison of ceftriaxone and penicillin. *Lancet* **1**, 1191-1194 (1988).

7 Pfister, H. W. *et al.* Randomized comparison of ceftriaxone and cefotaxime in Lyme neuroborreliosis. *J Infect Dis* **163**, 311-318 (1991).

8 Steere, A. C. *et al.* Treatment of Lyme arthritis. *Arthritis Rheum* **37**, 878-888 (1994).

9 Kalish, R. A. *et al.* Evaluation of study patients with Lyme disease, 10-20-year follow-up. *Journal of Infectious Diseases* **183**, 453-460 (2001).

10 Kindstrand, E., Nilsson, B. Y., Hovmark, A., Pirskanen, R. & Asbrink, E. Peripheral neuropathy in acrodermatitis chronica atrophicans - effect of treatment. *Acta Neurol Scand* **106**, 253-257 (2002).

11 Berglund, J., Stjernberg, L., Ornstein, K., Tykesson-Joelsson, K. & Walter, H. 5-y Follow-up study of patients with neuroborreliosis. *Scand J Infect Dis* **34**, 421-425, doi:10.1080/00365540110080421 (2002).

12 Dattwyler, R. J. *et al.* A comparison of two treatment regimens of ceftriaxone in late Lyme disease. *Wien Klin Wochenschr* **117**, 393-397 (2005).

13 Borg, R. *et al.* Intravenous ceftriaxone compared with oral doxycycline for the treatment of Lyme neuroborreliosis. *Scand J Infect Dis* **37**, 449-454, doi:10.1080/00365540510027228 (2005).

14 Oksi, J. *et al.* Duration of antibiotic treatment in disseminated Lyme borreliosis: a double-blind, randomized, placebo-controlled, multicenter clinical study. *Eur J Clin Microbiol Infect Dis* **26**, 571-581, doi:10.1007/s10096-007-0340-2 (2007).

15 Weber, K. *et al.* Antibiotic therapy of early European Lyme borreliosis and acrodermatitis chronica atrophicans. *Ann N Y Acad Sci* **539**, 324-345 (1988).

16 Strle, F. *et al.* Azithromycin versus doxycycline for treatment of erythema migrans: clinical and microbiological findings. *Infection* **21**, 83-88 (1993).

17 Dattwyler, R. J. *et al.* Ceftriaxone compared with doxycycline for the treatment of acute disseminated Lyme disease. *N Engl J Med* **337**, 289-294 (1997).

18 Picha, D., Moravcova, L., Lasikova, S., Holeckova, D. & Maresova, V. Symptoms of post-Lyme syndrome in long-term outcome of patients with neuroborreliosis. *Scand J Infect Dis* **38**, 747-748 (2006).
19 Aucott, J. N., Rebman, A. W., Crowder, L. A. & Kortte, K. B. Post-treatment Lyme disease syndrome symptomatology and the impact on life functioning: is there something here? *Qual Life Res* **22**, 75-84, doi:10.1007/s11136-012-0126-6 (2013).
20 Steere, A. C. *et al.* Treatment of the early manifestations of Lyme disease. *Ann Intern Med* **99**, 22-26 (1983).
21 Dattwyler, R. J., Volkman, D. J., Conaty, S. M., Platkin, S. P. & Luft, B. J. Amoxycillin plus probenecid versus doxycycline for treatment of erythema migrans borreliosis. *Lancet* **336**, 1404-1406 (1990).
22 Weber, K. *et al.* A randomized trial of ceftriaxone versus oral penicillin for the treatment of early European Lyme borreliosis. *Infection* **18**, 91-96 (1990).
23 Shadick, N. A. *et al.* The long-term clinical outcomes of Lyme disease. A population-based retrospective cohort study. *Ann Intern Med* **121**, 560-567 (1994).
24 Asch, E. S., Bujak, D. I., Weiss, M., Peterson, M. G. & Weinstein, A. Lyme disease: an infectious and postinfectious syndrome. *J Rheumatol* **21**, 454-461 (1994).
25 Hunfeld, K. P., Ruzic-Sabljic, E., Norris, D. E., Kraiczy, P. & Strle, F. In vitro susceptibility testing of Borrelia burgdorferi sensu lato isolates cultured from patients with erythema migrans before and after antimicrobial chemotherapy. *Antimicrob Agents Chemother* **49**, 1294-1301 (2005).
26 Klempner, M. S. *et al.* Two controlled trials of antibiotic treatment in patients with persistent symptoms and a history of Lyme disease. *N Engl J Med* **345**, 85-92, doi:10.1056/NEJM200107123450202 (2001).
27 Fallon, B. A. *et al.* A randomized, placebo-controlled trial of repeated IV antibiotic therapy for Lyme encephalopathy. *Neurology* (2007).
28 Aguero-Rosenfeld, M. E., Wang, G., Schwartz, I. & Wormser, G. P. Diagnosis of lyme borreliosis. *Clin Microbiol Rev* **18**, 484-509, doi:10.1128/CMR.18.3.484-509.2005 (2005).
29 Wilske, B., Fingerle, V. & Schulte-Spechtel, U. Microbiological and serological diagnosis of Lyme borreliosis. *FEMS Immunol Med Microbiol* **49**, 13-21 (2007).
30 Fleming, R. V. *et al.* Pre-treatment and post-treatment assessment of the C(6) test in patients with persistent symptoms and a history of Lyme borreliosis. *Eur J Clin Microbiol Infect Dis* (2004).
31 Marques, A. R., Hornung, R. L., Dally, L. & Philipp, M. T. Detection of immune complexes is not independent of detection of antibodies in Lyme disease patients and does not confirm active infection with Borrelia burgdorferi. *Clin Diagn Lab Immunol* **12**, 1036-1040, doi:10.1128/CDLI.12.9.1036-1040.2005 (2005).
32 Krupp, L. B. *et al.* Study and treatment of post Lyme disease (STOP-LD): a randomized double masked clinical trial. *Neurology* **60**, 1923-1930 (2003).
33 IDSA. *From the President: IDSA Stands Up For Lyme Disease Guidelines.*, < http://www.idsociety.org/newsArticle.aspx?id=4262> (2007).
34 Bockenstedt, L. K., Mao, J., Hodzic, E., Barthold, S. W. & Fish, D. Detection of attenuated, noninfectious spirochetes in Borrelia burgdorferi-infected mice after antibiotic treatment. *J Infect Dis* **186**, 1430-1437, doi:10.1086/345284 (2002).

35 Straubinger, R. K., Summers, B. A., Chang, Y. F. & Appel, M. J. Persistence of Borrelia burgdorferi in experimentally infected dogs after antibiotic treatment. *J Clin Microbiol* **35**, 111-116 (1997).
36 Straubinger, R. K., Straubinger, A. F., Summers, B. A. & Jacobson, R. H. Status of Borrelia burgdorferi infection after antibiotic treatment and the effects of corticosteroids: An experimental study. *J Infect Dis* **181**, 1069-1081, doi:10.1086/315340 (2000).
37 Yrjanainen, H. *et al.* Anti-tumor necrosis factor-alpha treatment activates Borrelia burgdorferi spirochetes 4 weeks after ceftriaxone treatment in C3H/He mice. *J Infect Dis* **195**, 1489-1496, doi:10.1086/513873 (2007).
38 Hodzic, E., Feng, S., Holden, K., Freet, K. J. & Barthold, S. W. Persistence of Borrelia burgdorferi following antibiotic treatment in mice. *Antimicrob Agents Chemother* **52**, 1728-1736, doi:10.1128/AAC.01050-07 (2008).
39 Embers, M. E. *et al.* Persistence of Borrelia burgdorferi in rhesus macaques following antibiotic treatment of disseminated infection. *PLoS One* **7**, e29914, doi:10.1371/journal.pone.0029914 (2012).
40 Barthold, S. W. *et al.* Ineffectiveness of tigecycline against persistent Borrelia burgdorferi. *Antimicrob Agents Chemother* **54**, 643-651, doi:10.1128/AAC.00788-09 (2010).
41 Liveris, D. *et al.* Comparison of five diagnostic modalities for direct detection of Borrelia burgdorferi in patients with early Lyme disease. *Diagn Microbiol Infect Dis* **73**, 243-245, doi:10.1016/j.diagmicrobio.2012.03.026 (2012).
42 Liveris, D. *et al.* Quantitative detection of Borrelia burgdorferi in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. *J Clin Microbiol* **40**, 1249-1253 (2002).
43 Nowakowski, J. *et al.* Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. *Clin Infect Dis* **33**, 2023-2027, doi:10.1086/324490 (2001).
44 Schwartz, I. *et al.* Diagnosis of early Lyme disease by polymerase chain reaction amplification and culture of skin biopsies from erythema migrans lesions. *J Clin Microbiol* **30**, 3082-3088 (1992).
45 Iyer, R. *et al.* Detection of Borrelia burgdorferi nucleic acids after antibiotic treatment does not confirm viability. *J Clin Microbiol* **51**, 857-862, doi:10.1128/JCM.02785-12 (2013).
46 Hodzic, E., Imai, D., Feng, S. & Barthold, S. W. Resurgence of persisting non-cultivable Borrelia burgdorferi following antibiotic treatment in mice. *PLoS One* **9**, e86907, doi:10.1371/journal.pone.0086907 (2014).
47 Bockenstedt, L. K., Gonzalez, D. G., Haberman, A. M. & Belperron, A. A. Spirochete antigens persist near cartilage after murine Lyme borreliosis therapy. *J Clin Invest* **122**, 2652-2660, doi:10.1172/JCI58813 (2012).
48 Shih, C. M., Chao, L. L. & Yu, C. P. Chemotactic migration of the Lyme disease spirochete (Borrelia burgdorferi) to salivary gland extracts of vector ticks. *Am J Trop Med Hyg* **66**, 616-621 (2002).
49 Marques, A. *et al.* Xenodiagnosis to detect Borrelia burgdorferi infection: a first-in-human study. *Clin Infect Dis* **58**, 937-945, doi:10.1093/cid/cit939 (2014).
50 Hu, L. Lyme arthritis. *Infect Dis Clin North Am* **19**, 947-961, doi:10.1016/j.idc.2005.07.007 (2005).

51 Steere, A. C. *et al.* Successful parenteral penicillin therapy of established Lyme arthritis. *N Engl J Med* **312**, 869-874 (1985).
52 Nocton, J. J. *et al.* Detection of Borrelia burgdorferi DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *N Engl J Med* **330**, 229-234, doi:10.1056/NEJM199401273300401 (1994).
53 Steere, A. C., Schoen, R. T. & Taylor, E. The clinical evolution of Lyme arthritis. *Ann Intern Med* **107**, 725-731 (1987).
54 El Khoury, M. Y. *et al.* Diagnosis of acute deer tick virus encephalitis. *Clin Infect Dis* **56**, e40-47, doi:10.1093/cid/cis938 (2013).
55 Hinten, S. R. *et al.* Increased recognition of Powassan encephalitis in the United States, 1999-2005. *Vector Borne Zoonotic Dis* **8**, 733-740 (2008).
56 Krause, P. J. *et al.* Human Borrelia miyamotoi infection in the United States. *N Engl J Med* **368**, 291-293, doi:10.1056/NEJMc1215469 (2013).
57 Gugliotta, J. L., Goethert, H. K., Berardi, V. P. & Telford, S. R., 3rd. Meningoencephalitis from Borrelia miyamotoi in an immunocompromised patient. *N Engl J Med* **368**, 240-245, doi:10.1056/NEJMoa1209039 (2013).
58 Telford, S. R., 3rd & Wormser, G. P. Bartonella spp. transmission by ticks not established. *Emerg Infect Dis* **16**, 379-384, doi:10.3201/eid1603.090443 (2010).
59 Reis, C. *et al.* Vector competence of the tick Ixodes ricinus for transmission of Bartonella birtlesii. *PLoS Negl Trop Dis* **5**, e1186, doi:10.1371/journal.pntd.0001186 (2011).
60 Gillespie, J. J. *et al.* A Rickettsia genome overrun by mobile genetic elements provides insight into the acquisition of genes characteristic of an obligate intracellular lifestyle. *J Bacteriol* **194**, 376-394, doi:10.1128/JB.06244-11 (2012).
61 Rounds, M. A. *et al.* Identification of endosymbionts in ticks by broad-range polymerase chain reaction and electrospray ionization mass spectrometry. *J Med Entomol* **49**, 843-850 (2012).
62 Tokarz, R. *et al.* Virome analysis of Amblyomma americanum, Dermacentor variabilis, and Ixodes scapularis ticks reveals novel highly divergent vertebrate and invertebrate viruses. *J Virol* **88**, 11480-11492, doi:10.1128/JVI.01858-14 (2014).
63 Kurtti, T. J. *et al.* Rickettsia buchneri sp. nov.: A rickettsial endosymbiont of the blacklegged tick Ixodes scapularis. *Int J Syst Evol Microbiol*, doi:10.1099/ijs.0.000047 (2015).
64 Nakayama, Y. & Spielman, A. Ingestion of Lyme disease spirochetes by ticks feeding on infected hosts. *J Infect Dis* **160**, 166-167 (1989).
65 Edlow, J. A. Tick paralysis. *Curr Treat Options Neurol* **12**, 167-177, doi:10.1007/s11940-010-0068-7 (2010).
66 Gorman, R. J. & Snead, O. C. Tick paralysis in three children. The diversity of neurologic presentations. *Clin Pediatr (Phila)* **17**, 249-251 (1978).
67 Ribeiro, J. M. *et al.* An annotated catalog of salivary gland transcripts from Ixodes scapularis ticks. *Insect biochemistry and molecular biology* **36**, 111-129, doi:10.1016/j.ibmb.2005.11.005 (2006).
68 Van Nunen, S. A., O'Connor, K. S., Clarke, L. R., Boyle, R. X. & Fernando, S. L. An association between tick bite reactions and red meat allergy in humans. *Med J Aust* **190**, 510-511 (2009).

69 Commins, S. P. *et al.* The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose-alpha-1,3-galactose. *J Allergy Clin Immunol* **127**, 1286-1293 e1286, doi:10.1016/j.jaci.2011.02.019 (2011).
70 Commins, S. P. & Platts-Mills, T. A. Delayed anaphylaxis to red meat in patients with IgE specific for galactose alpha-1,3-galactose (alpha-gal). *Curr Allergy Asthma Rep* **13**, 72-77, doi:10.1007/s11882-012-0315-y (2013).
71 Brown, A. F. & Hamilton, D. L. Tick bite anaphylaxis in Australia. *Journal of accident & emergency medicine* **15**, 111-113 (1998).
72 Valls, A., Pineda, F., Belver, M., Caballero, T. & Lopez Serrano, M. C. Anaphylactic shock caused by tick (Rhipicephalus sanguineous). *J Investig Allergol Clin Immunol* **17**, 279-280 (2007).
73 Acero, S., Blanco, R. & Bartolome, B. Anaphylaxis due to a tick bite. *Allergy* **58**, 824-825 (2003).
74 Kleine-Tebbe, J. *et al.* Bites of the European pigeon tick (Argas reflexus): Risk of IgE-mediated sensitizations and anaphylactic reactions. *J Allergy Clin Immunol* **117**, 190-195, doi:10.1016/j.jaci.2005.08.056 (2006).
75 Van Wye, J. E., Hsu, Y. P., Lane, R. S., Terr, A. I. & Moss, R. B. IgE antibodies in tick bite-induced anaphylaxis. *J Allergy Clin Immunol* **88**, 968-970 (1991).
76 Burke, G. *et al.* Hypersensitivity to ticks and Lyme disease risk. *Emerg Infect Dis* **11**, 36-41, doi:10.3201/eid1101.040303 (2005).
77 Krause, P. J. *et al.* Dermatologic changes induced by repeated Ixodes scapularis bites and implications for prevention of tick-borne infection. *Vector Borne Zoonotic Dis* **9**, 603-610, doi:10.1089/vbz.2008.0091 (2009).
78 Alguire, P. C. & Mathes, B. M. Skin biopsy techniques for the internist. *J Gen Intern Med* **13**, 46-54 (1998).
79 Aucott, J. N., Crowder, L. A. & Kortte, K. B. Development of a foundation for a case definition of post-treatment Lyme disease syndrome. *Int J Infect Dis*, doi:10.1016/j.ijid.2013.01.008 (2013).

APPENDIX 1: FOLLOWING PUNCH BIOPSY OF THE SKIN

1. Keep the area dry and the dressing on for the first 24 hours.

2. Thereafter, remove the dressing prior to showering.

3. If adhesive steri-strips (which look like small pieces of tape) were used to close the incision, do not remove them. They will gradually fall off on their own.

4. If sutures were placed, reapply Vaseline and a Band-Aid daily as needed. You will return 4 to 7 days following the procedure for removal of the sutures.

5. If you experience discomfort at the biopsy site, you can take acetaminophen (brand name: Tylenol), two 325 mg tablets every 4 to 6 hours as needed (do not take more than 12 tablets in 24 hours) or ibuprofen (brand names: Advil, Motrin), one to two 200 mg tablets 4 to 6 hours as needed (do not exceed 6 tablets in 24 hours).

6. If the biopsy site begins to bleed, apply direct pressure for 10 minutes. If it continues to bleed, call us.

7. Skin infection can follow any surgical procedure. If you develop increased pain, redness, pus or swelling at the biopsy site, call us.

Contact numbers:
Hospital
Dr. _____
Page Operator

APPENDIX 2: CARE OF THE XENODIAGNOSIS SITE

Instruction Sheet

Tick placement and care of site:

- Your dressing is to remain in place for 4 to 6 days. The dressing keeps the ticks in place. Please do not open or remove the dressing during the study period.
- Do not scratch the dressing site if it itches. You may take oral diphenhydramine (Benadryl) for itching.
- When showering, please cover the dressing site with AquaGuard. Remove the AquaGuard promptly after showering. Gently pat dry the dressing if necessary. Please check that the dressing is firmly in place.
- Refrain from aerobic exercise or any activity that would induce heavy perspiration.
- Please refrain from high-risk tick exposure activities (hiking, gardening, etc.)
- Please do not take garlic supplements for the next 4 to 6 days. Garlic as seasoning in food is okay.
- Check the dressing daily to make sure all edges are sealed. Call the study personnel if there is a problem with the dressing. You can contact _____ at phone number _____.

Diary Card:

- Day 1 is the date ticks were placed.
- Complete the diary card each day.
- Please note any new medications taken during the study period.

Scheduled visit date and time for tick removal:_____.

APPENDIX 3: TOXICITY GRADING SCALE

(Adapted from Guidance for Industry: Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials (Food and Drug Administration, Center for Biologics Evaluation and Research, September 2007).

Local Reaction	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially Life Threatening (Grade 4)
Pain	Does not interfere with activity	Repeated use of non-narcotic pain reliever >24 hours or interferes with activity	Any use of narcotic pain reliever or prevents daily activity	Emergency room (ER) visit or hospitalization
Tenderness	Mild discomfort to touch	Discomfort with movement	Significant discomfort at rest	ER visit or hospitalization
Erythema/Redness	2.5-5 cm	5.1-10 cm	>10 cm	Necrosis or exfoliative dermatitis
Induration/Swelling	2.5-5 cm and does not interfere with activity	5.1-10 cm or interferes with activity	>10 cm or prevents daily activity	Necrosis

Vital Signs*	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially Life Threatening (Grade 4)
Fever (°C) (°F) **	38.0-38.4 100.4-101.1	38.5-38.9 101.2-102.0	39.0-40 102.1–104	>40 >104
Tachycardia-beats per minute	101-115	116-130	>130	ER visit or hospitalization for arrhythmia
Bradycardia-beats per minute***	50-54	45-49	<45	ER visit or hospitalization for arrhythmia
Hypertension (systolic)-mm Hg	150-160	161-170	>170	ER visit or hospitalization for malignant hypertension
Hypertension (diastolic)-mm Hg	95-100	101-105	>105	ER visit or hospitalization for malignant hypertension
Hypotension (systolic)-mm Hg	85-89	80-84	<80	ER visit or hospitalization for hypotensive shock

* Subject should be at rest for all vital sign measurements.
** If oral temperature; no recent hot or cold beverages or smoking.
*** When resting heart rate is between 60-100 beats per minute. Use clinical judgment when characterizing bradycardia among some healthy subject populations, for example, conditioned athletes.

Systemic (General)	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially Life Threatening (Grade 4)
Nausea/vomiting	No interference with activity or 1-2 episodes/24 hours	Some interference with activity or >2 episodes/24 hours	Prevents daily activity, requires outpatient IV hydration	ER visit or hospitalization for hypotensive shock
Diarrhea	2-3 loose stools or <400 gms/24 hours	4-5 stools or 400-800 gms/24 hours	6 or more watery stools or >800gms/24 hours or requires outpatient IV hydration	ER visit or hospitalization
Headache	Mild increase over baseline.	Moderate increase over baseline, with repeated use of non-narcotic pain reliever >24 hours and some interference with activity	Significant increase over baseline; any use of narcotic pain reliever or prevents ADL	Disabling, ER visit or hospitalization
Fatigue	Mild fatigue over baseline	Moderate fatigue over baseline or causing difficulty performing some activities of daily living (ADL)	Severe fatigue over baseline, interfering with ADL	Disabling, ER visit or hospitalization
Myalgia	Mild increase over baseline	Moderate increase over baseline, or causing difficulty with ADL.	Significant increase over baseline; interfering with ADL	Disabling, ER visit or hospitalization