Global Lyme Alliance

Persister bacteria & Lyme disease: a connection?

PRESENTED BY:
THE GLA SCIENCE TEAM
GLOBAL LYME ALLIANCE

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MORE THAN FORTY YEARS AFTER THE INITIAL DESCRIPTION OF LYME DISEASE, IT'S RECOGNIZED THAT FOR MANY PATIENTS WHO ARE PROPERLY DIAGNOSED EARLY IN INFECTION, RECOVERY IS POSSIBLE AFTER A STANDARD 21-DAY ANTIBIOTIC TREATMENT PROTOCOL.

Unfortunately, even among those diagnosed and treated in the acute stages of the disease, 10% to 20% continue to suffer chronic, even debilitating, symptoms which can last 6 months or more, and include joint and muscle pain, fatigue, and neurocognitive impairment Figure 1).[1] This chronic illness, termed post-treatment Lyme disease (PTLD), may affect large numbers of people, as shown in a recent mathematical model estimating that by 2020, there were between 81,713 to 1,944,189 individuals with PTLD in the US.[2] Researchers do not clearly understand what causes PTLD, with one suspect being antibiotic-tolerant 'persister' bacteria.[3-5]

Numerous pathogens, including *Borrelia burgdorferi*, the spirochete bacterium that causes Lyme disease, have the ability to form persisters, which are dormant cells that have developed antibiotic tolerance.[4,5] Based on evidence seen in vitro (in lab culture), exposure to antibiotics kills the majority of bacterial cells, but a small number survive. This subpopulation is made up of persister cells, which are tolerant, but not resistant to antibiotics.[4] In this white paper, we will examine general themes of bacterial persistence. We will also discuss evidence of *Borrelia* persisters in vitro and in vivo (animals and humans), along with the potential contribution of these persister cells to symptoms experienced in PTLD. Although patient genetic and epigenetic factors, or immune dysfunction, could contribute to PTLD, those are the subject of separate articles.

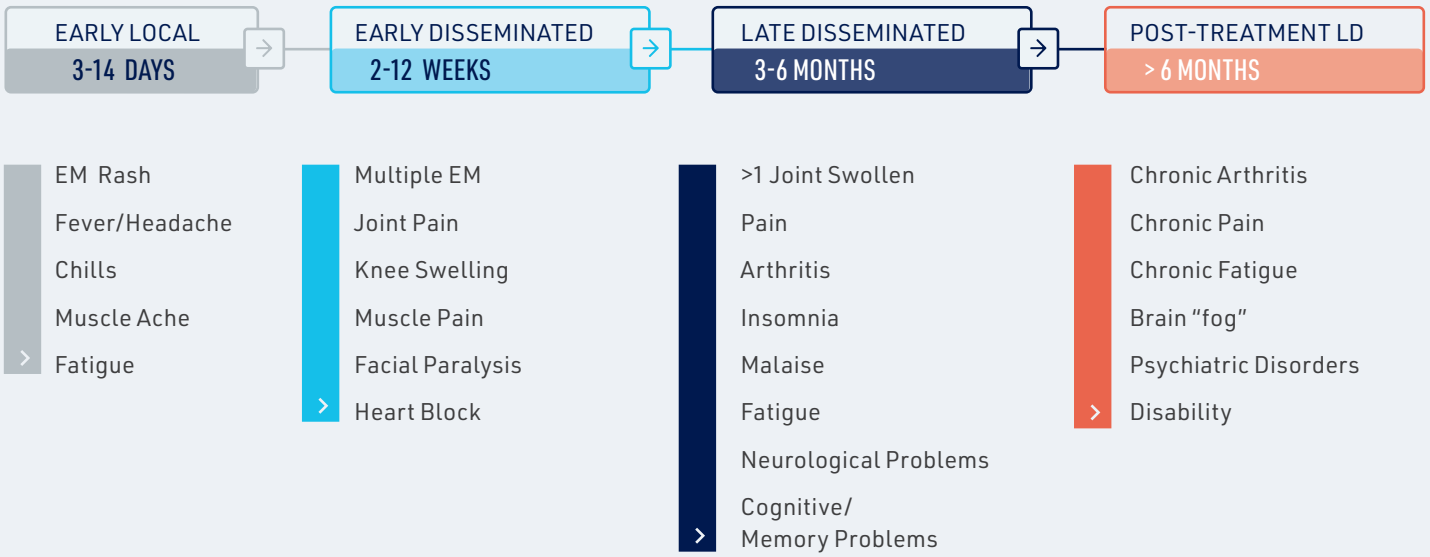


FIGURE 1. THE STAGES OF LYME DISEASE

Lyme disease early symptoms start at the bite site in most, but not all cases. An expanding rash known as *erythema migrans* (EM) may be accompanied by flu-like symptoms. If untreated, disease progresses through early and late disseminated stages, with new symptoms appearing. For those who are diagnosed and treated, 10-20% may continue to suffer debilitating symptoms, known as post-treatment Lyme disease.

BORRELIA BURGDORFERI: A MASTER OF SURVIVAL

B. burgdorferi is a biological marvel, having evolved to survive and evade host immune defenses as well as antibiotic treatment - via numerous mechanisms. These bacteria, which take the form of spiral-shaped spirochetes, have adapted to vastly different host environments, including tick, bird and mammal, including humans. They have thus evolved adaptations to survive under diverse and sometimes harsh environments,[6] including forming persister cells.[4,5]

B. burgdorferi is a motile bacterium whose movement resembles a corkscrew drilling or boring (Figure 2). After initially colonizing the skin after a tick bite, it spreads to many parts of the body, including the joints, heart, and central and peripheral nervous system.[7] Patients whose treatment with antibiotics may be delayed as a result of missed or misdiagnosis, allows the disease to progress. They may be at risk for more complicated disease, especially involving tissues where antibiotic concentrations may be limited, such as the brain. When treatment is delayed, arthritis, carditis, and meningitis can become more severe, and arthritis in particular can be chronic and unresponsive to antibiotic therapy.[8] Given the ineffectiveness of current antibiotic regimens to kill drug tolerant persisters, they are suspected as a potential cause in the development of PTLD and its associated chronic symptoms.

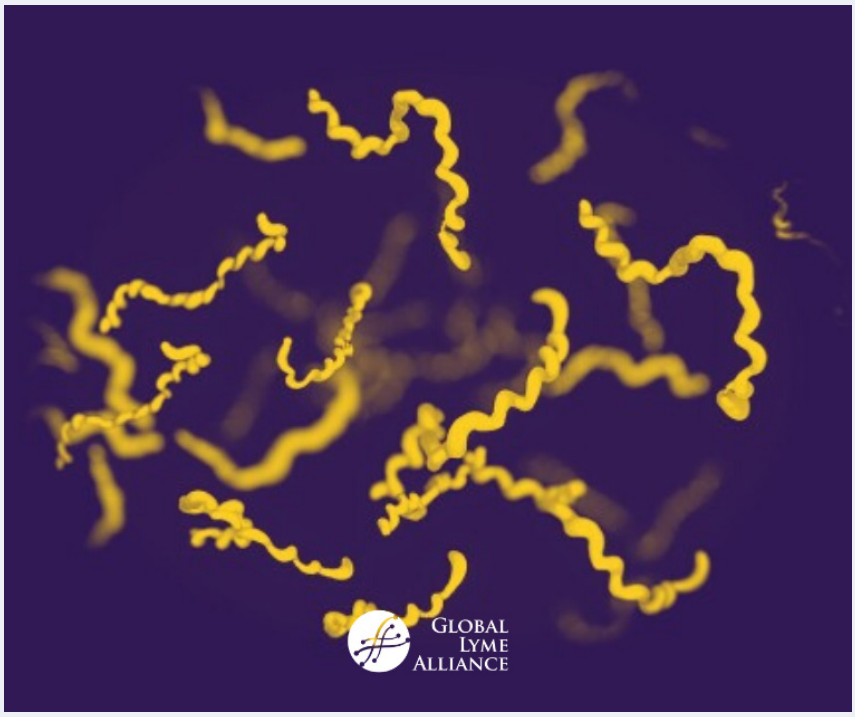


FIGURE 2

Borrelia burgdorferi, the cause of Lyme disease is a motile, corkscrew-shaped bacterium.

Persistence of bacteria after standard treatment also may be attributed to additional survival mechanisms that *B. burgdorferi* and other pathogens possess. For example, there is in vitro evidence that *B. burgdorferi* can form biofilm-like colonies of spirochetes that may protect them against the effects of antibiotics.[9-13] While these studies have been highly publicized among the lay community and touted as a target for treatment, there is scant evidence for their existence in vivo. The in vitro evidence for biofilm formation in Lyme disease and other diseases will be examined here. *B. burgdorferi* also has some intriguing mechanisms to avoid host cellular and humoral immune defenses.[14] Normally, pathogens display antigens on their cell surface, a red flag for the immune system to "seek and destroy" through the action of specific antibodies that are produced. However, *B. burgdorferi* has an elaborate mechanism for altering its cell-surface antigens in response to immune attack, thus effectively camouflaging itself against the immune system (Figure 3).[14-16]

B. burgdorferi also can protect itself against clearance mechanisms associated with the complement system[17], and can hijack the host's ability to suppress inflammation, which would normally limit tissue damage, but may also allow spirochetes to persist unhindered.[18]

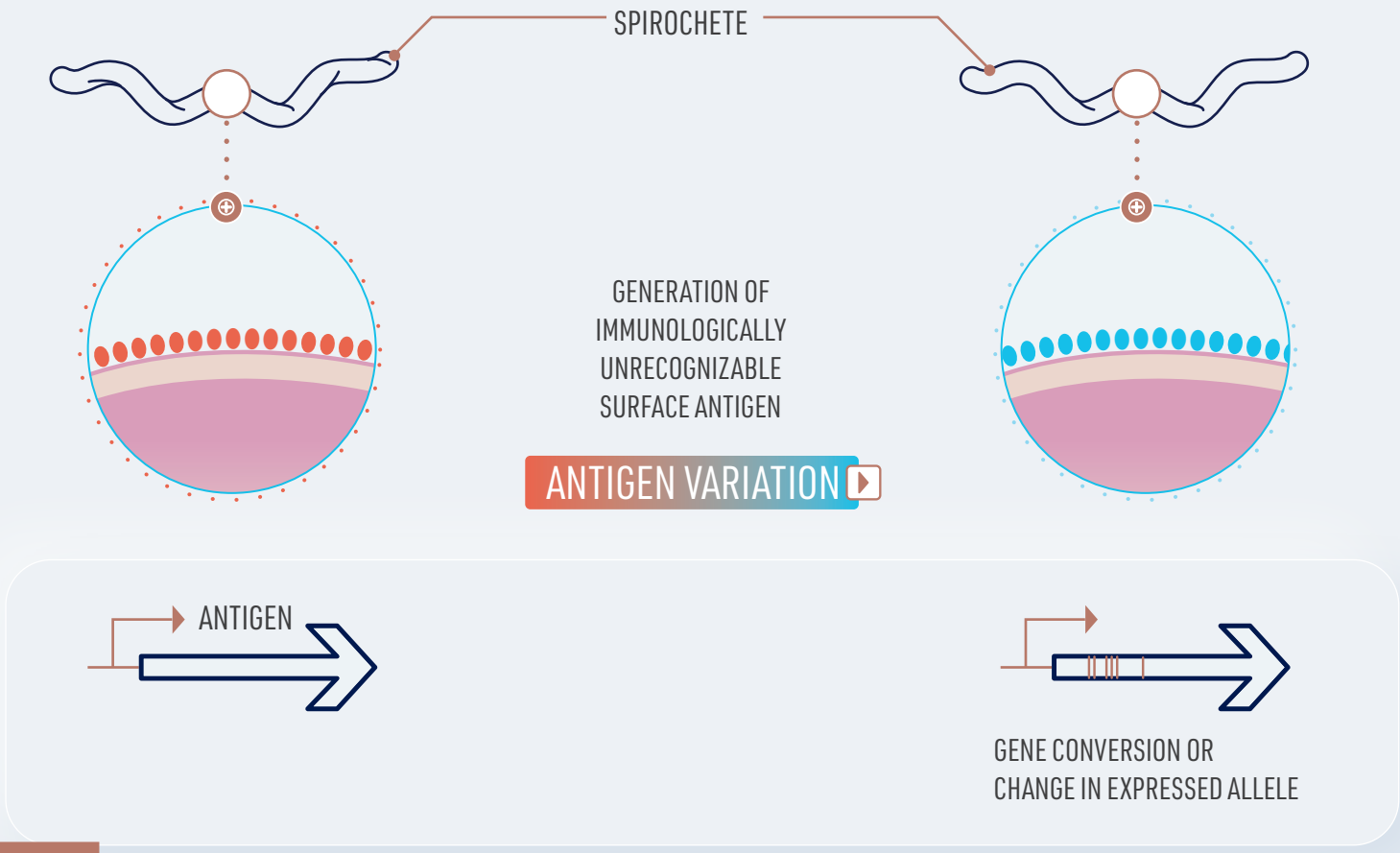


FIGURE 3

Illustration of antigen variation. Some pathogens thwart their recognition by the host immune system by continually changing a prominent surface antigen through changes in the allele expression or gene conversion events to modify the expressed allele. In the schematic, the changing surface antigen of a pathogen is depicted by the red and blue ovals. Modified and reused from Chaconas G, 2020, ref 52, under terms of the Creative Commons CC-BY license.

PERSISTERS IN OTHER CHRONIC INFECTIONS

As research is still ongoing to prove or disprove the presence and contribution of persisters in PTLD, it's useful to look at the impact of persisters in other diseases, which utilize mechanisms that may be similar to that used by *B. burgdorferi*. One such example occurs in most patients with late-stage cystic fibrosis (CF), who ultimately succumb to infection with the bacteria *Pseudomonas aeruginosa* despite prolonged antibiotic treatment.[19] Like many other bacteria, *P. aeruginosa* forms a biofilm, which confers protection against antibiotics.[20]

Biofilms, which are communities of microorganisms that attach to each other and to surfaces, are a common survival mechanism used by bacteria to protect themselves against environmental stressors such as extreme pH or temperatures, host defenses, and antibiotics (Figure 4). They often form on indwelling medical devices, such as implants or catheters.[21] Bacteria aggregate together in a complex mesh, secreting an extracellular polymeric substance which forms a protective slimy mucus layer around the bacterial colony.[10] *P. aeruginosa* survives antibiotic treatment as a biofilm, remaining dormant during treatment, but becoming active afterward, replicating and repopulating the biofilm. In studies by Dr. Kim Lewis and colleagues at Northeastern University, persister variants of *P. aeruginosa* were isolated from CF patients, and this post-antibiotic remnant was proposed to hinder full eradication of *P. aeruginosa* infection.[19] In another study by the same group, drug tolerant persister strains of the yeast, *Candida albicans* were isolated from patients who had an 8 week-long infection of chronic oral thrush. These persister fungal pathogens also formed biofilms, with an enhanced tolerance to an antifungal drug.[22]

While there is evidence in humans of biofilms forming and contributing to persistence in other diseases (e.g., CF and oral thrush), in the case of *B. burgdorferi*, the evidence of biofilms mainly comes from in vitro studies, and thus its relevance to human disease is unknown.

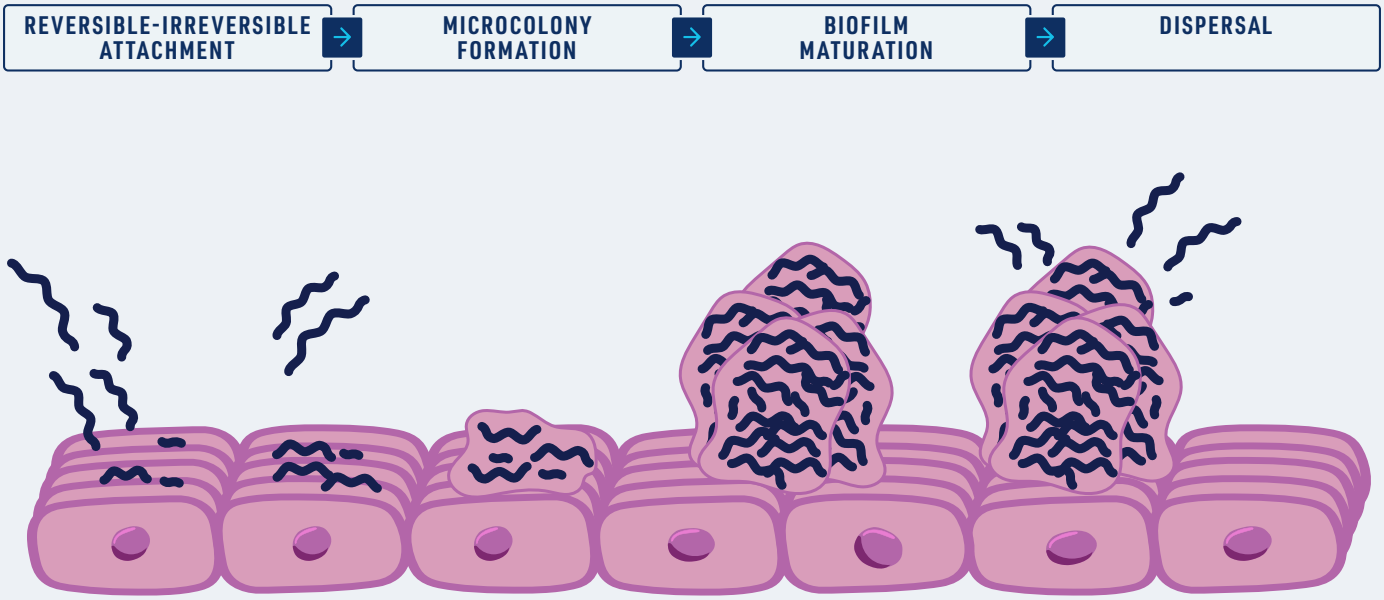


FIGURE 4

Potential biofilm development by *B. burgdorferi*.
Modified and reused from Kostakioti M, 2013, Ref. 53, with permission from ©Cold Spring Harbor Laboratory Press.

Eva Sapi et al. have published some of this work evaluating *B. burgdorferi* biofilms.[10,11] They showed that *B. burgdorferi* appears to form biofilms in culture medium and that these biofilms have characteristic features similar to well-established biofilms produced by other microorganisms.[10] In addition, Sapi evaluated 5 antibiotics against biofilm-like colonies of *B. burgdorferi*, reporting that 70%-85% of viable organisms remained after antibiotic treatment.[11] However, their in vitro studies with one of these antibiotics gave conflicting results from that of another researcher's study in mice.[23] Thus, in vitro evidence either is contradictory or remains to be confirmed by other researchers. And, with only one in vivo study,[24] which also awaits independent confirmation, there clearly is a need for more research in this area.

Another survival strategy used by persisters is to invade host cells. For example, dormant as well as replicating *Salmonella* persisters have been found after engulfment by macrophages.[25] Another such example is dormant *Mycobacterium tuberculosis* persisters in lung lesions in tuberculosis patients. This bacterium persists in a latent state in untreated or incompletely treated tuberculosis patients, and drugs that are very effective in vitro require long treatment periods to be effective.[26-28] The lesions are a source of new infection, as persister mycobacteria can transition from a dormant to an actively growing state. Interestingly, *B. burgdorferi* has also been found in vitro and in vivo inside of cells, specifically in macrophages, neurons, glial cells, and keratinocytes in the skin.[2] The same is true for *Treponema pallidum*, the spirochete bacterium that causes syphilis. It too has been found inside neurons, glial cells, and macrophages. However, it is important to note that only in studies of *B. burgdorferi* invasion of non-professional phagocytes (e.g., fibroblasts and endothelial cells) were researchers able to recover viable spirochetes from antibiotic-treated cells.[29,30] The general consensus in the field is that internalization of spirochetes by professional phagocytes (e.g., neutrophils, monocytes/macrophages, and dendritic cells) results in killing of the spirochetes, both in vitro and in vivo.[31]

Other intriguing traits shared by *B. burgdorferi* and *T. pallidum* that may contribute to persistence and chronic disease include the ability of these spirochetes to morph from their typical spiral form into other forms.[2,32,33] There is some evidence that, as a result of adverse conditions, both can form vesicles in vitro that bud from their cellular membranes, producing chains of free vesicles or granules (also called cysts).[2,33] In the case of *B. burgdorferi*, these tiny spore-like granules, of 0.1–0.3 micrometers in diameter, contain DNA and surface proteins specific to the spirochete form.[2] Another similarity to note is that just as *T. pallidum* occurs in the brain in syphilis patients, *B. burgdorferi* may possibly persist in the brain of patients with chronic Lyme neuroborreliosis[2] and has been proposed, but not conclusively shown to contribute to dementia.[34] These preliminary findings open the door to further studies that would include rigorous testing in animal models, in addition to well-controlled, carefully documented post-mortem histopathological studies.

Studies of other pathogenic bacteria also suggest that adopting alternate morphologies provides survival advantages, allowing them to disseminate through different tissues and to potentially persist even after therapeutic treatment and despite challenges from the host immune response. *B. burgdorferi* is a master shape-shifter, changing from a familiar corkscrew-shaped spirochete form to a variety of other forms that include granules and L-forms (with spheroplast and protoplast subtypes).[2,35-37] Cysts, propagules, round, and spherical blebs have also been used to describe atypical forms of *B. burgdorferi*. However, these names are scientifically inaccurate as they have other definitions.[37] There is likely an overlap in some of these atypical forms described by different researchers.[36] A few in vitro studies have shown that atypical forms can convert back into a motile spirochete form.[35,36] *B. burgdorferi* mobile spirochetes introduced into spinal fluid transform into dormant "cysts", specifically spheroplast L-forms, within a 1-24 hour time frame.[35] Transferring these spheroplast L-forms to a suitable culture medium then allows their transition back to to motile spirochetes after 9 to 17 days. However, such studies must be done with great care to ensure that absolutely no motile spirochetes are transferred from one growth medium to another. If these findings are borne out by further experiments, they would have ramifications for diagnostic testing. For example, cases of neuroborreliosis (a neurological manifestation of Lyme disease) might require screening for both spheroplast L-forms and spirochetes through imaging techniques or other procedures.[35] Culturing *B. burgdorferi* has revealed a wide variety of forms the bacteria can adopt and their existence has led to speculation these atypical forms may contribute to the spirochetes' ability to persist and perhaps cause chronic disease. However, a systematic review of *B. burgdorferi* morphologic variants does not support a role in chronic Lyme disease.[37]

In addition to in vitro evidence, limited small-scale post-mortem studies provide evidence of atypical and cystic forms of spirochetes in brain cells. Specifically, in 2008, atypical and cystic forms identical to in vitro forms were found in the brains of a very small case study of three patients with Lyme neuroborreliosis and concurrent Alzheimer's disease.[2] It is hypothesized that these atypical forms and the intracellular location in glial cells and neurons may contribute to the persistence of *B. burgdorferi* in these infected tissues. However, rigorous controls documenting an absence of these forms in normal brains were not presented. In addition, the staining techniques used for detection were not strictly controlled and no evidence was provided that the atypical forms were metabolically active let alone capable of replication. At best, the limited scope of this study suggested a possible association between *B. burgdorferi* atypical forms and dementia. However, further in-depth studies with updated methods and larger sample sizes might generate more conclusive evidence and shed further light on this intriguing question. Clearly, there is much more research to be done in animals and humans to confirm the existence of these atypical forms in the context of natural infection and what, if any, role they play in Lyme disease pathogenesis.

ERADICATING *B. BURGDORFERI* PERSISTERS: IN VITRO STUDIES

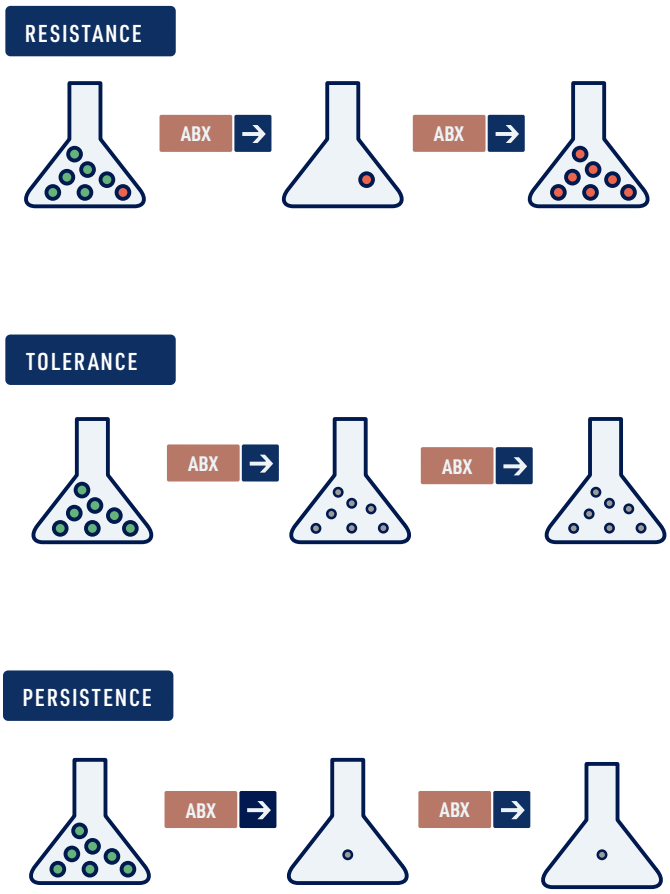
Although the "jury may still be out" on the presence and role of persisters during human infection, identifying drugs that can kill persister forms of *B. burgdorferi* and testing their efficacy in vitro and in animal models would be of potential interest. Persister cells can be formed naturally in culture media if spirochetes are allowed to grow to high enough densities or can be induced through antibiotic treatment.[38] Antibiotics generally will kill a large number of *B. burgdorferi* in culture, or in the case of bacteriostatic antibiotics such as doxycycline, prevent *B. burgdorferi* from replicating, so that the immune system can kill the bacteria. However it's possible that, after antibiotic treatment, a small population remains, and these are the persister cells that tolerate (but are not resistant to) antibiotics (Figure 5).[4] Generally, persisters are thought to be slow-growing or dormant forms of bacteria that return to normal growth when antibiotics are removed. Researchers have found that persisters share common mechanisms of survival after antibiotic treatment.

FIGURE 5

Resistance Resistance occurs when a rare genetic mutant within a bacterial population survives the addition of an antibiotic. This mutant is able to replicate in the presence of the antibiotic, and its progeny carry a resistance gene as a heritable trait. Bacteria can also acquire resistance genes through gene transfer from other bacteria. Resistant mutants become the dominant bacteria within the population, and higher concentrations of antibiotics are needed to kill them than the original susceptible population.

Tolerance Tolerance occurs when bacteria switch to slow growth or dormancy in the presence of antibiotics. Although the same concentration of drug kills them as susceptible bacteria, the time required to kill them may be increased. Tolerance is generally not due to heritable gene changes, but due to changes in the expression of genes.

Persistence: Persistence occurs when a very rare subset (typically < 1%) of bacteria survives and continues to replicate very slowly while in the presence of antibiotics. They are killed at a much slower rate than susceptible bacteria. Persistence is not heritable, but persistent cells may be resuscitated into normal replicating cells after removal of antibiotics. These populations, after another exposure to antibiotics, may again be mostly killed, with a small subpopulation of persisters that survive.



For example, using *Escherichia coli* as model bacteria, Dr. Ying Zhang and colleagues at Johns Hopkins University reported that persisters that form in response to different antibiotics (tetracycline and rifampin) exhibit an upregulation of common DNA repair pathways, as well as common genes corresponding to metabolism (folate and energy), flagella biosynthesis (for bacterial locomotion), cellular transporters, etc.[38] This type of information on common pathways provides researchers insight into the mechanisms of how these bacteria become antibiotic-tolerant.

To study the mechanism of persister formation in *Borrelia burgdorferi*, Dr. Zhang and colleagues analyzed RNA transcription by stationary-phase bacteria grown in the presence of doxycycline or amoxicillin[5]. This study, funded by GLA, suggests how genes are differently expressed in persister bacteria compared to normally replicating bacteria grown in the absence of antibiotics. Figure 6 shows gene transcription changes in persister *B. burgdoreri*.

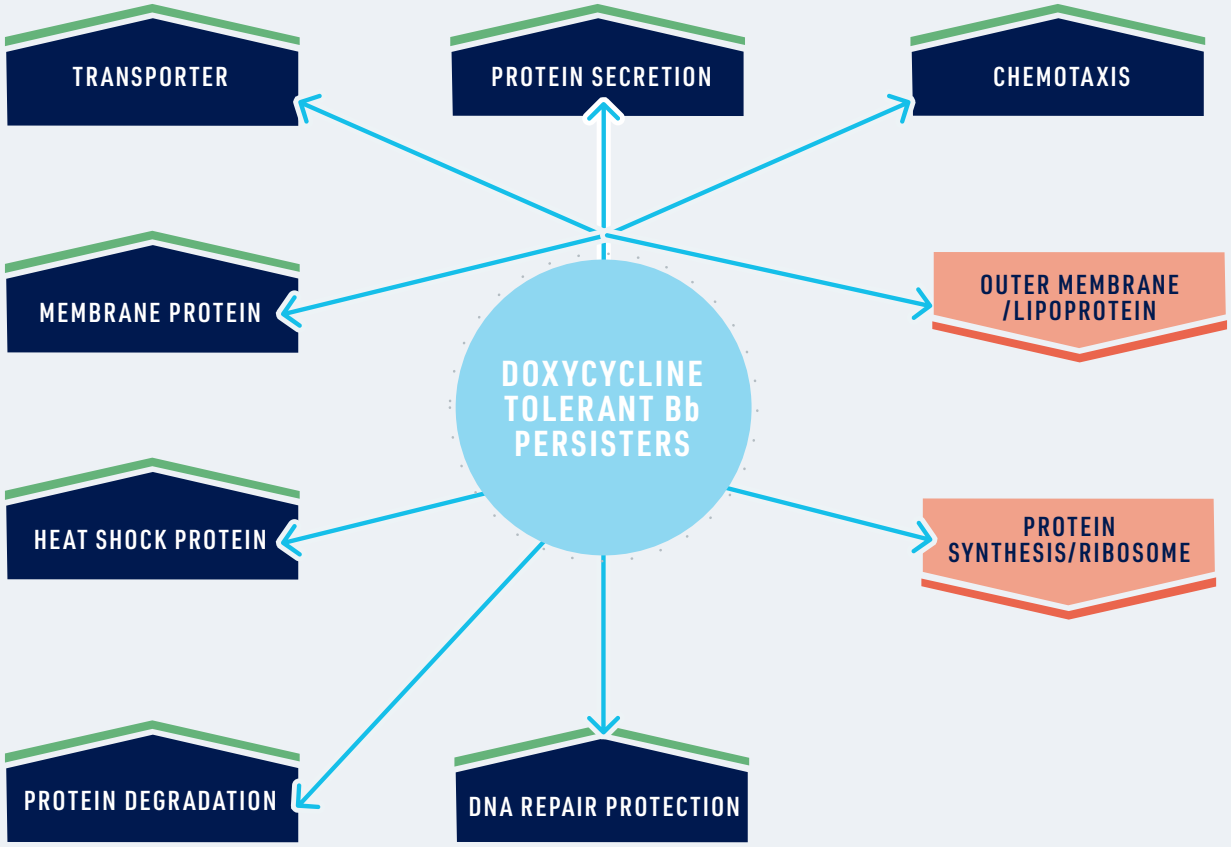


FIGURE 6

Differentially expressed genes in doxycycline tolerant *B. burgdorferi* persister grouped into pathways. The blue upward pentagons indicate upregulated genes, and the red downward pentagons indicate downregulated genes. Modified and reused from Feng J, 2015, ref 5, under terms of the Creative Commons CC-BY license.

In a study by Dr. Kim Lewis and colleagues using *B. burgdorferi* grown in culture, antibiotic treatment with either amoxicillin or ceftriaxone killed off the majority of the spirochete population, but persister cells survived. These remaining bacteria were tested for possible antibiotic tolerance or antibiotic resistance, and were found to be tolerant, which meant they had not developed or acquired a genetic basis for resistance.[4] In studies funded by GLA, Lewis' group also tested a variety of antibiotics in culture medium to see which were most effective in eradicating persister cells. What they found was that the anticancer agent mitomycin C killed multiple forms of *B. burgdorferi*, including persisters as well as growing and stationary cultures of spirochetes. Successful elimination of all live persisters also was obtained by pulse dosing with ceftriaxone, i.e., by treating with the antibiotic, washing it away to allow the persisters to reproduce, and adding the antibiotic again, through four rounds.[3]

Another class of agents that show promise of antimicrobial activity are plant-derived essential oils, some of which have anti-persister activity. Dr. Zhang's group tested 34 essential oils in vitro against *B. burgdorferi* and found three that completely eliminated persister population re-growth: clove bud, oregano, and cinnamon bark.[13] Some essential oils even had anti-biofilm activity.

Collectively, these studies have identified possible strategies to eliminate persister forms of *B. burgdorferi* in controlled, laboratory culture environments. Extending these findings to humans will need careful validation in animal models, experiments to determine safety, and a thorough understanding of how these compounds are metabolized and interact in vivo.

ANIMAL AND HUMAN STUDIES OF *B. BURGDORFERI* PERSISTERS

While studies in culture medium have provided promising preliminary evidence of persisters and the means of their eradication, studies in animals and humans are key to gaining a complete biological picture. Living organisms have an immune system and other physiological factors that can influence the development and progression of Lyme disease. There is evidence from studies with mice[39,40], dogs[41] and monkeys[42] of persistence of *B. burgdorferi* DNA after antibiotic treatment, even though scientists cannot cultivate the persisting spirochetes directly from the animals. Using a mouse model of Lyme borreliosis, one to three months after treating mice with the antibiotic ceftriaxone for one month, researchers found that *B. burgdorferi* DNA was present in the mice and that spirochetes could be seen using sensitive and specific imaging techniques.[39] While the presence of DNA does not alone confirm live *B. burgdorferi*, the evidence is stronger when combined with imaging.

In another mouse study, the researchers followed the levels of *B. burgdorferi* DNA in tissues two, four, and eight months after antibiotic treatment for Lyme disease, and found declining DNA, as expected. However, after 12 months, several tissues had a resurgence in *B. burgdorferi* DNA, with about the same amount of DNA as found in control (saline-treated) mice.[40] Spirochetes were observed in mouse tissue through imaging as well as by using a diagnostic technique in which uninfected ticks were allowed to feed on the infected animal. This is known as xenodiagnosis, which tests the ability of uninfected ticks to acquire *B. burgdorferi* in blood meals from infected, antibiotic-treated animals.[40] Successful tick acquisition of spirochetes from these animals, after a clear resurgence of bacteria following antibiotic treatment, provides strong evidence that these are *B. burgdorferi* persisters.

In another small-scale study, monkeys were infected with *B. burgdorferi* and treated aggressively with antibiotics, and the animals were monitored to see if *B. burgdorferi* persisted. Six to 12 months after treatment, the animals were euthanized and *B. burgdorferi* DNA, RNA, and antigen were detected in the tissues of treated animals. Small numbers of intact spirochetes also were visualized by fluorescence microscopy is some tissues, including brain, and were recovered via xenodiagnosis (Figure 7), though not by direct culture methods.[42]

Despite evidence of antibiotic-tolerant *B. burgdorferi* persisters in animals, more needs to be done to reproduce and expand these preliminary results in other labs. The presence of bacterial DNA or antigens suggests there may be fragments of bacteria, or intact bacteria that are viable, but not culturable after removal from animals or humans. Development of techniques other than xenodiagnosis that allow recovery of live persisters directly from animal tissues would be helpful. Additionally, larger studies could provide a greater level of statistical support for the hypothesis that antibiotic-tolerant spirochetes contribute to persistent symptoms.

Thus far, evidence in humans for persisters is even more sparse and indirect than in animals. One study conducted two systematic reviews of the literature through 2013 to identify reported cases of round morphologic variants of *B. burgdorferi* isolated from patients.[37] The researchers found six studies obtained from 32 patients in which round atypical forms or morphological variants were observed. Another 29 studies described the typical corkscrew-shaped spirochete form isolated from patients with Lyme disease. The authors noted that no study established causal relationships between the atypical forms and treatment failure and severity or chronicity of

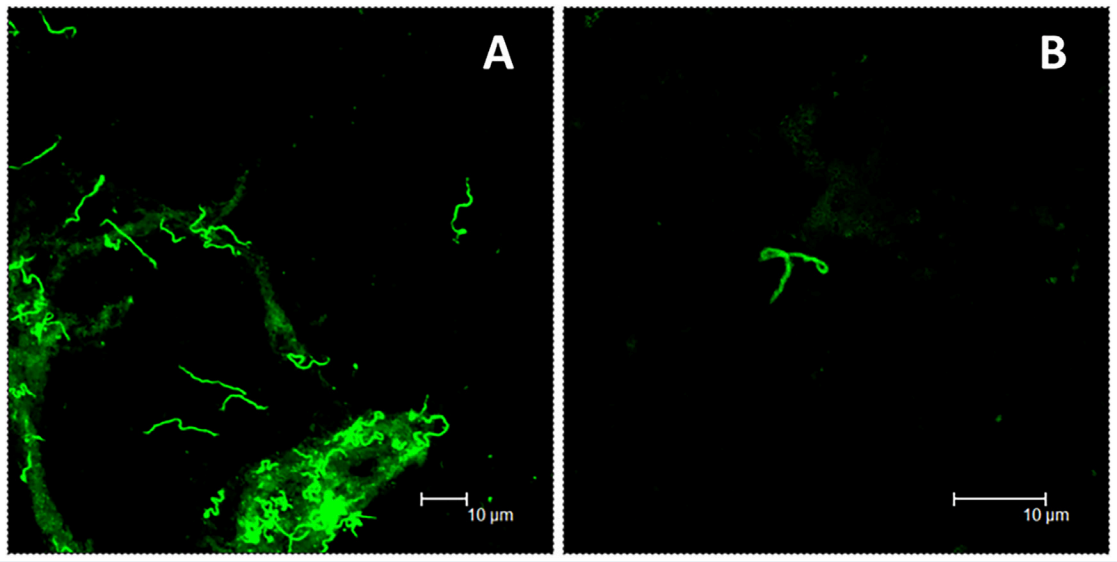


FIGURE 7.

Spirochetes recovered by xenodiagnosis from animals treated in the disseminated phase of infection. Images from direct fluorescent staining of *B. burgdorferi* spirochetes found in xenodiagnostic tick midgut culture (A) or tick midgut preparation (B) from antibiotic-treated macaques. Modified and reused from Embers M, 2012 ref. 42, under terms of the Creative Commons Attribution license.

clinical disease.[37] Also, they found no association between the presence of atypical forms and patients with only subjective symptoms such as fatigue or pain, which are commonly reported by patients with PTLD. This review revealed some gaps that can provide guidance for more clinical research. One study since this systematic review did reveal a patient with PTLD who was positive for *B. burgdorferi* DNA, as recovered through xenodiagnosis.[43] However, neither live *B. burgdorferi* in an atypical or spirochetal form were recovered.

A recent post-mortem study[55] analyzed central nervous system (CNS) tissues from a patient with a history of Lyme disease, who was treated with antibiotics and years later, developed neurodegenerative symptoms. Pathological examination of autopsy tissues revealed Lewy body dementia, consistent with the patient's gradual worsening, and concurrent with antibodies against *B. burgdorferi*. Using polymerase chain reaction, *B.burgdorferi* DNA was found in the spinal cord and amygdala samples. Additionally, careful imaging revealed an intact spirochete in the spinal cord, close to blood vessels. This study was better-controlled than previous studies examining *B. burgdorferi* in autopsied CNS samples. Negative controls were post-mortem CNS tissues from a tissue collection, and positive controls were nonhuman primate CNS tissues that were cultured with *B. burgdorferi*. In addition, the researchers found that RNA degradation rendered *B. burgdorferi* RNA detection unfeasible, which was not unexpected given the extreme fragility of RNA.

While these findings do not prove a causal relationship between Lyme disease and dementia, they are indications that bacteria persisted in the CNS even after repeated antibiotic treatment. Since the presence of live, replicating *B. burgdorferi* has not been definitively established or excluded in PTLD patients[44], more sensitive and better controlled methods will be instrumental in answering this question. The field awaits improvements in technology.

DO ANTIBIOTICS ALLEVIATE LONGTERM SYMPTOMS?

If PTLD is caused by residual bacterial replication, then it follows that antibiotic therapy might kill them and alleviate symptoms. Multiple clinical trials have explored using additional rounds of antibiotics to kill presumed persistent bacteria. Ideally, such studies would be double-blinded, placebo-controlled randomized trials that monitor clinical outcomes. Sufficiently lengthy follow-up would determine whether any improvements in health are sustained. An influential article published in 2001 by Klempner and colleagues detailed two trials, in which patients with well-documented Lyme disease had been previously treated, but continued to suffer symptoms that caused considerable impairment of quality of life. These included neurocognitive deficit, musculoskeletal pain, dysesthesia (abnormal sensations) and fatigue.[45] One trial, of 78 patients, had IgG-seropositive individuals, while the other, with 51 patients, was composed of seronegative patients. Patients were randomly assigned to receive intravenous antibiotics for 30 days, followed by oral antibiotics for 60 days. Control-treated individuals received matching intravenous and oral placebos for the same length of time. The researchers found no difference in symptom improvement between the antibiotic-treated and placebo-treated patients. While 37% of the patients who received antibiotics reported improvement, 40% in the placebo group also experienced improved symptoms. These results indicate that 40% of patients eventually improved without any additional treatment beyond the initial standard treatment.

The outcome measures for these studies relied on patient responses to the SF-36, a survey of subjective measures of health quality of life. However, a careful statistical analysis of this study suggested that the number of patients per group was insufficient to detect a minimum clinically important difference in symptom improvement using this method[54]. The authors of the statistical review suggested that diseases with similar levels of disability as PTLD measure the improvements of SF-36 scores differently, and that the Klempner study was underpowered to detect meaningful clinical improvements. Thus, they argued that larger studies of antibiotics to treat lingering Lyme disease are still warranted.

Impaired cognitive function is a frequently reported symptom of PTLD. A study of 129 previously treated Lyme disease patients evaluated the effect of 90 days of antibiotics, with a focus on cognitive functioning, but also tracked pain, attention, memory and executive function[46]. Over the course of the study, antibotic and placebo treated groups had improvements in symptoms, but there was no statistical difference between the two.

An important study addressing the effect of antibiotic therapy on neurocognitive functioning, pain and fatigue was led by Dr. Brian Fallon, a GLA grantee, at Columbia University. In this trial, patients were randomized to receive either 10 weeks of intravenous ceftriaxone or placebo, followed by no treatment[47], a regimen that targets neuroborreliosis. Subjects were followed for 24 weeks, and it was found that while antibiotic-treated subjects had cognitive improvement by week 12, it was not sustained at week 24, at which time point the antibiotic-treated subjects had been without drug for 14 weeks. However, among patients with more severe pain and physical impairment at baseline, there was sustained improvement of these symptoms by week 24, an important finding, since these are significant symptoms. The authors pointed out that this benefit must be balanced against the observation that 26% of patients had adverse effects attributed to the intravenous ceftriaxone, which should not be considered as an effective approach for sustained improvement of cognitive function.

An obstacle to obtaining credible data in clinical trials of post-acute antibiotic therapy is the difficulty in enrolling sufficient numbers of patients with validated evidence of past Lyme disease. This reflects the problems with diagnosis. Many patients lack concrete clinical or serology-based diagnoses, and thus do not meet the inclusion criteria for enrollment in a clinical trial. For example, in the Fallon study[47], 3,368 patients were initially screened, but 1,828 (54%) did not meet CDC diagnostic criteria or have seropositive Western blot tests. Recruiting more patients with validated diagnoses, along with precise characterization of symptom severity would increase the strength of these studies.

CONCLUSIONS: MUCH MORE WORK REMAINS

Whether persister bacteria contribute to chronic symptoms of Lyme disease remains unclear, as current research is inconclusive in patients. What is clear is that in vitro, persister bacteria have been documented after antibiotic exposure, and *Borrelia* bacteria have characteristics that provide them antibiotic tolerance. Their relative contribution to long term symptoms, perhaps in combination with systemic immune dysregulation, should be evaluated through further study.

There also are an amazing variety of unique characteristics of *B. burgdorferi* that may help it survive, thrive, and persist, such as its ability to morph into different forms, become dormant, potentially invade different cells and organs, camouflage itself from the immune defenses, and possibly form biofilms. All of these characteristics have been observed in vitro, with some limited evidence of a few of these atypical forms in vivo. Again, further studies done in meaningful, well-controlled ways, would reveal whether these are a contributing factor to longterm illness.

Clearly, there are bacterial persisters that hinder effective antibiotic treatment of certain chronic diseases. For example, it is widely accepted that antibiotic-tolerant persisters contribute to the difficulty in treating tuberculosis, a known intracellular pathogen.[27,28] In the case of Lyme disease, to date, there is only limited evidence that persisters contribute to chronic symptoms. Most of the evidence from the limited small-scale animal studies is via imaging, the detection of *B. burgdorferi* DNA, or the presence of *B. burgdorferi* in ticks used in xenodiagnostics. What is needed is direct isolation of persisters in well-controlled animal experiments. Spirochetes and atypical forms are technically difficult to isolate from tissue, and newer tests could potentially distinguish between active and prior infection. Another gap is that, in general, much of the research done both in vitro and in vivo remains to be reproduced by other research groups.

Prolonged treatment with antibiotics for persistent symptoms post-treatment has been controversial and widespread among many patients, but currently the clinical evidence does not support its efficacy. Clinical studies carried out to date have revealed that at least under the conditions tested, there is no sustained benefit in reducing chronic symptoms after initial standard treatment. However, a variety of conventional antimicrobials, essential oil regimens, and combinatorial treatment strategies of varying duration have yet to be tested in humans. Also, better diagnostics are needed, to increase the numbers of patients included in trials and to increase their statistical power. The development of identifiable, measurable treatment endpoints would also help in the determination of efficacy of novel treatment strategies.

More evidence-based research in vitro and in clinical trials will lead to a greater understanding of persisters and tackle the chronic symptoms that result from infection by this highly complex and exquisitely adapted pathogen.

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CONTACT

1290 East Main Street
3rd Floor
Stamford, CT 06902
U.S.A

+1 203.969.1333 | INFO@GLA.ORG | GLA.ORG